ISSN 1949-8454 (online)

World Journal of *Biological Chemistry*

World J Biol Chem 2021 January 27; 12(1): 1-14

Published by Baishideng Publishing Group Inc

WJBC

World Journal of Biological Chemistry

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Bimonthly Volume 12 Number 1 January 27, 2021

ORIGINAL ARTICLE

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World Journal of Biological Chemistry

Bimonthly Volume 12 Number 1 January 27, 2021

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INDEXING/ABSTRACTING

The *WJBC* is now abstracted and indexed in PubMed, PubMed Central, China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database.

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Production Editor: *Yu-Jie Ma*; **Production Department Director:** *Yun-Xiaojian Wu*; **Editorial Office Director:** *Yun-Xiaojiao Wu*.

NAME OF JOURNAL
World Journal of Biological Chemistry

ISSN
ISSN 1949-8454 (online)

LAUNCH DATE
July 26, 2010

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Vsevolod Gurevich, Chun-Peng Wan

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1949-8454/editorialboard.htm

PUBLICATION DATE
January 27, 2021

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World J Biol Chem 2021 January 27; 12(1): 1-14

DOI: 10.4331/wjbc.v12.i1.1

ISSN 1949-8454 (online)

ORIGINAL ARTICLE

Basic Study

Inhibition of matrix metalloproteinase-9 secretion by dimethyl sulfoxide and cyclic adenosine monophosphate in human monocytes

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Author contributions: Nichols MR conceived the ideas and supervised the project; Denner DR and Udan-Johns ML planned and carried out the experiments; Nichols MR wrote the manuscript in consultation with Denner DR and Udan-Johns ML. All authors have read and approve the final manuscript.

Supported by The University of Missouri-St. Louis, Alzheimer's Association, No. NIRG-06-27267; and the Missouri Alzheimer's and Related Disorders Research Program.

Institutional review board statement: The THP-1 human monocytic cell line was acquired commercially from American Type Culture Collection, thus no human subjects were used in this study.

Conflict-of-interest statement: There are no conflicts of interest to declare by any of the authors.

Data sharing statement: No additional data are available.

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Abstract

BACKGROUND

Matrix metalloproteinases (MMPs), including MMP-9, are an integral part of the immune response and are upregulated in response to a variety of stimuli. New details continue to emerge concerning the mechanistic and regulatory pathways that mediate MMP-9 secretion. There is significant evidence for regulation of inflammation by dimethyl sulfoxide (DMSO) and 3',5'-cyclic adenosine monophosphate (cAMP), thus investigation of how these two molecules may regulate both MMP-9 and tumor necrosis factor α (TNFα) secretion by human monocytes was of high interest. The hypothesis tested in this study was that DMSO and cAMP regulate MMP-9 and TNFα secretion by distinct mechanisms.

AIM

To investigate the regulation of lipopolysaccharide (LPS)-stimulated MMP-9 and tumor necrosis factor α secretion in THP-1 human monocytes by dimethyl sulfoxide and cAMP.

METHODS

The paper describes a basic research study using THP-1 human monocyte cells. All experiments were conducted at the University of Missouri-St. Louis in the Department of Chemistry and Biochemistry. Human monocyte cells were grown, cultured, and prepared for experiments in the University of Missouri-St. Louis Cell Culture Facility as per accepted guidelines. Cells were treated with LPS for selected exposure times and the conditioned medium was collected for analysis of MMP-9 and TNFα production. Inhibitors including DMSO, cAMP regulators, and anti-TNFα antibody were added to the cells prior to LPS treatment. MMP-9 secretion was analyzed by gel electrophoresis/western blot and quantitated by ImageJ software. TNFα secretion was analyzed by enzyme-linked immuno

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Manuscript source: Unsolicited manuscript

Specialty type: Immunology

Country/Territory of origin: United States

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

Received: June 24, 2020
Peer-review started: June 24, 2020
First decision: November 4, 2020
Revised: November 17, 2020
Accepted: December 30, 2020
Article in press: December 30, 2020
Published online: January 27, 2021

P-Reviewer: Exbrayat JM, Rangel-Corona R
S-Editor: Zhang H
L-Editor: A
P-Editor: Ma YJ

sorbent assay. All data is presented as the average and standard error for at least 3 trials. Statistical analysis was done using a two-tailed paired Student *t*-test. *P* values less than 0.05 were considered significant and designated as such in the Figures. LPS and cAMP regulators were from Sigma-Aldrich, MMP-9 standard and antibody and TNFα antibodies were from R&D Systems, and amyloid-β peptide was from rPeptide.

RESULTS

In our investigation of MMP-9 secretion from THP-1 human monocytes, we made the following findings. Inclusion of DMSO in the cell treatment inhibited LPS-induced MMP-9, but not TNFα, secretion. Inclusion of DMSO in the cell treatment at different concentrations inhibited LPS-induced MMP-9 secretion in a dose-dependent fashion. A cell-permeable cAMP analog, dibutyryl cAMP, inhibited both LPS-induced MMP-9 and TNFα secretion. Pretreatment of the cells with the adenylyl cyclase activator forskolin inhibited LPS-induced MMP-9 and TNFα secretion. Pretreatment of the cells with the general cAMP phosphodiesterase inhibitor IBMX reduced LPS-induced MMP-9 and TNFα in a dose-dependent fashion. Pre-treatment of monocytes with an anti-TNFα antibody blocked LPS-induced MMP-9 and TNFα secretion. Amyloid-β peptide induced MMP-9 secretion, which occurred much later than TNFα secretion. The latter two findings strongly suggested an upstream role for TNFα in mediating LPS-stimulate MMP-9 secretion.

CONCLUSION

The cumulative data indicated that MMP-9 secretion was a distinct process from TNFα secretion and occurred downstream. First, DMSO inhibited MMP-9, but not TNFα, suggesting that the MMP-9 secretion process was selectively altered. Second, cAMP inhibited both MMP-9 and TNFα with a similar potency, but at different monocyte cell exposure time points. The pattern of cAMP inhibition for these two molecules suggested that MMP-9 secretion lies downstream of TNFα and that TNFα may a key component of the pathway leading to MMP-9 secretion. This temporal relationship fit a model whereby early TNFα secretion directly led to later MMP-9 secretion. Lastly, antibody-blocking of TNFα diminished MMP-9 secretion, suggesting a direct link between TNFα secretion and MMP-9 secretion.

Key Words: Matrix metalloproteinase-9; Inflammation; Human monocytes; Tumor necrosis factor alpha; Cyclic adenosine monophosphate; Dimethyl sulfoxide

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Core Tip: This article describes the stimulation of matrix metalloproteinase-9 (MMP-9) and tumor necrosis factor α (TNFα) secretion from human monocyte cells by lipopolysaccharide, and the inhibition of this process by dimethyl sulfoxide and increased intracellular levels of 3',5'-cyclic adenosine monophosphate. The experimental findings demonstrated that dimethyl sulfoxide concentration must be carefully controlled in inflammatory studies, that MMP-9 secretion occurred downstream from TNFα secretion and that the two processes were distinct, and that TNFα is an integral component of the pathway leading to MMP-9 secretion.

Citation: Denner DR, Udan-Johns ML, Nichols MR. Inhibition of matrix metalloproteinase-9 secretion by dimethyl sulfoxide and cyclic adenosine monophosphate in human monocytes. *World J Biol Chem* 2021; 12(1): 1-14
URL: https://www.wjgnet.com/1949-8454/full/v12/i1/1.htm
DOI: https://dx.doi.org/10.4331/wjbc.v12.i1.1

INTRODUCTION

Matrix-metalloproteinases (MMPs) are zinc-dependent extracellular matrix-degrading

enzymes that have a key role in a wide variety of physiological[1] and pathological[2,3] processes. Physiological processes include embryonic development, morphogenesis, angiogenesis, tissue repair, and immune responses while pathological processes include arthritis, cancer, neurological disease, breakdown of the blood–brain barrier, and cerebral hemorrhage. MMP subgroups are comprised of collagenases, gelatinases, stromalysins, and membrane-type and their activity is controlled by gene expression, zymogen activation, and endogenous tissue inhibitors[4,5]. MMP-1, MMP-8, MMP-13, and MMP-18 are collagenases that cleave interstitial collagen type I, II, and III and digest some extracellular matrix and non-extracellular matrix proteins. MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are gelatinases that degrade both collagens and gelatins[3].

MMPs have been described as a type of inflammatory cytokine since they are significantly expressed, secreted, and activated upon stimulation of inflammatory cells by various endogenous or exogenous factors[2]. MMP-9 is an important MMP in myeloid/monocyte cells and substantial work has been done on understanding the types of stimuli and mechanistic pathways that lead to MMP-9 secretion. Stimulation of inflammatory cells with lipopolysaccharide (LPS), cytokines, amino acids, and protein aggregates such as amyloid-β (Aβ) triggers signaling pathways that involve nuclear factor κB, protein kinase Cα, extracellular signal-regulated kinase 1 and 2 and mitogen-activated kinase p38, and lead to MMP-9 secretion[6-9]. MMP-9 secretion, and inflammation in general, can be regulated in a number of ways and there have been previous reports of regulation by both dimethyl sulfoxide (DMSO)[9-11] and the intracellular signaling molecule 3',5'-cyclic adenosine monophosphate (cAMP)[7].

DMSO is commonly used as a vehicle for delivering hydrophobic compounds, such as enzyme inhibitors or signaling regulators to cells, thus its role as an inflammatory regulator is of significant interest. DMSO inhibited production of MMP-9 in TNFα-stimulated human keratinocytes at concentrations of 0.75% and higher[9]. DMSO also decreased interleukin-1β (IL-1β)-induced interleukin-6 and macrophage chemoattractant protein-1 secretions in a dose-dependent manner in differentiated-Caco-2 cells[10]. Suppression of many pro-inflammatory cytokines/chemokines by DMSO in *Escherichia coli*- and herpes simplex virus-1-stimulated whole human blood has also been demonstrated[11]. Cyclic AMP is a critical signaling molecule and is involved in a myriad of physiological cellular processes including regulation of inflammatory processes. Support for this regulation includes the observation that adenosine dose-dependently decreased MMP-9 secretion in isolated human neutrophils regardless of whether the cells were stimulated by *N*-formylmethionyl-leucyl-phenylalanine, LPS, or H_2O_2[7]. Adenosine increased intracellular cAMP levels and it was concluded that the effect was mediated through a cAMP-mediated pathway[7].

MMP-9 and TNFα are important markers and mediators of inflammatory processes in immune cells. In this report we sought to further examine temporal and regulatory mechanisms of MMP-9 secretion in THP-1 human monocytes after stimulation with LPS. Specifically, dose-dependent regulation of MMP-9 and TNFα by the aprotic solvent DMSO and the intracellular signaling molecule cAMP. The findings demonstrated distinct regulatory effects by DMSO and cAMP on MMP-9 and TNFα secretion. These distinct, but significant and efficacious, effects by DMSO and cAMP revealed a probable role for TNFα in the pathway leading to MMP-9 secretion.

MATERIALS AND METHODS

Cellular assays

THP-1 monocytic cells were obtained from ATCC (Manassas, VA, United States) and maintained in RPMI-1640 culture medium (HyClone, Logan, UT, United States) containing 2 mmol/L L-glutamine, 25 mmol/L HEPES, 1.5 g/L sodium bicarbonate, 10% fetal bovine serum (HyClone), 50 U/mL penicillin, 50 mg/mL streptomycin (HyClone), and 50 mmol/L β-mercaptoethanol at 37 °C in 5% CO_2. For cell assays, THP-1 cells were centrifuged at 500 g for 10 min, washed, and resuspended in reduced fetal calf serum (2%) growth medium. Cell concentrations were adjusted to 1.0×10^6 cells/mL and 0.3 mL (3.0×10^5 cells) was added to individual wells of a 48-well sterile culture plate. For experiments evaluating the regulatory effect of DMSO on LPS-stimulated MMP-9 and TNFα secretion, suspension THP-1 monocyte cells were treated with DMSO (Thermo Fisher Scientific) at concentrations ranging from 0.016%-2% for 5 min prior to subsequent treatment with 1 μg/mL *Escherichia coli* bacterial 026.B6 LPS (Sigma-Aldrich, St. Louis). Cells were incubated for 72 h at 37 °C in 5%

CO_2. DMSO concentrations were varied from 0.016%-2% to evaluate the effect on cell response and specific concentrations for each experiment is detailed in each figure legend. Control cell treatments without LPS but in the presence of DMSO were done for all studies. For experiments assessing the regulatory role of cAMP on LPS-stimulated MMP-9 and TNFα secretion, THP-1 monocytes were treated with cAMP regulators dibutyryl 3',5'-cyclic adenosine monophosphate (Bt_2-cAMP), forskolin (Fsk), and 3-isobutyl-1-methylxanthine (IBMX) for 5 min prior to subsequent treatment with 1 μg/mL LPS. Cells were incubated for 6-72 h at 37 °C in 5% CO_2. In one experiment, cells were stimulated with 0.3 μg/mL LPS. Concentrations or concentration ranges used for the regulators were 0.3 mmol/L for Bt_2-cAMP, 0.01-0.1 mmol/L for Fsk, and 0.01-0.3 mmol/L for IBMX. Control cell treatments without LPS but in the presence of cAMP regulators were done for all studies. For experiments assessing the effect of an anti-human TNFα monoclonal antibody (MAB610, R&D Systems, Minneapolis, MN, United States) on LPS-stimulated MMP-9 and TNFα secretion, THP-1 monocytes were treated with the antibody for 5 min prior to subsequent treatment with 1 μg/mL LPS. Cells were incubated for 48 h at 37 °C in 5% CO_2. Control cell treatments with or without LPS and/or in the presence or absence of the antibody, were done for these studies. After all treatments, cells were removed from each well, centrifuged at 2500 g for 10 min, and the supernatant was collected and frozen at −20 °C for subsequent analysis.

Western blot

Supernatants from individual wells of treated THP-1 monocytes were mixed 1:1 with Laemmli SDS sample buffer (Bio-Rad) containing 5% β-mercaptoethanol. Samples and 10 μL recombinant human MMP-9 western blotting standard (WBC018, R&D Systems, Minneapolis, MN, United States) were heated at 95 °C for 5 min and separated on 7.5% Tris-HCl gels (Ready Gel, Bio-Rad) under denaturing conditions (25 mmol/L Tris, 192 mmol/L glycine, 0.1% SDS at pH 8.3) using a Mini Protean 3 Cell (Bio-Rad). Pre-stained SDS-PAGE standards (BioRad) were loaded directly into gel wells. Gels were transferred to Immobilon-P PVDF membrane (Millipore) in transfer buffer containing 25 mmol/L Tris base, 192 mmol/L glycine, and 10% methanol at pH 8.3 using a Tank VEP-2 electroblotting system (Owl Separation Systems). Following protein transfer, the membrane was blocked for 1 h at 25 °C with PBS containing 0.2% Tween 20 (PBST) supplemented with 5% nonfat dry milk, and probed for 1 h at 25 °C with 1 μg/mL goat anti-human MMP-9 antibody (AB911, R&D Systems) in PBST containing 1% milk. After washing membrane 3× in PBST, the membrane was incubated with a 1:1000 dilution of goat anti-mouse IgG-HRP secondary antibody (R&D systems) in PBST containing 1% milk for 1 h at 25 °C. Protein detection was accomplished using ECL Western Blotting Substrate reagents (Pierce) and exposure to film (Kodak). Densitometry of immunoblot TIFF images was done using ImageJ 1.52a.

Enzyme-linked immuno sorbent assay

Measurement of secreted TNFα in the supernatants was determined by enzyme-linked immuno sorbent assay (ELISA). Briefly, 0.1 mL of 4 mg/mL monoclonal anti-human TNFα capture antibody (MAB610, R&D Systems) was added to 96-well plates for overnight incubation at room temperature. Wells were washed with PBS (HyClone) containing 0.05% Tween-20 and blocked with 0.3 mL PBS containing 1% bull serum albumin (BSA), 5% sucrose and 0.05% NaN_3 for 1 h at room temperature. After washing, successive additions of 0.05 mL samples or standards (2 h), 0.1 mL biotinylated polyclonal anti-human TNFα detection antibody (BAF210, R&D Systems) in 20 mmol/L Tris with 150 mmol/L NaCl and 0.1% BSA (2 h), 0.1 mL streptavidin-HRP (R&D Systems) diluted 200 times with PBS containing 1% BSA (20 min), and 0.1 mL of equal volumes of 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide (KPL, Gaithersburg, MD, United States) (30 min). The reaction was stopped by the addition of 1% H_2SO_4 solution. The optical density of each sample was analyzed at 450 nm with a reference reading at 630 nm using a SpectraMax 340 absorbance platereader (Molecular Devices, Union City, CA, United States). A standard curve was constructed by sequential dilution of a TNFα standard from 2000-15 pg/mL. The concentration of TNFα in the experimental samples was calculated from the standard curve

Preparation of Aβ

Amyloid-β peptide (Aβ42) (rPeptide, Bogarth, GA, United States) was dissolved in 100% hexafluoroisopropanol (Sigma) for 1 h, aliquotted into sterile microcentrifuge tubes, dried in a vacuum centrifuge, and stored at -20 °C. Prior to cell treatment the lyophilized peptides were resuspended in sterile water to 100 μmol/L peptide

concentration and incubated at 4 °C for 24 h. Cells were exposed to a final concentration of 17 μmol/L Aβ42 for selected times. For experiments conducted on Aβ42-stimulated MMP-9 and TNFα secretion, THP-1 monocytes were treated with Aβ42 at a final concentration of 17 μmol/L, and incubated for 6, 24, or 48 h at 37 °C in 5% CO_2. Control cell treatments without Aβ42 were done with the same volume of sterile water.

Statistical analysis

Two-tailed paired Student *t*-tests were performed for experiments when applicable to determine the confidence limit at which two measurements were statistically different. Analysis was applied to each data set and *P* values less than 0.05 were considered significant and are designated in the Figures with a superscript a ([a]) for ($P < 0.05$) or superscript b ([b]) for ($P < 0.01$).

RESULTS

In the course of investigating MMP-9 secretion by THP-1 human monocytes after exposure to LPS, it was observed that DMSO had an inhibitory effect on this process. Western blot analysis showed that MMP-9 secretion was sensitive to LPS concentration, in that 1 μg/mL LPS triggered greater MMP-9 protein secretion compared to 0.1 μg/mL LPS (Figure 1A). The inclusion of 2% DMSO almost completely abolished the release of MMP-9 from THP-1 cells after stimulation with LPS at either concentration (Figure 1A). Densitometry analysis of the immunoblot is provided in Figure 1B. TNFα is a classical proinflammatory cytokine and LPS-induced TNFα secretion in immune cells has been well-studied. Surprisingly, this process was not inhibited by DMSO. The same samples in Figure 1A were analyzed for TNFα levels by ELISA. As expected, 1 μg/mL LPS triggered greater TNFα secretion compared to 0.1 μg/mL in THP-1 cells (Figure 1C). 2% DMSO did not diminish the LPS-induced TNFα response indicating a MMP-9-selective effect by DMSO.

The observation that DMSO inhibited MMP-9 secretion in THP-1 monocytes prompted additional investigation of concentration dependence. THP-1 cells were exposed to increasing concentrations of DMSO prior to stimulation with 1 μg/mL LPS. MMP-9 secretion, measured by immunoblot, was slightly diminished at 0.2% DMSO and almost completely inhibited at 1% DMSO (Figure 2A). Densitometry analysis of the Figure 2A immunoblot allowed non-linear curve fitting of the dose-dependent DMSO effect on secreted MMP-9. The inhibition curve fit yielded an IC_{50} value of 0.64% DMSO (Figure 2B). Analysis of the same LPS/DMSO samples for TNFα levels again showed no inhibitory effect of DMSO on LPS-stimulated TNFα secretion (Figure 2C).

Addition of Bt_2-cAMP, a cell-permeable cAMP analog that mimics the activity of cAMP[12], to THP-1 monocytes prior to LPS treatment resulted in an inhibition of MMP-9 secretion (40%), but only at the later cell-exposure time points (Figure 3A and B). The effect of Bt_2-cAMP on TNFα was very different in that TNFα secretion was significantly inhibited (> 77%) at earlier and later time points. Fsk, an adenylyl cyclase activator that can also raise intracellular cAMP levels[13], was tested for its ability to alter LPS-induced MMP-9 and TNFα secretion. Due to the hydrophobic nature of Fsk, DMSO was used as a vehicle in this 72 h LPS cell treatment experiment. The DMSO concentration was carefully determined and controlled to fully ascertain the effect of Fsk. DMSO did not inhibit LPS-induced MMP-9 secretion at 0.016% and 0.048%, but 0.16% DMSO did inhibit 50% of the control LPS monocyte treatment (Figure 4A and B). Fsk potently inhibited MMP-9 secretion far in excess of any DMSO inhibition that was observed at 30 μmol/L and 100 μmol/L, but not 10 μmol/L, Fsk (Figure 4A and B). As in the earlier data, DMSO did not inhibit LPS-induced TNFα secretion (Figure 4C). However, Fsk dose-dependently inhibited TNFα secretion, with 39% inhibition at 10 μmol/L, 54% inhibition at 30 μmol/L, and 66% inhibition at 100 μmol/L (Figure 4C). The Fsk inhibition values were determined relative to the corresponding DMSO-treated THP-1 monocytes. Fsk also inhibited LPS-induced TNFα secretion at an early time point (6 h), with 57% and 47% inhibition observed at 30 μmol/L and 100 μmol/L respectively (data not shown).

In order to increase intracellular cAMP levels by a different pathway, the cell-permeable general cAMP phosphodiesterase (PDE) inhibitor IBMX was tested. Treatment of THP-1 monocytes with a concentration range of IBMX prior to application of LPS produced a dose-dependent inhibition of MMP-9 secretion (Figure 5A). Quantitation of the immunoblot by densitometry yielded an inhibition

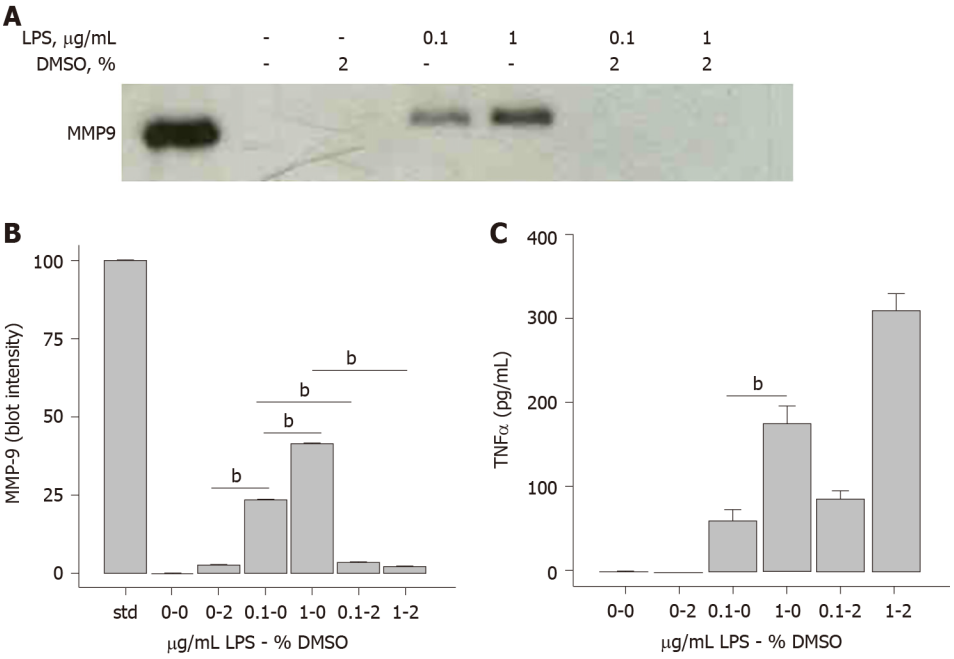

Figure 1 Dimethyl sulfoxide inhibits lipopolysaccharide-induced matrix-metalloproteinase-9, but not tumor necrosis factor α, secretion. THP-1 monocytes were treated in the absence or presence of dimethyl sulfoxide (2%) and with either sterile water or lipopolysaccharide (0.1 μg/mL or 1 μg/mL) for 72 h. A: Cells were collected, centrifuged at 500 g for 10 min and supernatant assessed for matrix-metalloproteinase-9 (MMP-9), SDS-PAGE/immunoblot of cell supernatants. Lane 1 is an MMP-9 Western blotting standard (10 μL); B: Densitometry of Panel A. Each data bar is the average ± standard error for three independent densitometry measurements; and C: TNFα levels were determined in cell supernatants by enzyme-linked immuno sorbent assay and each data bar is the average ± std error for three independent measurements, Tumor necrosis factor α (TNFα). Statistical differences ($^{a}P < 0.05$ or $^{b}P < 0.01$) for 0.1 μg/mL or 1 μg/mL lipopolysaccharide and dimethyl sulfoxide inhibition of MMP-9 or TNFα secretion are shown. DMSO: Dimethyl sulfoxide; LPS: Lipopolysaccharide; MMP-9: Matrix-metalloproteinase-9; TNFα: Tumor necrosis factor α.

plot that was then analyzed by nonlinear curve fitting. The fit produced an IC_{50} value of 0.05 mmol/L for IBMX inhibition of MMP-9 secretion (Figure 5B, circles). The effect of IBMX on TNFα secretion was also assessed in the same supernatant samples by ELISA. A very similar dose-dependent inhibition was observed for TNFα as for MMP-9 with an IC_{50} value of 0.08 mmol/L (Figure 5B, diamonds). The temporal and connected relationship between LPS-induced secretion of TNFα and MMP-9 strongly suggested that TNFα played a contributory role in MMP-9 secretion. This idea was tested by the inclusion of an anti-TNFα antibody prior to LPS treatment of THP-1 monocytes. The presence of the TNFα antibody reduced the secretion of MMP-9 when analyzed by immunoblot (Figure 6A). Quantitative analysis by densitometry showed 47% inhibition of MMP-9 by blocking TNFα (Figure 6B, left plot). As expected, the presence of the TNFα antibody significantly reduced TNFα levels in the cell supernatant (Figure 6B, right plot).

In an effort to examine MMP-9 and TNFα secretion by a cell-stimulator other than LPS, the ability of Aβ42 oligomers to trigger THP-1 monocyte MMP-9 secretion was examined. Hexafluoroisopropanol-treated and vacuum-dried Aβ42 was reconstituted in sterile H_2O and incubated for 24 h at 4 °C to allow oligomer formation. THP-1 cells exposed to the Aβ42 oligomers began to secrete MMP-9 at 24 h of treatment, with a much larger level by 48 h (Figure 7A). Comparison of the immunoblot densitometry quantitation and ELISA measurement of secreted TNFα indicated that maximum TNFα secretion occurred prior to MMP-9 secretion (Figure 7B). The temporal relationship between to Aβ42 oligomer-induced TNFα secretion and MMP-9 secretion was similar to that observed by LPS. Significant analysis was performed to rule out any LPS contamination within the Aβ42 samples and delineate differences between LPS and Aβ42 stimulation of monocytes. These analytical measures were described in a previous publication[14]. The two pathogens, one exogenous (LPS) and one endogenous (Aβ), both trigger pathways leading to MMP-9 secretion.

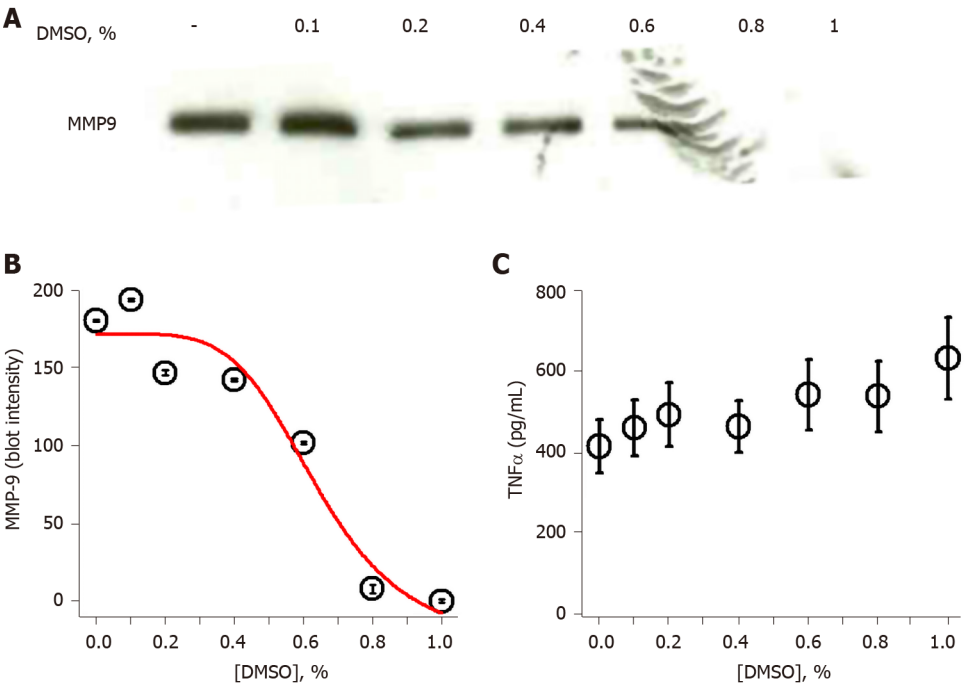

Figure 2 Dimethyl sulfoxide inhibits lipopolysaccharide-induced matrix-metalloproteinase-9 in a dose-dependent fashion. A: SDS-PAGE/immunoblot of cell supernatants; B: Densitometry of Panel A. Each data bar is the average ± standard error for three independent densitometry measurements. Data were fit to a nonlinear inhibition equation, which produced an IC_{50} of 0.64%; and C: Tumor necrosis factor α levels were determined in cell supernatants by enzyme-linked immuno sorbent assay and each data bar is the average ± standard error for three independent measurements. THP-1 monocytes were treated with a concentration range of dimethyl sulfoxide (0.1%-1%) followed by lipopolysaccharide (1 μg/mL) for 72 h. Cells were collected, centrifuged at 500 g for 10 min and supernatant assessed for (A) matrix-metalloproteinase-9 and (C) tumor necrosis factor α. DMSO: Dimethyl sulfoxide; MMP-9: Matrix-metalloproteinase-9; TNFα: Tumor necrosis factor α.

DISCUSSION

LPS triggers Toll-like receptor 4 (TLR4) activation and downstream signaling pathways that include myeloid differentiation protein 88 and NF-kB-mediated transcription and expression of innate immune genes such as TNFα and IL-1β[15-17]. LPS also activates extracellular signal-regulated kinases 1/2, mitogen-activated kinase p38, mitochondrial reactive oxygen species, and MMP-9 expression[6,18].

DMSO has long been recognized to have anti-inflammatory and analgesic properties[19]. Although controversial, it has been used in a variety of formulations for the treatment of interstitial cystitis, cutaneous scleroderma, and wound healing, with an additional potential as an anti-viral[19]. Furthermore, as a polar, aprotic solvent miscible with water, DMSO is widely used as a vehicle for hydrophobic compounds in cellular assays. Here we show that application of low concentrations (0.2%-0.8%) of DMSO to human monocytes inhibited the secretion of MMP-9. The mechanism by which the inhibition occurred was not a generalized process as DMSO did not inhibit secretion of TNFα, another inflammatory product and mediator. Cyclic AMP, on the other hand, inhibited both TNFα and MMP-9 secretion. The manner in which cAMP was generated in the monocytes did not influence the inhibitory outcome, whether by direct application of a cell-permeable cAMP analog, cAMP synthesis by activation of adenylyl cyclase, or boosting cAMP levels by inhibition of PDE.

Our findings indicated that DMSO concentration should be restricted to 0.4% or below to avoid inadvertent inhibition of MMP-9 secretion. Furthermore, there are specific distinctions in the mechanistic pathways by which MMP-9 and TNFα are secreted, and the inhibitory effect of DMSO on MMP-9 secretion reveals one or more areas of these mechanistic distinctions. Our DMSO inhibitory observations and effective concentration were consistent with other studies. Majtan and Majtan[9] previously demonstrated DMSO inhibition of MMP-9 transcription and synthesis in TNFα-treated human keratinocytes[9]. We expanded our investigation by stimulating monocytes further upstream with LPS and comparing both DMSO and cAMP regulation of TNFα and MMP-9 secretion. The findings showed that DMSO did not alter LPS-induced TNFα levels indicating a distinctive effect for MMP-9. Previous studies have shown DMSO inhibition of MMP-2 in human aorta endothelial cells at

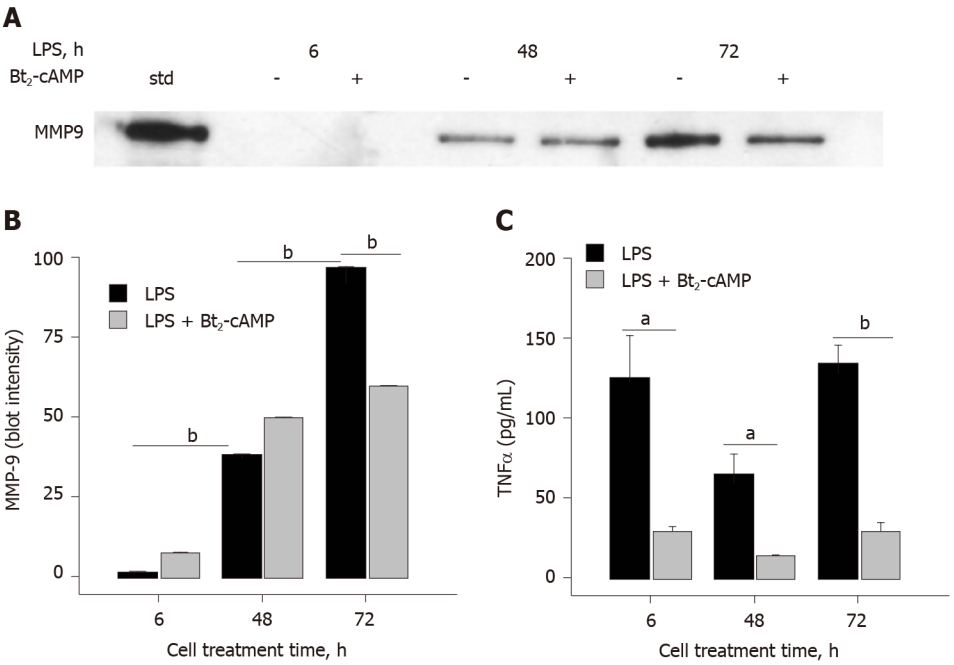

Figure 3 Cyclic adenosine monophosphate inhibits lipopolysaccharide-induced matrix-metalloproteinase-9 and tumor necrosis factor α secretion. A: SDS-PAGE/immunoblot of cell supernatants. Lane 1 is a matrix-metalloproteinase-9 (MMP-9) standard; B: Densitometry of Panel A. Each data bar is the average ± standard error for three independent densitometry measurements; and C: Tumor necrosis factor α (TNFα) levels were determined in cell supernatants by enzyme-linked immuno sorbent assay and each data bar is the average ± standard error for three independent measurements. THP-1 monocytes were treated with 3',5'-cyclic adenosine monophosphate (300 μmol/L) followed by lipopolysaccharide (0.03 μg/mL) for 6, 48, and 72 h. Cells were collected, centrifuged at 500 g for 10 min and supernatant assessed for (A) MMP-9 and (C) TNFα. Statistical differences ($^{a}P < 0.05$ or $^{b}P < 0.01$) between MMP-9 secretion at different lipopolysaccharide treatment times and 3',5'-cyclic adenosine monophosphate inhibition of either MMP-9 or TNFα secretion are shown. LPS: Lipopolysaccharide; MMP-9: Matrix-metalloproteinase-9; TNFα: Tumor necrosis factor α; Bt_2-cAMP: 3',5'-cyclic adenosine monophosphate.

slightly higher concentrations of 1%-3%[20]. For other inflammatory molecules, Elisia *et al*[11] found that 0.5%-2% DMSO inhibited expression of numerous cytokines in bacterial-stimulated whole blood samples and in the joints of arthritic mice[11]. The findings on TNFα were mixed, with some inhibition observed at 2% DMSO in whole blood, but no inhibition in the arthritic mouse paws. Hollebeeck *et al*[10] reported DMSO inhibition of the intestinal Caco-2 cell inflammatory response in the range of 0.1%-0.5%. The cells were stimulated with IL-1β or a proinflammatory cocktail consisting of IL-1β, TNFα, interferon-γ and LPS. Differential effects by DMSO were observed, whereby DMSO inhibited transcription of IL-6, IL-1α, IL-1β, and cyclooxygenase-2, but not IL-8 or TNFα.

Cyclic-AMP is a ubiquitous second messenger molecule that can modulate a number of processes by direct binding to molecular targets or by activation of cAMP-dependent protein kinase (PKA) and subsequent downstream phosphorylation. Adenylyl cyclase and PDE tightly control cAMP levels by regulating synthesis and degradation respectively. Cyclic AMP is well-recognized as an inflammation modifier in a variety of cell types and an important therapeutic target (reviewed in[21,22]). Attenuation of LPS-induced TNFα production by agents that raise cAMP levels was specifically demonstrated in monocyte/macrophage cells[15,23]. Furthermore, elevation of cAMP levels in neutrophils inhibited MMP-9 secretion and implicated cAMP-dependent PKA in the process[7].

The mechanism by which cAMP regulates cytokine pathways has been widely researched and there are multiple points at which this occurs. Two of these areas in monocytes/macrophages include the orphan nuclear receptor Nr4a1 (Nur77) and the nuclear factor κB transcriptional complex. Elevated cAMP levels induce Nur77 expression leading to repression of numerous inflammatory genes reviewed in[24]. Either incomplete or excessive phosphorylation of cAMP response element binding protein can suppress TNFα transcription[25]. PKA can also regulate cAMP response element binding protein/deoxyribonucleic acid binding in ganglioside and plasminogen-induced microglial cytokine production[26]. It has been proposed for some time that cAMP has a role as an inflammation gate-keeper[23]. Our results were consistent with previous findings and showed that increased cAMP levels attenuated LPS-induced MMP-9 and TNFα secretion.

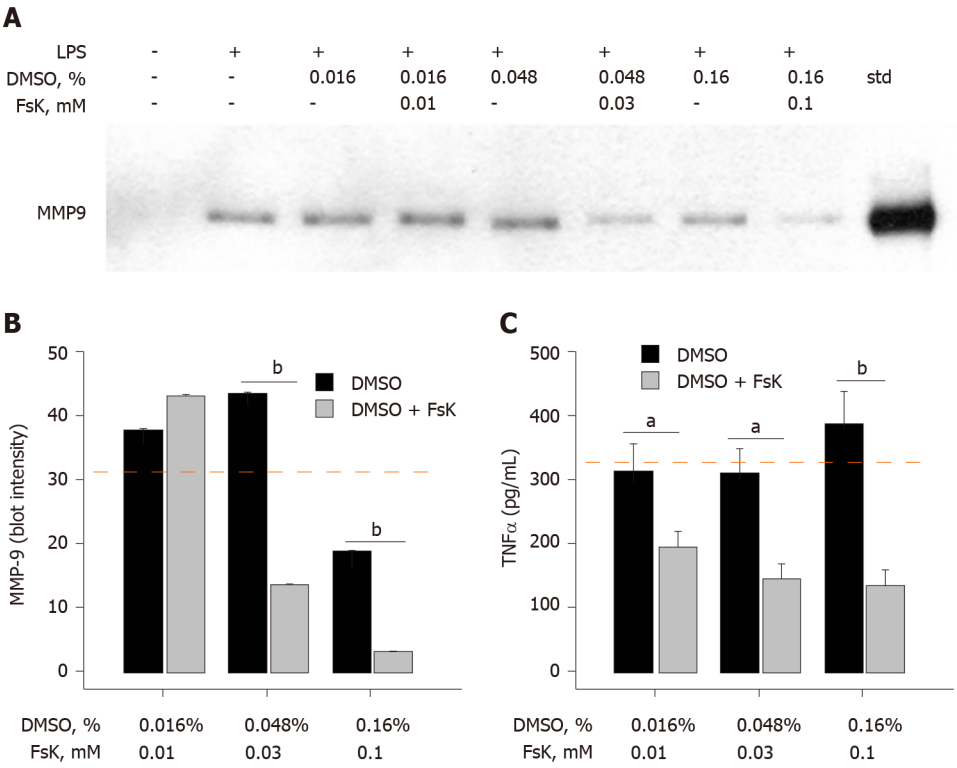

Figure 4 Activation of adenylyl cyclase inhibits lipopolysaccharide-induced matrix-metalloproteinase-9 and tumor necrosis factor α secretion. A: SDS-PAGE/immunoblot of cell supernatants. Lane 9 is a matrix-metalloproteinase-9 standard; B: Densitometry of Panel A. Each data bar is the average ± standard error for three independent densitometry measurements; and C: Tumor necrosis factor α (TNFα) levels were determined in cell supernatants by enzyme-linked immuno sorbent assay and each data bar is the average ± standard error for three independent measurements. THP-1 monocytes were treated with three forskolin (Fsk) concentrations that contained different percentages of dimethyl sulfoxide (DMSO), followed by lipopolysaccharide (1 μg/mL) for 72 h. Cells were collected, centrifuged at 500 g for 10 min and supernatant assessed for matrix-metalloproteinase-9 and TNFα. The red dashed lines in Panels B and C represent TNFα secretion induced by lipopolysaccharide (1 μg/mL) in the absence of DMSO or Fsk. Statistical differences ($^aP < 0.05$ or $^bP < 0.01$) between DMSO and DMSO + Fsk are shown. LPS: Lipopolysaccharide; DMSO: Dimethyl sulfoxide; MMP-9: Matrix-metalloproteinase-9; TNFα: Tumor necrosis factor α; Fsk: Forskolin.

Several aspects of our data indicated that MMP-9 secretion was a distinct process from TNFα secretion and occurred downstream. First, DMSO inhibited MMP-9, but not TNFα, suggesting that the MMP-9 secretion process was selectively altered. Second, cAMP inhibited both MMP-9 and TNFα with a similar potency, but at different monocyte cell exposure time points. The pattern of cAMP inhibition for these two molecules suggested that MMP-9 secretion lies downstream of TNFα and that TNFα may a key component of the pathway leading to MMP-9 secretion. This temporal relationship fit a model whereby early TNFα secretion directly led to later MMP-9 secretion. Lastly, antibody-blocking of TNFα diminished MMP-9 secretion, suggesting a direct link between TNFα secretion and MMP-9 secretion.

Bacterial LPS stimulation of human monocytic cells has been well-studied and evokes a classical innate immune response to exogenous pathogens *via* TLR4. It is now widely-recognized that endogenous pathogens, such as Aβ in Alzheimer's disease, can trigger similar pathways *via* TLR4 and other TLRs[14,27-29]. Stimulation of MMP-9 secretion by Aβ42 has been shown previously in THP-1 monocytes[30], murine microglia[31], rat astrocytes[32], and human retinal pigment epithelial cells[33]. Furthermore, MMP-9 levels are significantly elevated in plasma from Alzheimer's disease patients[34] and in 5xFAD/MBP$^{-/-}$ transgenic mice[35]. Our findings show that Aβ42 oligomer-induced MMP-9 secretion appears to follow the same temporal pathway as LPS in that significant MMP-9 levels were not observed until later cell exposure time points and well after TNFα release. The LPS and Aβ42 results fit a mechanism whereby induced TNFα secretion and subsequent autocrine stimulation of TNF receptor type 1 leads to MMP-9 production and secretion[36].

CONCLUSION

The cumulative data indicated that MMP-9 secretion was a distinct process from TNFα

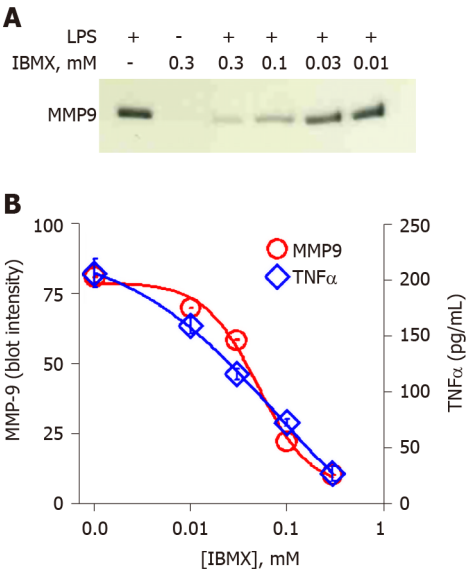

Figure 5 Inhibition of cyclic adenosine monophosphate phosphodiesterase inhibits lipopolysaccharide-induced matrix-metalloproteinase-9 and tumor necrosis factor α in a dose-dependent fashion. A: SDS-PAGE/WB of cell supernatants; and B: Densitometry of the matrix-metalloproteinase-9 (MMP-9) immunoblot in Panel A (circles). Each data point is the average ± standard error for three independent densitometry measurements. Tumor necrosis factor α (TNFα) levels (diamonds) were determined in cell supernatants by enzyme-linked immuno sorbent assay and each data point is the average ± standard error for three independent measurements. THP-1 monocytes were treated with a concentration range of 3-isobutyl-1-methylxanthine (0.01-0.3 mmol/L) followed by lipopolysaccharide (1 μg/mL) for 48 h. Cells were collected, centrifuged at 500 g for 10 min and supernatant assessed for (A) MMP-9 and (B) TNFα. MMP-9 and TNFα data points were fit to a nonlinear inhibition equation, which produced IC_{50} values of 0.05 mmol/L and 0.08 mmol/L for MMP-9 and TNFα inhibition respectively. LPS: Lipopolysaccharide; MMP-9: Matrix-metalloproteinase-9; TNFα: Tumor necrosis factor α; IBMX: 3-Isobutyl-1-methylxanthine.

secretion and occurred downstream. First, DMSO inhibited MMP-9, but not TNFα, suggesting that the MMP-9 secretion process was selectively altered. Second, cAMP inhibited both MMP-9 and TNFα with a similar potency, but at different monocyte cell exposure time points. The pattern of cAMP inhibition for these two molecules suggested that MMP-9 secretion lies downstream of TNFα and that TNFα may a key component of the pathway leading to MMP-9 secretion. This temporal relationship fit a model whereby early TNFα secretion directly led to later MMP-9 secretion. Lastly, antibody-blocking of TNFα diminished MMP-9 secretion, suggesting a direct link between TNFα secretion and MMP-9 secretion.

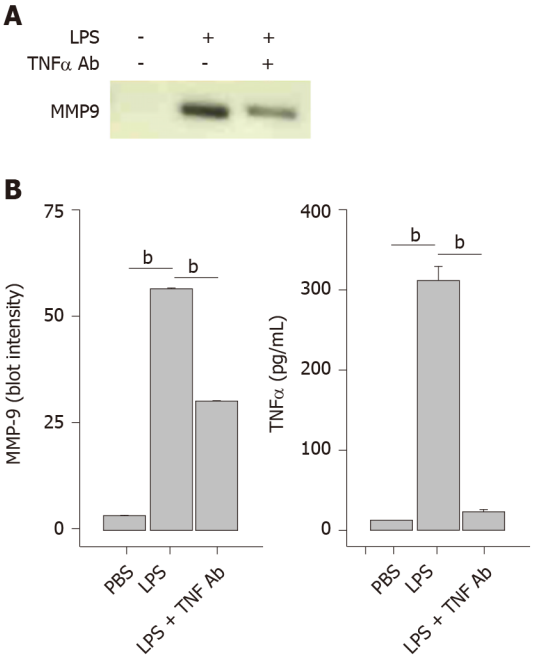

Figure 6 Antif- tumor necrosis factor α antibody blocks lipopolysaccharide-induced matrix-metalloproteinase-9 secretion. THP-1 monocytes were treated with a tumor necrosis factor α (TNFα) antibody (1 μg/mL) followed by lipopolysaccharide (1 μg/mL) for 48 h. Cells were collected, centrifuged at 500 *g* for 10 min and supernatant assessed for matrix-metalloproteinase-9 and TNFα. A: SDS-PAGE/WB of cell supernatants; and B: Densitometry of Panel A (left plot). Each data bar is the average ± standard error for three independent densitometry measurements. TNFα levels (right plot) were determined in cell supernatants by enzyme-linked immuno sorbent assay and each data bar is the average ± standard error for three independent measurements. Statistical differences ($^{a}P < 0.05$ or $^{b}P < 0.01$) between lipopolysaccharide-stimulated matrix-metalloproteinase-9 or TNFα secretion compared to PBS buffer control and in the absence or presence of an anti-TNFα antibody are shown. LPS: Lipopolysaccharide; MMP-9: Matrix-metalloproteinase-9; TNFα: Tumor necrosis factor α.

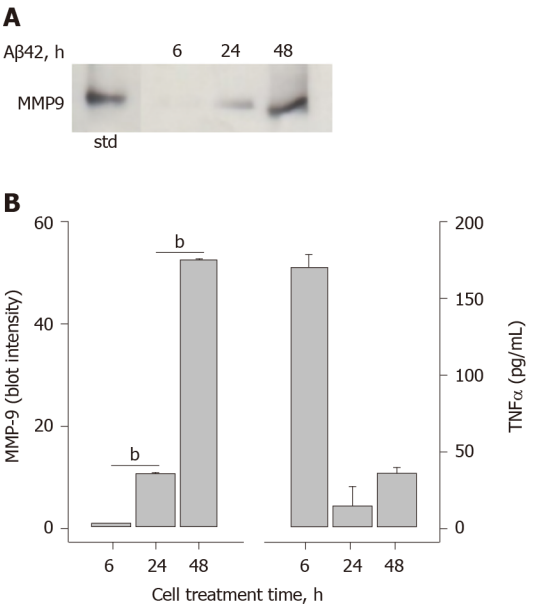

Figure 7 Amyloid-β peptide-induced matrix-metalloproteinase-9 secretion occurs much later than tumor necrosis factor α secretion. THP-1 monocytes were treated with amyloid-β peptide (17 μmol/L) for 6, 24 and 48 h. Cells were collected, centrifuged at 500 *g* for 10 min and supernatant assessed for matrix-metalloproteinase-9 (MMP-9) and tumor necrosis factor α (TNFα). A: SDS-PAGE/WB of cell supernatants. Lane 1 is an MMP-9 standard; and B: Densitometry of Panel A (left plot). Each data bar is the average ± standard error for three independent densitometry measurements. Enzyme-linked immuno sorbent assay determination of TNFα levels in cell supernatants (right plot). Each data bar is the average ± standard error for three independent measurements. Statistical differences ($^{a}P < 0.05$ or $^{b}P < 0.01$) between MMP-9 or TNFα secretion at different amyloid-β peptide treatment times are shown with an asterisk. Aβ42: Amyloid-β peptide; MMP-9: Matrix-metalloproteinase-9; TNFα: Tumor necrosis factor α.

ARTICLE HIGHLIGHTS

Research background

Matrix metalloproteinases (MMPs), including MMP-9, are an integral part of the immune response and are upregulated in response to a variety of stimuli. New details continue to emerge concerning the mechanistic and regulatory pathways that mediate MMP-9 secretion. There is significant evidence for regulation of inflammation by dimethyl sulfoxide (DMSO) and 3',5'-cyclic adenosine monophosphate (cAMP), thus investigation of how these two molecules may regulate both MMP-9 and tumor necrosis factor α (TNFα) secretion by human monocytes was of high interest. The hypothesis tested in this study was that DMSO and cAMP regulate MMP-9 and TNFα secretion by distinct mechanisms.

Research motivation

The objective of this study was further examine temporal and regulatory mechanisms of MMP-9 secretion in THP-1 human monocytes after stimulation with lipopolysaccharide (LPS). Specifically, dose-dependent regulation of MMP-9 and TNFα by the aprotic solvent DMSO and the intracellular signaling molecule cAMP.

Research objectives

The objective of this study was further examine temporal and regulatory mechanisms of MMP-9 secretion in THP-1 human monocytes after stimulation with LPS. Specifically, dose-dependent regulation of MMP-9 and TNFα by the aprotic solvent DMSO and the intracellular signaling molecule cAMP.

Research methods

The paper describes a basic research study using THP-1 human monocyte cells. All experiments were conducted at the University of Missouri-St. Louis in the Department of Chemistry and Biochemistry. Human monocyte cells were grown, cultured, and prepared for experiments in the University of Missouri-St. Louis Cell Culture Facility as per accepted guidelines. Cells were treated with LPS for selected exposure times and the conditioned medium was collected for analysis of MMP-9 and TNFα production. Inhibitors including DMSO, cAMP regulators, and anti-TNFα antibody were added to the cells prior to LPS treatment. MMP-9 secretion was analyzed by gel electrophoresis/western blot and quantitated by ImageJ software. TNFα secretion was analyzed by enzyme-linked immuno sorbent assay. All data is presented as the average and standard error for at least 3 trials. Statistical analysis was done using a two-tailed paired Student *t*-test *P* values less than 0.05 were considered significant and designated as such with an asterisk in the figures ($P < 0.05$). LPS and cAMP regulators were from Sigma-Aldrich, MMP-9 standard and antibody and TNFα antibodies were from R&D Systems, and amyloid-β peptide was from rPeptide.

Research results

In our investigation of MMP-9 secretion from THP-1 human monocytes, we made the following findings. Inclusion of DMSO in the cell treatment inhibited LPS-induced MMP-9, but not TNFα, secretion. Inclusion of DMSO in the cell treatment at different concentrations inhibited LPS-induced MMP-9 secretion in a dose-dependent fashion. A cell-permeable cAMP analog, dibutyryl cAMP, inhibited both LPS-induced MMP-9 and TNFα secretion. Pretreatment of the cells with the adenylyl cyclase activator forskolin inhibited LPS-induced MMP-9 and TNFα secretion. Pretreatment of the cells with the general cAMP phosphodiesterase inhibitor reduced LPS-induced MMP-9 and TNFα in a dose-dependent fashion. Pre-treatment of monocytes with an anti-TNFα antibody blocked LPS-induced MMP-9 and TNFα secretion. Amyloid-β peptide-induced MMP-9 secretion and occurred much later than TNFα secretion. The latter two findings strongly suggested an upstream role for TNFα in mediating LPS-stimulate MMP-9 secretion.

Research conclusions

The cumulative data indicated that MMP-9 secretion was a distinct process from TNFα secretion and occurred downstream. First, DMSO inhibited MMP-9, but not TNFα, suggesting that the MMP-9 secretion process was selectively altered. Second, cAMP inhibited both MMP-9 and TNFα with a similar potency, but at different monocyte cell exposure time points. The pattern of cAMP inhibition for these two molecules suggested that MMP-9 secretion lies downstream of TNFα and that TNFα may a key

component of the pathway leading to MMP-9 secretion. This temporal relationship fit a model whereby early TNFα secretion directly led to later MMP-9 secretion. Lastly, antibody-blocking of TNFα diminished MMP-9 secretion, suggesting a direct link between TNFα secretion and MMP-9 secretion.

Research perspectives

Regulation of MMP-9 is a significant important in many disease processes including arthritis and Alzheimer's disease. Further understanding of the pathways leading to MMP-9 secretion and regulation of this process will be important for managing human health.

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ISSN 1949-8454 (online)

World Journal of *Biological Chemistry*

World J Biol Chem 2021 March 27; 12(2): 15-37

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NAME OF JOURNAL
World Journal of Biological Chemistry

ISSN
ISSN 1949-8454 (online)

LAUNCH DATE
July 26, 2010

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Vsevolod Gurevich, Chun-Peng Wan

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1949-8454/editorialboard.htm

PUBLICATION DATE
March 27, 2021

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World J Biol Chem 2021 March 27; 12(2): 15-37

DOI: 10.4331/wjbc.v12.i2.15

ISSN 1949-8454 (online)

REVIEW

Understanding the function of the tumor microenvironment, and compounds from marine organisms for breast cancer therapy

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Author contributions: All authors design, collect, wrote, and edit the paper.

Conflict-of-interest statement: None.

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Manuscript source: Invited manuscript

Specialty type: Biochemistry and molecular biology

Country/Territory of origin: United States

Peer-review report's scientific

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Abstract

The pathology and physiology of breast cancer (BC), including metastasis, and drug resistance, is driven by multiple signaling pathways in the tumor microenvironment (TME), which hamper antitumor immunity. Recently, long non-coding RNAs have been reported to mediate pathophysiological developments such as metastasis as well as immune suppression within the TME. Given the complex biology of BC, novel personalized therapeutic strategies that address its diverse pathophysiologies are needed to improve clinical outcomes. In this review, we describe the advances in the biology of breast neoplasia, including cellular and molecular biology, heterogeneity, and TME. We review the role of novel molecules such as long non-coding RNAs in the pathophysiology of BC. Finally, we provide an up-to-date overview of anticancer compounds extracted from marine microorganisms, crustaceans, and fishes and their synergistic effects in combination with other anticancer drugs. Marine compounds are a new discipline of research in BC and offer a wide range of anti-cancer effects that could be harnessed to target the various pathways involved in BC development, thus assisting current therapeutic regimens.

Key Words: Breast cancer; Tumor microenvironment; Long non-coding RNAs; Growth; Metastasis; Angiogenesis; Marine compounds

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quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: December 23, 2020
Peer-review started: December 23, 2020
First decision: January 11, 2021
Revised: January 13, 2021
Accepted: February 20, 2021
Article in press: February 20, 2021
Published online: March 27, 2021

P-Reviewer: Gazouli M
S-Editor: Gao CC
L-Editor: A
P-Editor: Yuan YY

Core Tip: Breast cancer (BC) is an aggressive and heterogenous disease. The BC tumor microenvironment contributes to immune evasion and chemoresistance in BC. Long non-coding RNAs contribute to BC pathophysiology and are potential BC biomarkers. Marine compounds display promising anticancer activity against BC. The use of novel bioengineering tools will enormously help in improving their production and clinical development for the treatment of BC and other cancers.

Citation: Malla RR, Farran B, Nagaraju GP. Understanding the function of the tumor microenvironment, and compounds from marine organisms for breast cancer therapy. *World J Biol Chem* 2021; 12(2): 15-37
URL: https://www.wjgnet.com/1949-8454/full/v12/i2/15.htm
DOI: https://dx.doi.org/10.4331/wjbc.v12.i2.15

INTRODUCTION

According to GLOBOCAN (2018), 2.1 million females were diagnosed with breast cancer (BC) in 2018, accounting for 11.6 % of total cancer patients. As per this report, BC accounts for nearly 1 in 4 cancer cases among women, illustrating its high frequency. In fact, the incidence of BC ranks first among women-specific cancers worldwide. The elevated prevalence of BC compared to other cancers is due to its risk factors associated with menstruation, reproduction, intake of hormones, nutrition, and anthropometry, postponement of childbearing, *etc.* Additionally, the prevalence rate of BC increases in successive generations of high-risk populations due to inherited genetic predisposition[1].

The characterization of pathophysiology and treatment of BC is highly challenging due to its heterogeneous nature. BC displays rapid visceral as well as distant metastatic ability. Some BC subtypes can be highly aggressive and exhibit unique proliferation markers, endothelial growth factor receptor (EGFR) and endocrine receptors. They differ in metabolism, cell proliferation, protein synthesis, and communication between cells. The combined action of mutations, as well as changes in copy number, define the type of BC, while alterations in epigenetic regulation primarily initiate the development of cancer[2]. BC patients develop drug resistance, recurrence, or therapy failure. The high mortality of BC patients is mainly due to aggressive nature, heterogeneity, metastasis, drug resistance, and recurrence as well as late diagnosis.

Breast tumors consist of cancer cells, surrounding stromal cells, and blood cells in the tumor microenvironment (TME), which are critical for cancer progression and metastasis. For example, suppressor immune cells, soluble factors, and remodeled extracellular matrix (ECM) collectively hamper the anti-tumor immunity but help metastasis. Stromal cells with altered molecular mechanisms as well as abnormal signaling pathways and their interplay with other components of the TME are associated with poor prognosis of BC. Additionally, the ECM can induce changes in biochemical and molecular mechanisms. Furthermore, the molecular signature of the stroma-ECM influences BC progression as well as impacts the drug resistance cancer phenotypes towards hormone-based as well as cytotoxic based therapeutics. Similarly, the cancer-associated stroma (CAS) overexpresses genes encoding ECM, cell cycle-related proteins, matrix metalloproteinases (MMPs) (MMP-2, -11 and -14), and mitochondrial ribosomal proteins during the change to invasive phenotype from pre-invasive phenotype. CAS also correlates with enhanced expression of genes related to immune response[3]. The molecular mechanisms promoting obesity-associated changes in the TME support BC growth by altering functions of adipose tissue, inducing adipocyte demise, and imposing low-grade chronic inflammation. The inflamed TME allows remodeling and infiltration of immune cells and stimulates protumor after-effects to sustain irreversible metastatic progression[4].

Tumor angiogenesis is a hallmark of solid tumors and an alternative target of metastatic BC. Metastatic breast tumors create unusual vascularization around the tumor by secreting abnormal levels of proangiogenic factors. The vascular network in tumors is highly disorganized and permeable. The abnormal vascularization leads to the development of poorly perfused tumors in the hypoxic TME. This hypoxic environment selects highly invasive and aggressive cancer cells with the ability to

escape from tumor-destroying immune cells for metastasis. Furthermore, vascular endothelial growth factor (VEGF) increases angiogenesis by suppressing immunity. In addition, abnormal perfusion decreases the efficacy of chemotherapy and radiation therapy. Apart from cancer cells, tumor-associated leukocytes and stromal cells also promote tumor angiogenesis by secreting extracellular proteases, chemokines, and cytokines as well as exosomes.

Given the complex biology of BC, novel personalized therapeutic strategies that address its diverse pathophysiologies are needed to improve clinical outcomes. Marine sources offer tremendous potential for the detection and advancement of new therapeutic regimens that can assist in BC treatment. Since 1980, advances in biotechnology have enabled new avenues of research exploring marine organisms and their therapeutic potential for novel drug development. This research is dynamic and utilizes advanced technological tools[5], and has led to an increased interest in exploiting marine microbial, crustacean, and natural fish product scaffolds for rational drug discovery. Marine bioactive compounds represent an accessible source of chemical entities, novel drugs as well as drug leads. They are the basis for nearly 80% of the chemotherapeutics approved by the United States Food and Drug Administration (FDA), and more than half of all drugs. Marine compounds are currently used for the treatment of 87% of diseases, including cancer as they can induce cytotoxicity in cancer cells by targeting mediators of oncogenic transduction pathways or by inhibiting the growth of cancer cells[6,7]. Recent advances in technology and research focusing on marine compounds have allowed the discovery of novel anticancer agents, which are currently undergoing clinical trials.

In the present review, we describe advances in the biology of breast neoplasia, including cellular and molecular biology and heterogeneity as well as epidemiology. Furthermore, we summarize recent knowledge of BC pathophysiology, including the role of TME components in metastasis, angiogenesis, and drug resistance. We then provide an up-to-date review of anticancer compounds originating from marine microorganisms, crustaceans, and fishes and their synergistic effect on anticancer drugs. Finally, we present a summary of the pathophysiology of BC and delineate future directions in the development of marine-based therapeutics for BC.

BC BIOLOGY

The biology of BC encompasses a unique signature of histopathology, cellular and molecular heterogeneity and responses, which allows a deeper understanding of BC pathophysiology. The biology of breast tumors in women varies with age. BC in younger women is highly complex and accounts for 15% of all BC subtypes in developing countries. Breast tumors in younger women are classified as high-frequency subtypes with unique signatures of histopathology, genetics, molecular biology, and genomics. Substantial literature validates the function of patient immunosurveillance in affecting response to treatment in some subtypes of BC like triple-negative breast cancer (TNBC) and human epidermal growth factor receptor 2 (HER2)+, but its role in estrogen receptor (ER)+ remains unclear because it is less immunogenic[8], highlighting the need for in-depth clinical studies to improving management strategies in ER+ BC.

AGE SIGNATURE OF BC PATIENTS

BC in younger women exhibits differential gene expression, definite genomic signs, and elevated genetic susceptibility, compared to aged females with BC[9]. In addition, differences in breast stroma, changes in the breast during pregnancy or breastfeeding contribute to the difference in the biology of tumors in younger women[10]. Furthermore, BCs in younger women are multicentric, grade 3, triple-negative, and have a high proliferative index, Ki-67[11]. Furthermore, radiation treatment following BCS can decrease local recurrence from 19.5% to 10.2% in younger BC patients[12]. Clinical investigations evaluating five years of endocrine therapy in TEXT and SOFT trials and extended therapy with tamoxifen in ATTom and ATLAS trials suggest that adjuvant endocrine therapy is an effective treatment for younger BC patients[13].

BCs in adolescents account for 5.6% of all invasive BC and are likely to be driven by genes responsible for familial cancer predisposition, which cause larger tumors with distinct metastasis. These patients need a multidisciplinary approach of care and treatment regimens to minimize premature menopause, as well as osteoporosis and

sexual health[14]. In aged women, BCs found at an initial stage are treatable and have prolonged disease-free survival. Furthermore, adjuvant chemotherapy, radiation or endocrine therapy can reduce the danger of BC recurrence and improve survival[15]. Population-based studies conducted in Asia have recognized that adjuvant modalities, including endocrine and chemotherapy regimens, have significantly improved the overall survival of older women with BC[16], thus illustrating the beneficial role of clinical studies in ameliorating patient outcomes.

CELLULAR AND MOLECULAR BIOLOGY OF BC

The role of pathological examination in predicting etiology, pathogenesis, and clinicopathological correlations of BCs is the gold standard. Despite many biochemical and histochemical biomarkers, ER, progesterone receptor (PR), and HER-2 are the vital components of clinical examination. Initially, these markers were used for the prognosis of BCs, but also for prediction of treatment outcome. However, high throughput experimentation shows molecular heterogeneity in histologically similar subtypes. For example, high throughput data has facilitated the classification of invasive ductal carcinoma into four categories such as luminal, HER2 amplification type, basal-like and normal breast-like. Among the subtypes, 75% of BCs belong to the luminal type, which exhibits highly complex endocrine resistance due to genetic and epigenetic mechanisms. Epigenetic changes in receptors or their signaling pathways, such as the pattern of DNA methylation, histone modifications, and altered expression of miRNAs, are heritable and reversible in luminal BCs[17]. Lobular carcinoma of invasive (ILC) type of BC accounts for 5%-15% of ductal carcinoma of invasive type. This subtype shows an endocrine response but responds poorly to chemotherapy. Some studies suggest that systemic therapy might be useful for patients with ILC[18].

Genomic analysis has identified seven BC epitypes (ET1 to ET7). One subtype is characterized by *BRCA1* mutations and is linked to basal-like tumors, while another subtype has a complex genome with several additional amplifications such as *ERBB2* amplification. A third subtype exhibits a normal epithelial cell-like methylation profile. In contrast, the remaining four epigenetic subtypes look like luminal BCs but differ in genomic instability, promoter, and global hypermethylation as well as the rate of proliferation[19]. Furthermore, high throughput studies have uncovered an interaction between genetic and epigenetic alterations among BC subtypes[20]. The diverse and complex biology of these various BC subtypes highlights the need for personalized targeted therapies that address the specificities of each subtype to enhance patient outcomes. In an effort to identify new biomarkers of each subtype, a microarray study by Zheng *et al*[21] found differentially expressed genes associated with immune response, cell differentiation, and cell adhesion in various BC types. The genes engage in a protein-protein network with EGFR and spleen-associated tyrosine kinase, which are critical for BC metastasis, and could thus serve as promising novel biomarkers[21] that could improve early BC diagnosis.

CELLULAR AND MOLECULAR HETEROGENEITY OF BC

As the previous section shows, breast tumor consists of genetically, pathologically, and clinically distinct subtypes. Based on the characteristics of histopathology, BC can be classified into three subtypes, including BCs positive for ER/PR, TNBC, and amplified with HER2. TNBCs account for 15% of all BCs, enriched with heterogeneity in terms of *BRCA1/2* mutations, expression of androgen receptor (AR) along with rare genomic changes, and a high degree of lymphocyte infiltration. Furthermore, gene expression studies have demonstrated six clinically distinct subsets of TNBC, counting basal-like-1 and -2, immunomodulatory, mesenchymal, luminal AR and mesenchymal stem-like[3]. Breast tumors display hierarchical heterogeneity due to the presence of a high degree of cell plasticity elicited by activated oncogenes, which allows the cells to differentiate into diverse cell types. Also, the differentiation of tumor-initiating cells or stem cells, such as the $CD44^{high/+}/CD24^{low}$ subtypes, in breast tumors underlies tumor heterogeneity and the emergence of aggressive and resistant neoplastic niches[22].

A BC subclone carrying mutations in *AKT*, *FGFR*, *PIK3CA*, and *TP53* and increased chemoresistance, invasiveness, and metastatic ability has been characterized using whole genome and targeted genome sequencing. Single-cell technology has identified a unique claudin-low subclone displaying an epithelial stem cell phenotype and high resistance to chemotherapy[23]. Additionally, a change in the copy number can cause

intratumor heterogeneity in BC. For example, HER2 copy numbers can vary in different BC patients. The accumulation of copy number variations due to complex genomic rearrangements or bursts of chromosome changes can induce the development of metastable, pseudodiploid or chromosomic subpopulations in TNBC[24]. Copy number changes are also responsible for the emergence of immune suppressive phenotypes in some T-and B-lymphocytes as well as M2 macrophages[25].

Molecular heterogeneity in ER has been described in estrogen and androgen hormone responsive BCs, which differ in distant recurrence risk and disease-free survival. Mutational analysis uncovered ER^+/PR^- BC subpopulations enriched with mutations in *TP53*, *GATA3*, *CDH1*, *HER2*, and *BRAF*[26]. Whole exome sequencing studies also reported heterogeneity in ER+/HER2- BCs, which differ in mutational alleles in *TP53*, *CDH1*, *MYC* and have poor overall survival[27]. As these studies show, cellular and molecular heterogeneity pose great challenges in treating metastatic BCs and overcoming drug resistance.

EPIDEMIOLOGY OF BC

Epidemiological investigations have established unmodifiable risk factors such as race, family history of BC, genetic makeup and ethnicity, as well as variable factors such as consumption of alcohol, high-fat diet, female reproductive factors, hormone therapy, obesity as BC causative factors. Additionally, menarche at a younger age, pregnancy at a late age, parity, and nulliparity can also influence BC risk *via* by affecting sex hormones. Genetic variants and gene mutations in proteins tangled in DNA repair have been implicated as additional risk promoters in BC[28]. Molecular and genetic studies have identified high-risk BC patients with mutations in *BRCA1* and *BRCA2*, along with moderate change of BC-susceptibility genes including *PTEN* and *TP53*[29]. Furthermore, *GWAS* have established the combination of genetic weakness loci, reproductive risk factors, the interaction of gene-environment with obesity and lifestyle as a potential risk factor. Additionally, gene-environment interaction studies have identified BC subtypes in high-risk women[30]. Physical activity can enhance anti-tumor immunity in BC patients by increasing cytotoxic monocytes, natural killer (NK) cells, and cytokines[31]. Recent technological advances have thus allowed an improved characterization of the various BC subtypes and their molecular and genetic underpinnings, which can enable enhanced detection strategies.

PATHOPHYSIOLOGY OF BC

The current understanding of the pathophysiology of the breast TME is that the aggressive and metastatic phenotypes arise through a series of molecular alterations at the cellular and molecular level. Advanced studies have also elucidated the role of sustained angiogenesis in the pathophysiology of TME and the involvement of CSCs and redox biology in drug resistance in BC. The following sections explore the role of the TME in the development and progression of breast tumors.

TME components in BC metastasis

Metastasis is the last step of BC progression and is a common cause of BC mortality. Recent studies show that the components of the TME, including immune suppressive cells, soluble factors, and remodeled ECM, can reciprocally communicate with each other to promote BC metastasis (Figure 1). The breast TME is characterized by unusual signaling pathways and characteristic molecular changes. Tumor associated stomal cell interactions with BC cells promote metastasis through the induction of cytokine (CXCL8) production, which activates the expression of notch1 *via* tumor necrosis factor (TNF) α-induced p65 activation[32]. Cancer-associated fibroblasts (CAFs) are the most prominent stromal cells of the breast TME and accelerate metastasis by associating with other TME cells. Zhou *et al*[33] reported that CAFs promote lymph node metastasis of TNBC cells by interacting with tumor-associated macrophages (TAMs). This study also demonstrated that activated CAFs can increase lymphatic metastasis by increasing the intrusion of polarized macrophages in TNBC patients. CAFs promoted lung metastasis of TNBC cells in the homograft tumor model by activating transforming growth factor (TGF) β[34], thus indicating their key role in BC progression.

Neutrophils can also accelerate BC metastasis. Coffelt *et al*[35] reported that

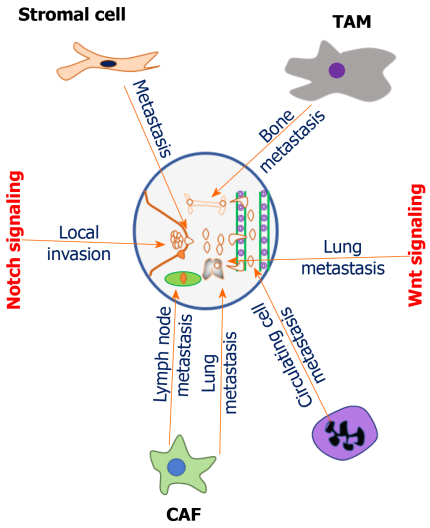

Figure 1 Role of tumor microenvironment components in promoting breast cancer metastasis. Breast tumor microenvironment is typified with different types of components with typical cellular and molecular functions. Tumor associated stromal cells promote metastasis through the induction of cytokine (CXCL8) production by activating the expression of notch1 *via* TNFα-induced p65 activation. Cancer-associated fibroblasts (CAFs) accelerate lymph node metastasis of triple-negative breast cancer (TNBC) cells by increasing the intrusion of polarized macrophages in TNBC patients. Tumor associated macrophages promote bone metastasis by secreting interleukin 12. CAFs promoted lung metastasis of TNBC cells in the homograft tumor model by activating transforming growth factor β. Cancer associated neutrophils mediates metastasis by leukotrienes. Neutrophils enhance metastatic ability of circulating breast cancer (BC) cells. Notch signaling promotes local invasion of BC cells. Wnt signaling modulates metastasis of BC cells to the lung. TAM: Tumor-associated macrophage; CAF: Cancer-associated fibroblast.

neutrophils are expanded and polarized by interleukin (IL) 17 from γδT-cells of breast tumor in mice. The polarized neutrophils promote metastasis by suppressing $CD8^+$ cytotoxic T cells. IL-17 neutralization using specific antibodies reduced the lung metastasis of BC cells by decreasing the T-cell suppressive neutrophils[35]. Another study reported that neutrophils can also mediate BC metastasis through leukotrienes. This study observed that blocking leukotriene synthesizing enzyme 5-lipooxygenase can reduce metastasis by abrogating the prometastatic activity of neutrophils[36]. Additionally, neutrophils can increase the metastatic ability of BC cells by eliciting cell cycle progression *via* modulating the expression of cytokine receptors and genes associated with cell-cell junctions[37]. Notch signaling also promotes local invasion of BC cells. Furthermore, the Wnt signaling component LGR4 (the fourth member of the leucine-rich repeat-containing GPCR family) is over expressed in BC stem cells (BCSC) and can modulate the metastasis of BC cells to the lung by promoting epithelial to mesenchymal transition (EMT) *via* increasing the expression of N-cadherin and transcription factor snail and decreasing the expression of E-cadherin[38]. Therapeutic strategies that target and reprogram the TME are thus required to improve treatment outcomes in BC patients.

TME components in BC angiogenesis

In the TME, angiogenesis is modulated by innate immune cells and other communicators (Figure 2), including neutrophils, macrophages, dendritic cells, myeloid-derived cells, γδTcells, mast cells, and NK cells[39]. Lin *et al*[40] reported that TAMs help angiogenesis after infiltrating into the breast TME. This study correlated the expression of CCL18, CD34, and microvascular density by double immuno-histochemical staining of breast tumor tissues. It also demonstrated the induction of angiogenesis by chemokine, CCL18 in the coculture of TAMs, and endothelial cells. Additionally, the study reported that blocking of CCL18 putative receptor (PITPNM3) inhibited CCL18-promoted migration of endothelial cells as well as tube formation. In contrast, the treatment of endothelial cells with CCL18 stimulated the transition of endothelial cells to mesenchymal cells by activating the Akt/GSK-3β/Snail and ERK (extracellular regulated protein kinases) signaling[40]. In the TME, TAMs induced angiogenesis by remodeling ECM for promoting invasion and BC cell modeling into stem cells for evading host immunity as well as recruiting immune-suppressive immune cells[41]. This study indicates that TAMs can induce angiogenesis and promote the progression of BC.

Similar to TAMs, CAFs promote tumor angiogenesis by various mechanisms. Du *et al*[42] demonstrated that miRNA-205/YAP1 signaling induces tumor formation as

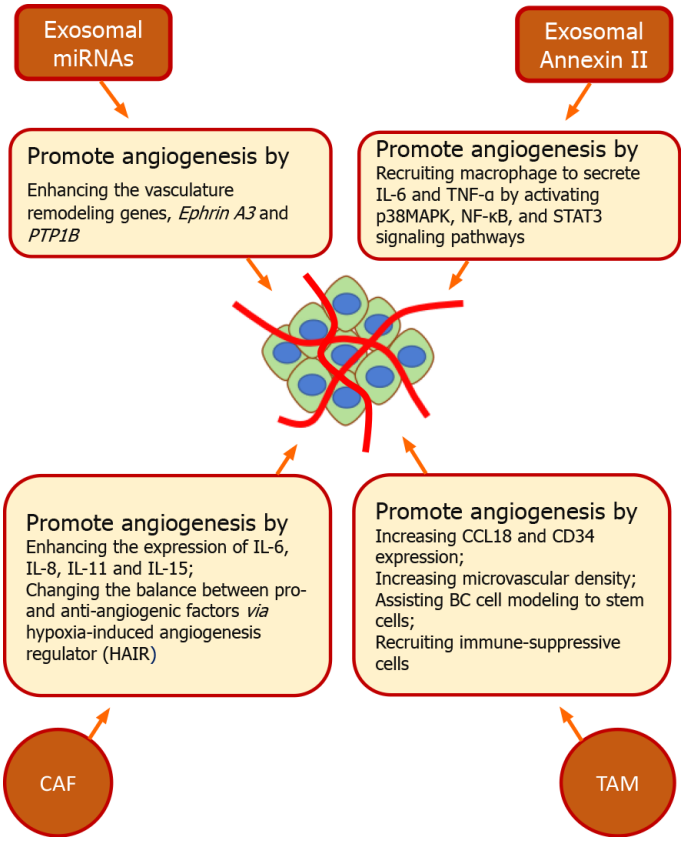

Figure 2 Modulation of angiogenesis by innate immune cells and other communicators in breast tumor microenvironment. In breast tumor microenvironment, innate immune cells and other communicators modulate angiogenesis. Tumor-associated macrophages promote angiogenesis by increasing the expression of CCL18, CD34, and microvascular density and assisting breast cancer cell modeling into stem cells for recruitment of immune-suppressive cells. Cancer-associated fibroblasts promote tumor angiogenesis by enhancing the expression of interleukin (IL) 6, IL-8, IL-11 and IL-15 as well as by changing the balance between pro-and anti-angiogenic factors *via* hypoxia-induced angiogenesis regulator. Tumor-derived exosomal Annexin II induce angiogenesis by recruiting macrophages to secrete IL-6 and tumor necrosis factor-α by activating p38MAPK, nuclear factor-κappa beta, and STAT3 signaling pathways. Exosomal miRNAs contribute to the development of tumor angiogenesis by enhancing the vasculature remodeling genes, *Ephrin A3* and *PTP1B*. TAM: Tumor-associated macrophage; CAF: Cancer-associated fibroblast; HAIR: Hypoxia-induced angiogenesis regulator; NF-κβ: Nuclear factor-κappa beta; TNF: Tumor necrosis factor; IL: Interleukin; BC: Breast cancer.

well as the sprouting of endothelial cells by promoting the transformation of normal fibroblasts into CAFs *via* enhancing the expression of IL11 and IL15. However, blocking of miRNA-205 in CAFs inhibited angiogenesis. Additionally, the study reported that CAFs release IL11 and 15 by stimulating STAT3 signaling in endothelial cells. However, blocking of IL11 and 15 halted CAF-induced angiogenesis as well as BC metastasis by inactivating STAT3 signaling[42], thus highlighting its therapeutic potential in BC. Furthermore, Eiro *et al*[43] reported that CAFs can enhance the levels of invasion and angiogenesis-associated genes in BC. The study demonstrated that CAFs derived from MMP11 positive stromal mononuclear inflammatory cells increased the transcript levels of IL-6 and -8 in BC cells in the co-culture model[43]. However, in co-culture of hypoxic CAFs and endothelial cells, CAFs induced abnormal blood vessels formation by changing the balance between anti- and pro-angiogenic factors. This study also revealed that hypoxia altered the expression of hypoxia-induced angiogenesis regulator (HAIR) in CAFs. Furthermore, knockdown of HAIR inhibited CAF-induced migration of endothelial cells by reducing the release of VEGF as well as inhibiting VEGF/VEGFR signaling[44]. These various studies show that CAFs can strongly influence BC development, suggesting that therapeutic strategies that target CAFs in the TME could improve therapeutic responses in BC patients.

Tumor-derived exosomes have emerged as key mediators of tumor-induced angiogenesis. Annexin II (Anx II) is one of the most highly expressed exosomal proteins associated with BC pathogenesis. The exosomal Anx II (Exo-AnxII) reshapes the environment for supporting metastasis, including promoting tPA-mediated angiogenesis. This study reported that Exo-Anx II induces macrophages to secrete IL6 and TNF-α by activating p38 MAPK (mitogen-activated protein kinase), nuclear factor-κappa beta (NF-κB), and STAT3 signaling pathways[45]. Also, exosomal miRNAs from hypoxic tumor cells contribute to the development of tumor angiogenesis. They

promote angiogenesis by enhancing the vasculature remodeling genes, including *Ephrin A3* and *PTP1B*, *via* transferring miRNA-210 to adjacent tumor cells in TME[46]. Exosomes orchestrate angiogenesis for BC metastasis after activation by Notch signaling. The interaction of aspartate β-hydroxylase (ASPH) with Notch signaling components promotes the synthesis and release of exosomes in murine BC models[47]. Exosomes from CXCR4 overexpressing BC cells enhance the expression of stemness-related genes in recipient cells and increase their proliferative, migratory, and invasive ability[48]. In contrast, exosomes from mesenchymal stem cells can reduce angiogenesis in BC by decreasing the VEGF expression *via* targeting miRNA-16[49]. These studies support the role of exosomes in the modulation of angiogenesis in BC and highlight their importance as novel potentials diagnostic and therapeutic tools in BC.

TME components in drug resistance of BC

In BC, drug resistance and recurrence are mediated by BCSC as well as redox molecules (Figure 3). A fine orchestration between BCSC and TME cells is involved in the process of drug resistance. This interaction promotes drug resistance by upregulating BC resistance protein (BCRP), which mediates the drug efflux mechanism in BC cells. Stem cells from bone marrow, adipose tissue, and fibroblasts also enhance the metastatic potential of BC cells by activating developmental pathways *via* secreting chemokines and cytokines. Wnt signaling of BCSC along with glutathione overexpressing genes mediates resistance[50]. These mechanisms can reshape the TME leading to increased drug evasion and survival, thus challenging the success of current therapeutic regimens.

Additionally, emerging scenarios have recognized that redox signals promote TME mediated drug resistance in BC. Redox mechanisms orchestrate pathophysiology of BC to promote abnormal proliferation, metastasis, and drug resistance through reactive oxygen species (ROS) signals. The hypoxic condition in the TME promotes drug resistance by inducing the remodeling of cells from a low ROS mesenchymal state to a high ROS epithelial state. Cancer cells with high ROS induce drug resistance by producing chemokines that elicit the infiltration of monocytes into the TME and activate macrophages to promote angiogenesis[51]. ROS also induces drug resistance by promoting the immunosuppressive phenotype of TAMs, which release immuno-suppressive chemokines by increasing the levels of programmed death ligand-1 (PD-L1) *via* NF-κB signaling[52]. In the breast TME, multinucleated cells promote drug resistance by increasing VEGF secretion and macrophage migration inhibitory factor (MIF) *via* RAS/MAPK pathway-dependent hypoxia-inducible factor (HIF)-1α[53]. Macrophages induce TGF-1β expression in cancer cells by secreting IL-1β and IL-6. These cytokines promote EMT and migration of tumor cells by activating pCREB signaling *via* ROS. ROS facilitates drug resistance in metastatic cells by inducing MIF *via* promoting phosphorylation of ERK, which promotes the release of high-mobility group box 1 (HMGB1) into the cytosol from the nucleus by inducing caveolin-1 phosphorylation. The released HMGB1 then phosphorylates NF-κB *via* activating TLR4 (toll-like receptor 4) signaling. The activated NF-κB activates MMP2 and induces transcription of Snail and Twist. This MIF-mediated HMGB1 signaling regulates the CD11b+ immune cell recruitment in the breast TME and promotes drug resistance[54]. As these various studies show, the TME plays a key role in BC development and metastasis. Hence, novel therapeutic regimens that target the various components of the TME to overcome resistance mechanisms could unleash the native immune system against BCs and improve therapeutic outcomes.

LONG NON-CODING RNAS IN THE PATHOPHYSIOLOGY OF BC

Recently, long non-coding RNAs (lncRNAs) have been reported to mediate pathophysiological developments such as metastasis as well as immune suppression within the TME (Figure 4). Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a lncRNAs enriched in the nucleus and helps cancer growth and progression by regulating gene expression and post-translational modifications. In TNBC cells, MALAT1 drives tumor progression and metastasis. The expression levels are correlated with reduced disease-free survival in both HER2 and TNBC patients, who are negative for ER and lymph node[55]. MALAT1interacts with ER, and its increased expression levels correlate with ER and PR positive status in BCs with prognostic significance (cut-off value 75%) associated with relapse. It also promotes invasion and migration of TNBCs by targeting tumor suppressor miRNA-129-5p, but its silencing induced cell cycle arrest at G0/G1phage[56]. Hence, MALAT1 could be a

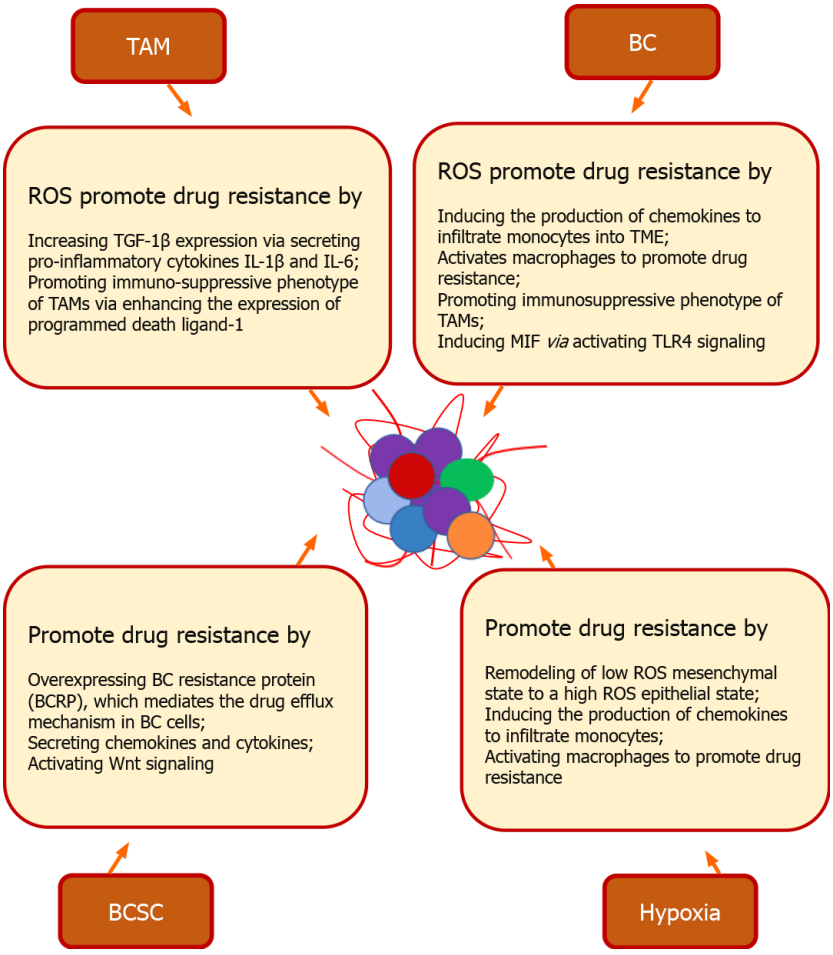

Figure 3 Cancer stem cell and redox mediated drug resistance in breast cancer. In breast tumor microenvironment (TME), a fine orchestration between breast cancer (BC) stem cells (BCSC) and TME cells promotes drug resistance. BCSC promote drug resistance by overexpressing BC resistance protein, which mediates the drug efflux mechanism in BC cells. Stem cells derived from bone marrow, adipose tissue, and fibroblast enhances the drug resistance by activating developmental pathways *via* secreting chemokines and cytokines. Wnt signaling of BCSC along with glutathione overexpressing genes mediates resistance. Hypoxic TME promotes drug resistance by remodeling of cells from a low reactive oxygen species (ROS) mesenchymal state to a high ROS epithelial state. High ROS from cancer cells induces the production of chemokines to infiltrate monocytes into TME and activates macrophages to promote drug resistance. ROS also induces drug resistance by promoting the immunosuppressive phenotype of tumor-associated macrophages *via* enhancing the expression of programmed death ligand-1 *via* NF-κB (nuclear factor-kappa beta) signaling. Multinucleated cells promote drug resistance by increasing the secretion of vascular endothelial growth factor and macrophage migration inhibitory factor (MIF) *via* RAS/MAPK pathway-dependent hypoxia-inducible factor-1α. Macrophage derived ROS induces drug resistance by increasing transforming growth factor 1β expression *via* secreting pro-inflammatory cytokines interleukin (IL)-1β and IL-6. ROS also facilitates drug resistance by inducing MIF *via* promoting phosphorylation of ERK. *via* activating TLR4 (toll-like receptor 4) signaling. TAM: Tumor-associated macrophage; BC: Breast cancer; ROS: Reactive oxygen species; TGF: Transforming growth factor; IL: Interleukin; TME: Tumor microenvironment; MIF: Macrophage migration inhibitory factor; TLR: Toll-like receptor; BCSC: Breast cancer stem cell.

potential diagnostic tool for BC (Figure 4)[57].

Silencing of MALAT1 reduced cancer growth and progression by increasing cell adhesion and decreasing migration of cancer cells in mouse mammary carcinoma model *via* altering the expression pattern of genes implicated in differentiation and protumorigenic signaling pathways[58]. The expression patterns of MALAT1 and transcription factor BACH1 were substantially associated with TNM stage, metastatic ability, and pathological stage as well as the survival of patients[59]. MALAT1 can possibly promote metastasis in association with ZEB2 by transcriptionally regulating disruptor of telomeric silencing 1-like in BC[60]. Furthermore, hypoxia induces MALAT1, which also enhances the metastasis of BCs by promoting the interaction of HIF-2α with the MALAT1 gene between the promoter region and putative enhancer-3 as well as chromatin interactions with putative enhancer-7[61]. In contrast, Kim *et al*[62] reported that overexpression of MALAT1 can reduce metastasis of BC cells in the xenograft model by hindering the interaction of TEAD with YAP *via* transcriptional inactivation. However, the silencing of MALAT1 enhances the metastatic ability of BC cells.

Another important lncRNA that promotes metastasis of BCs by reprogramming chromatin state is HOX antisense intergenic RNA (HOTAIR). It is highly expressed in

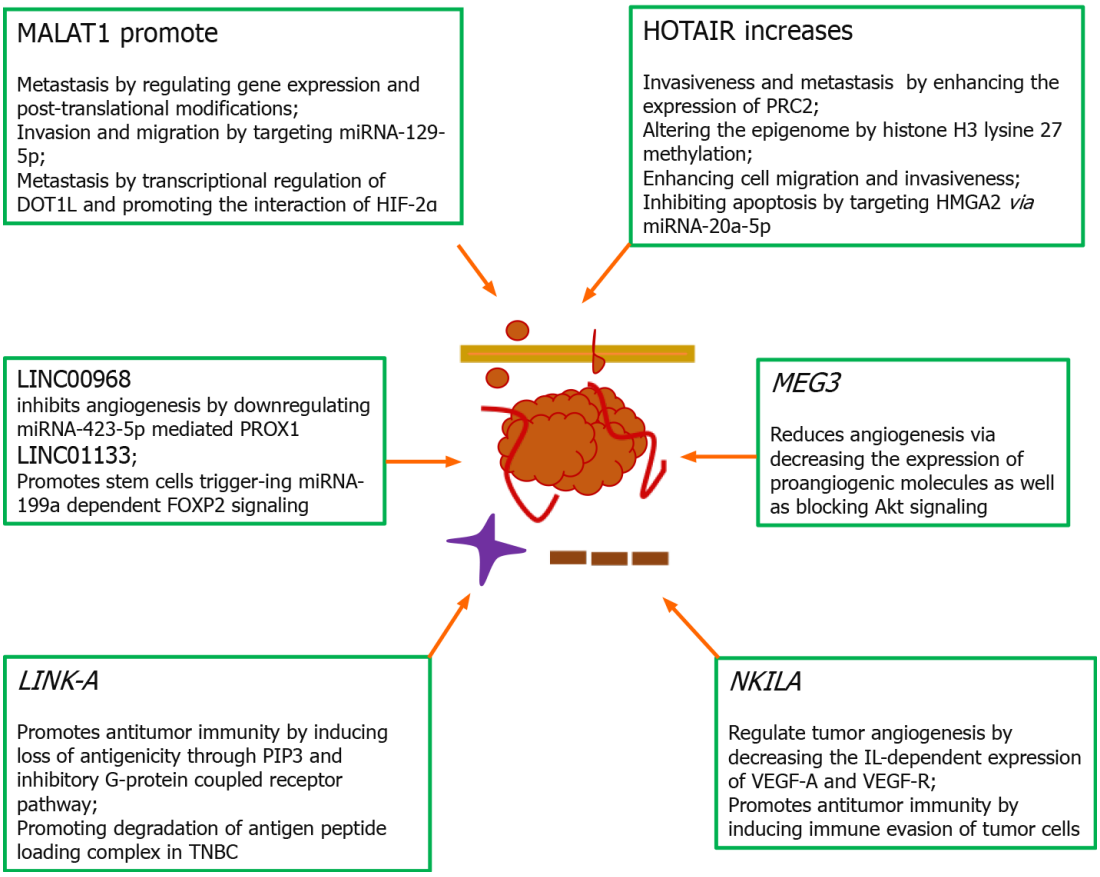

Figure 4 Role of long non-coding RNAs in the pathophysiology of breast cancer. Long non-coding RNAs are reported to mediate pathophysiological developments such as metastasis as well as immune suppression within the tumor microenvironment. Metastasis-associated lung adenocarcinoma transcript 1 promotes growth and metastasis by controlling gene expression and post-translational modifications, invasion and migration by targeting tumor suppressor miRNA-129-5p, metastasis by transcriptionally regulating disruptor of telomeric silencing 1-like and promoting the interaction of hypoxia-inducible factor-2α. HOTAIR (HOX antisense intergenic RNA) increases invasiveness and metastasis by enhancing the expression of polycomb repressive complex 2 and altering the epigenome by histone H3 lysine 27 methylation, enhancing cell migration and invasiveness and inhibiting apoptosis through targeting high mobility AT-hook 2 *via* miRNA-20a-5p. *MEG3* reduced angiogenesis by decreasing the expression of proangiogenic molecules as well as blocking Akt signaling. *NKILA* [nuclear factor-kappa beta (NF-κB) interacting lncRNA] negatively regulates tumor angiogenesis by decreasing the interleukin-dependent expression of vascular endothelial growth factor (VEGF)-A and VEGF-R through inhibiting NF-κB signaling and promotes antitumor immunity by inducing immune evasion of tumor cells *via* sensitizing T-cells through activation-induced cell death mechanism[57]. LINC00968 inhibits capillary formation by downregulating miRNA-423-5p mediated *PROX1*. LINC01133 promotes stem cell phenotypes by triggering miRNA-199a dependent FOXP2 signaling *via* modulation of Kruppel-like factor 4. Silencing of lncRNA-21 in tumor-associated macrophages induced apoptosis and reduced cell migration and invasion. *LINK-A* promotes antitumor immunity by inducing loss of antigenicity through PIP3 and inhibitory G-protein coupled receptor pathway as well as attenuating protein kinase A-dependent phosphorylation of E3 ubiquitin ligase TRIM71 and degradation of antigen peptide loading complex in triple-negative breast cancer. MALAT1: Metastasis-associated lung adenocarcinoma transcript 1; DOT1L: Disruptor of telomeric silencing 1-like; HIF: Hypoxia-inducible factor; HOTAIR: HOX antisense intergenic RNA; PRC2: Polycomb repressive complex 2; HMGA2: High mobility AT-hook 2; TNBC: Triple-negative breast cancer; IL: Interleukin; VEGF: Vascular endothelial growth factor.

both primary as well as metastatic breast tumors. Its expression is strongly correlated with lymph node metastasis and the expression of the AR. Mechanistically, HOTAIR upregulation using overexpressing plasmid enhanced the expression of polycomb repressive complex 2 (PRC2) and altered the epigenome by histone H3 lysine 27 methylation. It also enhanced invasiveness and metastasis in BCs, while its silencing inhibited invasiveness in PRC2 overexpressing BCs[63]. HOTAIR can also promote progression by increasing migration and invasiveness and inhibiting apoptosis through targeting high mobility AT-hook 2 (HMGA2) *via* miRNA-20a-5p in BC cells[64].

The overexpression of *MEG3* reduced angiogenesis in BC by reducing the capillary formation of endothelial cells *via* decreasing the expression of proangiogenic molecules as well as blocking Akt signaling[65]. However, overexpression of lnc RNA *NKILA* (NF-κB interacting lncRNA) significantly reduced tumor angiogenesis by reducing the IL-dependent VEGF-A and VEGF-R expressions through inhibiting NF-κB signaling and IkBα phosphorylation and nuclear translocation[66]. Recently, Sun *et al*[67] reported that overexpression of lncRNA (LINC00968) reduced capillary formation by downregulating miRNA-423-5p mediated *PROX1* in BC cells. Another lnRNA, LINC01133 promoted stem cell phenotypes in TNBC cells by triggering

miRNA-199a dependent FOXP2 signaling *via* modulation of Kruppel-like factor 4[68]. TME induced stemness in BC cells by silencing lncRNA-HAL through chromatin level and transcriptional regulation[69].

Additionally, lncRNAs can regulate tumor immunity through various mechanisms. Huang *et al*[57] reported that *NKILA* promotes antitumor immunity by inducing immune evasion of tumor cells *via* sensitizing T-cells through activation-induced cell death mechanism. Silencing of lncRNA-21 in TAMs induced apoptosis in BC cells and reduced metastasis[70]. In contrast, lncRNA (*LINK-A*) promotes antitumor immunity by inducing loss of antigenicity through PIP3 and IGPCR pathway in TNBC[71]. Hence, lncRNAs can modulate metastasis at different molecular levels, including epigenetic modification and multiple signaling pathways. Future studies could lead to the development of clinical applications based on lncRNAs as potential diagnostic biomarkers or drug targets.

BC THERAPY

As the previous sections show, the complex pathophysiology poses a challenge to the development of effective therapeutic regimens. Novel personalized strategies that target the BC TME are required to improve clinical responses. Natural compounds (*e.g.*, cytarabine, eribulin mesylate) from marine organisms have been approved by the FDA for cancer treatment and could represent valuable clinical modalities for BC. Several marine compounds with antineoplastic activity are currently undergoing different phases of evaluation in clinical trials (*e.g.*, plinabulin). Additionally, the anticancer activity of more than 1500 marine compounds has been evaluated using *in vivo* models while the activity of 10000 compounds has been tested using *in vitro* models[72]. Marine compounds malformin A (cyclic pentapeptide), kuanoniamine D (pyridoacridine alkaloid), hymenialdisine (C11–N5 alkaloid), and gallic acid (GA, phenolic acid) exert potentially high anticancer activity against MCF-7 BC cells by inducing apoptosis and promoting nuclear fragmentation, membrane protrusion, blebbing and chromatin segregation[73]. The following paragraphs will examine some of the best-studied marine compounds and explore their relevance to BC treatment.

Marine compounds with potential anti-BC activity from microorganisms and algae

Marine compounds from microorganisms have potential anticancer activity (Table 1). For example, GA can stimulate apoptosis by changing p53, Mcl, and p21 expression. GA can also stimulate cell cycle arrest in BC cells by altering the cyclin-dependent kinases (CDKs) expression. Mechanistically, MAP38 kinase is intricated in GA-stimulated cell cycle arrest and apoptosis *via* downregulating cyclin Da/CDK4 and cyclin E/CDK2. However, a combination of GA with curcumin stimulated apoptosis in BC cells by overexpressing the expression of Bax as well as activating caspase 3 and poly (ADP ribose) polymerase (PARP) and decreasing Bcl2 along with arresting at sub-G1 phase[74]. In addition, a nanocomposite of iron oxide magnetite nanoparticles (NPs) and polyethylene glycol (PEG) enhanced the anticancer activity of GA. Moreover, GA conjugated Gold NPs suppressed the metastasis of MDA-MB cells by blocking EGF dependent MMP-9 expression *via* suppressing stabilization of p300 as well as activating NF-κB/c-Jun pathway. However, overexpression of MEK1 and Akt reduced the inhibitory activity of GA on the EGF-dependent upregulation of MMP-9[75]. Crambescidin 800 is a heteropenta cyclic guanidine alkaloid. Crambescidin 800 stimulated arrest at the G2M phase by decreasing the cyclin D1, CDK-4, and -6 expression in TNBC cells *via* modulating the Akt/NF-κB/MAPK pathway[76] and thus warrants further clinical investigation.

The polysaccharide EPS11, isolated from marine bacteria, inhibits lung metastasis by inhibiting the cell adhesion protein CD99. It also inhibits cancer cell growth by inducing anoikis *via* inducing the Akt pathway-dependent expression of βIII-tubulin[77]. The polysaccharide fractions, SWP1, and SWP2 from brown seaweed decreased the proliferation of BC cells by stimulating apoptosis and activating caspase 3/9 and disrupting the mitochondrial membrane *via* generation of ROS[78]. Carrageenan, a polysaccharide from red algae, inhibited BC cells at 50 μg by inducing apoptosis *via* promoting condensation of the nucleus and fragmentation of DNA as well as activating caspase 8, an extrinsic apoptotic protein[79]. Exopolysaccharides from marine algae inhibited the growth of BC cells by reducing the cyclin D1 and E transcript levels, while inducing the proliferation of B-cells and decreasing the IL-6 and TNF-1α production in T-cells[80]. Hence this compound displays promising immunomodulatory effects that warrant further investigation.

Table 1 Marine compounds with potential anticancer activity against breast cancer from microorganisms and algae

Marine compound	Chemical nature	Mechanism of action	Ref.
Gallic acid (GA)	Phenolic compound	(1) Altered the expression of P53, Mcl and p21 as well as cell cycle regulators; and (2) MAP38 kinase involved in GA induced cell cycle arrest and apoptosis *via* downregulating cyclin Da/CDK4 and cyclin E/CDK2	Moghtaderi *et al*[74]
GA	Phenolic compound	(1) In combination with curcumin stimulated apoptosis by increasing the Bax expression, activating PARP and caspase 3; (2) Decreased Bcl2 expression; and (3) Arrested at sub-G1 stage	
GA	Phenolic compound	Conjugated to Gold NPs, suppressed metastasis by blocking EGF dependent MMP-9 expression *via* suppressing stabilization of p300 and activation of NF-κB/c-Jun pathway	Chen *et al*[75]
Crambescidin 800	Heteropenta cyclic guanidine alkaloid	Induced cell cycle at the G2M phase by decreasing the cyclin D1, CDK-4, and -6 expression in TNBC cells *via* modulating Akt/NF-κB/MAPK pathway	Moon *et al*[102]
EPS11	Polysaccharide	(1) Inhibited lung metastasis by inhibiting cell adhesion protein CD99; and (2) Inhibited cancer cell growth by inducing anoikis *via* inducing Akt pathway-dependent expression of βIII-tubulin	Cao *et al*[77]
SWP1 and SWP2	Polysaccharide	Inhibited proliferation by inducing apoptosis, activating caspase 3/9 and disrupting the mitochondrial membrane *via* generation of ROS	Vaikundamoorthy *et al*[78]
Carrageenan	Polysaccharide	Induced apoptosis *via* promoting condensation of the nucleus and fragmentation of DNA as well as activating caspase 8, an extrinsic apoptotic protein	Murad *et al*[79]
Exopolysaccharide	Polysaccharide	(1) Inhibited the cell growth by decreasing the cyclin D1 and E expression; and (2) Induced the proliferation of B-cells and decreased production of IL-6 and TNF-1α in T-cells	Park *et al*[80]
Ilmycin C	Cyclic peptide	Inhibited migration and invasion by inducing apoptosis *via* Bax/Bcl-2 dependent caspases as well as inhibiting MMP-2 and -9 *via* blocking IL-6 dependent phosphorylation of STAT3	Xie *et al*[81]
		Inhibited growth by inducing apoptosis through activation of SR stress and reducing Bcl2 in a CHOP dependent manner	Zhou *et al*[82]
Molassamide	Cyclic depsipeptide	(1) Abrogated elastase-dependent migration of highly metastatic TNBC cells; and (2) Inhibited the activity of elastase and the migration of TNBC cells by targeting the expression of ICAM-1 *via* inhibiting the NF-κB pathway	Al-Awadhi *et al*[83]
Kempopeptin C	Cyclic depsipeptide	Inhibited invasion and migration by decreasing the cleavage of matriptase substrates CDCP1 and sesmoglein-2	Al-Awadhi *et al*[84]
Cyclic leucylproline	Cyclic peptide	Inhibited migration by inhibiting cell proliferation, inducing cell arrest *via* DNA damage. Mechanistically, CLP induced cell cycle arrest by blocking the expression of cyclin C, CDK4, PAK, RAC1, and p27kiP1 *via* targeting CD151 and EGFR signaling axis in TNBC cells	Kgk *et al*[85]
Galaxamide	Cyclic pentapeptide	Elicited apoptosis in BC cells by arresting at the G1 phase as well as reducing mitochondrial membrane potential *via* the generation of ROS	Lunagariya *et al*[86]
Brintonamide D	Linear peptide	Reduced the CCL27 and stimulated proliferation and progression of metastatic BC cells by targeting serine protease kallikrein 7 (KLK7). This study reported that brintonamide D targeted KLK7 by modulating CCR10, the receptor of CCL27 in BC cells	Al-Awadhi *et al*[87]
Iturin A	Lipopeptide	(1) Induced apoptosis by increasing sub-G1 cell population, fragmentation of DNA *via* inhibiting FGF-mediated phosphorylation of Akt, FoxO3a and GSK3β; (2) And reduced tumor growth by promoting translocation of FoxO3a *via* downregulating MAPK and Akt kinase in the xenograft model	Dey *et al*[88]
Halilectin-3	Sugar-binding lectin protein	Inhibited proliferation by inducing arrest at the G1 phase and apoptosis by increasing the activity of caspase 9 and autophagy by inducing the expression of light chain 3	do Nascimento-Neto *et al*[89]
Sinularin	Terpenoid	Reduced cell viability by halting at the G2M phase and stimulating apoptosis through activation of caspase-3 and -8	Huang *et al*[90]

		as well as PARP. In addition, it also induced DNA damage by generating ROS *via* stimulating oxidative stress	
Sipholenol A	Triterpene	Reduced the metastatic ability of TNBC cells by inhibiting protein tyrosine kinase 6, a key mediator of growth factor-dependent migration	Foudah *et al*[91]
Agelasine B	Diterpene alkaloid	(1) Induced apoptosis by inhibiting ER Ca^{2+} -ATPase (SERCA) activity *via* releasing Ca^{2+} from ER and inducing DNA fragmentation; (2) Reduced the Bcl2 expression and enhanced the caspase 8 expression; and (3) Induced cell death in an ER-mediated extrinsic apoptotic pathway	Pimentel *et al*[92]
Hirsutanol A	Sesquiterpene	(1) Reduced cell growth by inhibiting proliferation; (2) Induced apoptosis, and autophagy *via* generating ROS; and (3) Silenced Atg7 with siRNA and blockade of autophagy using bafilomycin A1 synergistically increased the efficacy of hirsutanol A in inducing apoptosis and inhibiting cell proliferation	Yang *et al*[93]
Dehydrothyrsiferol	Triterpenoid	Induced apoptosis by causing DNA fragmentation and arrest at S-phase and G2M phase	Pec *et al*[94]
Sodwanone	Triterpene	(1) Induced cytotoxicity to BC cells; and (2) Inhibited hypoxia-induced HIF-1α	Dai *et al*[95]
Pseudopterosin	Diterpene glycoside	(1) Reduced the production of IL-6, TNF-1α, and MCP-1 *via* blocking p65 and IkB phosphorylation; and (2) Promoted translocation of glucocorticoid receptor from nucleus to cytosol	Sperlich *et al*[96]
Quinazoline	Heterocyclic compound	(1) Induced apoptosis in HER+ve BC cells by reducing the Bcl2 expression and increasing the Bax expression; and (2) Promoted cell death *via* ROS-dependent extrinsic or intrinsic apoptotic pathways without systemic toxicity in the mouse model	De *et al*[97]
(3β)-Cholest-5-en-3-ol	Cholesterol	Induced cell death by activating caspase 3 and 8 as well as increasing the Bax expression and decreasing the Bcl2 expression	Sharifi *et al*[98]
3β,11-dihydroxy-9,11-secogorgost-5-en-9-one	Sterol	(1) Inhibited cell growth by inducing apoptosis *via* activation of caspase 3 and PARP and cell cycle arrest *via* targeting cyclin D1 and CDK6 through blocking the p38/ERK signaling pathway; and (2) Induced autophagy *via* generating ROS and DNA damage by increasing the expression of *H2AX*	Weng *et al*[99]
4-methyenedioxy-β-nitrostyrene	β-nitrostyrene derivatives	Inhibited migration by disrupting the focal adhesion complex as well as a network of actin stress fibers *via* reducing β1 integrin-dependent phosphorylation of FAK and paxillin	Chen *et al*[100]

CDK: Cyclin-dependent kinase; PAPR: Poly (ADP ribose) polymerase; NPs: Nanoparticles; EGF: Endothelial growth factor; MMP: Matrix metalloproteinase; NF-κβ: nuclear factor-κappa beta; TNBC: Triple-negative breast cancer; MAPK: Mitogen-activated protein kinase; ROS: Reactive oxygen species; IL: Interleukin; TNF: Tumor necrosis factor; ICAM-1: Intercellular adhesion molecule-1; CDCP1: CUB-domain containing protein 1; CLP: Cyclic dipeptide of leucine and proline; EGFR: Endothelial growth factor receptor; HIF: Hypoxia-inducible factor; MCP-1: Monocyte chemotactic protein-1; BC: Breast cancer; HER: Human epidermal growth factor receptor; FAK: Focal adhesion kinase.

The marine compound, Ilmycin C, is a cyclic peptide, that inhibits the invasion and migration of BC cells by inducing apoptosis *via* Bax/Bcl-2 dependent caspases as well as inhibiting MMP-2 and -9 *via* blocking IL-6 dependent phosphorylation of STAT3[81]. Ilamycin E can also inhibit TNBC growth by inducing apoptosis through activating SR stress and reducing Bcl2 in a CHOP dependent manner[82]. Cyclic depsipeptide, molassamide can abrogate elastase-dependent migration of highly metastatic TNBC cells. It can also inhibit the activity of elastase and the migration of TNBC cells by targeting the expression of ICAM-1 (intercellular adhesion molecule-1) *via* decreasing NF-κB[83]. Another cyclic depsipeptide, kempopeptin C from marine cyanobacteria can inhibit the invasion and migration of TNBC cells by reducing the cleavage of matriptase substrates CDCP1 (CUB-domain containing protein 1) and sesmoglein-2[84]. However, cyclic dipeptide of leucine and proline (CLP) decreased TNBC cells migration by inhibiting proliferation and inducing G2/M arrest *via* DNA damage. Mechanistically, CLP induced cell cycle arrest by blocking cyclin C, CDK4, PAK, RAC1, and p27kiP1 expression *via* targeting CD151 and EGFR signaling axis in TNBC cells[85]. The marine cyclic pentapeptide galaxamide and its analogs elicited apoptosis in BC cells by arresting the G0/G1 phase as well as reducing mitochondrial membrane potential *via* the generation of ROS[86]. These various studies illustrate the wide ranging molecular and immunomodulatory effects of marine compounds, highlighting their

versatility as potential sources of TME modulating molecules with therapeutic benefits.

Brintonamide D is a linear peptide that can reduce CCL27-stimulated proliferation and migration of metastatic BC cells by targeting serine protease kallikrein 7 (KLK7). This study reported that brintonamide D targeted KLK7 by modulating CCR10, the receptor of CCL27 in BC cells[87]. Iturin A, a marine bacterium derived lipopeptide, stimulated apoptosis in BC cells by increasing sub-G1 cell population, fragmentation of DNA *via* inhibiting FGF-mediated phosphorylation of Akt, FoxO3a and GSK3β. This study also reported that Iturin A reduces tumor growth by promoting translocation of FoxO3a *via* inactivating MAPK and Akt kinase in the xenograft model[88]. Furthermore, Halilectin-3, a sugar-binding lectin protein from marine sponges, inhibited BC cell proliferation by stimulating the G0/G1 arrest. It also promoted apoptosis by increasing the activity of caspase 9 and autophagy by inducing the expression of the light chain 3[89]. These various marine compounds display promising anti-tumor activity that could be harnessed to target the BC TME in a non-invasive and non-toxic fashion.

The marine terpenoid sinularin can decrease the viability of TNBC cells by halting the the G2/M phase and inducing apoptosis through activation of caspase-3 and -8 and PARP. In addition, it can also induce DNA damage by generating ROS *via* stimulating oxidative stress[90]. Sipholenol A, a triterpene from marine sponges, reduced the metastatic ability of TNBC cells by inhibiting protein tyrosine kinase 6, a key mediator of growth factor-dependent migration[91]. Agelasine B is a diterpene alkaloid from marine sponges that can stimulate apoptosis in BC cells by inhibiting ER Ca^{2+}-ATPase (SERCA) activity *via* releasing Ca^{2+} from ER and inducing DNA fragmentation. It also reduced Bcl2 expression and increased caspase 8 levels. This study demonstrated that Agelasine B can induce cell death in BC cells through an ER-mediated extrinsic apoptotic pathway[92]. Hirsutanol A, a sesquiterpene from marine fungus, also reduced the growth of BC cells by inhibiting proliferation and stimulating autophagy and apoptosis *via* generating ROS. Silencing of Atg7 with siRNA and blockade of autophagy using bafilomycin A1 synergistically increased the efficacy of hirsutanol A in inducing apoptosis and inhibiting cell proliferation. This study indicates that hirsutanol A can induces autophagy and programmed cell death (PCD) by enhancing ROS accumulation in BC cells[93]. Dehydrothyrsiferol (DT) is a triterpenoid that promotes apoptosis in BC cells by DNA fragmentation and arrest at S-phase and G2M phase[94]. Sodwanone, a marine triterpene, is highly toxic to BC cells and can inhibit hypoxia-induced HIF-1α[95]. Pseudopterosin, a marine diterpene glycoside, also decreased the production of IL-6, TNF-1α, and MCP-1 (monocyte chemotactic protein-1) in BC cells *via* blocking p65 and IkB phosphorylation as well as promoting translocation of glucocorticoid receptor from nucleus to cytosol[96].

Quinazoline, a six-membered nitrogen-containing heterocyclic compound from marine sponge *Hyrtios erectus*, stimulated apoptosis in HER-positive BC cells by decreasing Bcl2 expression and increasing Bax. This study reported that quinazoline promoted cell death *via* ROS-dependent extrinsic or intrinsic apoptotic pathways without systemic toxicity in the mouse model[97]. Sharifi *et al*[98] showed that marine compounds (3β)-Cholest-5-en-3-ol (cholesterol), 2-hexadecanol and hexadecanoic acid from sea pen stimulated cell death in TNBC cells by activating caspase 3 and 8 as well as increasing Bax expression and reducing Bcl 2 levels. Furthermore, a sterol from soft corals inhibited BC cell growth by stimulating apoptosis *via* activation of caspase 3 and PARP. It also elicited Go/G1 arrest by reducing cyclin D1 and CDK6 and blocking p38/ERK signaling. Furthermore, it induced autophagy *via* generating ROS and DNA damage through *H2AX* upregulation[99]. Another marine compound, 4-methyenedioxy-β-nitrostyrene (MNS) also inhibited the migration of TNBC cells by disrupting the focal adhesion complex as well as a network of actin stress fibers *via* reducing β1 integrin-dependent phosphorylation of FAK (focal adhesion kinase) and paxillin[100]. Yang *et al*[101] reported that leucine aminopeptidase-3 (LAP-3) can inhibit the growth, invasion and migration of TNBC cells by targeting LAP-3 dependent expression of fascin and MMP-2/9. As these studies illustrate, marine compounds display a wide range of antineoplastic effects that could be exploited to develop novel drug modalities for BC.

Marine compounds with potential anti-BC activity from crustaceans

The crustacean shell is a rich source of chitin. Crab, the major crustacean, is highly recommended for cancer treatment in traditional medicine due to chitin, carotenoids (astaxanthin, AST), lutein, and β-carotene (Table 2)[102]. The hydro-alcoholic extract of crab shells can reduce the proliferation of MCF-7 cells by stimulating apoptosis and decreasing nitric oxide production. This study reported that chitosan and its

Table 2 Marine compounds with potential anticancer activity against breast cancer from crustaceans

Marine compound	Chemical nature	Mechanism of action	Ref.
Hydro-alcoholic extract of crab	Chitosan and Astaxanthin	Reduced proliferation by inducing apoptosis and decreasing nitric oxide production	Moghtaderi *et al*[74]
Chitosan	Polysaccharide	(1) Reduced proliferation without affecting normal fibroblasts by inducing arrest at the G2M phase; and (2) Induced apoptosis by decreasing the expression of Bcl2 *via* elevation of p53 level	Resmi *et al*[104] and Mohamed *et al*[105]
Astaxanthin	Oligosaccharide	Induced PCD in BC cells *via* alteration in the cyclin D1, p53, Bax, and Bcl2 expression through inducing arrest at G0/G1 stage	Resmi *et al*[104] and Mohamed *et al*[105]
Chondroitin sulfate	Muco-polysaccharide	Inhibited angiogenesis by reducing tube formation *via* inhibiting the expression of VEGF	Chen *et al*[75]
β-carotene	Terpenoids	Stimulated apoptosis in BC cells by inducing the release of cytochrome C, increasing PPAR-γ, and p21 (WAF1/CIP1) expression and decreasing cyclooxygenase-2 expression through ROS generation	Moon *et al*[102]

PCD: Programmed cell death; BC: Breast cancer; VEGF: Vascular endothelial growth factor; ROS: Reactive oxygen species; PPAR-γ: Peroxisome proliferator-activated receptor gamma; WAF1/CIP1: Cyclin-dependent kinase inhibitor p21.

oligosaccharides, AST as well as selenium could be the principle compounds accountable for the anticancer activity of hydro-alcoholic extract of crab[103]. Chitosan from shrimp shell waste reduced the proliferation of MCF-7 cells without affecting normal fibroblasts by stimulating G2/M arrest and apoptosis, decreasing Bcl2 expression and upregulating p53 levels[104,105]. AST from shrimps, a marine crustacean, induced PCD in BC cells *via* alteration in expression of cyclin D1, Bax, p53, and Bcl2 through arrest at G0/G1 phase[106]. Chondroitin sulfate from shrimps inhibited angiogenesis by reducing tube formation *via* inhibiting the expression of VEGF[107]. β-carotene stimulated apoptosis in BC cells by inducing the release of cytochrome C, increasing PPAR-γ (peroxisome proliferator-activated receptor gamma), and p21(WAF1/CIP1) expression and decreasing cyclooxygenase-2 expression through ROS generation[108]. These studies suggest that bioactive compounds from marine crustaceans are potential targets of BC.

Marine compounds with potential anti-BC activity from marine fishes

The muscle and live tissues of marine fishes have been described as a source of anticancer compounds (Table 3). Epidemiological studies show that high consumption of fish and seafood in Asian countries correlates with a low prevalence of BC. A meta-analysis studies reported that high consumption of marine fish can reduce the risk of BC due to the presence of n-3 polyunsaturated fatty acids (PUFA) as well as α-linolenic acid[109]. Another epidemiological study reported a positive association between the reduction of invasive BC risk and intake of fatty fish but not with the intake of lean fish[110]. Some *in vitro* and animal studies also confirmed the inhibitory effect of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and n-3 PUFA from marine fish on BC growth. The intake of fish containing EPA and DHA showed a dose-dependent reduction of BC risk[111]. Two peptides (K092A and K092B) from marine fish stimulated apoptosis in BC cells by altering the cytoskeleton *via* targeting actin and tubulin, halting at the G2M phase and decreasing mitochondrial activity[112]. A tetrahydroisoquinoline alkaloid, trabectedin from tunicate, induced death receptor-mediated apoptosis by increasing the expression of TNF-related apoptosis-inducing ligand (TRAIL)-R1, -R2, Fas, TNF RI, and FADD (fas-associated protein with death domain) in BC cells[113]. Hence, marine compounds extracted from marine fish can display a wide and divers' range of anti-cancer effects and warrant further study to exploit their potential therapeutic properties in the context of BC.

Combination of marine compounds and chemotherapeutics

Marine compounds can resensitize resistant BC cells to chemotherapy (Table 4). For instance, marine-derived cyclic peptide, elisidepsin, and cisplatin or paclitaxel showed synergistic toxicity on BC cells by reducing the phosphorylation of Akt and inhibiting the MAPK pathway *via* targeting *ERBB* expression[114]. Sipholenol A increased the sensitivity of paclitaxel in BC cells by inhibiting P-glycoprotein (P-gp) and multidrug-resistant associated protein (MRP1)[115]. Permethyl ningalin B, resensitized BC cells to paclitaxel by inhibiting drug efflux activity of P-gp and increasing drug accumu-

Table 3 Marine compounds with potential anticancer activity from marine fishes and invertebrates against breast cancer

Marine compound	Chemical nature	Mechanism of action	Ref.
n-3 PUFA, α-linolenic acid	Fatty acids	Decreased the risk of BC	Moghtaderi *et al*[74]
EPA, DHA, n-3 fatty acids	Fatty acids	(1) Decreased the risk of BC; and (2) Inhibited growth BC cells	Resmi *et al*[104] and Mohamed *et al*[105]
K092A and K092B	Peptides	Induced apoptosis by altering the cytoskeleton *via* targeting actin and tubulin and halting cell cycle at the G2M and decreasing mitochondrial activity	Resmi *et al*[104] and Mohamed *et al*[105]
Tetrahydro-isoquinoline	Alkaloid	(1) Induced death receptor-mediated apoptosis by increasing the expression of TRAIL-R1, -R2, Fas, TNF RI, and FADD; and (2) Induced mitochondrial-mediated apoptosis by decreasing the of Bcl2 and Bcl-XL expression and increasing the Bax, Bad, cytochrome C and caspase 3 expression *via* increasing the ROS generation	Chen *et al*[75]

BC: Breast cancer; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; PUFA: Polyunsaturated fatty acids; TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand; TNF: Tumor necrosis factor; FADD: Fas-associated protein with death domain; ROS: Reactive oxygen species.

Table 4 Marine compounds with potential anticancer activity and chemotherapeutics against breast cancer

Marine compound	Chemical nature	Mechanism of action	Ref.
Elisidepsin	Cyclic peptide	With combination of cisplatin or paclitaxel showed synergistic toxicity on BC cells by reducing the phosphorylation of Akt and inhibiting the MAPK pathway *via* targeting ErbB expression	Moghtaderi *et al*[74]
Sipholenol A	Triterpene	Increased the sensitivity of paclitaxel in BC cells by inhibiting P-gp and MRP1	Resmi *et al*[104] and Mohamed *et al*[105]
Permethyl ningalin B	Pyrrole-containingcompound	Sensitized BC cells to paclitaxel by inhibiting drug efflux activity of P-gp and increasing drug accumulation	Resmi *et al*[104] and Mohamed *et al*[105]
Trabectedin	Alkaloid	Combination with cisplatin or paclitaxel or doxorubicin showed an additive effect in the preclinical system	Chen *et al*[75]
Marine polysaccharide	Polysaccharide	In combination with cisplatin, synergistically inhibited the proliferation and migration by blocking the MMP-2 and MMP-9 expression	Chen *et al*[75]
Iturin A	Cyclo-lipopeptide	In combination with docetaxel, substantially sensitized docetaxel-resistant TNBC cells by reducing proliferation *via* massive arresting at the G1 stage and activating caspase 3 as well as inhibiting Akt and its downstream signaling pathways	Dey *et al*[119]
Renieramycin M	Tetrahydro-isoquinoline	Sensitized MCF-7 cells synergistically to doxorubicin by promoting doxorubicin-induced DNA damage, cell cycle arrest, and apoptosis *via* downregulating ErbB/PI3K-Akt, integrin, and focal adhesion signaling	Tun *et al*[120]
Sulfated polysaccharide	-	In combination with pH-sensitive DOX releasing nanosystem inhibited growth and metastasis of BC cells in tumor-bearing mice	Zhang *et al*[121]
Papuamine	Pentacyclic alkaloid	Inhibited colony formation of BC cells by targeting activation of JNK	Kanno *et al*[122]

BC: Breast cancer; MAPK: Mitogen-activated protein kinase; P-gp: P-glycoprotein; MRP: Multidrug-resistant associated protein; MMP: Matrix metalloproteinase; TNBC: Triple-negative breast cancer; DOX: Doxorubicin; JNK: c-Jun N-terminal kinase.

lation[116]. Preclinical studies reported that trabectedin (ET-743) used with a combination of cisplatin or paclitaxel or doxorubicin displayed an additive effect in the preclinical system[117]. Moreover, marine polysaccharides, in combination with cisplatin, synergistically inhibited TNBC cells growth and migration by blocking MMP-2 and MMP-9 expression[118]. Iturin A, combined with docetaxel, substantially sensitized docetaxel-resistant TNBC cells by reducing proliferation *via* massive arrest at the G1 phase, increasing caspase 3 levels and inhibiting Akt and its downstream signaling pathways[119]. Renieramycin M, a tetrahydroisoquinoline from blue sponges, sensitized MCF-7 cells synergistically to doxorubicin by promoting doxorubicin-induced DNA damage, cell cycle arrest, and apoptosis *via* downregulating ERBB/PI3K-Akt, integrin, and focal adhesion signaling[120]. Zhang *et al*[121] demonstrated that marine sulfated polysaccharide in a combination of pH-sensitive doxorubicin releasing nanosystem inhibited growth and progression of BC cells in tumor-bearing mice. Furthermore, papuamine, a marine compound from sponges,

inhibited the colony-forming ability of BC cells at a subtoxic concentration by targeting activation of JNK[122]. Hence, marine compounds can resensitize refractory BC cells to chemotherapy and display promising potential as therapeutic companion tools to overcome drug resistance in BC.

CONCLUSION

As this in-depth review shows, BC is a highly complex and heterogenous disease and remains one of the major causes of female mortality worldwide. Hence, novel diagnostic tools and drug targets are needed to develop effective management and treatment regimens that overcome chemoresistance and increase survival. The search for novel treatment BC strategies has prompted the clinical investigation of various anticancer compounds extracted from marine microorganisms. As per our detailed survey, marine compounds display promising antineoplastic therapeutic potential for BC. In fact, their enormous genetic diversity supports a wide range of anti-cancer and immunomodulatory mechanisms of action that could be harnessed for BC treatment. Future pharmaceutical development is required to improve the pharmacokinetic properties of these compounds and evaluate their efficacy. Although marine compounds represent a relatively young discipline, their chemical diversity, eco-friendliness and therapeutic properties render them a promising new source of anticancer drugs. The use of novel bioengineering tools will enormously help in improving their production and clinical development for the treatment of BC and other cancers.

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ISSN 1949-8454 (online)

World Journal of *Biological Chemistry*

World J Biol Chem 2021 May 27; 12(3): 38-51

Published by Baishideng Publishing Group Inc

WJBC

World Journal of Biological Chemistry

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Bimonthly Volume 12 Number 3 May 27, 2021

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World Journal of Biological Chemistry

Bimonthly Volume 12 Number 3 May 27, 2021

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INDEXING/ABSTRACTING

The *WJBC* is now abstracted and indexed in PubMed, PubMed Central, China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Ying-Yi Yuan*; **Production Department Director:** *Yun-Xiaojian Wu*; **Editorial Office Director:** *Yun-Xiaojiao Wu*.

NAME OF JOURNAL
World Journal of Biological Chemistry

ISSN
ISSN 1949-8454 (online)

LAUNCH DATE
July 26, 2010

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Vsevolod Gurevich, Chun-Peng Wan

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1949-8454/editorialboard.htm

PUBLICATION DATE
May 27, 2021

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World Journal of Biological Chemistry

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World J Biol Chem 2021 May 27; 12(3): 38-51

DOI: 10.4331/wjbc.v12.i3.38

ISSN 1949-8454 (online)

ORIGINAL ARTICLE

Basic Study

Polyglutamylase activity of tubulin tyrosine ligase-like 4 is negatively regulated by the never in mitosis gene A family kinase never in mitosis gene A -related kinase 5

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Author contributions: Melo-Hanchuk TD performed the experiments and interpreted them together with Kobarg J; both authors wrote the manuscript; Kobarg J supervised the project.

Supported by Fundação de Amparo à Pesquisa do Estado São Paulo (FAPESP; São Paulo, Brazil) through Grant Temático, No. 2017/03489-1.

Institutional review board statement: The study was reviewed and approved by the Institutional review board of UNICAMP.

Conflict-of-interest statement: Both authors declare they have no conflict of interest.

Data sharing statement: Data will be made available upon reasonable request.

Open-Access: This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative

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Abstract

BACKGROUND

Tubulins, building blocks of microtubules, are modified substrates of diverse post-translational modifications including phosphorylation, polyglycylation and polyglutamylation. Polyglutamylation of microtubules, catalyzed by enzymes from the tubulin tyrosine ligase-like (TTLL) family, can regulate interactions with molecular motors and other proteins. Due to the diversity and functional importance of microtubule modifications, strict control of the TTLL enzymes has been suggested.

AIM

To characterize the interaction between never in mitosis gene A-related kinase 5 (NEK5) and TTLL4 proteins and the effects of TTLL4 phosphorylation.

METHODS

The interaction between NEK5 and TTLL4 was identified by yeast two-hybrid screening using the C-terminus of NEK5 (a.a. 260–708) as bait and confirmed by immunoprecipitation. The phosphorylation sites of TTLL4 were identified by mass spectrometry and point mutations were introduced.

RESULTS

Here, we show that NEK5 interacts with TTLL4 and regulates its polyglutamylation activity. We further show that NEK5 can also interact with TTLL5 and TTLL7. The silencing of NEK5 increases the levels of polyglutamylation of proteins by increasing the activity of TTLL4. The same effects were observed after the expression of the catalytically inactive form of NEK5. This regulation of TTLL4

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Manuscript source: Unsolicited manuscript

Specialty type: Biochemistry and molecular biology

Country/Territory of origin: Brazil

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: October 25, 2020
Peer-review started: October 25, 2020
First decision: December 24, 2020
Revised: January 6, 2021
Accepted: February 25, 2021
Article in press: February 25, 2021
Published online: May 27, 2021

P-Reviewer: Grawish M
S-Editor: Zhang L
L-Editor: Webster JR
P-Editor: Yuan YY

activity involves its phosphorylation at Y815 and S1136 amino acid residues.

CONCLUSION

Our results demonstrate, for the first time, the regulation of TTLL activity through phosphorylation, pointing to NEK5 as a potential effector kinase. We also suggest a general control of tubulin polyglutamylation through NEK family members in human cells.

Key Words: Kinase; Polyglutamylation; Never in mitosis gene A-related kinase 5; Tubulin tyrosine ligase-like 4; Microtubules; Post translational regulation

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Core Tip: Tubulins are modified extensively by post-translational processes such as polyglutamylation. Considering the diversity of microtubule polyglutamylation and the existence of many non-tubulin substrates, it is important to understand how the effector enzymes, the tubulin ligase-like (TTLL) proteins, are regulated. TTLL4 interacts with never in mitosis gene A (NIMA)-related kinase 5, a member of the mitotic NIMA-related kinases. We demonstrate that NIMA-related kinase 5 is a potential regulator of polyglutamylation through the control of TTLL4 activity. Here we show, for the time, the regulation of TTLL4 activity through phosphorylation, and demonstrate the potential control of polyglutamylation through NEK family members in human cells.

Citation: Melo-Hanchuk TD, Kobarg J. Polyglutamylase activity of tubulin tyrosine ligase-like 4 is negatively regulated by the never in mitosis gene A family kinase never in mitosis gene A - related kinase 5. *World J Biol Chem* 2021; 12(3): 38-51
URL: https://www.wjgnet.com/1949-8454/full/v12/i3/38.htm
DOI: https://dx.doi.org/10.4331/wjbc.v12.i3.38

INTRODUCTION

The microtubule cytoskeleton is essential for the internal organization of eukaryotic cells and is involved in cell division, differentiation and active transport processes. The great diversity of tubulin inside the cells is due to the expression of tubulin isotypes and a large array of post-translational modifications including acetylation/deacetylation, removal and addition of tyrosines to the C-terminal tail of αα-tubulin, phosphorylation, polyglycylation and polyglutamylation[1-5].

Polyglutamylation was initially discovered in tubulins and consists of the addition of glutamate side chains to acceptor glutamate residues in the main chain of the modified proteins[1,2]. Glutamylation is found on microtubules of cilia and flagella[6], centrioles, basal bodies and centrosomes[7]. The levels of glutamylated tubulin of the mitotic spindle are increased during cell division[8-10].

In vitro experiments have shown that polyglutamylation of α- or β-tubulin can act as a regulator of microtubule interactions with microtubule-associated proteins[11,12] and motor proteins[11,13-15]. The enzymes responsible for polyglutamylation are members of the tubulin ligase-like (TTLL) family[16,17]. Their name is derived from tubulin tyrosine ligase[18], a related tubulin-modifying enzyme[19], with which they share a strong sequence homology. A total of nine enzymes that can catalyze polyglutamylation have been identified[16,17]. TTLL4, 5, 6, 7, 11, and 13 generate tubulin glutamylation when overexpressed in mammalian cells. Studies of the catalytic activity of TTLL family members revealed that these enzymes have intrinsic preferences for either α- or β-tubulin and for the generation of either short or long glutamate chains[17]. Polyglutamylation is not restricted to tubulins. Several substrates have been identified, including nucleosome assembly proteins such as nucleosome assembly protein (NAP) 1 and 2 among others[20,21]. From the family of glutamylases, TTLL4 and TTLL5 glutamylases have also been demonstrated to glutamylate non-tubulin proteins[21].

Kinases are key regulators of many cellular processes, and could thus also be potential regulators of polyglutamylases. The never in mitosis gene A (NIMA)-related

kinases (NEKs) are mammalian enzymes, which were identified by their high identity (40%-45%) to the *Aspergillus nidulans* mitotic protein NIMA within their catalytic domain[22-24]. In humans, the NEK family is represented by 11 members that have been functionally associated to one of the three core functions established for this family in mammals: (1) Centrioles/mitosis; (2) Primary ciliary function/ciliopathies; and (3) Deoxyribonucleic acid (DNA) damage response[25].

The participation of NEKs in the microtubule-related process is broadly described; however, the first link with polyglutamylation was identified during the purification of polyglutamylase from the protist *Crithidia fasciculata*[26]. Purified extracts of NIMA-related kinase were capable of glutamylating tubulins *in vitro*. The later discovery of TTLLs as tubulin polyglutamylases strongly suggested that extracts of NIMA-related kinase were associated with a *Crithidia fasciculata* TTLL enzyme.

Here, we describe the identification and confirmation of the interaction between NEK5 and TTLL4. This prompted us to investigate the activity of TTLL4. In a broader context, our analysis showed, for the first time, a mechanism for direct regulation of a glutamylase from the TTLL family through phosphorylation, pointing to NEK5 as a potential candidate as an effector kinase.

MATERIALS AND METHODS

DNA constructs, mutagenesis, short hairpin ribonucleic acid and stable cells

TTLL4 full size, TTLL4 C347-1193, TTLL4 C555-1193, TTLL4 C606-1193, TTLL5, TTLL5 N800 and TTLL7 genes were amplified from mouse brain or testis complementary DNA (cDNA) and previously cloned in a vector containing a C-terminal enhanced yellow fluorescent protein (EYFP) tag[17]. The TLL domains of TTLL4, TTLL5 and TTLL7 have many conserved residues (Supplementary Figure 1). TTLL5 N800 corresponds to the first 800 N-terminal amino acids and TTLL5 is the full size, but both are active versions of TTLL5. Point mutations were introduced by the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, LaJolla, CA, United States) to generate the NEK5-kinase-dead (NEK5-KD) (K33A-inactive mutant of NEK5), TTLL4-T813E, TTLL4-T815A, TTLL4-Y815F, TTLL4-Y815A, TTLL4-S912E, TTLL4-S960E, TTLL4-S960A, TTLL4-T968E, TTLL4-S1136A, TTLL4-S1139E and TTLL4-S1136A. All mutants were confirmed by DNA sequencing.

The production of HEK293 silenced for NEK5 was carried out by short hairpin ribonucleic acid (shRNA) lentiviral particles (Santa Cruz Biotechnology, Incorporated). Stable cells were obtained with the Flp-In. System. The procedure to obtain all the cell lines has been described previously[27]. Stable cells expressing NEK5 were used as controls.

Antibodies

The following antibodies were used for both immunoprecipitation and Western blot assay: mouse anti-NEK5 (SC130492), mouse anti-green fluorescent proteins (GFP) (G1546), mouse GT335 (Adipogen) and anti-TTLL4 (Novus Biologicals).

Yeast two-hybrid screen

The cDNA encoding the C-terminus of NEK5 (a.a. 260–708) was cloned in the pGBKT7. The yeast two-hybrid screen was performed following the Matchmaker™ Gold Yeast Two-Hybrid System (Clontech Laboratories, Incorporated, Mountain View, CA, United States) according to the manufacturer's instructions. Selective medium without tryptophan, leucine, adenine and histidine but containing aureobasidin A antibiotic and X-α-Gal was used to screen interactors from the human universal cDNA library. To identify the "prey" genes, the DNA was extracted and sequenced.

DNA transfection

TTLL genes amplified from mouse brain or testis cDNA were used because mouse proteins show a better expression level. Mouse and human TTLL4 protein are very similar and share 79.98% identity and 85.80% similarity (Supplementary Figure 2). Expression plasmids were transfected using JetPEI (Polyplus transfection) or homemade PEI.

Immunoprecipitation

Cells were lysed in RIPA buffer supplemented with a protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany), subjected to freeze/thaw, submitted to

three cycles of 5 min in an ultrasound bath (UltraSonic Clear 750, UNIQUE) for complete pellet suspension and centrifuged at 20000 × g for 30 min at 4°C. Protein concentration was determined using the Bradford method[28]. The supernatants were used for immunoprecipitation. Briefly, the supernatant was added to GFP-Trap® (ChromoTek GmbH, Germany) coupled to agarose beads or anti-NEK5, previously coupled to G-sepharose beads and incubated overnight. The beads were collected, washed five times with wash buffer (10 mmol/L Tris/Cl pH: 7.5; 150 mmol/L NaCl; 0.5 mmol/L ethylene diamine tetraacetic acid) and then eluted with 2 × sodium dodecyl sulfate (SDS)-sample buffer (120 mmol/L Tris/Cl pH: 6.8; 20% glycerol; 4% SDS, 0.04% bromophenol blue; 10%β-mercaptoethanol). The proteins were immunoblotted onto an Immobilon-P membrane (Millipore Corporation, Bedford, MA, United States) and probed with antibodies. Blots were developed using an emitter coupled logic (ECL) chemiluminescence kit (Amersham Biosciences).

Immunoblotting analysis

50 μg of protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto an Immobilon-P membrane (Millipore Corporation, Bedford, MA, United States). In the case of mammalian tubulin, a special protocol was used, as described in Eddé *et al*[1]. The protein bands were probed using the following primary antibodies diluted in 3% bovine serum albumin blocking solution: mouse anti-GFP (1:1000, Sigma), mouse anti-NEK5 (1:500, Santa Cruz Biotechnology) and rabbit anti-TTLL4 (1:500, Novus Biologicals). The monoclonal mouse GT335 (anti-polyglutamylation, 1:1000) and monoclonal mouse 12G10 (anti-tubulin, 1:500) were produced in the laboratory[17,29] and were used at 1:1000 dilutions. Detection of the protein bands was performed with HRP-labeled anti-rabbit or anti-mouse IgG (1:5000; Sigma), followed by the chemiluminescent ECL Western Blotting System (ECL Western blot detection kit; GE Healthcare). Protein bands were quantified using ImageJ software (National Institutes of Health, United States).

Mass spectrometry analysis

The proteins from the GFP affinity-purified fraction were separated by SDS-PAGE and stained using Colloidal blue. Protein bands at the expected size for TTLL4 and TTLL5 were excised from the gel and submitted to in-gel trypsin or chymotrypsin digestion. Peptides were concentrated and analyzed by MS/MS on a Q-TOF II mass spectrometer (Micromass Limited, Manchester, United Kingdom). Data analysis was performed with Mascot (Matrix Science Limited, London, United Kingdom) against the National Center for Biotechnology Information database.

Flow cytometry and sorting

Transfected control and shRNA-NEK5 cells were collected and dissociated using trypsin. Cells were suspended in saline solution, passed through a 0.45 μmol/L filter and analyzed by flow cytometry. Gates were created to separate single cells using the FSC and SSC parameters followed by a new gate for yellow fluorescent protein (YFP) positive cells. The sorting was performed by separation in positive and negative YFP cells on a binding domain-FACS Aria flow cytometer using FACS Diva 6.0 software and data were analyzed using FlowJo (Tree Star Incorporated, Ashland, OR, United States).

RESULTS

TTLL4 and NEK5 physically interact

The molecular functions of NEK5 are still not fully elucidated. In order to identify putative functional partners of NEK5, the yeast two-hybrid experiment was performed. The C-terminal fragment of NEK5 (a.a. 260-708) was used as a bait protein fused to the Gal4-DNA binding domain against a human cDNA library. The prey proteins from the library were expressed as a fusion to a Gal4-activation domain (AD)[30,31]. In this stringent protein-protein interaction screen, four independent reporter genes must be activated (*AUR1-C*, *ADE2*, *HIS3*, and *MEL1*). The screening resulted in the identification of TTLL4 (amino acid residues 895 to 1189) as NEK5's interactor (Figure 1A). The controls of yeast two-hybrid screening are presented in the supplementary material (Supplementary Figure 3). The interaction between endogenous TTLL4 and NEK5 was confirmed only for the C-terminal regulatory (260-708) region of NEK5 (Figure 1A). This is possibly due to a closed folded state of full-length

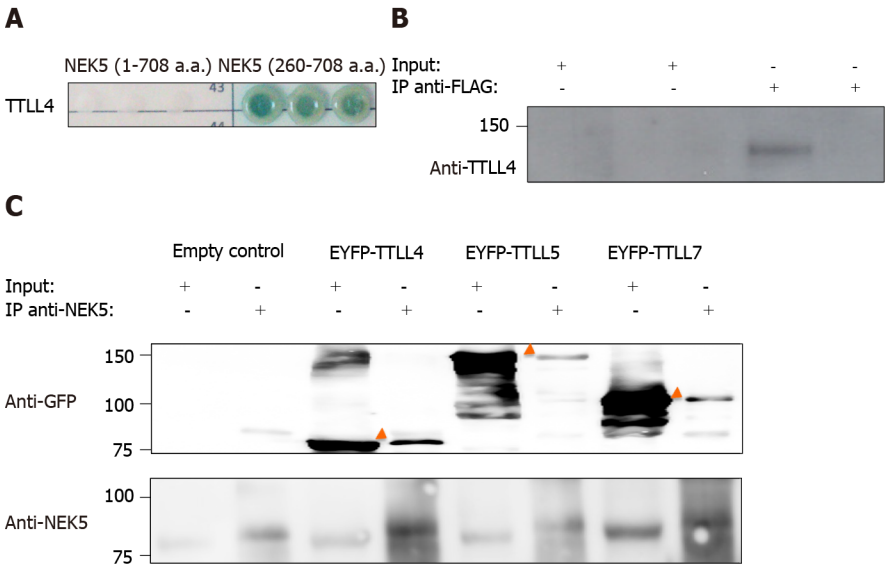

Figure 1 Never in mitosis gene A-related kinase 5 interacts with tubulin tyrosine ligase-like 4. A: Yeast colonies grew in selective medium SD-WLAH with aureobasidin and X-α-Gal. In addition to the reporter gene *Auri-C* we also activated the genes *MEL1*, *ADE2* and *His3* as indicators of interaction between the proteins. Controls of the yeast two-hybrid assay are presented in Supplementary Figure 3; B: Stable cells expressing never in mitosis gene A-related kinase 5 (NEK5)-FLAG were induced with 2 μg/mL of tetracycline and fractionated in cytoplasm and mitochondria. 1 μg of protein was used to immunoprecipitate NEK5 using an anti-FLAG resin, after elution the proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. Tubulin tyrosine ligase-like (TTLL) 4 was co-immunoprecipitated with NEK5 as assessed with anti-TTLL4 antibody. Controls of cell fractioning are presented in Supplementary Figure 5; and C: NEK5 interacts with TTLL4, TTLL5 and TTLL7 by immunoprecipitation. HEK293T were transfected with EYFP-TTLL4, EYFP, TTLL5 and EYFP-TTLL7. Endogenous NEK5 was immunoprecipitated using anti-NEK5 antibody. Immunoprecipitated proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. The identification of TTLLs was assessed by an anti-green fluorescent protein antibody to confirm interaction between the proteins. NEK5: Never in mitosis gene A-related kinase 5; GFP: Green fluorescent proteins; EYFP: Enhanced yellow fluorescent protein; TTLL: Tubulin tyrosine ligase-like.

NEK5, where the N-terminal kinase domain folds back to the regulatory C-terminal region (site of interaction with TTLL4), thereby preventing interaction. Such an auto-inhibitory conformation has been described for several other NEKs (*e.g.* NEK8 and NEK9). Additional interactions or post-translational modification may also be required to expose the C-terminus of NEK5 thus allowing the interaction with TTLL4. In summary, the most plausible explanation for the lack of interaction of TTLL4 with the full-length NEK5 in the yeast two-hybrid screen is the possibility that yeast lacks the biochemical apparatus necessary to induce the putative NEK5 conformational changes necessary for the interaction to occur. Using immunoprecipitation we confirmed the interaction of endogenous full-length TTLL4 and full-length NEK5 fused to FLAG (Figure 1B).

NEK5 interacts with other members of the TTLL family

Polyglutamylase from the TTLL enzyme family displays reaction preferences such as the length of side chains (long or short) and substrates (α- or β-tubulin)[17,32]. TTLL4, TTLL5 and TTLL7 are involved in the initiation of the polyglutamylation chain but with different substrate specificity. While TTLL4 has a preference for α-tubulin and NAP proteins, TTLL5 and TTLL7 have a preference for α- and β-tubulin, respectively[17]. The diversity of specificity suggests alternative interaction partners as well as mechanisms of regulation. To investigate if the interaction of NEK5 with TTLLs is exclusive for TTLL4, HEK293T cells were transfected with EYFP-TTLL4, EYFP-TTLL5 and EYFP-TTLL7 and the proteins were immunoprecipitated by the anti-NEK5 antibody. The protein extract was analyzed by Western blot using the anti-GFP antibody in order to identify interaction of NEK5 with other TTLL members. Surprisingly TTLL4, 5 and 7 were immunoprecipitated with NEK5 as observed in Figure 1C, showing that NEK5 interacts not only with TTLL4 but also with other members of this family and its potential effects on regulation of TTLL activity may not be exclusive for this protein but more related to the process of polyglutamylation as a whole. Therefore, this work opens up a vast perspective for more detailed studies related to the mechanisms underlying the regulation of TTLLs through phosphorylation triggered by NEK kinases.

NEK5 interferes negatively with TTLL4 and TTLL5-mediated polyglutamylation

TTLLs are responsible for the polyglutamylation of tubulin as well as other proteins. As we have shown that NEK5 and TTL4 interact (Figure 1A and B), we hypothesized that NEK5 could regulate TTLL4 activity. To confirm this, cells stably expressing the inactive version of NEK5-KD, as well as cells silenced for NEK5 (shRNA-NEK5) and control cells were transfected with TTLL4 and TTLL5 and the polyglutamylation profiles of cell lysates were analyzed by Western blot using GT335, a glutamylation-specific antibody.

Cells expressing NEK5 were used as controls and showed low levels of all the polyglutamylated protein bands (Figure 2A, lane 1). The catalytically inactive NEK5-KD cells, on the other hand, showed increased levels of protein polyglutamylation (Figure 2A, lane 2). The difference in polyglutamylation status between controls *vs* either shNEK5 knockdown or NEK5-KD was more pronounced upon overexpression of TTLL4 (Figure 2A, compare lane 4 with lane 5 and 6) or TTLL5 (Figure 2A, compare lane 7 with lanes 8 and 9).

TTLL4 and TTLL5 have preferences for glutamylation of β and α-tubulin, respectively[17]. The ratio of α and β glutamylated-tubulin was also altered after TTLL4 and TTLL5 transfection in the presence or absence of functional NEK5. For example, despite the preference of TTLL5 for α-tubulins, upon decreased expression of functional NEK5, cells transfected with TTLL5 showed that not only the level of polyglutamylation of all protein substrates was increased, but β-tubulin also turned out to be an additional new target (Figure 2A, lanes 7 *vs* 9). TTLL4 on the other hand, even in the absence of NEK5 (Figure 2A, lane 5) or in the presence of catalytically non-functional NEK5 (lane 6) continued to prefer β-tubulin as substrate.

Although only TTLL4 has been identified as a NEK5 interactor in the yeast two-hybrid screen, the inhibitory effects of NEK5 on TTLL activity were also observed on TTLL5, suggesting that, NEK5 may play a regulatory role on more than one member of the TTLL family (Figure 2A). Polyglutamylation levels of other than tubulins have also been altered in the absence of functional NEK5 and upon overexpression of TTLL4 or TTLL5 (Figure 2A and B).

The knockdown of NEK5 increases the levels of polyglutamylated proteins

The putative catalytic region of TTLL4 is subdivided into two sub-domains: one common to all TTLLs and TTL, called "core TTL domain" (green region in Figure 3A) and a conserved bipartite upstream region called the "extended TTL domain" (purple and light blue regions in Figure 3A), which is necessary for TTLL4 activity[17].

The TTLL4 protein fragment found to interact with NEK5 in the yeast two-hybrid screen is localized in the C-terminal region between residues 895-1189. This suggests that the possible regulatory region of TTLL4 partially coincides with the catalytic TTL domain (a.a. 599-942). In order to map regions of TTLL4 required for its polyglutamylation activity and to test the influence of NEK5 on the activity of TTLL4 we compared the activity of the full-length protein and 3 deletion mutants.

Expression of the catalytically inactive NEK5 (Figure 3C) or the depletion of NEK5 expression (Figure 3D) resulted in an increased polyglutamylation activity for all TTLL4 truncated versions, except for the mutation C606-1193 (Figure 3C and D, lane 5), which is the inactive version, because it is missing the "extended domain" (Figure 3B-D, lane 5).

The overexpression of TTLL4 leads to the polyglutamylation of tubulin and many non-tubulin substrates in the presence of NEK5-KD and shRNA-NEK5. On the other hand, TTLL7, TTLL5 full size or its shortest version (TTLL5-N800) overexpression lead to more pronounced differences in polyglutamylation levels of tubulins (Figure 3C and D). The presence of NEK5 reduced the polyglutamylation of tubulins not only after TTLL4 transfection, but also TTLL7 and TTLL5, suggesting that the regulation by NEK5 may not be exclusive for TTLL4.

In order to confirm the regulatory role of NEK5 on the enzymatic activity of TTLL4 and TTLL5 toward tubulins, we performed an *in vitro* polyglutamylation assay. Purified tubulins from mouse brains were subjected to *in vitro* polyglutamylation assays with extracts from control and shRNA-NEK5 expressing cells, transiently transfected with EYFP-TTLL4 and EYFP-TTLL5. Once again the activity level of TTLL4 was increased in shRNA-NEK5-cell extract and reduced in the presence of NEK5 (Supplementary Figure 4). Inactive versions of TTLL4 and TTLL5 were used as a control in this experiment. Furthermore, NEK5 did not affect the activity level of TTLL5 using the *in vitro* assay (Supplementary Figure 4).

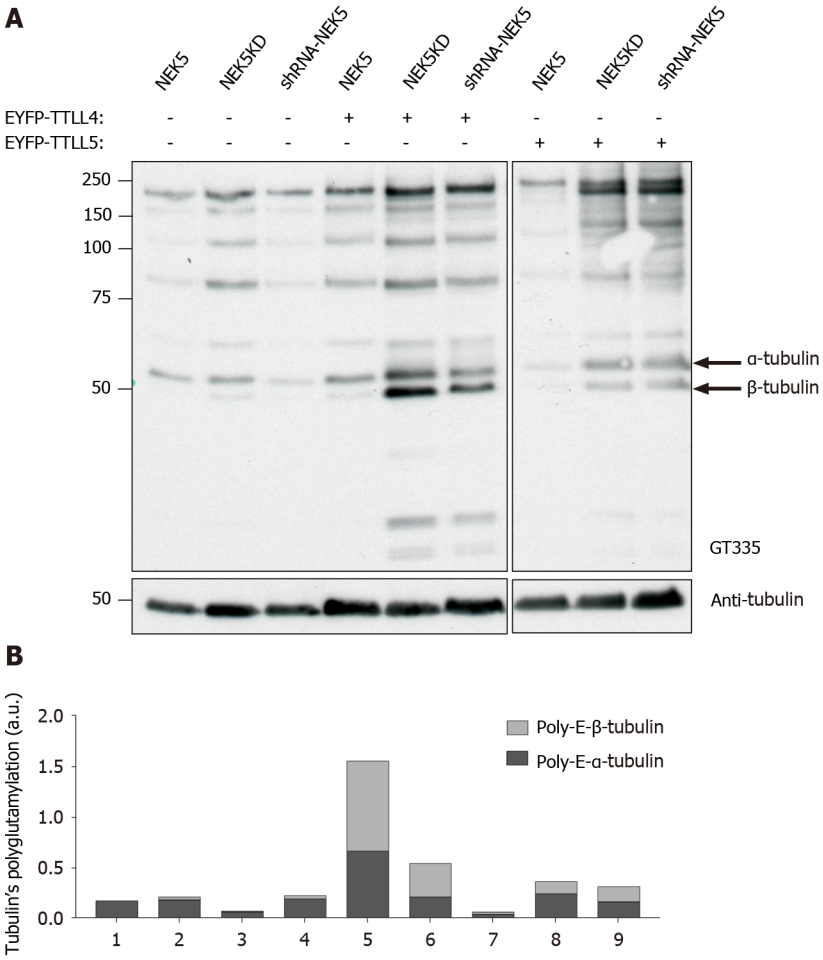

Figure 2 Never in mitosis gene A-related kinase 5 changes the tubulin tyrosine ligase-like 4 and tubulin tyrosine ligase-like 5-mediated polyglutamylation. A: Analysis by Western blot of tubulin tyrosine ligase-like 4 and 5 in polyglutamylation in cells that stably express either never in mitosis gene A-related kinase 5 (NEK5) or NEK5-kinase-dead (catalytically inactive) or cells silenced for NEK5 expression. The graph next to the Western blot corresponds to the intensity of polyglutamylated α- and β-tubulins selected among the bands labeled by GT335 antibody which were measured and normalized to total α-tubulin. The arrows indicate α- and β-tubulins polyglutamylated used in this graph; and B: The intensity (area of the bands) of Western blot of the figure presented in "c" was measured using ImageJ software. NEK5: Never in mitosis gene A-related kinase 5; EYFP: Enhanced yellow fluorescent protein; TTLL: Tubulin tyrosine ligase-like.

TTLL4 and TTLL5 are phosphorylated at Ser, Thr and Tyr sites

According to the Uniprot database, TTLL4 is phosphorylated on S691 and S696 residues and using the Kinase-specific Phosphorylation Site Prediction tool GPS 3.0[33] several other residues could also be phosphorylated. The amino acid residues with the highest score for phosphorylation were S1184, S1117, S1125 and S1151.

The prediction of TTLL4 phosphorylation sites associated with its inactivation in the presence of NEK5 suggests that NEK5 could phosphorylate TTLL4 directly or indirectly (through the phosphorylation/interaction with the effector kinase), thereby inhibiting its activity. To identify the possible phosphorylation sites in TTLL4 and TTLL5, stably NEK5-expressing cells and shRNA-NEK5 cells were transiently transfected with EYFP-TTLL4 or EYFP-TTLL5 (Figure 4A, only the TTLL4 experiment is shown). As the efficiency of the transfection is low, transfected cells were submitted to sorting by flow cytometry to enrich the cells expressing EYFP-TTLL4 or EYFP-TTLL5. The positive (TTLL4$^+$ or TTLL5$^+$) and negative (TTLL4$^-$ or TTLL5$^-$) cells were collected in different tubes, lysed and the proteins separated by SDS-PAGE and analyzed for polyglutamylation levels by Western blot (Figure 4A).

TTLL4$^+$ cells showed an increase in polyglutamylation levels, especially of tubulins, when NEK5 was depleted (Figure 4, compare lane 3 with lane 2). As expected, cells negative for YFP signal (TTLL4$^-$) showed undetectable levels of polyglutamylation (Figure 4A, lanes 1 and 4). In the presence of NEK5, TTLL4, detected by the anti-GFP antibody, migrates as two bands, suggesting a phosphorylation event or another type of post-translational modification. The lower of those two bands is lost in cells in which NEK5 has been silenced (Figure 4A, upper panel, compare lanes 2 and 3). This could suggest the loss of phosphorylation of TTLL4 by NEK5.

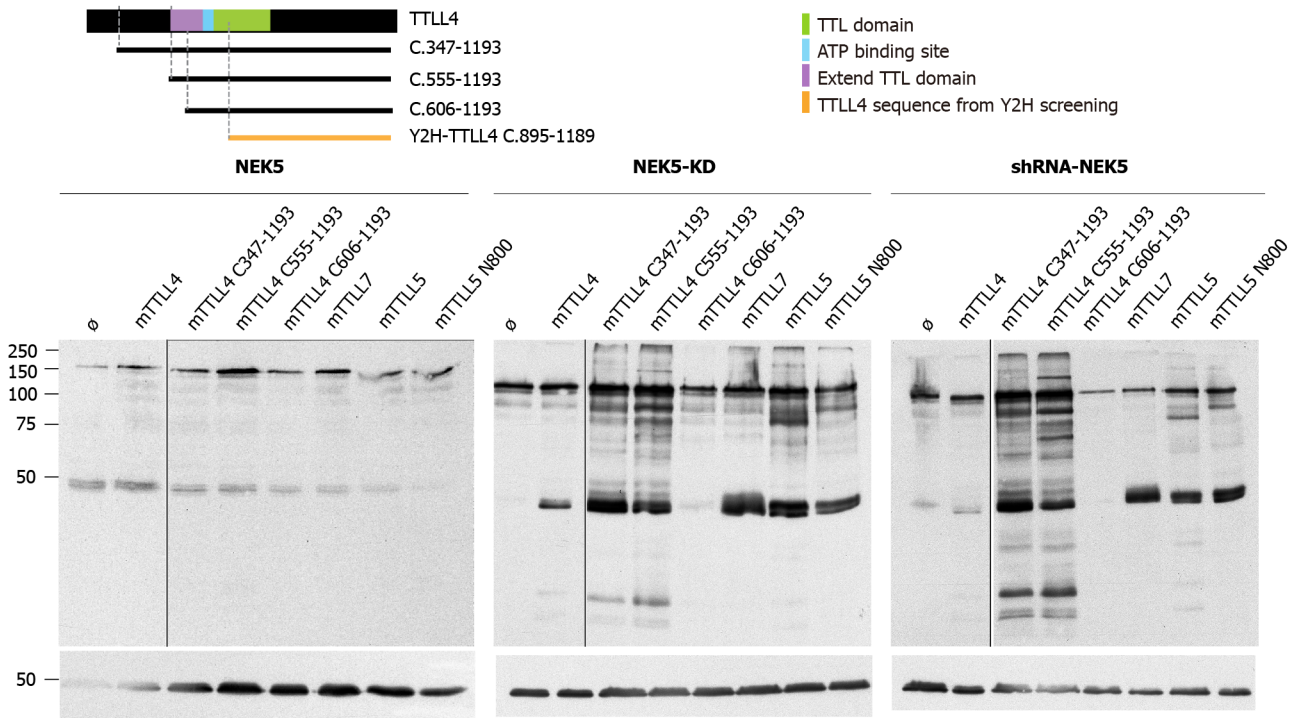

Figure 3 The effect of never in mitosis gene A-related kinase 5 on the activity of different truncations of the tubulin tyrosine ligase-like 4.

A: Schematic representation of the tubulin tyrosine ligase-like (TTLL) 4 constructs used. The sequences expressed for each truncated version used in this work are depicted as black bars underneath the pictograms of the full-length TTLL proteins. Domains unnecessary or required for autonomous activities are shown. Green area corresponds to tubulin tyrosine ligase domain (599-942 a.a.), the purple area is extended tubulin tyrosine ligase domain, the blue area is the ATP binding site and the orange construction is the TTLL4 sequence identified by yeast two-hybrid screening. See text for more details; and B-D Analysis by Western blot of truncations of TTLL4 and TTLL5 promoting polyglutamylation in (b) control, (c) cells stably expressing never in mitosis gene A-related kinase 5-kinase-dead or (d) cells silenced for expression of never in mitosis gene A-related kinase 5. NEK5: Never in mitosis gene A-related kinase 5; KD: Kinase-dead; shRNA: Short hairpin ribonucleic acid; EYFP: Enhanced yellow fluorescent protein; TTLL: Tubulin tyrosine ligase-like.

In order to identify the phosphorylation sites in EYFP-TTLL4 and EYFP-TTLL5 proteins, the proteins of YFP positive cells were immunoprecipitated using anti-GFP beads and separated by SDS-PAGE (Figure 4B). The bands corresponding to the expected sizes of TTLL4 and TTLL5 (identified with the arrows in Figure 4B) were excised, submitted to trypsin and chymotrypsin digestion and analyzed by mass spectrometry. The analysis of the TTLL4 amino acid sequence allowed the identification of amino acid residues T813, S912, S960, T968, S1136 and S1139 as phosphorylated. Some of the residues identified as phosphorylated in TTLL4 are conserved in TTLL5 and TTLL7 (Supplementary Figure 1).

The activity of TTLL4 is regulated through phosphorylation

To evaluate the polyglutamylation activity of phosphorylated TTLL4, the residues T813, S912, S960, T968 and S1139 of EYFP-TTLL4 were mutated to glutamic acid (E), mimicking phosphorylation. For inactivation of the phosphosites the residues T813, Y815, S960, S1136 and S1139 were mutated to alanine (A) or phenylalanine (F) for the residue Y815. The mutated residues, as well as their location in the full-length protein, are represented in Figure 5A. To investigate the *in vivo* enzyme activity of TTLL4 mutants, we expressed EYFP-tagged TTLL4 mutants in HEK293T cells and the levels of polyglutamylation were analyzed by Western blot using the GT335 antibody (Figure 5B).

The expression of unphosphorylated mutants TTLL4-Y815F, TTLL4-Y815A and TTLL4-S1136A in HEK293T cells caused an important decrease in the polyglutamylation levels (Figure 5B, lanes 5, 6 and 11 compared to lane 2). In HeLa cells, the active TTLL4 has α-tubulin as the preferential substrate. However, here we showed that TTLL4-Y815F, TTLL4-Y815A and TTLL4-S1136A mutants reduced the polyglutamylation of not only α-tubulin but also β-tubulin and other substrates of TTLL4. The results indicate that the amino acid residues Y815 and S1136 are potential key residues in the regulation of TTLL4 activity through phosphorylation, switching on or off its polyglutamylation activity. Especially for the TTLL4-S1136A mutant, the polyglutamylation levels of α - and β-tubulin drop to undetectable levels, compared to untrans-

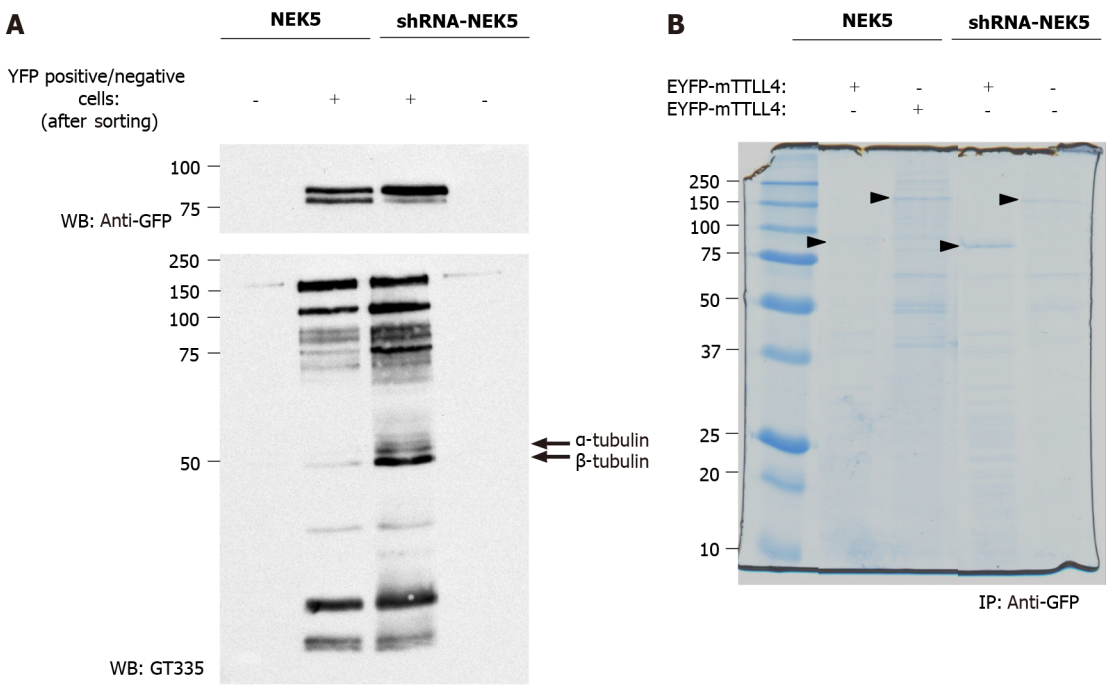

Figure 4 Presence of never in mitosis gene A-related kinase 5 decreases tubulin tyrosine ligase-like 4 activity in a phosphorylation dependent manner. Short hairpin ribonucleic acid-never in mitosis gene A-related kinase 5 and control cells transfected with enhanced yellow fluorescent protein (EYFP)-tubulin tyrosine ligase-like (TTLL) 4 or EYFP-TTLL5 were sorted in TTLL5/TTLL4^{+} and TTLL5/TTLL4^{-} cells by flow cytometry. A: Sorted EYFP-TTLL4 transfected cells were lysed and analyzed by Western-blot. Stable cells expressing never in mitosis gene A-related kinase 5 were used as controls; and B: The protein extracts from EYFP-mTTLL-4 or -5 positive cells were immunoprecipitated by anti-green fluorescent protein-beads for TTLL4 or TTLL5 enrichment. The bands indicated by the black arrow correspond to TTLL4 and TTLL5, respectively, and were cut, digested and submitted to mass spectrometry. NEK5: Never in mitosis gene A-related kinase 5; GFP: Green fluorescent proteins; shRNA: Short hairpin ribonucleic acid; EYFP: Enhanced yellow fluorescent protein; TTLL: Tubulin tyrosine ligase-like.

fected cells. Together, these data suggest that the regulation of TTLL4 may occur directly or indirectly through phosphorylation and/or interaction with another effector kinase intermediated by NEK5.

DISCUSSION

Tubulin polyglutamylation has been suggested to regulate the interactions of some microtubule-associated proteins and molecular motors with microtubules, thus selectively controlling specific microtubule functions inside cells[32]. To control these complex modification patterns in time and space, strict control of the polyglutamylation enzymes (TTLL) is expected. For instance, aberrations in the fine regulation of the polyglutamylation profile contribute to defects in ciliary beating in *Chlamydomonas reinhardtii*[34], *Tetrahymena*[35] and zebrafish[36]. Hyperglutamylation has been associated with neurodegeneration in mouse models and can be reversed by TTLL1 inhibition[37].

Despite the importance of controlling polyglutamylation in cells, the regulatory mechanism that controls the activity of TTLL enzymes has not yet been identified. In this study we show that NEK5 interacts and has the potential to phosphorylate TTLL4, regulating its polyglutamylation activity. The expression of the enzymatically inactive version of NEK5 as well as shRNA-NEK5 cells showed increased levels of polyglutamylation when transfected with TTLL4. We further showed that the phosphorylation of residues serine 1136 and tyrosine 815 of TTLL4 are key for its activity. When the phosphorylation of these residues was prevented, the activity of TTLL4 was dramatically reduced. Thus, we demonstrated a mechanism for the regulation of the activity of TTLL enzymes.

NEK kinases have multiple biological functions but, until now, information regarding their substrates was scarce. Although they were first described as serine/threonine kinases, recent studies are classifying some members of the family as tyrosine-kinases as well[38]. The phosphorylation of TTLL4 at the tyrosine 815 site seems to control its activity and our data pointed to NEK5 as the potential effector

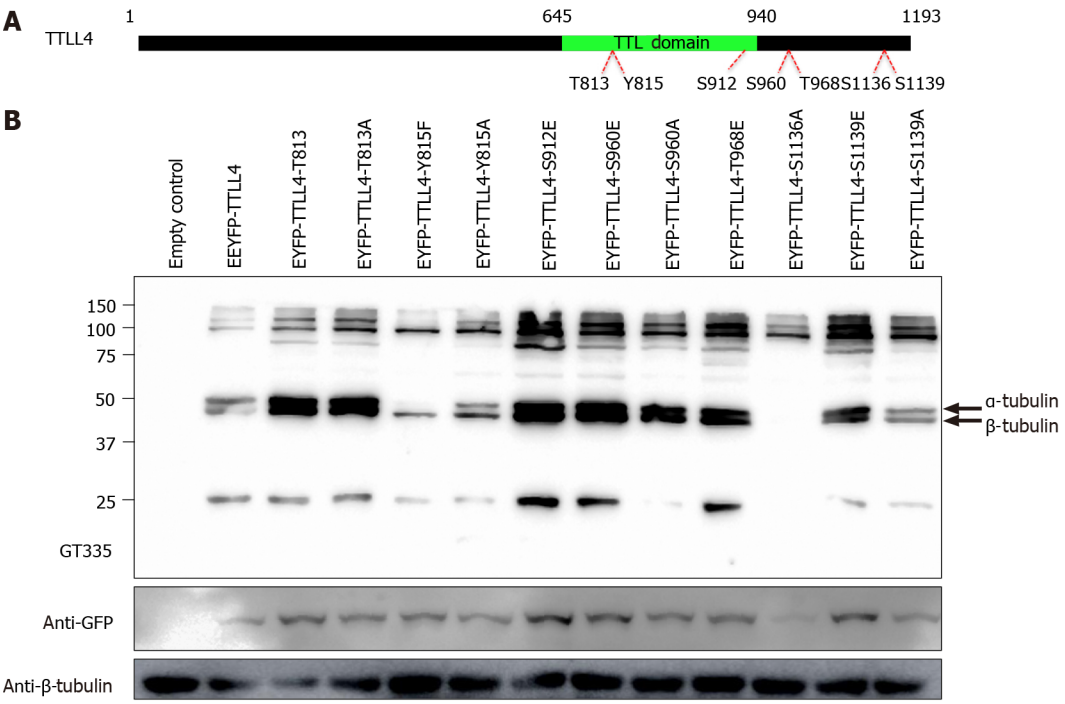

Figure 5 Tyrosine 815 is important for tubulin tyrosine ligase-like 4 activity. Based on the residues identified by mass spectrometry, phosphomimetic or inactivating point mutants (Ser/Thr/Tyr to Alanine, Phenylalanine) were generated. A: Schematic representation of the tubulin tyrosine ligase-like 4 and sites of target amino acids for point mutations. The green region corresponds to the tubulin tyrosine ligase domain; and B: HEK293T proteins of cells transfected with enhanced yellow fluorescent protein-tubulin tyrosine ligase-like 4 containing different mutations were separated by sodium dodecyl sulfate- polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes and stained with GT335, anti-green fluorescent proteins and anti-tubulin antibodies. GFP: Green fluorescent proteins; shRNA: Short hairpin ribonucleic acid; EYFP: Enhanced yellow fluorescent protein; TTLL: Tubulin tyrosine ligase-like; TTL: Tubulin tyrosine ligase.

kinase. Similar effects of enzyme inactivation by phosphorylation were observed for Protein-tyrosine Phosphatase 1B in response to insulin[39].

Our results indicate that the implications of NEKs in the regulation of TTLL enzymes could be of general importance for the control of tubulin glutamylation in cells. NEKs and TTLLs are often located or have functions in cilia, cytoplasmic microtubules or centrosomes. NEK kinases are extensively related to centrosomes and primary cilia[25,40-48]. Prosser *et al*[49] demonstrated that NEK5 is located at the centrosomes and is involved in ensuring its integrity during interphase and its separation during mitosis[49]. Centrosomes and basal bodies are highly polyglutamylated with long glutamate chains, and these modifications play a role in generating or maintaining the stability of this organelle[7,50].

Strikingly, we found that interactions between NEK and TTLL family members are of a general nature, which implies that the NEK5 enzyme might be involved in regulation of the activity of different TTLL members. Thus, specific localization of the NEK kinases, together with locally-controlled interactions with TTLL is expected to control how and when a NEK kinase phosphorylates a TTLL enzyme.

Our work thus delivers a strong incentive to further explore the relationship between NEK kinases and members of the TTLL family. The centrosomal localization, the role of tubulin polyglutamylation in centrosome stability[7], associated with the fact that some kinases from different organisms are involved in centrosome maturation and integrity[51,52], all indicate that the relation between these two families need to be analyzed more profoundly especially in the cell cycle context, midbody formation and ciliary functions. Understanding the role of NEK kinases in the tubulin code will be essential in understanding the signaling networks controlling this complex regulatory mechanism.

CONCLUSION

In conclusion, our data suggest a mechanism for regulation of TTLL activity through phosphorylation by a member of the NEK family. shRNA knockdown of NEK5 or

expression of a "KD" NEK5 in cells transfected with TTLL4 showed increased polyglutamylation levels, while HEK293T cells resulted in low polyglutamylation of proteins, especially tubulins. The regulation of TTLL4 occurs in the C-terminal region through the phosphorylation of its Y815 and S1136 residues and NEK5 emerged as the potential effector kinase.

ARTICLE HIGHLIGHTS

Research background

Enzymes of the tubulin tyrosine ligase-like (TTLL) family are responsible for the polyglutamylation of tubulins and many other protein substrates. The never in mitosis gene A-related kinases (NEKs) are protein kinases involved in diverse aspects of regulation of the cell cycle, microtubules, primary cilia and the deoxyribonucleic acid damage response. Previous data from the literature and protein interaction data between TTLLs and NEKs suggested a possible crosstalk and regulatory connection between these two protein families.

Research motivation

In a yeast two-hybrid assay for protein interactors of human NEK5, TTLL4 was identified as a partner. Additionally, a previously report showed that purified extracts of NEK in *Crithidia fasciculata* was capable of glutamylating tubulins *in vitro*. Here, we set out to confirm the interaction between NEK5 and TTLL4 and to explore possible functional consequences of this interaction.

Research objectives

Confirm and map the interaction between TTLL4 and NEK5 proteins and explore a possible regulation mechanism of TTLL4 through phosphorylation.

Research methods

We used transient transfection of full-length TTLL4, deletions and point mutants in cells with stable expression of NEK5 as well as knock down of NEK5 expression by short hairpin ribonucleic acid. Site-directed mutagenesis was used to generate a series of point mutants of TTLL4. The polyglutamylation activity of TTLL4 variants was assessed by Western blot, using antibody GT335, which detects polyglutamylation of protein substrates.

Research results

We confirmed the interaction between TTLL4 and NEK5 through yeast two hybrid screening and imunoprecipitation. Furthermore, we showed that expression of NEK5 interferes negatively in the polyglutamylation activity of TTLL4 towards tubulins and other protein substrates, whereas NEK5 knock down or over-expression of a kinase dead variant of NEK5 result in the contrary: An increase in TTLL4 activity. Mass spectrometry showed phosphorylation of TTLL4 on specific Thr, Ser and Tyr residues. Modification of some of these residues affected TTLL4 activity.

Research conclusions

We describe, for the first time, the interaction between members of the NEK and TTLL families. A mechanism for regulation of TTLL4 activity through phosphorylation has emerged and NEK5 is a potential effector kinase, affecting polyglutamylation of many substrates.

Research perspectives

This is the first evidence of a functional and regulatory crosstalk between TTLL and NEK protein families. Members of both families have localization and important functions at microtubules, primary cilia and centrosomes. The functional interplay of the protein families in the context of the cell cycle and microtubule functions should be explored in further detail. This work opens a new perspective of study on the NEK family, mainly in areas related to polyglutamylation, such as cilia, neuronal, blood and muscle disorders.

ACKNOWLEDGEMENTS

We would like to thanks Annemarie Wehenkel, Magiera MM and Janke C (Institut Curie, Orsay, France) for technical support, insightful suggestions and critical discussion of the manuscript.

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ISSN 1949-8454 (online)

World Journal of *Biological Chemistry*

World J Biol Chem 2021 July 27; 12(4): 52-56

Published by Baishideng Publishing Group Inc

WJBC

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World Journal of Biological Chemistry

Bimonthly Volume 12 Number 4 July 27, 2021

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INDEXING/ABSTRACTING

The *WJBC* is now abstracted and indexed in PubMed, PubMed Central, China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Jia-Hui Li*; **Production Department Director:** *Yu-Jie Ma*; **Editorial Office Director:** *Yun-Xiaojiao Wu*.

NAME OF JOURNAL
World Journal of Biological Chemistry

ISSN
ISSN 1949-8454 (online)

LAUNCH DATE
July 26, 2010

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Vsevolod Gurevich, Chun-Peng Wan

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1949-8454/editorialboard.htm

PUBLICATION DATE
July 27, 2021

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World Journal of Biological Chemistry

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World J Biol Chem 2021 July 27; 12(4): 52-56

DOI: 10.4331/wjbc.v12.i4.52

ISSN 1949-8454 (online)

OPINION REVIEW

Stem cells and the pursuit of youth, a tale of limitless possibilities and commercial fraud

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Author contributions: Al-Sowayan BS was responsible for the concept and article drafting; Al-Shareeda AT carried out the revision and final approval of the manuscript.

Conflict-of-interest statement: The authors declare no conflict of interests.

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Manuscript source: Invited manuscript

Specialty type: Biochemistry and molecular biology

Country/Territory of origin: Saudi Arabia

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Abstract

This article examines the hype generated around the term "stem cell", and the capitalization of the stem cell craze by the cosmetic industry. It started by introducing product lines containing active ingredients derived from plant stem cells. Then, evolved to using own cells for skin regeneration and hair loss treatment, and allogenic cells for the manufacturing of stem cell-derived products. This article also discusses the missing links for safe and reliable stem cell applications in cosmetics, and why local regulatory bodies, members of the industry and consumers must all work together to stop the illegitimate use of the "stem cell" good name in unsafe or fraudulent commercial practices.

Key Words: Stem cell; Stem cell-derived products; Cosmetics; Regulations

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Core Tip: The capitalization of the stem cell craze by the cosmetic industry. Products and procedures utilizing plant stem cells-extracts, a person's own cells or allogenic laboratory grown stem-like cells, are all being offered as direct-to-consumer options for tissue regeneration. What are the missing links for safe and reliable stem cell applications in cosmetics, and why is it important to address these issues?

Citation: Al-Sowayan BS, Al-Shareeda AT. Stem cells and the pursuit of youth, a tale of

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: February 21, 2021
Peer-review started: February 21, 2021
First decision: April 20, 2021
Revised: May 17, 2021
Accepted: July 7, 2021
Article in press: July 7, 2021
Published online: July 27, 2021

P-Reviewer: Ventura C
S-Editor: Fan JR
L-Editor: Webster JR
P-Editor: Li X

limitless possibilities and commercial fraud. *World J Biol Chem* 2021; 12(4): 52-56
URL: https://www.wjgnet.com/1949-8454/full/v12/i4/52.htm
DOI: https://dx.doi.org/10.4331/wjbc.v12.i4.52

INTRODUCTION

The term "stem cell" first appeared in the literature in the 1860s, in an attempt to explain the Darwinian Theory of Evolution. The German biologist Ernest Heckel constructed a phylogenetic tree, and used the term "*StammZelle*" (Stem Cell), to describe the original single cell organism from which, he suggested, all other multicellular organisms evolved[1]. Since then, the "stem cell" concept went through diligent polishing, to be crystallized in the restricted and descriptive term of a biological entity that we use now. Scanning through the history of stem cell research, a major milestone worth mentioning occurred in 2010, when embryonic stem cells were used for the first time to treat a patient with a spinal injury[2]. This marked the conquering of theoretical, technical, and ethical challenges facing the advancement of stem cell-based regenerative claims from bench to bedside. Subsequently, the transitional applications of stem cells expanded rapidly to include therapeutic and non-therapeutic applications, and stem cell research news started to be broadcasted in the mainstream media coverage. Even though specialists in the field are still contending with the evolving nomenclature of the stem cell sub-types, the general public do share a broad understanding of what a stem cell is, its functional traits and the promising potential. This has initiated a widespread capitalization of stem cells. Within the cosmetic industry, manufacturers and clinics started introducing new product lines and procedures with the term "stem cell" in the label, to capture the customer's attention in this highly competitive market.

PLANT STEM CELL

The first consumer encounter with stem cell cosmetics began with skin and hair care products containing active ingredients derived from plant stem cells. The manufacturers of these products often failed to disclose that the term "stem cell" in the label of their under eye cream or night serum, referred to stem cell extracts of plant origin. More than a decade ago, research groups presented evidence supporting the regenerative effects of different plant stem cells and their extracts on fibroblasts, keratinocytes and isolated hair follicles[3-5]. Yet, it is doubtful whether the actual benefits of the hundreds of commercial products containing plant stem cell extracts, mixed with various ingredients and preservatives, can be consistent. The validity of the anti-aging and regenerative claims varies vastly between these products, depending on the formulation. Fortunately, the worst case scenario when buying such products is the possibility of being a victim of false advertising, as no serious side effects are caused by the plant extracts. Still, we posit that introducing the plant-based products to market as "stem cell-products" had two major consequences. Firstly, manufacturers realized that including "stem cell" in a product's label supports a steep price increase. For example, a tomato anti-oxidant cream is 10$, but a stem cell anti-oxidant cream, made of tomato plant stem cell extracts, can be 50$, although both creams are made of the same basic ingredients. Such commercialization opportunities increased the industry's appetite for developing more direct-to-consumer stem cell products and services. Secondly, flooding the market with allegedly "stem cell" containing products desensitized the public regarding the term and its significance. For example, if a person can buy a shampoo claiming to contain "stem cells" to control hair loss, this person will consent to injecting stem cells directly into his/her scalp, not realizing that the first is a plant extract, and the latter is a living biological entity. In other words, the mislabeling of plant extracts as stem cell-off-the-shelf products made the current direct-to-consumer marketing of mammalian stem cell-based products/procedures acceptable to the public, who often miss the small print.

AUTOLOGOUS STEM CELL

The next consumer encounter with stem cell-cosmetics was with mammalian stem

cells, in the form of procedures using autologous stem cells. The most promoted and widely used autologous regenerative procedure is platelet rich plasma (PRP). Although this procedure does not involve actual stem cells, it is often marketed as a stem cell-based regenerative treatment. The regulatory challenges of PRP and autologous stem cell use in cosmetic applications are similar. PRP is separated from the person's own blood *via* centrifugation, to obtain the platelets, plasma proteins and growth factor-rich fraction. Theoretically, the application of PRP to skin or hair follicles could induce cell proliferation and extracellular matrix synthesis. Current literature do report the regenerative effects of PRP on skin and hair[6-8], but large controlled clinical trials are still lacking. In addition, there is no standardization in terms of a sample collection and processing protocol, PRP volume and concentration, and the method of administration for each intended purpose, which makes consistent and reliable results unattainable. These two major concerns of PRP, a procedure that has been used for years, is a clear indication that the premature introduction of a cosmetic procedure to market is acceptable to both practitioners and consumers. The actual autologous stem cells used are mostly adipose tissue-derived mesenchymal stem cells for skin regeneration or tissue augmentation, and follicular stem cells for hair loss. As in PRP, there is evidence supporting the claims; however, large controlled clinical trials are still required[9,10]. Also required, is standardization of the procedure, as the differences in the techniques used cause significant variations in the results for patients in different clinics.

ALLOGENEIC STEM CELL

Allogenic stem cell use in cosmetics is primarily using stem cells of human or animal origin for the mass production of stem cell-derived products. Currently, there is a number of products made from stem cell condition media, or a specific fraction, in the form of tropical creams or serums or intradermal injections. This is based on the claims that the constituents of the condition media, including cytokines, growth factors and exosomes, will initiate the required cellular pathways for tissue regeneration. Studies on the mechanism of action of stem cell therapies are confirming that the transplanted cells exert their regenerative effects through the release of paracrine factors. These factors stimulate endogenous cells and promote local angiogenesis at the damaged tissue site[11-13]. For skin and hair, in particular, there is a considerable number of publications supporting the claim that stem cell condition media have a measurable effect when used to treat skin damage or hair loss[14-17]. Notably, these studies also report significant inconsistencies in the outcomes depending on the method used for cell isolation, medium collection, medium processing, and mode of application[18]. On the other hand, allogenic whole-stem cell use for cosmetic applications is still in it is infancy. It is projected to be laboratory grown, genetically manipulated, and ready to inject cells with stem cell characteristics. These cells will be sold by manufacturers to cosmetic clinics for "heavy duty" cosmetic applications, such as severe facial deflation or advance pattern baldness. It is projected that, similar to previous applications, implementation in practice will occur prior to the completion of appropriate clinical investigation, or establishing clear regulations. The concern is that this category has an increased risk of serious side effects, specifically different types of immune reactions. When using whole cells, an additional concern is unspecific differentiation or tumorigenicity, which occurred in stem cell therapeutics, especially when certain sub-types such as embryonic and induced pluripotent stem cells are used[19,20] (Figure 1).

STEM CELL BUSINESS

Thousands of businesses globally are engaged in direct-to-consumer marketing of stem cell-derived products or stem cell-based procedures. Some of which are mislabeled as stem cell-related, while others do involve stem cells, but are not yet clinically proven or adequately regulated. Mammalian stem cells, especially from an allogenic source, and their paracrine factors are biological products and must be considered with extreme caution. Firstly, there should be strict requirements for the laboratories that prepare stem cells for the purposes of generating cell-derived products or whole cell use. Secondly, it is crucial to establish detailed protocols for manufacturing *i.e.* tissue source and collection, cell isolation, cell culture and manipulation, phenotypic profile, concentration or number of cells administered and mode of administration for each intended purpose. Thirdly, adequate clinical trials must be

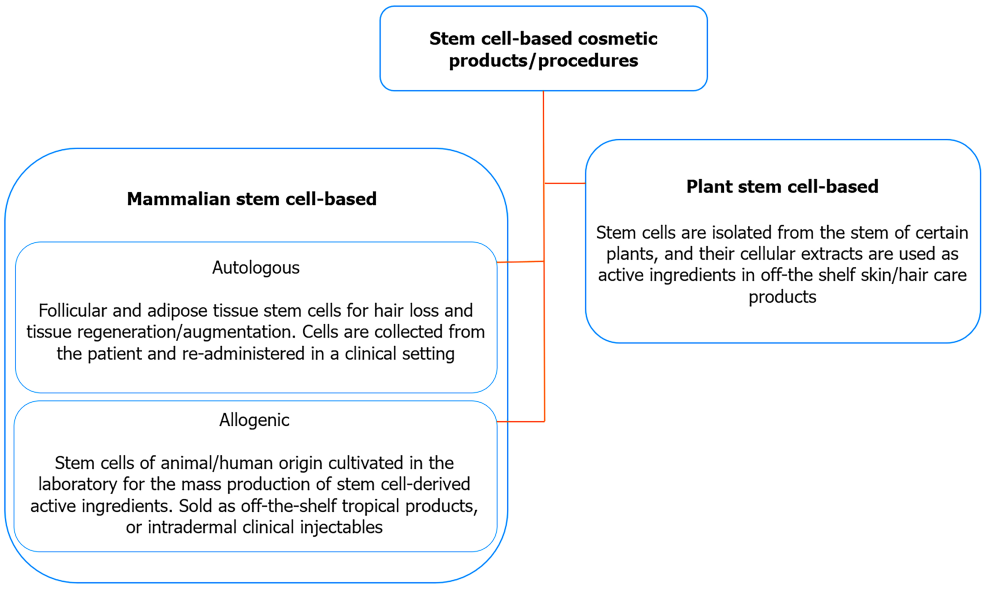

Figure 1 Categorization of currently available stem cell-based cosmetic products/procedures available through direct-to-consumer marketing.

conducted for each application, to confirm safety and effectiveness. It is crucial that providers of a given product or procedure do not cause harm to their consumers, or practice deception with unproven claims. Stem cell products and procedures must comply with both the local Drug and Medical Devices Authorities guidelines for safety, as well as the truth-in-advertising laws. It is also worth mentioning that this inadequate regulation is not unique to stem cell cosmetics. In the cosmetic industry, a procedure is frequently introduced to market prior to adequate clinical testing. This is due to the regulatory bodies' oversight being unable, or under equipped, to manage this rapidly paced industry. The cosmetic industry has to offer the latest social media cosmetic hypes, even unregulated or unproven products or procedures, to supply the demand and not lose market share. Therefore, it is vital to inform consumers about the requirements and the processes of a cosmetic product or procedure approval, as well as the gaps within the system. For example, an approved laser machine for cellulite removal indicates that the machine is relatively safe, but it does not mean that it actually eliminates cellulite! This is important so that consumers can inform and educate themselves about the product or procedure they intend to purchase, and report any suspected malpractices.

CONCLUSION

In conclusion, some final remarks. Using stem cells in cosmetic applications cannot be dismissed as trivial. Improving peoples' aesthetic concerns can significantly improve their quality of life. Conditions such as facial acne scars or lupus bald patches can be detrimental to a person's self-perception and body image, which could have serious consequences. The addition of a profitable, commercial arm will push the entire field of stem cell research forward. Due to the growing demand, companies within the cosmetic industry are investing more in research and development of new stem cell related products and applications. When the cosmetic industry becomes well regulated, and provides evidence-based claims by following proper scientific research practices, the outcomes of their research will contribute to the pool of stem cell knowledge. This means that the data from cosmetic-related research could be used as a proof-of-concept for other therapeutic applications, where funding is scarce. The second remark is that the East Asian market is the biggest market for stem cell-cosmetics. A study investigating online direct-to-consumer marketing of stem cell therapies, reported that 83% of the websites in the Japanese online stem cell market were promoting cosmetic procedures, compared to only 14% in the United States of America[21]. The Drug and Medical Devices regulatory authorities in East Asia will benefit from taking the lead in governing the market practices of manufacturing, marketing and use of stem cells for cosmetic purposes. This will not only offer safety

and avoid commercial fraud for local consumers, but will also establish East Asia as the number one destination for affordable, high quality cosmetic solutions.

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7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
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E-mail: bpgoffice@wjgnet.com
Help Desk: https://www.f6publishing.com/helpdesk
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ISSN 1949-8454 (online)

World Journal of *Biological Chemistry*

World J Biol Chem 2021 September 27; 12(5): 57-103

Published by Baishideng Publishing Group Inc

WJBC

World Journal of Biological Chemistry

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Bimonthly Volume 12 Number 5 September 27, 2021

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World Journal of Biological Chemistry

Bimonthly Volume 12 Number 5 September 27, 2021

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INDEXING/ABSTRACTING

The *WJBC* is now abstracted and indexed in PubMed, PubMed Central, China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Ying-Yi Yuan*; **Production Department Director:** *Yu-Jie Ma*; **Editorial Office Director:** *Yun-Xiaojiao Wu*.

NAME OF JOURNAL
World Journal of Biological Chemistry

ISSN
ISSN 1949-8454 (online)

LAUNCH DATE
July 26, 2010

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Vsevolod Gurevich, Chunpeng Craig Wan

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1949-8454/editorialboard.htm

PUBLICATION DATE
September 27, 2021

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https://www.wjgnet.com/bpg/gerinfo/204

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https://www.f6publishing.com

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E-mail: bpgoffice@wjgnet.com https://www.wjgnet.com

World Journal of Biological Chemistry

Submit a Manuscript: https://www.f6publishing.com

World J Biol Chem 2021 September 27; 12(5): 57-69

DOI: 10.4331/wjbc.v12.i5.57

ISSN 1949-8454 (online)

REVIEW

Proteomics: Concepts and applications in human medicine

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Author contributions: Al-Amrani S drafted the first draft and approved the final version; Al-Jabri Z, Al-Zaabi A, Alshekaili J, and Al-Khabori M critically edited the manuscript and approved the final version.

Conflict-of-interest statement: All authors declare no conflict of interest.

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Manuscript source: Invited manuscript

Specialty type: Biochemistry and

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Abstract

Proteomics is the complete evaluation of the function and structure of proteins to understand an organism's nature. Mass spectrometry is an essential tool that is used for profiling proteins in the cell. However, biomarker discovery remains the major challenge of proteomics because of their complexity and dynamicity. Therefore, combining the proteomics approach with genomics and bioinformatics will provide an understanding of the information of biological systems and their disease alteration. However, most studies have investigated a small part of the proteins in the blood. This review highlights the types of proteomics, the available proteomic techniques, and their applica-tions in different research fields.

Key Words: Proteomics; Biomarker; Mass spectrometry; Two-dimensional electrophoresis; Drug discovery

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Core Tip: Proteomics is the complete evaluation of the structure and function of proteins to understand an organism's nature. Mass spectrometry is an essential tool that is used to profile proteins in the cell. However, biomarker discovery remains the major challenge of proteomics because of the complexity and dynamicity. This review highlights the types of proteomics, the available proteomic techniques, and their applications in different research fields.

molecular biology

Country/Territory of origin: Oman

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

Received: February 22, 2021
Peer-review started: February 22, 2021
First decision: April 20, 2021
Revised: May 4, 2021
Accepted: July 15, 2021
Article in press: July 15, 2021
Published online: September 27, 2021

P-Reviewer: Suriyaphol G
S-Editor: Gao CC
L-Editor: Filipodia
P-Editor: Yuan YY

Citation: Al-Amrani S, Al-Jabri Z, Al-Zaabi A, Alshekaili J, Al-Khabori M. Proteomics: Concepts and applications in human medicine. *World J Biol Chem* 2021; 12(5): 57-69
URL: https://www.wjgnet.com/1949-8454/full/v12/i5/57.htm
DOI: https://dx.doi.org/10.4331/wjbc.v12.i5.57

INTRODUCTION

Proteomics is a new type of 'omics' that has rapidly developed, especially in the therapeutics field. The word proteome was created by Marc Wilkins in 1995[1]. Proteomics is the study of the interactions, function, composition, and structures of proteins and their cellular activities[2]. Proteomics provides a better understanding of the structure and function of the organism than genomics. However, it is much more complicated than genomics because the protein expression is altered according to time and environmental conditions[3]. It is estimated that there are almost one million human proteins, many of which contain some modifications such as post-translational modifications (PTMs). However, it is also estimated that the human genome codes for about 26000-31000 proteins[4]. There are a variety of proteomics techniques including one-dimensional (1D) and two-dimensional (2D) gel electrophoresis (2-DE)[5], as well as gel-free high-throughput screening technologies such as multidimensional protein identification technology[6], stable isotope labeling with amino acids in cell culture[7], isotope-coded affinity tag, and isobaric tagging for relative and absolute quantitation [8]. Shotgun proteomics[9], 2D difference gel electrophoresis (2D-DIGE)[10], and protein microarrays[11] can be used in tissues, organelles, and cells. Large-scale western blot assays[12], multiple reaction monitoring assays[13], and label-free quantification of high mass resolution liquid chromatography (LC)-tandem mass spectrometry (MS) are commonly used for high-throughput processing. In the last decade, proteomics has been classified into protein expression mapping and protein interaction mapping[14]. The former method uses 2-DE combined with MS for quantitative proteome expression in cells, body fluids, or tissues. Protein expression mapping can provide an understanding of the PTMs of expressed proteins under different environmental conditions or disease states[14]. Protein-protein interaction mapping uses the yeast two-hybrid system coupled with MS to determine the interaction partners for each cell's encoded proteins and the proteome-wide scale[15].

Proteomics is a multi-step technique in which every step should be very well controlled to avoid non-biological factors interfering with protein expression and interaction. Sample preparation is the most important step because it solubilizes all proteins in the sample and eliminates all interfering inhibitory compounds such as lipids. Adequate sample preparation is crucial to obtain reliable, accurate, and reproducible results[16]. PAGE is the most widely used method for protein separation and isolation[17]. High-performance LC (HPLC)[18], 1-DE, and 2-DE are the methods used to separate proteins[19]. Proteins are isolated using 1-DE based on their molecular mass. Protein solubility is rarely an issue since proteins are solubilized in sodium dodecyl sulfate (SDS).

Furthermore, 1-DE is easy to use, repeatable, and capable of resolving proteins with molecular masses ranging from 10 kDa to 300 kDa[17]. As 1-DE gel has minimal resolving power, it is most commonly used to characterize proteins after being purified. However, in more complex protein mixtures, such as a crude cell lysate, 2-DE may be used. In 2-DE, proteins are determined by their net charge and their molecular mass[17].

Proteomics can analyze the expression of a protein at different levels allowing the assessment of specific quantitative and qualitative cellular responses related to that protein[20]. Qualitative and quantitative proteomes are measured at post-transcriptional, transcriptomic, and genomic levels[21]. According to the conditions, qualitative proteomics utilizes microarrays, 2-DE, and 2D-LC to monitor protein mixture composition and protein expression changes[20]. In addition, it can provide information on the molecular mechanisms of diseases and compare two groups such as patients with healthy controls[20]. Quantitative proteomics can also provide deep insights into disease mechanisms, cellular functions[22], and biomarker discovery[23]. Several new strategies are used in quantitative proteomics, such as post-extraction or metabolic stable-isotope labeling alone or in combination with affinity labeling[24,25]. MS identifies compounds by sorting cations according to their mass-to-charge ratio[26].

The study of proteomics has many applications in different fields such as medicine, oncology, food microbiology, and agriculture. This review will shed light on proteomics, their techniques, some of its applications, and the challenges currently faced in this field.

TYPES OF PROTEOMICS

Proteomics has three main types: expression proteomics, functional proteomics, and structural proteomics[27].

Expression proteomics

Expression proteomics is a novel approach that studies the quantitative and qualitative expression of proteins. It aims to specify the difference in protein expression between two conditions such as patients and controls[28]. In addition, it can identify disease-specific proteins and new proteins in signal transduction[17]. Expression proteomics experiments are usually used to study the patterns of protein expression in different cells. For example, a tumor tissue sample is compared to a normal tissue sample to identify differences in the levels of proteins[26]. Variations in protein expression, which are present or missing in tumor tissue compared to normal tissue, are detected using 2-DE and MS techniques[29].

Structural proteomics

Nuclear magnetic resonance spectroscopy and X-ray crystallography are used in structural proteomics to determine the three-dimensional structure and structural complexities of functional proteins. It specifies all protein interactions such as membranes, cell organelles, and ribosomes in the mixture[30]. The study of the nuclear pore complex is an example of structural proteomics[31].

Functional proteomics

This type of proteomics studies the protein functions and molecular mechanisms in the cell and determines the protein partner's interactions. In particular, it investigates the interaction of an unknown protein with partners from a specific protein complex involved in a particular process. This may indicate the biological role of the protein [32]. In addition, the elucidation of protein-protein interactions *in vivo* can lead to comprehensive descriptions of cellular signaling pathways[33].

PROTEOMICS WORKFLOW

Two methods can be used in proteomics: top-down and bottom-up workflows. The bottom-up method is sometimes called peptide-based proteomics. Here, the protein is digested by trypsin and separated by a specific column, followed by analysis of the peptides by MS[15]. The bottom-up approach can be classified into two groups according to the fractionation step. The first approach uses 2-DE to isolate the proteins from the gel. Then the proteins are digested into peptides that MS can identify. The second approach is called "shotgun" proteomics. Here, the digestion of protein occurs without fractionation, and LC is used to separate the peptides identified by MS[34]. In top-down proteomics, whole proteins or polypeptides are immediately assessed by MS. The molecular mass of proteins is sometimes calculated by using electrospray ionization (ESI) followed by matrix-assisted laser desorption/ionization (MALDI) MS [35]. Top-down proteomics can identify proteins with a molecular mass of > 200 kDa [36]. Both approaches have various advantages and limitations. In the bottom-up approach, there is low percentage coverage of the protein sequence, because the recovered sample includes small and inconsistent fractions of total peptides. This results in missing a large proportion of alternative splice variants and PTMs. However, in top-down proteomics, all characteristics of proteins are protected, and almost all existing modifications and correlations can also be recovered. Moreover, in top-down proteomics, the results of the exclusion of protein digestion with time are preserved [37]. The major challenge in top-down proteomics is the poor solubility of proteins compared to small peptides. Some proteins in the membrane have high solubility but need to be washed with SDS; however, SDS cannot be used in ESI[38]. Proteomics workflows involve sample preparation and analytical flow. The latter include separation of proteins, protein identification, and validation.

Sample preparation

Proteomics experiments highly depend on the accuracy of sample preparation, in addition to a well-designed pre-analytical workflow. There is no standard technique for sample preparation in proteomics. Each method depends on the number of proteins in the sample, the sample's complexity, and the study's objectives. Extraction of proteins from the mixture is the most vital step in the preparation of samples. To maximize protein extraction and solubilization, the extraction should include organic solvents and detergents followed by a tissue disruption technique. The organic solvents and detergents can be removed by lyophilization[39]. In previous detergent-based methods, the extraction of 2,2,2-trifluoroethanol (TFE) macro-scale (> 100 μg) materials and nano-scale (30 μg)-based lysis have provided comparable protein detection rates[40].

Separation and isolation of protein

Gel-based and chromatography-based approaches are used for the separation and isolation of proteins from the mixture.

Gel-based approach

The best technique for protein isolation and detection is PAGE[41]. For separation, 1-DE and 2-DE can be used. Furthermore, 2D-DIGE and SDS-PAGE are examples of 2D variations used in gel electrophoresis[42].

1-DE

1-DE, can isolate proteins with a molecular weight of 10 kDa to 300 kDa. It uses SDS, a detergent that denatures secondary and non-disulfide-linked tertiary structures, and combines them with a negative charge proportional to their volume. This allows the calculation of molecular weights[43]. SDS-PAGE can be used to verify the purity of samples, test protein purification, and calculate molecular weights for unknown proteins[44].

2-DE

2-DE differentiates proteins better than 1-DE due to the variation in molecular weight and isoelectric point of protein molecules[43]. It also has a better resolution than 1-DE because the protein is separated into two different dimensions. In 1-DE, the protein is separated based on net charge, but in 2-DE, protein separation is based on the molecular mass and isoelectric point. Thus, this method can detect different forms of proteins such as PTMs and phosphorylation. Some proteins that arise from different proteolysis processes and splicing of alternative mRNA can be resolved by 2-DE[45]. There are many applications of 2-DE, including protein expression profiling and cell map proteomics. Protein expression profiling can be used for comparing normal and diseased tissues. Mapping proteins in 2-DE can be used in cellular organelles[46], protein complexes[19], and microorganisms[47]. 2-DE can help catalog proteins, and the database can be created on the World Wide Web[48]. However, 2-DE cannot detect proteins at a low molecular weight and the limits of separation by isoelectric point and size[49].

Chromatography-based approach

Chromatography of affinity, size exclusion chromatography (SEC), and ion-exchange chromatography (IEC) techniques can be used to purify protein-based chromatography. In addition, western blotting and the enzyme-linked immunosorbent assay are used to identify selective proteins[50].

IEC

IEC is used to purify proteins according to their charges. This technique allows separating proteins according to their charge nature, which is not possible by other approaches. The charge accepted by the molecule of interest can be readily used by altering the pH of the buffer. The IEC technique is low cost and can persist in variable buffer conditions[30].

SEC

SEC can be used to separate different compounds according to their size (hydrodynamic volume) measured by how efficiently they enter the stationary phase's pores. However, this technique is not as useful as other proteomics techniques[51]. Two basic versions of SEC are utilized: gel permeation chromatography (GPC) using organic

solvents, which is used for polymer analysis; and gel filtration, which is performed using aqueous solvents.

Affinity chromatography

Affinity chromatography is the process of protein separation according to its interaction with an immobilized ligand. In 2-DE and non-2-DE, affinity chromatography helps decrease the protein complexity[52]. There are three types of affinity chromatography: separation of protein before 2-DE, affinity chromatography of protein before MS, and affinity chromatography of peptides before MS.

LC

LC is a powerful technique that can separate proteins from a complex mixture and can analyze large and fragile biomolecules. When combined with MS, it can be used for determining the peptides in the mixture[53]. LC can help researchers discover novel biomarkers and understand the mechanisms of carcinogenesis according to the modification of proteins. For example, some researchers use LC-MS/MS to rapidly monitor congenital adrenal hyperplasia from dried filter-paper blood samples[54].

Protein identification and characterization

The identification of proteins is a critical step in proteomics. MS can be used after the separation of the proteins by chromatography or electrophoresis[55]. Other techniques can also identify proteins such as Edman sequencing and protein microarray[17].

Edman sequencing

Edman sequencing has been used to detect the sequence of amino acids in peptides or proteins. This technique includes the reaction of chemicals, which remove and determine amino acid residues present at the N-terminus of the polypeptide chain. Thus, it plays a significant role in assessing biopharmaceutical quality and therapeutic proteins[17].

MS

MS is the best analytical tool for rapidly facilitating the sequencing of proteins[56]. It can also be used to detect the molecular weight of proteins. In this technique, protein molecules are ionized, and their mass is calculated according to mass-to-charge ratios. The mass spectrometer has three main components: an analyzer, an ion source, and a detector. The methods used for ionization are ESI and MALDI[57]. In MALDI, a chemical matrix is mixed with the peptides, and spotted onto a metal multiwall microliter plate to make a crystal lattice. The matrix chemicals pass the energy to the samples after absorbing it. Then peptide ions are detected by a mass analyzer. MALDI creates mostly singly charged ions that help to determine the m/z value[58]. In ESI, the power is activated in the protein sample to create charged droplets that increase gaseous ion production, which then are analyzed with a mass analyzer[59]. The advantages of ESI are its high reproducibility and high elasticity to combine many categories of MS. Furthermore, ESI can be fixed to time-of-flight (TOF)-MS, quadruple, ion traps, and fourier transform ion cyclotron resonance. On the other hand, the disadvantages of ESI are that it cannot be applied for molecular imaging, it requires a large quantity of samples, and multiple peaks are produced due to the many charged ions that result in the complexity of MS/MS spectra[60].

Protein identification and validation

Sequent, Mascot, Comet, and Tandem are instruments currently available for database searching[61]. However, most search devices do not produce matching data as they operate on differentiation algorithms and recording functions, creation integration, and data comparison from many studies and experiments. As a result, the identification of peptides by data search needs additional time[62]. High-quality data makes the data search more effective and less time consuming. Moreover, using accurate mass to measure ion fragments can shorten database explorations and produce more accurate results[63].

BIOINFORMATICS IN PROTEOMICS

Bioinformatics analyses use novel proteomics algorithms to manage the large and varied data in the process of marker discovery[64]. Controlling this massive quantity

of data and finding the association between other omics technologies (*e.g.*, metabolomics and genomics) remain difficult. The analyses of proteomics data is challenging because of the parameters used in processing, quality valuation, and shortage of standards for data formats. The big challenge is how to analyze massive data and create real biological understanding[65]. Protein pathways are a collection of internal cell reactions that have a specific biological impact. For protein pathways, a variety of tools and databases are available[66]. The Kyoto Encyclopedia of Genes and Genomes, BioCarta, Pathway Knowledge Base Reactome and Ingenuity pathway databases have extensive information on metabolism, signaling, and interactions[67,68]. Unique databases for signal transduction pathways, such as GenMAPP or protein analysis through evolutionary relationships (PANTHER), have been created[69,70]. Furthermore, databases such as Netpath, which include cancer-related pathways, have been created to detect proteins unique to a specific cancer type[71]. Details about protein interactions in complexes can be found in databases including BioGRID, IntAct, MINT, and HRPD [72-74]. The STRING database links to various other databases for literature mining and is commonly used for protein interaction. Furthermore, using the STRING database, protein networks can be drawn based on the list of genes given and the available interactions[75,76].

APPLICATIONS OF PROTEOMICS IN MEDICINE

Proteomics is a revolutionary technique that has been used in medicine, including drug and biomarker discovery. Proteomics can identify and monitor biomarkers by analyzing the proteins in the body fluids such as urine, serum, exhaled breath and spinal fluid. Proteomics can also facilitate drug development by providing a comprehensive map of protein interactions associated with disease pathways[77].

Biomarker discovery

A biomarker is an assessable pointer of a normal or abnormal biological state in the body[78]. In clinical settings, cancer development and its response to therapy are measured by cancer biomarkers[79]. 2D-PAGE is used for the discovery of biomarkers. It can also compare the profiles of proteins in normal and diseased cells such as tumor tissues and body fluids[80]. Cancer biomarkers are divided into three classes, predictive, prognostic and diagnostic, based on their uses. Predictive biomarkers can predict the response to therapy. For instance, in breast cancer, the activation and the positivity of human epidermal growth factor receptor 2 can predict the response to trastuzumab[81]. In addition, in colorectal cancer, mutation of Kirsten rat sarcoma virus gene can predict resistance to treatment with epidermal growth factor receptor inhibitors (*e.g.*, cetuximab)[82].

On the other hand, prognostic biomarkers can provide physicians with a prediction of the clinical outcomes. For example, the 21-gene repetition mark predicts breast cancer relapse and complete survival in node-negative, tamoxifen-treated breast cancer[83]. The third group of biomarkers is the diagnostic biomarker, which indicates if a patient has a specific disease condition. For example, in colorectal cancer, a stool DNA test is used as a diagnostic biomarker[84]. These biomarkers can be found in tissues, serum, blood, and urine. The body-fluid sampling for proteomics is thus less invasive and low cost. The discovery of biomarkers has progressed in many diseases such as acquired immune deficiency syndrome, cardiovascular diseases, diabetes, cancer, and renal diseases[85,86]. However, the highly complex mixtures of proteins and the high range of protein dynamics are examples of challenges in fluid sampling for proteomics. Each type of sample has a different usage according to the disease conditions. For instance, in kidney disease, the urine sample is used to assess urine proteins, reflecting changes in kidney functions[87]. In other human diseases, blood is also used for biomarker discovery. There are some challenges for using the plasma in biomarker discovery, such as protein dynamicity, the variation of the patient[87], and the low abundance of biomarkers in plasma. These challenges in biomarker discovery have yet to be addressed[88]. Most biomarker discovery studies are focused on cancer-related diseases due to their clinical importance. For instance, many biomarkers are associated with tumors that can be used to follow up with the patients[89].

Drug discovery

Drug discovery is a complex process with many different stages including chemical, functional, and clinical proteomics-based approaches. The application of proteomics in drug discovery has been developed to include patients' treatment and care[90]. 2-DE

cannot be used in drug discovery because it fails to separate the membrane proteins that characterized about 50% of important drug targets[91]. Moreover, 2-DE cannot detect low-abundance proteins[90]. In drug discovery proteomics, understanding the function of proteins and their interactions in the mixture is very important. Also, the methods should be able to detect low-abundance proteins and their activity. Therefore, many technologies such as MS and protein-chip have been used to identify and separate phage proteins. In addition, other techniques such as activity-based assays and two-hybrid assays can be used for the same purpose[92]. Using 2D-PAGE-MALDI-TOF/TOF, Lavandula angustifolia was used as a drug to treat Alzheimer's disease in rats[93].

Oncology

The application of proteomics in cancer is called oncoproteomics. Oncoproteomics can be used to identify anticancer drugs and the personalization of cancer management [94]. Microarrays and laser capture microdissection (LCM) of the tumor tissue can classify proteins in cancer. Oncoproteomics applications are used in many tissues such as the colon, breast, rectum, prostate, and brain. In addition, proteomics can be used to diagnose cancer and discover novel therapies[95]. Many proteomics techniques can be used to detect biomarkers in cancer such as aptamer-based molecular probes, cancer immunomics, tissue microarrays, nano-proteomics (to isolate signatures of autoantibodies), and antibody microarrays[94].

Two approaches can be used in tumor proteomics, LCM and MS imaging (MSI)[96]. LCM can separate the target proteins from the areas within the tumor before analysis with MS. In addition, this approach can help to determine proteins that correlate with tumor progression in the early and late stages of the disease using the proteinChip SELDI system®[97]. However, fewer studies use tumor tissues than serum due to the technical difficulties and low throughput using tumor tissues.

The second approach is using MSI. This direct tissue technique allows placing a small amount of MALDI matrix mixed directly with a fresh piece of the tumor[98]. This approach can help to map small molecules and proteins in a 3D view. This approach was to map eight normal lung tissues with 42 lung tumors[99]. Additionally, MSI can predict diagnosis, categorize lung cancer histology, and organize 85% of the nodal connections[96].

Leukemia

Proteomics was used to discover many leukemia biomarkers that could determine types of leukemia. Examples of these biomarkers include catalase, annexin 1, alpha-enolase, annexin A10, tropomyosin, tropomyosin 3, peroxiredoxin 2, and RhoGDI2. These biomarkers help to predict the diagnosis and outcome of the disease[100]. In addition, the proteomics approach can help developing new treatment pathways for leukemias using their proteomics profiles[101]. However, a major limitation of this approach is that important proteins controlling key cellular elements are present in low abundance and may not be readily detected.

Acute myeloid leukemia and proteomics

Acute myeloid leukemia (AML) is an aggressive blood cancer. Patients reach complete remission after intensive chemotherapy given as induction and consolidation[102]. However, relapsed AML may acquire at least one specific mutation such as FLT3, RUNX1, or ASXL1. Mutations in signaling genes such as KIT, NRAS, PTPN11, and NPM1 are less frequent[103]. The use of proteomics in AML may guide the post-induction strategy of either chemotherapy or allogeneic stem cell transplantation. Moreover, proteomics can help discover new or modified therapy options for AML patients[104]. Since the 1980s, many studies have focused on finding biomarkers in AML. For example, Hanash *et al*[105] used 2-DE to identify the cell of origin in acute leukemia.

While the prognosis of AML patients has improved through the years, especially in younger patients, mortality remains the highest among all other cancers[106]. Proteomics can assist the development of personalized therapy in AML[104]. Kwak *et al* [107] used 2-DE and MS to identify eight differentially expressed proteins between 12 healthy people and 12 patients with AML. Proteasome 26S ATPase subunit, immunoglobulin heavy-chain variant, and haptoglobin-1 were upregulated, while five proteins (unknown protein, lipoprotein C-III, RBP4 gene product, SP-40 and α-2-HS-glycoprotein) were downregulated[107]. Another study identified seven other proteins. These proteins were annexin A10, alpha-enolase, tropomyosin 3, lipocortin 1 (annexin 1), peroxiredoxin 2, RhoGDI2, and catalase[108].

In a recent study, BCL11A expression was found to play a role in AML. The study included 292 AML patients. The study found a significant association between the laboratory variables and the levels of BCL11A. However, BCL11A was not associated with survival and complete remission[109].

Most studies in proteomics in AML were performed on peripheral blood cells and bone marrow samples at an early stage. However, one study compared AML at diagnosis, remission, and relapse. It concluded that the proteome expression at diagnosis and relapse is similar at a high protein concentration[79,110]. Another study that focused on AML (subtypes M1 and M2) compared patients with healthy individuals. Twenty-five proteins were characterized in the peripheral blood and bone marrow samples. The study found that 6-phosphogluconate dehydrogenase, Annexin III and L-plastin were only found in the M2 subtype. The annexin I and actin gamma 1 levels were found to correlate with drug resistance at relapse[111].

CHALLENGES OF PROTEOMICS

There are many challenges in proteomics. The major challenge is the broad change in protein expression with the environment and cell type[112]. In addition, there is no comparable proteomics method, unlike genomics, that uses polymerase chain reaction [113]. Moreover, protein activities are highly regulated post-transnationally, which adds difficulty is proteomics[114]. Finally, the type of samples and sample preparation techniques are other challenges in proteomics that can significantly change the quality of MS data. For example, the protein and phosphoprotein levels in breast cancer tumor samples were affected by the sample manipulation technique and bio-specimen type [115].

CONCLUSION

Proteomics is a fast, sensitive technology that provides high proteome coverage. Expression proteomics, functional proteomics, and structural proteomics are the three major types of proteomics. There are two different workflows in proteomics: top-down and bottom-up proteomics. In addition, there are increasing uses of proteomics in the majority of biological sciences. Finally, proteomics can assist in finding new biomarkers in different diseases and discover new therapies.

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World Journal of Biological Chemistry

Submit a Manuscript: https://www.f6publishing.com

World J Biol Chem 2021 September 27; 12(5): 70-86

DOI: 10.4331/wjbc.v12.i5.70

ISSN 1949-8454 (online)

REVIEW

Culprits or consequences: Understanding the metabolic dysregulation of muscle in diabetes

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Author contributions: O'Reilly CL wrote the majority of the review; Uranga S contributed to the writing, designed and produced the figures; Fluckey JD contributed to the writing and edited the manuscript.

Conflict-of-interest statement: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Manuscript source: Invited

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Abstract

The prevalence of type 2 diabetes (T2D) continues to rise despite the amount of research dedicated to finding the culprits of this debilitating disease. Skeletal muscle is arguably the most important contributor to glucose disposal making it a clear target in insulin resistance and T2D research. Within skeletal muscle there is a clear link to metabolic dysregulation during the progression of T2D but the determination of culprits *vs* consequences of the disease has been elusive. Emerging evidence in skeletal muscle implicates influential cross talk between a key anabolic regulatory protein, the mammalian target of rapamycin (mTOR) and its associated complexes (mTORC1 and mTORC2), and the well-described canonical signaling for insulin-stimulated glucose uptake. This new understanding of cellular signaling crosstalk has blurred the lines of what is a culprit and what is a consequence with regard to insulin resistance. Here, we briefly review the most recent understanding of insulin signaling in skeletal muscle, and how anabolic responses favoring anabolism directly impact cellular glucose disposal. This review highlights key cross-over interactions between protein and glucose regulatory pathways and the implications this may have for the design of new therapeutic targets for the control of glucoregulatory function in skeletal muscle.

Key Words: Insulin resistance; Skeletal muscle; Mammalian target of rapamycin; Glucose uptake; Glucose regulation; Insulin signaling

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Core Tip: The prevalence of type 2 diabetes (T2D) continues to rise despite the amount of research dedicated to finding the culprits of this debilitating disease. Within skeletal muscle there is a clear link to metabolic dysregulation during the progression of T2D but the determination of culprits *vs* consequences of the disease has been elusive. Emerging evidence in skeletal muscle implicates influential cross talk between the

manuscript

Specialty type: Biochemistry and molecular biology

Country/Territory of origin: United States

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: March 31, 2021
Peer-review started: March 31, 2021
First decision: June 7, 2021
Revised: June 21, 2021
Accepted: August 3, 2021
Article in press: August 3, 2021
Published online: September 27, 2021

P-Reviewer: Zhang LL
S-Editor: Fan JR
L-Editor: A
P-Editor: Yuan YY

mammalian target of rapamycin (mTOR) complexes (mTORC1 and mTORC2) during insulin stimulated glucose uptake. This review highlights interactions between protein and glucose regulatory pathways and the implications this may have for the control of glucoregulatory function in skeletal muscle.

Citation: O'Reilly CL, Uranga S, Fluckey JD. Culprits or consequences: Understanding the metabolic dysregulation of muscle in diabetes. *World J Biol Chem* 2021; 12(5): 70-86
URL: https://www.wjgnet.com/1949-8454/full/v12/i5/70.htm
DOI: https://dx.doi.org/10.4331/wjbc.v12.i5.70

INTRODUCTION

Globally, 462 million individuals are affected by type 2 diabetes (T2D) and it is ranked as the 9th leading cause of mortality[1]. The prevalence of diabetes over the past few decades has continued to rise with no sign of this changing[1]. T2D is characterized by insulin resistance and hyperglycemia and can lead to various other outcomes and comorbidities reducing quality of life in those effected. While the pathogenesis and progression of T2D is still widely debated, it is clear that a complex interplay between the pancreas and peripheral tissues is dependent for maintenance of glucose homeostasis. Peripheral tissues account for 80%-90% of glucose disposal[2,3] and of those tissues skeletal muscle is a large contributor to glucose disposal[4,5] and arguably the most important for glucose clearance[6,7]. Within skeletal muscle there is clear link to metabolic dysregulation during the progression of T2D, but the definition of causes *vs* consequences within the development of this disease is difficult. Identifying clear relationships, interactions and feedback loops within the insulin signaling cascade and other metabolic pathways in skeletal muscle is imperative to our understanding for the development, its progression and ultimately a cure for this disease. To that end, this review will present the canonical understanding of insulin signaling, the influential connections between mammalian target of rapamycin (mTOR) complexes (mTORC1 and mTORC2) and the current intertwined implications of these signaling paradigms in skeletal muscle metabolic dysregulation.

INSULIN SIGNALING

The insulin signaling cascade involves both glucoregulatory and anabolic processes which is outlined in Figure 1. Insulin responsive tissues have insulin receptors (IR) on the cell surface plasma membrane. These IR contain subunits where insulin can bind as well as residues that provide docking sites for downstream signaling molecules including the IR substrates (IRS). The two predominant insulin receptor substrates are IRS1 and IRS2 with similar sequences but specific signaling roles[8,9]. IRS1 appears to be the insulin receptor substrate protein whose primary responsibility is glucose regulation, including glucose transporter 4 (GLUT-4) translocation[8] with speculation that IRS2 is more involved with fatty acid metabolism, currently known to occur in adipose tissue[9]. IRS1 is a clear mediator of insulin signaling through a specific intermediate phosphatidylinositol 3 kinase (PI3K). Interaction of PI3K to IRS produces membrane phosphatidylinositol 3,4,5-triphosphates (PIP3) which is necessary for the recruitment and localization of Protein Kinase B, also known as AKT[10].

Upstream glucose related substrates

This serine/threonine kinase is part of the AGC protein family and is known for its diverse function in growth, survival, proliferation and most importantly substrate metabolism[11-13] AKT is often referred to as one molecule but actually comprises of three distinct isoforms (AKT1, AKT2, AKT3) , while all isoforms are present in skeletal muscle, AKT2 is the most prevalent[12], but varies from low to immeasurable amounts in skeletal muscle[14,15]. While defining the variation and overlap between the AKT isoforms is important and needed, it is beyond the scope of this review but what is known currently can be found in these reviews[12,16] It is important to note that AKT2 is expressed primarily in insulin responsive tissues like fat and skeletal muscle and is

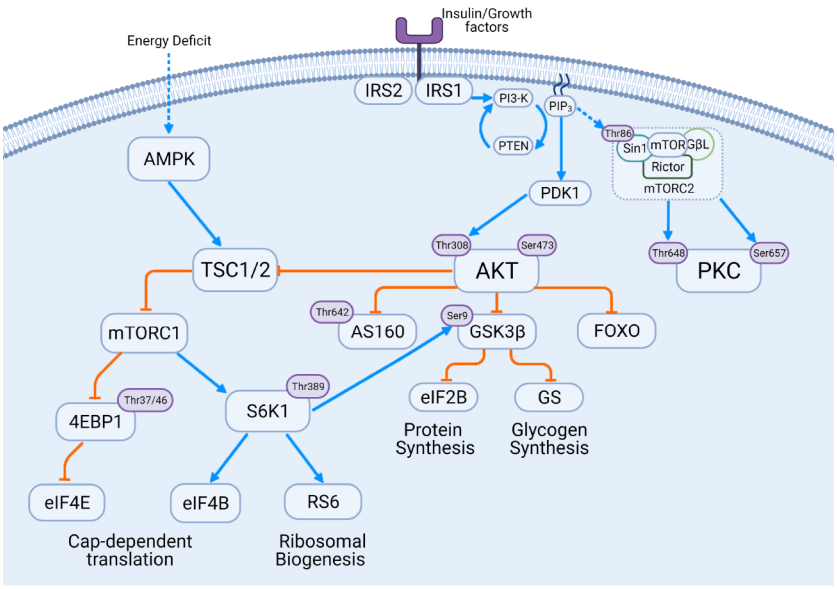

Figure 1 Insulin signaling cascade involving both glucoregulatory and anabolic pathways. Phosphorylation sites of interest indicated on figure. Blue arrows (→) indicate activation of the substrate, orange bars (⊥) indicate inhibitory action on the substrate. Figure created with BioRender.com. mTOR: Mammalian target of rapamycin; mTORC: mTOR complex; S6K1: S6 kinase beta-1; IRS: Insulin receptor substrates; PKC: Protein kinase C; AMPK: AMP-activated protein kinase; TSC: Tuberous sclerosis complex; GSK-3β: Glycogen synthase kinase 3β; PIP3: phosphatidylinositol 3,4,5-triphosphates.

the most abundant isoform in skeletal muscle[14,15,17,18]. AKT is as a critical regulator of insulin sensitive glucose uptake as well as anabolic signaling through mTORC1 making it a prime target in understanding metabolic dysregulation.

The upstream regulation of AKT, in its most simple iteration, appears to be very similar across isoforms. The two common phosphorylation sites of AKT are Ser473 (Ser474 in AKT2) and Thr308. The insulin receptors IRS1 and IRS2 will activate the PI3K-dependent conversion of PIP2 to PIP3, and PIP3 will recruit Pyruvate Dehydrogenase Kinase 1 (PDK1) and AKT to the membrane where colocalization will allow for phosphorylation at the Thr308 by PDK1[12,13]. Further, some evidence suggests that mitogen-activated protein kinase-associated protein 1 (mSin1) of the mTORC2 complex is brought to the membrane by PIP3 (binding with the pH domain) that promotes colocalization of mTORC2 to the membrane[19,20], which is the major kinase for the Ser473 phosphorylation site of AKT.

The regulation of mTORC2 activity by mSin1 phosphorylation is controversial. It has been proposed that PIP3) promotes mTORC2 activity directly[21,22]. Recent work has indicated a positive feedback loop between AKT and mTORC2 *via* phosphorylation of mSin1[23,24]. Those studies in adipocytes and Hela cells indicated that phosphorylation of mSin1 at Thr86 by AKT (*via* Thr308) increased mTORC2 activity and phosphorylation of AKT on Ser473[20,23]. This positive feedback loop provides an avenue for mTORC2 control *via* growth factors; however, the total impact of this feedback loop on mTORC2 activity and downstream substrates like AKT Ser473 is currently unknown. It is well established that PDK1 and mTORC2 are the major kinases involved upstream of AKT and that AKT is involved in a large scale, insulin sensitive pathway, but the distinct actions of these two phosphorylation sites are still not well understood.

There is also considerable debate over what the phosphorylation of specific AKT sites implicates for AKT activity and substrate specificity. Much of the early work in AKT reported a requirement of phosphorylation at Ser473 for full activation[25-28]. However, more recent work in platelets[29], HEK cells[27,30], and skeletal muscle[31,32] demonstrated that not all downstream substrates are impacted by Ser473 phosphorylation. There is some evidence to support that these changes in activity and substrate *via* phosphorylation site may be isoform specific[33,34] but more work needs to be done in this area.

The implications of Ser473 phosphorylation *via* mTORC2 has been studied in various tissues. In mSin1 knockout mouse embryonic fibroblasts, a regulator of mTORC2 complex formation and stability, Forkhead box 01/03 (FOX01/3a) phosphorylation was inhibited but tuberous sclerosis complex 2 (TSC2) and glycogen synthase kinase 3 (GSK-3) phosphorylation was unaffected[35]. In adipose tissue[36] and liver [37], rapamycin insensitive companion of mTOR (RICTOR) knockouts demonstrated

tissue specific differences in mTORC2 substrate specificity. When mTORC2 inhibitors were applied in skeletal muscle, phosphorylation of AKT at Thr308 was unaffected and the downstream phosphorylation of TSC1/2, S6 kinase beta-1 (S6K1) and GSK-3β, all associated with protein synthesis and growth, were also unaffected by the reduction of Ser473 phosphorylation[32]. However AKT substrate of 160 kDa (AS160), an enzyme associated with GLUT-4 translocation and glucose disposal as well as proteins in the FOXO family associated with apoptosis were negatively affected by Ser473 reduction[32]. That work demonstrated that there is some demarcation of substrate specificity within AKT of skeletal muscle. It may also indicate phosphorylation of Thr308 focuses AKT kinase activity towards substrates involved with growth and phosphorylation of Ser473 focuses on substrates involved in glucose regulation and cell survival. Alternatively, substrates unaffected by inhibition or downregulation of mTORC2 phosphorylation of AKT at Ser473 may be phosphorylated by other proteins. For example GSK-3 can be phosphorylated at the same phosphorylation site that AKT does Ser9 by S6K[38] and protein kinase C (PKC)[39]. Despite the alternative theory there is evidence for at least some context-dependent substrate specificity towards AKT's downstream targets. As for whether the activity of AKT is dependent on Ser473 for full activation, a recent study in adipose tissue purports that AKT2 activity is reduced by about 50% for its substrates TSC2, PRAS40, FOX01/3a and AS160[40]. Taken together, there may be argument for some combination of Ser473 impacting substrate specificity and activity, but to our knowledge this has not been validated in skeletal muscle and would need more systematic study in both AKT1 and AKT2 to truly define this regulatory mechanism.

Downstream glucose related substrates

As previously mentioned AKT has various downstream substrates that make the action of this kinase quite diverse in cell function. These substrates include members of the mTOR complexes Pras40 and Sin1, Glucose uptake proteins AS160 and GSK-3, Protein synthesis related Tuberous sclerosis 2, and apoptotic signaling through the FOX0 family. This section will focus on signal transduction related to glucose uptake.

GLUT-4 is the predominant isoform of the GLUT family found in skeletal muscle, and one of insulin's primary metabolic roles is to promote the translocation of GLUT-4 to the surface membrane. AKT has been linked to downstream substrates that impact insulin-dependent GLUT-4 translocation including GSK-3[41] as well as AKT Substrate of 160kd (AS160)[31,42,43] making it a prime target for understanding glucose uptake. GSK-3β is a well-known inhibitor of glycogen synthase, but is also an inhibitor of eiF2B which is a potent regulator of protein synthesis. When GSK-3β is phosphorylated at Ser79 its activity is inhibited, which allows for the activation of both glycogen synthase and eiF2B. Interestingly GSK-3 has been linked to mTORC2 regulation *via* RICTOR phosphorylation at Ser1235 which interferes with mTORC2 binding to AKT[44] and Ser1695[45] which marks RICTOR for degradation. Also been linked to AS160 is a substrate of AKT that contains a Rab-GTPase activating protein and has been associated with regulating glucose transport. In basal conditions AS160 maintains GLUT-4 containing vesicles in the cytosol (intracellular) through its gap domain[46, 47], when insulin is applied AS160 is rapidly phosphorylated which disengages AS160 from the vesicles allowing them to move to the membrane for exocytosis. In skeletal muscle, like fat[43,48], AS160 is phosphorylated in response to insulin in a dose dependent manner[49] and insulin stimulation of GLUT-4 exocytosis is dependent on AS160 phosphorylation[48]. AS160 can be phosphorylated by other proteins including AMP-activated protein kinase (AMPK) making it part of both insulin dependent and insulin independent translocation of GLUT-4[31,50].

Anabolic signaling

AKT phosphorylates TSC2 at Thr1462 which regulates the tuberin-hamartin complex and it's activity[51-53]. Phosphorylation at this site releases the tuberin-hamartin complex inhibition of the mTORC1 complex and allows for downstream targets to be phosphorylated[51]. mTORC1 is a prolific kinase with multiple downstream substrates, but Ribosomal protein S6K1 and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) are arguably the most well-known downstream targets. 4E-BP1 is known as a translation repressor protein because it inhibits cap-dependent mRNA translation by binding to peptide-chain initiation factor eIF4E. Phosphorylation of 4E-BP1 disrupts the interaction of 4E-BP1 and eIF4E, releasing it so that it may participate in translation by chaperoning specific cap-dependent transcripts to the translation apparatus[54]. S6K1 is best known for its action on ribosomal protein S6 (S6) which is involved in the translational control of 5′ oligopyrimidine tract (5′-TOP) mRNAs[55]. Phosphorylation of S6K1 at Thr389 is known to be critical for function of

the protein[55], as well as correlated with kinase activity *in vivo*[56]. The subsequent phosphorylation of S6 ribosomal protein correlates with increases in translation of cap-dependent proteins, that are necessary for the manufacture of ribosomal machinery and peptide-chain elongation factors necessary for mRNA translation[57,58]. The regulation of S6K1 activity is diverse but S6K1 activation has been shown to be elevated by hyperglycemia[59], hyperinsulinemia[60], and high fat diet in muscle and adipose tissue[61].

INSULIN SIGNALING AND DIABETES

It is generally agreed that glucose transport is the rate limiting step of glucose uptake, and the step most impacted by the progression of T2D. The consensus in diabetes research at large is that the translocation or trafficking of glucose transport molecules in skeletal muscle is impaired in T2D[43,62] but the culprit behind this impairment is still widely debated. In skeletal muscle GLUT-4 is the predominantly expressed isoform[63,64] and the localization of GLUT-4 has been confirmed with insulin[65], exercise[65,66] and hypoxia[67]. The first important finding with diabetes is that the limitation in glucose transport cannot be explained by production or maintenance of the GLUT itself, because total GLUT-4 protein is largely unchanged with diabetes[68-70]. This implies that the issue is not related to GLUT-4 expression, *per se*, but within the signaling cascades that assist in the translocation of GLUT-4 to the surface membranes.

As the initial step in the insulin signaling cascade, the insulin receptor was a primary target of research related to the breakdown of the glucoregulatory signals. While current data are conflicting on IR activity with some reporting impairment[62,71,72] and others reporting normal activity[73-77], it appears that the important signaling 'defects' of T2D are further down the signal cascade. Signaling defects in IRS1 phosphorylation[73,77-79] and PI3K[73,77,78,80,81], activity are consistently found in the diabetic model. More controversial is the activity of AKT with studies reporting significant reductions of insulin stimulated AKT phosphorylation on Ser473 or Thr308[69,75,82,83]. While others report not impact of T2D on insulin dependent phosphorylation[80,81]. Downstream substrates of AKT have also been presented in the diabetic model with reduced glycogen synthase activity with protein levels of GSK-3 reported as being elevated which would inhibit GS activity[84]. Additionally, insulin dependent phosphorylation of AS160 has also been reported to be higher in T2D[42], despite the fact that AKT phosphorylation was not different in the same study.

Despite the continued exploration and detailed understanding of what the signaling cascades are doing during diabetes, there is still no consensus on where these dysfunctions are originating. Molecular mechanisms that underlie this dysfunction of glucoregulatory processes associated with T2D as outlined above have been studied extensively, but the interaction of glucoregulatory processes with those of protein metabolism (protein turnover) are still lacking, despite the evidence that the two processes may be dependent on one another.

It is well documented that muscle mass and strength decline with T2D[85,86] and contribute to a decline in quality life over time. Interestingly despite a loss in muscle mass, there appears to be an upregulation of protein synthesis and the anabolic signal cascade in diabetic muscle[87,88]. Previously, studies assessing anabolic responses [fractional synthesis rate (FSR)] in diabetic skeletal muscle have been inconsistent, ranging from decreased[89,90], to normal[91,92] but more recently increased FSR has been confirmed by our lab[87,88,93,94] and others[95,96]. In Fatty Zucker rats, a well-documented model for T2D, upregulated protein synthesis in specific muscle fractions and increased phosphorylation of S6K1 were observed despite an overall decrease in muscle mass. This upregulation of S6K1 appears to be linked to a loss of control of upstream mTOR activation. While the hyperactive mTOR activity may be a result of the maintained state of hyperinsulinemia with glucose intolerance, we suspect something much more sinister for the progression of diabetes.

Our recent studies have demonstrated that the constitutive activation of mTOR may be a result of suppressed DEPTOR expression in the diabetic state. DEPTOR is one of the mTOR associated binding partners that can be a part of either mTORC1 or mTORC2 and is a negative regulator of mTOR activity. Similar to several lines of cancer[97]. DEPTOR is substantially lower in obese subjects[87,88]. Since DEPTOR is still a fairly new discovery in the mTOR signaling cascade, the implications of low DEPTOR and the regulation of mTORC1 are still speculative but the low DEPTOR

appears to allow the downstream anabolic signals to go unchecked[98] which has implications for mRNA translation[99], as well as glucoregulatory signaling cascades. This is unbridled mTORC1 activity without concomitant muscle mass accretion is indicative of high protein turnover[88], where it may not be warranted or wanted. It is also an important bridge between mTORC1 and mTORC2 which will be discussed in a later section.

CONNECTING ANABOLISM TO INSULIN RESISTANCE

A relatively recent but important discovery in the connection of anabolic and glucoregulatory signaling paths is an inhibitory pathway that directly links S6K1 to IRS1. IRS1 can be serine phosphorylated through many pathways including c-Jun NH2-terminal kinase, IkB kinase, PKC, and S6K1[100,101]. It is now known that the insulin receptor contains multiple phosphorylation sites[102] and even in a basal state it is highly phosphorylated[103]. Ser/Thr phosphorylation of IRS-1 has been linked to the degradation of IRS1 itself and the downstream signaling needed for glucose uptake. While the patterns and requirements of these phosphorylation's for the downstream signal disruption are still undefined it has been clearly demonstrated that chronic exposure of cells to insulin results in degradation of IRS-1 protein[104-106]. It was later found that AKT mediated the Ser/Thr phosphorylation of IRS-1 and that this was inhibited by rapamycin[107]. More specifically IRS1 phosphorylation at Ser307 and Ser636/639 were observed in moments of increased mTORC1 activation and this increase was absent in mice that were S6K1 deficient[61]. In support of this constitutive activation of S6K1 lead to IRS1 phosphorylation and degradation as well as inhibition of IRS-1 transcription[108,109]. It is now a well-supported conclusion that IRS1 phosphorylation by S6K1 (Figure 2), decreases insulin signaling through the insulin receptor substrate[61,100,103,110,111]. This critical role is highlighted in the elevated levels of activation in liver adipose and muscle of obese animals[61,87,88,112] and is further supported by S6K1 deficient mice being protected against diet-induced obesity and insulin resistance[61]. This clearly links mTORC1 and more specifically S6K1 to the general insulin signaling cascade making it a target molecule for alteration of insulin signaling.

While we are gaining perspective in the current literature about the interaction between mTORC1 signaling for protein synthesis and the disruption of insulin signaling for glucose disposal in skeletal muscle, far less is known about how the two mTOR complexes interact in this process. While the S6K1 connection to IRS1 is now fairly accepted, S6K1 also appears to have a role in the cross-talk between the two mTOR complexes that is not yet well defined but thought to play a role in insulin resistance. To date, very little is known about the regulation of mTORC2[113] despite its role in phosphorylation of AKT at Ser473. The role of AKT and its regulation through Ser473, both upstream and downstream is still quite controversial in the literature as discussed earlier in section 2.1 AKT/protein kinase B (PKB), despite its being a widely used marker of AKT activity[25-27]. The downstream targets of AKT include various substrates involved in glucose uptake so the choice of this important intermediate as a marker seems obvious; however, the interpretation of what phosphorylation of AKT at Ser473 truly implies remains ambiguous.

The mTORC2 complex is best known for its involvement in cell survival but is known to phosphorylate AKT through Ser473[25,114-117] as well as the PKC family [40,116-119]. This complex is composed of binding partners mSin1, DEPTOR, Protor1, mLST8 and RICTOR. While all of these binding partners play roles in mTORC2 activity, the RICTOR has currently demarcated mTORC2's role in signal transduction [25]. RICTOR aids in localization of mTOR to the plasma membrane as well as the binding of mSin1 to the mTORC2 complex[19], making it an important binding partner worthy of the interest it has received. While mTORC2 has been established as the kinase responsible for phosphorylation of AKT at Ser473 the mechanism behind this phosphorylation is controversial. Two binding partners, RICTOR and Sin1, have been established as important regulators of mTORC2 complex activity, and of interest is that both of these binding partners appear to be regulated by S6K1. RICTOR is prone to phosphorylation[114,120,121] and that phosphorylation may impact downstream targets like AKT, as indicated by phosphorylation at Ser473[115,122].

Work by others indicated that the muscle-specific deletion of RICTOR led to decreased Ser473 phosphorylation of AKT and was accompanied by reduced phosphorylation of AS160 at Thr642 and overall glucose intolerance[123]. That work lead to speculation that regulation of RICTOR through phosphorylation was responsible for

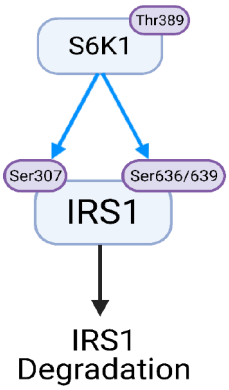

Figure 2 Downstream mammalian target of rapamycin complex 1 substrate S6 kinase beta-1 phosphorylation of insulin receptor substrates 1 at Ser307 and Ser636/639 leads to insulin receptor substrates 1 degradation. Blue arrows (→) indicate activation of the substrate, black arrow (→) indicates degradative pathway. Figure created with BioRender.com. S6K1: S6 kinase beta-1; IRS: Insulin receptor substrates.

the increases or decreases in Ser473 phosphorylation[115,122], and the concomitant responses of insulin-stimulated glucose homeostasis. Others determined that the phosphorylation of RICTOR at thr1135 (Figure 3) was responsible for inhibition of kinase activity toward AKT at Ser473[119,122,124,125]. Phosphorylation of RICTOR at Thr1135 was sensitive to both growth factors and rapamycin[124] and was the direct target, established through silencing and pharmacology, of S6K1[119]. Although the evidence connecting S6K1 to RICTOR regulation is compelling, the functional consequences of this phosphorylation are controversial. Some studies have indicated that this phosphorylation is a direct regulator of mTORC2 activity towards AKT[119,122], while others report no alteration in mTORC2 activity[124,125]. It must be noted that different experimental models were used across these studies, so it is possible that some of the differences observed were due to the differences in genetic models used to arrive at those conclusions. Despite those discrepancies, the S6K1-RICTOR interaction further supports the concept of crosstalk between the insulin glucoregulatory and protein synthesis pathways, as implicated by data demonstrating that mTORC1 regulation is important for Ser473 regulation. With mTORC1 and S6K1 activity being upregulated with diabetes, this connection to the insulin signaling pathways and the direct control mTORC1 may be critically important for further understanding of the metabolic dysregulation of T2D.

RESISTANCE EXERCISE

Exercise and physical activity are effective, low cost interventions for insulin resistance and T2D[126,127]. The benefits of aerobic exercise on glucose tolerance are well established[128-132] and the improvements are independent of improvements in general condition[132]. However many people with T2D are overweight and/or obese, have mobility issues and other neuropathies making aerobic-type exercises difficult to accomplish[133,134]. Resistance exercise has been proposed as a more feasible activity when aerobic exercise is inaccessible and there is a growing body of evidence to support that this form of exercise can be beneficial with regard to glucose tolerance [135,136]. Much of this work attributes the glucoregulatory improvements following resistance training are due to increased muscle mass[2,137,138] which may or may not be applicable to T2D. Additionally, acute resistance exercise appears to increase insulin clearance without a change in glucose tolerance[139], which was originally attributed to increases in insulin sensitivity *via* receptor number or a greater liver or tissue clearance following exercise.

It is often speculated that insulin-resistant skeletal muscle is desensitized or 'resistant' to the anabolic actions of exercise[88,140,141], making it difficult to achieve gains in muscle mass. Given the aforementioned hyperactivation of mTOR with insulin resistance, the current theory is that the 'anabolic resistance' observed with diabetes/obesity may really be due to an "anabolic ceiling" in skeletal muscle that has been achieved in the hyper-insulinemic state. In healthy tissue. resistance exercise is a potent stimulator of rates of protein synthesis in muscle and repeated bouts of resistance exercise lead to skeletal muscle hypertrophy[142]. It has also been established that insulin is a necessary component in elevated protein synthesis rates after re-

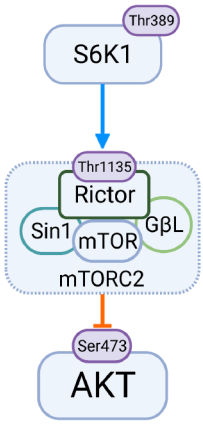

Figure 3 Downstream mammalian target of rapamycin complex 1 substrate S6 kinase beta-1 is the primary kinase responsible for phosphorylation of the mammalian target of rapamycin complex 2 component Rictor at Thr1135 which has been implicated in phosphorylation of AKT at Ser473. Blue arrow (→) indicates activation of the substrate, orange bar (⊥) indicates inhibitory action on the substrate. Figure created with BioRender.com. mTOR: Mammalian target of rapamycin; mTORC: mTOR complex; S6K1: S6 kinase beta-1.

sistance exercise and it is the combination of resistance exercise and insulin that causes this modulation[143,144]. This effect of insulin appears to be through a rapamycin sensitive pathway[145-148] at least in healthy unperturbed tissue, but en-gaging in a moderate to high intensity exercise bouts involving eccentric muscle actions lead to a transiently-reduced capacity of insulin to elevate glucose uptake[149,150]. The mechanisms behind this alteration are still not well defined, but speculation includes a diminished capacity for glycogen synthesis and reductions in GLUT-4 protein which may be fiber type specific[150]. Further, as noted above, there are circumstances where the activation of protein anabolism requires S6K1 activation, which may feedback on upstream signals that impair glucose uptake by insulin[61,87,88,111]. More work is warranted to better define these mechanisms.

Aside from insulin sensitivity, there are benefits to regular exercise, whether it is of an aerobic or anaerobic nature. It is important to note here that there are insulin independent pathways that trigger glucose uptake that are directly related to skeletal muscle contraction. This pathway is triggered by muscle contraction and involves a distinct subset of GLUT-4[66,151-153]. These pathways can involve nitric oxide[154] and activation of AMPK[155,156] as well as cytosolic calcium[130] but these effects are distinct and additive to those of insulin mediated glucose uptake[2,157-159]. Probably most important for T2D research is that these contraction mediated glucose pathways are not only present in T2D but are fully functional[160,161].

Interestingly, in insulin resistant muscle there seems to be a difference in the control of muscle protein synthesis. It appears that in tissue where the upstream activators of the mTORC1 pathway are impaired there are alterations to the use in protein synthesis. Unlike their lean counterparts obese Zucker rats administered insulin had augmented rates of muscle protein synthesis and that these actions persisted in the presence of rapamycin[94]. This suggest that the rapamycin sensitive mTORC1 pathway is not responsible for the increased muscle protein synthesis rates observed.

One key player that may have an impact on muscle protein synthesis in response to insulin is a serine/threonine kinase called PKC. PKC has long been considered as a regulatory contributor during mRNA translation in a number of tissues[162,163] but more recently specific isoforms of PKC have been implicated in the regulation of glucose uptake. Specifically, the conventional family of PKCs (α, β, γ) lead to attenuated insulin receptor tyrosine kinase and PI3K activity[164,165] which leads to reduced glucose disposal. It has been discovered that in diabetic tissue, when insulin complexes with its receptor PKC is activated which then impairs downstream insulin signal[93]. This phenomenon is not observed in muscle from lean humans who have normal glucose response, mirroring the observed changes in insulin induced protein synthesis not present in lean counterparts[94]. Additionally inhibition of PKC activity through pharmacology has been demonstrated to partially restore signal transduction and glucose disposal in otherwise insulin resistant muscle[164,166]

The regulation of PKC, like many of the enzymes related to insulin signal transduction and glucose uptake is complex. It is known that PKCα is a downstream substrate of mTORC2 at both its turn motif (Thr638) and is hydrophobic motif (Ser657) both of which are required for PKCα stability[40,116-119]. Deletion of RICTOR, abo-

lishes phosphorylation of the hydrophobic motif of PKCα[114,115] and deletion of either RICTOR or Sin1 dramatically reduces PKCα protein content[117], implicating that RICTOR, a component of mTORC2, plays a role in PKC activation much like it does for the activation of AKT at Ser473. This draws mTORC2 further into the complex crosstalk that impacts insulin signaling and provides a feasible opportunity for mTORC2 to assist in the bypass of normal insulin signaling with the upregulation of PKC. It is important to note that PKC activation does not rely on mTORC2 however because it can also be activated by Diacylglycerol[117] which would be high in the obese state.

CONCLUSION

Dysregulation of mTOR signaling is a key player in the development of many disease states including diabetes. While decades of research have been dedicated to understanding the insulin signaling cascade, many aspects of its regulation and control remain elusive. It is becoming clear that crosstalk between the two mTOR complexes is adding considerable complexity by impacting both hormone-mediated glucose uptake and the underlying pathogenesis of this disease. This emerging evidence now blurs their roles and responsibilities of fixtures in protein homeostasis. Research in this area has focused on specific culprits in the glucoregulatory pathway that are thought to cause the manifestation of the disease, but with all of the newly emerging anabolic/glucoregulatory cross talk that are involved with the manifestation of this disease, it is possible that the factors once viewed as culprits for this disease may actually be the consequence of anabolic/glucoregulatory cross talk. These recent findings offer exciting new targets for the control of insulin resistance.

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World Journal of Biological Chemistry

Submit a Manuscript: https://www.f6publishing.com

World J Biol Chem 2021 September 27; 12(5): 87-103

DOI: 10.4331/wjbc.v12.i5.87

ISSN 1949-8454 (online)

REVIEW

Alcoholic liver disease: Current insights into cellular mechanisms

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Author contributions: Petagine L performed the literature search, wrote the original draft, designed the figures; Petagine L, Patel VB and Zariwala MG reviewed and edited the final manuscript; all authors have read and agreed to the published version of the manuscript.

Conflict-of-interest statement: There is no conflict of interest.

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Manuscript source: Invited manuscript

Specialty type: Biochemistry and molecular biology

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Abstract

Alcoholic liver disease (ALD) due to chronic alcohol consumption is a significant global disease burden and a leading cause of mortality. Alcohol abuse induces a myriad of aberrant changes in hepatocytes at both the cellular and molecular level. Although the disease spectrum of ALD is widely recognized, the precise triggers for disease progression are still to be fully elucidated. Oxidative stress, mitochondrial dysfunction, gut dysbiosis and altered immune system response plays an important role in disease pathogenesis, triggering the activation of inflammatory pathways and apoptosis. Despite many recent clinical studies treatment options for ALD are limited, especially at the alcoholic hepatitis stage. We have therefore reviewed some of the key pathways involved in the pathogenesis of ALD and highlighted current trials for treating patients.

Key Words: Liver; Alcohol; Oxidative stress; Inflammation; Gut microbiome; Mitochondria

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Core Tip: Alcoholic liver disease (ALD) causes significant global disease burden inducing both cellular and molecular modifications in hepatocytes. Although the spectrum of disease is widely recognized, the precise disease pathogenesis is yet to be fully elucidated. In this review we summarize some of the key pathways responsible for the pathogenesis of ALD and highlight current available treatments.

Citation: Petagine L, Zariwala MG, Patel VB. Alcoholic liver disease: Current insights into cellular mechanisms. *World J Biol Chem* 2021; 12(5): 87-103

URL: https://www.wjgnet.com/1949-8454/full/v12/i5/87.htm

DOI: https://dx.doi.org/10.4331/wjbc.v12.i5.87

Country/Territory of origin: United Kingdom

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: April 30, 2021
Peer-review started: April 30, 2021
First decision: July 8, 2021
Revised: July 20, 2021
Accepted: August 12, 2021
Article in press: August 12, 2021
Published online: September 27, 2021

P-Reviewer: Naserian S
S-Editor: Fan JR
L-Editor: A
P-Editor: Yuan YY

INTRODUCTION

Alcoholic liver disease (ALD) is one of the most prevalent chronic liver diseases and causes significant mortality globally. Chronic alcohol consumption has been implicated in multiple medical conditions including cancer, diabetes, cardiovascular disease, liver and pancreatic disorders. However, no new treatment options have been developed for many years. At present, abstinence remains the most important treatment for ALD, however, there is a need to develop effective treatment options associated with alcohol misuse.

The spectrum of ALD is widely recognized ranging from simple liver steatosis; which can occur in up to 90% of heavy drinkers[1] to alcoholic hepatitis (AH), which develops in 10% to 35% of heavy drinkers[2]. AH can ultimately progress to fibrosis, where hepatic stellate cell (HSC) activation, collagen synthesis and accumulation of extracellular matrix proteins occurs due to the formation of protein adducts. Cirrhosis and lastly hepatocellular cancer are the final stages. Fibrosis/cirrhosis can cause hepatocyte inactivation, and is associated with abnormal DNA repair, damage to mitochondrial function and oxygen utilization disorders[3]. This can lead to hepatocellular failure and portal hypertension which ultimately requires a liver transplantation.

The molecular and biochemical mechanisms underlying the pathogenesis of ALD as well as the precise triggers for disease progression are not completely understood. Recent evidence suggests disease progression is thought to involve several pathological stages such as mitochondrial dysfunction, oxidative stress, altered methionine metabolism, iron dysregulation, gut dysbiosis, activation of inflammatory pathways and decreased production of antioxidants (Figure 1)[4]. Currently, no effective treatment for ALD exists due to the incomplete understanding of hepatic biochemical alterations and pathogenic mechanisms responsible for disease progression.

EPIDEMIOLOGY AND PREVALENCE

Alcohol misuse is a leading cause of liver disease worldwide. ALD is a global disease burden and results in approximately 3 million deaths *per* year[5]. In 2016, 5.3% of all deaths were caused by the harmful effects of alcohol worldwide[5]. Alcohol accounts for 5.1% of total disease burden worldwide, measured in disability-adjusted life years [5]. Total alcohol consumption *per* capita increased in 2005 from 5.5 L to 6.4 L in 2010 and was sustained at 6.4 L in 2016[5]. Europe has the highest *per* capita alcohol consumption and disability-adjusted life years[5], as well as binge drinking particularly, in France and England[6].

Although the overall prevalence of ALD has remained stable from 2001-2016 at between 8.1%-8.8%, the proportion of ALD with stage 3 fibrosis and above has increased from 2.2%-6.6%[7], such that in the United States chronic liver disease and cirrhosis is the 12th leading cause of death[7]. In the United States, the number of adults listed as waiting for liver transplants also increased by 63% from 2007 to 2017[7], where approximately one third of all liver transplants are due to alcohol-related disease[8]. Due to the high-risk drinking rates, the number of deaths due to alcohol-related liver disease in the United States is projected to increase by 84% from 2019-2040 [9]. In the United Kingdom, the number of deaths from ALD was reported at 5964 deaths in 2020 compared to 4954 deaths in 2019, increasing by 20%[10]. From 2001 the number of deaths due to ALD has increased by 72% from 2001-2020[10].

There are several factors which effect the development of ALD. There are significant differences in the amounts of alcohol consumed between males and females. It is well documented that men consume more alcohol than women, leading to a higher prevalence of alcohol related liver disease[11]. It is estimated 237 million men and 46 million women have alcohol use disorders[5]. Although men often consume more alcohol, women are more susceptible to the toxic effects of alcohol and have a higher risk of advanced liver disease[11], due to higher blood alcohol concentrations despite equal dosing between genders[11]. Therefore, female gender is an important risk factor for progression of ALD. Genomic data has also discovered that a variation in the *PNPLA3* gene is associated with increased hepatic fat content, increasing the risk of both ALD and non-alcoholic fatty liver disease, which has the highest frequency in Hispanics[12].

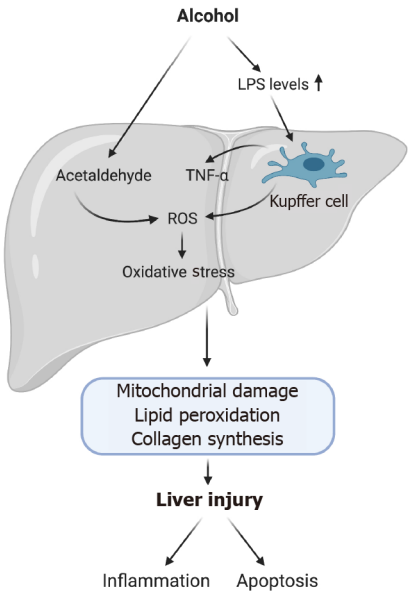

Figure 1 Alcohol-related induction of oxidative stress and liver injury. Alcohol misuse leads to loss of tight junctions in the gut increasing its permeability. This causes translocation of lipopolysaccharide into the liver activating toll-like receptor 4 on Kupffer cells (KCs). Activation of KCs can cause reactive oxygen species (ROS) production and pro-inflammatory cytokines such as tumor necrosis factor-α. ROS production also occurs due to the metabolism of alcohol. ROS production and inflammatory cytokines leads to inflammation and recruitment of inflammatory cells as well as activation of apoptotic pathways. (Figure created with BioRender.com). LPS: Lipopolysaccharide; ROS: Reactive oxygen species; TNF-α: Tumor necrosis factor-α.

DISEASE SPECTRUM

Sustained excessive alcohol consumption produces a vast range of hepatic lesions. The first stage is liver steatosis or alcoholic fatty liver, which can occur in up to 90% of heavy drinkers, emerging as early as 3 to 7 d after initial excessive alcohol consumption[1]. Steatosis is often asymptomatic with normal or only slightly increased liver enzymes levels. The deposition of microscopic fat droplets occurs initially in the centrilobular zone then spreads towards the periportal region of hepatocytes[13]. Steatosis, although reversible and originally thought to be a benign state, is a now a priming phase for AH, which develops in 10% to 35% of heavy drinkers and is a more severe stage of ALD. AH is characterized by hepatocyte ballooning, the formation of Mallory-Denk bodies, infiltration of white blood cells, Kupffer cell (KC) activation, and collagen deposition *via* once dormant HSCs[14], the latter playing an important role in fibrosis leading to cirrhosis. At this stage hepatocyte inactivation, abnormal DNA repair, damage to mitochondrial structures, oxygen utilization disorders, and the accumulation of extracellular matrix proteins occurs[3]. Continuation of hepatic scarring and the spread of collagen (bridging fibrosis) throughout the liver can lead to cirrhosis and in some cases hepatocellular cancer.

Although the pathogenesis of ALD is yet to be fully understood, it is thought to include multiple interplaying factors and pathways including the production of toxic ethanol metabolites, oxidative stress, innate and adaptive immune activation, fibrogenesis and cell death. Upon activation of these pathways tissue damage can occur leading to the progression of the disease. This review will focus on mechanisms involved in inflammation that predominantly occur at the AH stage.

ALCOHOL METABOLISM

Within the liver, alcohol dehydrogenase and cytochrome p450 2E1 (CYP2E1) are the main oxidative pathways of alcohol metabolism. Another minor pathway of alcohol metabolism in the liver is *via* the peroxisomal enzyme catalase[15]. A small proportion of ethanol may also be metabolized by non-oxidative pathways such as by interaction with fatty acids, generating fatty acid ethyl esters[16]. Alcohol dehydrogenase oxidatively metabolizes alcohol to acetaldehyde, a highly reactive and toxic by product that contributes to tissue damage. This conversion reaction requires the cofactor nicotinamide adenine dinucleotide (NAD^+), creating reduced NAD^+ in the process. Due to the toxic nature of acetaldehyde, it is further oxidized to acetate, catalyzed by the

enzyme mitochondrial aldehyde dehydrogenase-2[17]. Increased conversion of NAD^+ results in leakage of electrons and reactive oxygen species (ROS) production. The toxic metabolites produced during alcohol metabolism as well as increased ROS can also trigger endoplasmic reticulum (ER) stress. The second major pathway is *via* the microsomal ethanol-oxidizing system and involves CYP2E1, which is involved in ethanol oxidation to acetaldehyde[17]. The activity of CYP2E1 is induced by alcohol, increasing its hepatocellular content causing accumulation of CYP2E1[18]. Electron leakage from the CYP2E1 pathway, leads to ROS generation, including hydroxyethyl, superoxide anion and hydroxyl radicals[19]. ROS can also form lipid peroxides and DNA adducts such as N2-ethyldeoxyguanosine, which has been detected both in the livers of alcohol-fed rats as well as leukocytes in ALD patients[20].

AUTOPHAGY, MITOPHAGY AND INFLAMMASOMES

Alcohol metabolism increases ROS production and ER stress leading to calcium depletion, glycosylation and lipid overloading, triggering the unfolded protein response (UPR)[21]. The UPR can restore ER homeostasis by attenuating translation of proteins, increasing folding capacity and degrading unfolded proteins. However, a prolonged UPR causes inflammation, fat accumulation, mitochondrial stress and apoptosis *via* direct activation apoptosis signal-regulating kinase 1[22], nuclear factor-κB (NF-κB), c-Jun N-terminal kinases and P38[23]. Alcohol induced ER stress involving an impaired UPR was first identified in a model of intragastric alcohol fed mice [24].

Alcohol can induce autophagy, a self-degradative process which occurs by the action of lysosomes and can be selective only for damaged mitochondria (mitophagy). Evidence suggests that autophagy in ALD can have inhibitory effects on inflammation and steatosis as well as the ability to remove lipid droplets, Mallory-Denk bodies and damaged mitochondria[25]. Whilst tumor necrosis factor (TNF)-α induces autophagy, generation of ROS can lead to inhibition of TNF-α induced autophagy through activation of NF-κB[26]. This suggests dysfunction of autophagy is associated with ALD pathogenesis[27,28]. Multiple animal models have observed autophagy as a protective response in ALD, as well as confirming ameliorative effects in ALD. Acute ethanol feeding (6 g/kg bodyweight) increased autophagy as measured by autophagosome numbers, however, chronic ethanol feeding (5.2% ethanol by volume) inhibited hepatic autophagy[29], suggesting that this protective mechanism is lost with longer term alcohol consumption.

Mitophagy can also be induced as a protective response to both acute and chronic alcohol consumption due to accumulation of ROS or loss of mitochondrial membrane potential *via* elimination of dysfunctional mitochondria. The process of mitophagy depends on induction of autophagy and priming of damaged mitochondria for recognition and is mediated by phosphatase and tensin homolog-induced putative kinase 1-Parkin signalling pathway or Nip3-like protein X[30]. The duration of alcohol exposure can affect the mitophagy process[30]. In rats, binge-models of alcohol consumption have been shown to increase mitophagy, decreasing alcohol-induced hepatoxicity[30,31]. Acute ethanol consumption also increases transcription factor EB, a master regulator of lysosomal biogenesis, however, chronic ethanol exposure decreased transcription factor EB[29]. Accumulation of damaged mitochondria occurs in chronic ethanol models which releases mitochondrial damage-associated molecular patterns (DAMPs), which in turn promote inflammation and fibrogenesis contributing to accelerated disease state[30]. Mitochondrial DNA (a mitochondrial DAMP), can bind to toll-like receptor (TLR)-9 activating HSCs and fibrogenesis[31]. Therefore, targeting mitophagy may be a potential therapeutic for ALD.

Inflammasomes

Oxidative stress in response to alcohol metabolism can damage hepatocytes, releasing endogenous DAMPs. Recognition of DAMPs can induce inflammation by release of proinflammatory cytokines, immune cell localization and stimulation of the inflammasome[32]. Inflammasomes are expressed in hepatic cells and are multi-protein complex's containing a nucleotide-binding oligomerization domain-like receptor (NLR). Inflammasome activation is thought to be a two-step process. Inflammasome sensor molecules can trigger the assembly of inflammasomes, including NLR molecules, for example NOD-, LRR- and pyrin domain-containing 3 (NLRP3)[33]. Assembly is initiated by TLR and pathogen-associated molecular pattern (PAMP)/DAMP signaling which results in the NLR forming complexes with pro-caspase 1 with or

without an adaptor molecule, apoptosis-associated speck like CARD-domain containing protein (ASC)[4,33]. Inflammasome assembly initiates cleavage of procaspase-1 to its active form caspase-1. Activated caspase-1 then promotes the secretion and activation of pro-inflammatory cytokines *via* cleavage of pro-interleukin (IL)-1β and pro-IL-18 into their active forms IL-1β and IL-18[33]. IL-1β plays an important role in the infiltration of immune cells and IL-18 is important for the production interferon-gamma (IFN-γ)[33]. Inflammasome activation also leads to a pathway of cell death called pyroptosis.

NLRP3 can be activated by a variety of stimuli including bacterial toxins, mitochondrial dysfunction and production of ROS. Interestingly, increased levels of IL-1β, ASC and NLRP3 have been documented in the livers of ethanol fed mice, whereas elevated mRNA expression of IL-1β, IL-18 and caspase-1 has been documented in the liver of ALD patients which correlated with liver lesions[34] and has also been associated with the development of liver fibrosis. Blocking IL-1β activity strongly decreases liver inflammation and damage[35]. Higher levels of serum IL-1 has also been documented in patients with AH in comparison to healthy controls[36]. Caspase-1 knockout mice have also been shown to be protected from fibrosis as well as treatment with IL-1 receptor antagonist has been shown to attenuate steatosis and liver injury when administered 2 wk post-ethanol feeding[36]. Decreased inflammation, steatosis and IL-1β expression has been associated in NLRP3 deficiency. Research has also shown mice deficient in NLRP3 have protection against ethanol-induced inflammation including attenuation of steatosis and liver injury[37]. Previous research has also shown inflammasome components such as NLRP3 and ASC are present in HSCs and are required for the development of liver fibrosis by inducing changes including upregulation of transforming growth factor-β and collagen[38]. Therefore, this demonstrates the importance of IL-1β signaling, inflammasome components and activation in ALD.

INNATE AND ADAPTIVE IMMUNITY

Gut permeability

Increases in gut permeability due to alcohol has been confirmed in both clinical and experimental studies[39,40]. Increased gut permeability enables the entrance of PAMPs, such as lipopolysaccharide (LPS) in the portal circulation[41]. LPS is one of the exogenous ligands for TLR4, a pattern recognition receptor found on KCs. LPS interaction with TLR4 initiates downstream signaling *via* TIR-domain-containing adapter-inducing IFN-β and IFN regulatory factor 3, leading to production of proinflammatory cytokines proinflammatory cytokines such as TNF-α and IL-1β (Figure 2), the former can activate the extrinsic pathway of apoptosis *via* the TNF receptor 1 and TNF receptor 2 signaling[42]. High levels of these death receptors, including Fas, are expressed in all liver cells, therefore, the extrinsic pathway is the main apoptotic pathway in hepatocytes, such that hepatocyte apoptosis has been correlated with severity of disease in AH[43]. Serum TNF-α and IL-6 are also increased after exposure to alcohol and LPS[44], with TNF-α correlating with liver injury and mortality. However, chronic ethanol exposure to TNF-α knockout mice does not cause alcohol-associated inflammation and liver injury[45], therefore, mechanisms to reduce TNF-α may be an important tool in preventing inflammation.

Natural killer cells

LPS and HSCs can directly interact with immune cells such as natural killer (NK) cells, natural killer T (NKT) cells and T cells leading to disease progression[46]. NK cells can kill activated HSCs *via* TNF-α related apoptosis, however, HSCs isolated from ethanol-fed mice showed reduced sensitivity to NK cell killing[46]. Alcohol consumption also enhances splenic NK cell apoptosis and blocks NK cell release from the bone marrow, as well as accelerating progression to fibrosis due to reduced NK cell activity[46]. Gut-derived bacteria such as LPS may influence the activation of hepatic NKT cells leading to induction of HSCs and apoptosis, further exacerbating liver injury. Patients with ALD have shown a decreased number of circulating NK cells along with reduced cytotoxic activity resulting in decreased anti-viral, anti-fibrotic, and anti-tumor effects which can contribute to accelerated progression of disease state[47]. However, NK/NKT cells may inhibit fibrosis through deletion of activated HSCs and production of IFN-γ[48]. On the other hand, activation of NKT cells also promotes liver fibrosis *via* enhancing hepatocellular damage and promoting HSC activation[48]. Therefore, a balance is required between inhibitory and stimulatory effects for liver health.

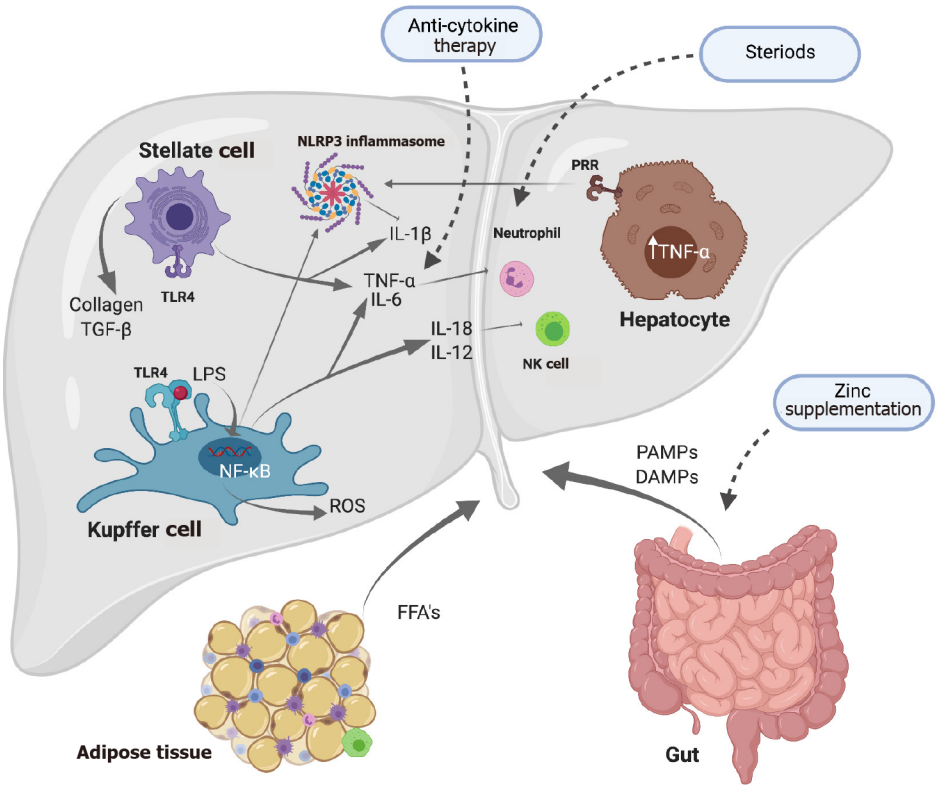

Figure 2 The inflammatory response during alcoholic liver disease. Excessive consumption of alcohol causes lipopolysaccharide release from the gut activating toll-like receptor 4 on Kupffer cells (KCs). Pattern recognition receptors become activated by pathogen-associated molecular patterns/damage-associated molecular patterns which induces inflammation *via* release of proinflammatory cytokines and inflammasome activation. Interleukin (IL)-18 production from KCs causes activation of natural killer cells. Toll-like receptor stimulation in hepatic stellate cells results in the expression of IL-6, transforming growth factor-β1 and tumor necrosis factor-α. (Figure created with BioRender.com). DAMPs: Damage-associated molecular patterns; FFA: Free fatty acids; PAMPs: Pathogen-associated molecular patterns; IL: Interleukin; LPS: Lipopolysaccharide; NF-κB: Nuclear factor-κB; NK: Natural killer; NLRP3: NOD-, LRR- and pyrin domain-containing protein 3; PRR: Pattern recognition receptor; ROS: Reactive oxygen species; TGF-β: Transforming growth factor β; TLR4: Toll-like receptor 4; TNF-α: Tumor necrosis factor-α.

Neutrophil activation

Neutrophils can be recruited in response to liver injury to form neutrophil extracellular traps (NETs), which generate ROS and undertake phagocytosis. Acute alcohol consumption leads to neutrophil imbalance in the liver releasing spontaneous NETs [49]. The scavenging ability of macrophages to eliminate NETs diminishes resulting in persistent inflammation *via* hepatocyte damage[49]. During AH, the infiltration of neutrophils is believed to occur *via* the activation of KCs, which recruit cytokines and chemokines including IL-8 and IL-17. In mouse models, blockade of inflammatory mediators such as IL-8 and IL-17, which are necessary for neutrophil infiltration, can ameliorate liver disease[50,51], which supports neutrophil dysfunction in disease progression. Patients with ALD have a decreased baseline function of neutrophils in the liver[14], which may provide an explanation for high rates of bacterial, fungal and viral infection as well as organ failure and mortality. Neutrophil dysfunction has been shown to be reversed in patients with AH following endotoxin removal[52]. In AH patients, extensive modification of albumin occurs, further activating neutrophil infiltration causing inflammation and oxidative stress[46,49]. Monocyte chemoattractant protein-1 also known as C–C motif chemokine ligand 2 is involved in proinflammatory cytokine activation and its levels have been found to be correlated with neutrophil infiltration and disease severity[46]. Therefore, neutrophils have been implicated in disease pathogenesis and a balance between anti-bacterial and anti-inflammatory functions is important for ALD patients.

Adaptive immunity

The adaptive immune response has also been implicated in pathogenesis of ALD (Figure 3). Early studies in both animals and humans have shown excessive alcohol consumption reduces peripheral T cell numbers, disrupts the balance between phenotypes, impairs function and promotes apoptosis[53]. Alcohol consumption can cause lymphopenia as well as disrupt the balance between T cell phenotypes, causing a shift from naïve populations to memory cells, experimentally and clinically[54-56]. Cy-

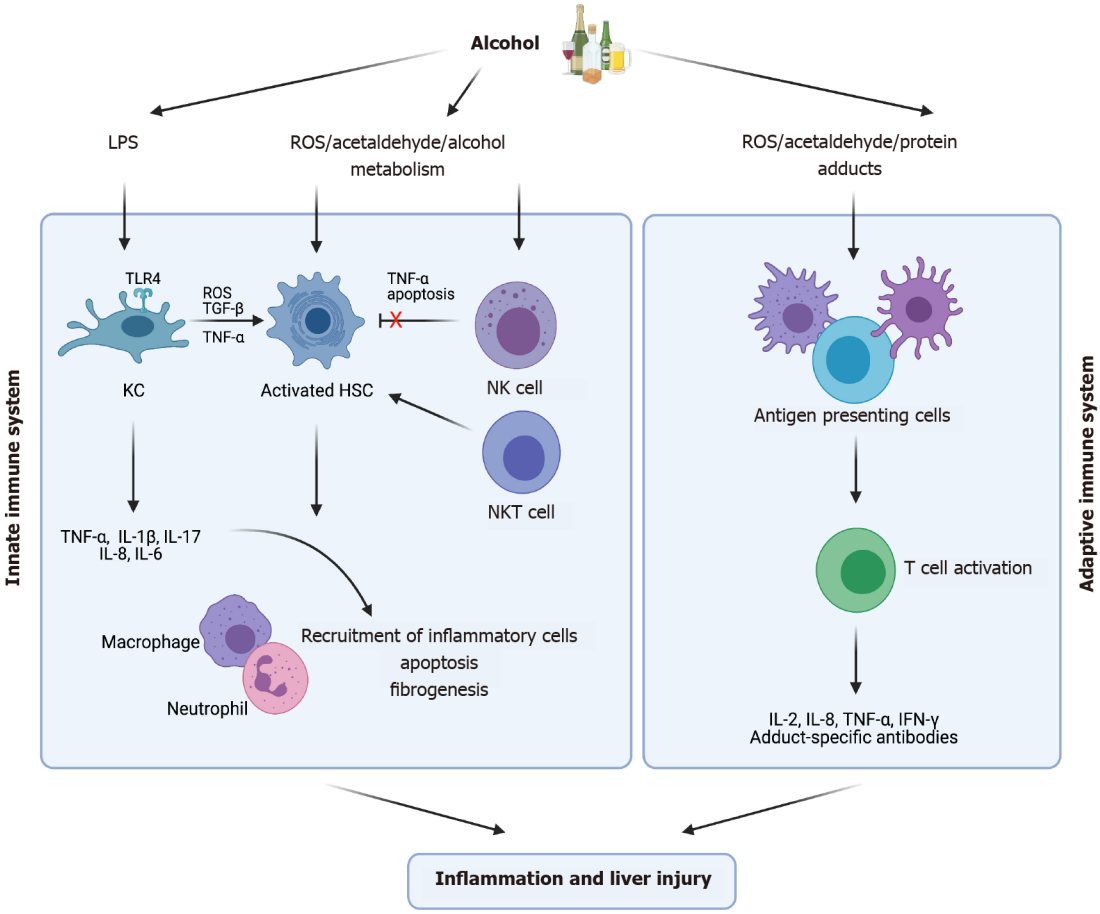

Figure 3 Innate and adaptive immune response to alcohol exposure. Kupffer cells, hepatic stellate cells and natural killer cells are components of the innate immune system which becomes activated following chronic alcohol consumption. This leads to the release of inflammatory cytokines, causing further recruitment of inflammatory cells. The adaptive immune system also becomes activated releasing inflammatory mediators as well as antibody generation to protein and Malondialdehyde-acetaldehyde adducts. Both immune response mechanisms eventually become dysregulated over time with alcohol consumption. (Figure created with BioRender.com). HSC: Hepatic stellate cell; IFN-γ: Interferon-γ; IL: Interleukin; KC: Kupffer cell; LPS: Lipopolysaccharide; NK: Natural killer; NKT: Natural killer T; ROS: Reactive oxygen species; TGF-β: Transforming growth factor β; TLR4: Toll-like receptor 4; TNF-α: Tumor necrosis factor-α.

totoxic CD8$^+$ T cells are greatly reduced, and this reduction was shown to correlate with stage of fibrosis and Child-Pugh (CTP) score, impairing cytotoxic functions leading to immune incompetence[56]. Decreased numbers of regulatory T cells are also associated with immune activation and increased inflammatory cytokines in AH[57].

More recently increasing phenotypes of T cells have been implicated in ALD. Chemokines such as CCL5, a chemoattractant for immune cells such as T lymphocytes, has been found to be upregulated in the liver[58]. Various proteins are expressed on the cell surface such as T-cell receptor, which recognize antigens and elicit a response. Infiltration and activation of both CD4$^+$ and CD8$^+$ T cells have been found to be increased in the livers of patients with ALD[58,59]. Until recently it had not been defined whether the increased activation of T cells in the livers of ALD patients were caused by bystander activation or due to an antigen-specific response[58,59]. Protein adducts derived from alcohol metabolism and lipid peroxidation have been identified in the liver of patients which act as neoantigens. These neoantigens are presented to CD4$^+$ T cells by antigen presenting cells inducing proliferation[58,59]. As well as antigen-specific activation, bystander activation of T cells can occur induced in the absence of *via* cytokines, DAMPs and PAMPs[59]. This infiltration of T cells has been found to be correlated with inflammation and necrosis in ALD as well as regeneration. Therefore, both antigen-specific and bystander activation may contribute to the progression of ALD but also provide a beneficial role, however, this requires further research.

Alterations in regulatory cells may also provide an explanation for disease pathogenesis, as oxidative stress is known to lower regulatory T cell populations in the liver[60]. Th17 cells have been identified in the livers of ALD patients and are critical for defense of bacterial infections[46]. These cells produce IL-17 and promote neutrophil infiltration. Mucosa-associated invariant T cells (MAIT), an innate-like subset

of T cells which inhibit bacterial infection, have been found to be reduced in ALD patients, consequentially increasing bacterial infection. Transcription factors (RORC/RORγt, ZBTB16/PLZF and Eomes) which control the differentiation of MAIT cells were lower in AH patients compared to heathy controls[61]. Therefore, MAIT cell function may provide an important therapeutic approach for the treatment of ALD.

During ALD a loss of peripheral B cells also occurs, as well as increased amounts of circulating immunoglobulin[53]. B cell numbers are documented to be lower in heavy drinkers (90 to 249 drinks/mo) compared to moderate (30 to 89 drinks/mo) to light drinkers (< 9 drinks/mo) as well as a loss or circulating B cells in patients consuming 164.9 to 400 g of alcohol/day on average. The differentiation of progenitor B cells can be affected by ethanol exposure *via* down-regulation of transcription factors (early B cell factor and Pax5) and cytokine receptors (IL-7R α)[62] and thus, alcohol use can affect subpopulations of B cells (B-1a, B-1b, B2-B)[53]. Exposure to 100 mmol/L of alcohol *in vitro* blocks the expression of transcription factors which has been shown to impair B cell differentiation[62]. Alcoholics cannot respond adequately to antigens which is likely due to a reduction in high-affinity antibody-producing B-2B subset[53]. Further, this decrease in B-2B subsets is typically associated with a decrease in the number of B-1a cells as well as a relative increase in the percentage of B-1b cells, important for T cell independent responses[53]. Although B cells numbers appear to be reduced in alcoholics, during cirrhosis, circulating levels of immunoglobulins (IgA, IgG, and IgE) may be increased against liver antigens. It has also been reported that IgG antibodies against CYP2E1 have developed in both alcohol fed rats and patients with advanced ALD[63]. The role of B cells in ALD requires further research.

DIAGNOSIS

Diagnosis of ALD is challenging as many patients present as asymptomatic. An ALD diagnosis is commonly made on a combination of clinical laboratory abnormalities, imaging and a history of alcohol abuse. Laboratory blood tests are used to identify abnormal aspartate aminotransferase (AST), alanine aminotransferase (ALT) level, gamma-glutamyl transpeptidase (GGT), mean corpuscular volume (MCV), carbohydrate-deficient transferrin (CDT) levels, albumin, prothrombin time (PT) (international normalized ratio), bilirubin and platelet counts. These blood tests are useful to suggest alcohol misuse but are inadequate at predicting alcohol use on their own or the disease severity[64]. Historically GGT was used alone as a marker for ALD, although elevated GGT alone has low sensitivity and specificity for alcohol abuse and may be limited by a high rate for false positives[64]. An AST/ALT ration above 2 is regarded as an indicator of ALD, although it is used less frequently to predict chronic alcohol abuse due to low sensitivity[64]. CDT is used as a biomarker for chronic ethanol intake (> 60 g ethanol/d) and has a higher specificity (sensitivity 46%-73%, specificity 70%) than conventional markers such as GGT and MCV. However, no individual biomarker alone provides suitable sensitivity and specificity for ALD diagnosis, therefore, a combination of these biomarkers, imaging and in some cases a biopsy can provide an improved diagnosis.

CLINICAL STAGING OF DISEASE SEVERITY

There are various algorithms used to assess the severity of liver disease as well as predicating survival and treatment options (Table 1). The first models developed were the CTP score and the model for end-stage liver disease (MELD) score. The CTP score identifies patients as class A, B or C determined by serum levels of bilirubin and albumin, prothrombin time, ascites, and encephalopathy[65]. These measures are scored 1-3, with 3 being the most severe. A CTP defined as Class A (5-6 points) indicates a 100% 1-year survival and 85% 2-year survival. Class B (7-9 points) has an 80% 1-year survival and 60% 2-year survival. Class C (10-15 points) has a 45% 1-year survival and a 35% 2-year survival[66]. The MELD score is determined by total bilirubin, creatinine, and international normalized ratio (INR) levels, and is a widely useful tool for evaluation of liver transplantation in patients[67]. MELD is calculated by the formula 9.57 × loge (creatinine) + 3.78 × loge (total bilirubin) + 11.2 × loge (INR) + 6.43[66]. Although these scores are useful in predicting mortality, they are less useful in assessing prognosis and treatment options. Research by Sheth *et al*[68] has shown that 30-d mortality predictions from the MELD scoring were 86% sensitive and 82% specific for MELD scores greater than 11[68].

Table 1 Models for clinical staging in alcoholic liver disease

Model	Stratification	Bilirubin	Albumin	Prothrombin Time	Ascites	Encephalopathy	INR	Creatinine	White blood cell count	Serum urea	Age
Child-Pugh[65, 66]	Severe: ≥ 10	+	+	+	+	+					
Model for End-Stage Liver disease[66]	Severe: ≥ 21	+					+	+			
Maddrey Discriminant Function score [66]	Severe: ≥ 32	+		+							
The Glasgow Alcoholic Hepatitis Score [66]	Poor prognosis: ≥ 9	+		+					+	+	+
Lille Model[66, 69]	≥ 0.45: Nonresponse. < 0.45: Response	+	+	+				+			+

INR: International normalized ratio.

The Maddrey Discriminant Function score (MDF) and The Glasgow AH Score (GAHS) were developed to determine disease severity and treatment options in AH patients. The MDF score assesses serum bilirubin and prothrombin time *via* the equation DF = {4.6 x [PT (sec) - control PT (sec)]} + (serum bilirubin) and classifies disease as either severe (MDF > 32) or non-severe (MDF < 32) and patients who fall in the severe category are most likely to benefit from steroid treatment[66]. The diagnostic sensitivity and specificity of the MDF scoring system was 86% and 48% when the DF was greater than 32[68]. The GAHS was developed to predict outcomes and to initiate therapy in AH patients. The GAHS includes age, white blood cell count, serum urea, bilirubin, and PT. Each variable is given a score and a final combined score between 5-12 is obtained. Patients with a GAHS above 9 have a poorer prognostic outcome. A study has shown patients with an MDF > 32 and a GAHS > 9 who were treated with steroids has a higher survival rate than those without treatment (59% compared to 38%)[67]. The Lille model is another prognostic model developed to identify response to corticosteroids in severe AH patients after 7 d of treatment[69]. Lille Model Score is defined as [exp(-R)]/[1 + exp(-R)]. Where r = 3.19 – 0.101*(age, years) + 0.147*(albumin day 0, g/L) + 0.0165* (evolution in bilirubin level, μmol/L) - 0.206*(renal insufficiency) - 0.0065*(bilirubin day 0, μmol/L) - 0.0096*(PT, seconds) [66]. It is useful for predicting short-term survival due to its high sensitivity and specificity and is able to identify patients at high risk of death at 6 mo[69].

THERAPEUTIC INTERVENTION

The consequences of excessive alcohol consumption causes significant morbidity and mortality with 704300 projected deaths due to alcohol-related liver disease in the United States between 2019-2040[9]. However, with a lack of new therapeutic options, abstinence is still regarded as the most important treatment, as well as treatments such as nutritional therapy, pharmacological therapy, combination therapy and transplantation (Table 2).

Abstinence

Abstinence is the most important treatment for patients with ALD[70]. Abstinence from alcohol improves overall survival and prognosis as well as preventing further disease progression[71]. A reduction in portal pressure, decreased progression to cirrhosis and an improvement in survival has been shown after a period of abstinence [70,72]. However, relapse rates are as high as 67%-81% in alcoholics[73]. Several drugs such as disulfiram, naltrexone and acamprosate have been trialed to sustain abstinence

Table 2 Current available treatments for alcoholic liver disease

Intervention	Objective	Treatment method	Treatment effects
Abstinence	Stop drinking	Abstinence combined with disulfiram, naltrexone or acamprosate	Improve overall survival at all stages[70]. Acamprosate has been shown to be effective in reducing withdrawal symptoms[77]
Nutritional Therapy	Replenish nutrition	1.5 g protein and 35 to 49 kcal *per* kg of body weight. Supplementation with vitamins	Nutritional support showed improved hepatic encephalopathy and reduced infections in AH patients[80]
Corticosteroid	Anti-inflammatory	40 mg daily for 28 d, then 20 mg daily for 7 d, and 10 mg daily for 7 d	Short-term histological improvement has been documented, however, no improvement in long term survival[83]
Pentoxifylline	Anti-cytokine	400 mg orally three times a day for 4 wk	Reduction in the levels of cytokines and lower mortality rate[86]
Infliximab	Anti-cytokine	Not confirmed. 5 mg/kg studied	Further studies required. Treatment has shown to predispose patients to higher rate of infections as well as higher likelihood of mortality[93]
Liver transplantation	Surgery	Healthy 'donor' liver transplanted. 6 mo abstinence required	Transplantation has been shown to improve in quality of life[95,96]
N-acetylcysteine	Antioxidant	Not confirmed	In animal models NAC has been shown to prevent relapse and improve injury[111]. Further research is required
SAM	Antioxidant	Not confirmed	SAM therapy has improved survival and delayed the need for transplants however other studies have not found evidence to support or refute its use[102,103]
Silymarin	Herbal	Eurosil 85® 420 mg a day	Treatment with Silymarin reduced mortality and improved 4-yr overall survival in cirrhotic patients as well as improving liver function[119]
Betaine	Nutrient	Not confirmed	Animal models have shown betaine supplementation can attenuate alcoholic fatty liver[106-108,120,121]

AH: Alcoholic hepatitis; NAC: N-acetylcysteine; SAM: S-adenosyl methionine.

and treat alcohol addiction. However, disulfiram has little evidence at improving abstinence[74]. Naltrexone, an opioid antagonist aimed to control alcohol cravings lowers the risk of relapse, although, it also has been shown to cause hepatocellular injury[71,75]. Acamprosate is used to minimize withdrawal symptoms when abstaining from alcohol[76]. It has been shown to reduce withdrawal in 15 controlled trials [77].

Nutritional therapy

Malnutrition is often correlated with disease severity in ALD patients[78]. Alcoholics suffer from deficiencies in several vitamins and minerals, including vitamin A, vitamin D, thiamine, folate, pyridoxine, and zinc[78]. Supplementation with zinc has shown to improve and prevent liver disease, as well as block mechanisms of liver injury including 'leaky gut', oxidative stress and apoptosis in animal models[79]. The recommended amount for ALD patients is 1.5 g of protein/kg body weight[2]. Supplementation with micronutrients may be necessary if an individual develops deficiencies. Nutritional support in AH has been reported to improve hepatic encephalopathy and reduce infections[80]. A reverse in both energy and protein deficits has been shown reduce morbidity and mortality in patients with acute AH and cirrhosis[81].

Steroid and anti-cytokine therapy

Steroids serve as the primary treatment for severe AH[82]. Treatment with glucocorticoids have decreased proinflammatory cytokines as well as inhibiting neutrophil activation[83]. Glucocorticoid therapy in AH patients showed short-term histological improvement and 28-d survival, however, long-term survival (beyond 1 year) was not improved[83].

Pentoxifylline, a phosphodiesterase inhibitor, is an anti-TNF-α agent. Pentoxifylline has been trialed in 101 patients with severe AH[84,85]. In-hospital mortality was 40% lower in those patients who were treated with pentoxifylline as well as reducing the likelihood of hepatorenal syndrome (HRS). 50% of deaths in the pentoxifylline treatment group were due to HRS, compared to 92% in the placebo group. Pentoxifylline also exhibited a higher 6-mo survival and a reduced incidence of HRS in patients with severe AH[86].

The Steroids or Pentoxifylline for AH trial (double-blind, randomized control trial) has evaluated the effects of both treatment with prednisolone and/or pentoxifylline

[87-89]. 1103 patients underwent randomization with 1053 suitable for primary end point analysis. The primary endpoint was mortality at 28 d[89]. Results showed primary end point mortality at 28 d was 17% in the placebo–placebo group, 14% in the prednisolone–placebo group, 19% in the pentoxifylline–placebo group, and 13% in the prednisolone–pentoxifylline group, showing that pentoxifylline did not improve patients overall survival[89]. Although not significant, the steroid group showed a trend toward reduced 28-d mortality[89]. However, in those patients who received steroid treatment the rate of serious infection was nearly doubled[89,90].

Another anti-cytokine therapy used in the treatment of ALD is infliximab, a monoclonal chimeric anti-TNF antibody. In a primary randomized study using infliximab, 20 AH patients were given either 5 mg/kg of infliximab as well as 40 mg/d of prednisone or prednisone alone[91]. The results indicated there was no change in overall mortality, however, combination therapy decreased cytokine levels[84]. In France, a clinical trial studied prednisolone (40 mg/d for 4 wk) treatment compared to prednisolone with infliximab (10 mg/kg, at study entry, 2 wk and 4 wk after entry) in 36 patients[92]. Unfortunately the trial was stopped early due to mortality and infection[84], and therefore, this study has received criticism for the dose of infliximab in the trial as this predisposed patients to infections[93]. These trials suggest that anti-cytokine treatment in ALD is associated with an increase likelihood of severe infections and mortality. Canakinumab, a licensed monoclonal antibody inhibitor of IL-1 is currently being studied to treat ALD, as IL-1 has the ability to mediate disease progression in ALD[58].

Liver transplantation

Liver transplantation is a common treatment for end stage chronic liver disease; however, it remains controversial due to the increasing demand for donor organs as well as concerns of relapse from abstinence. Prior to surgery patients must abstain from alcohol for a fixed period of 6 mo[94]. Studies have shown patients who receive transplants have a better quality of life[95,96], however, less than 20% of patients whom have an end-stage liver disease receive surgery[97].

Antioxidants

Oxidative stress is a major contributor to the pathogenesis of chronic liver disease; therefore, antioxidant therapy has been considered to be beneficial in the treatment of ALD. Antioxidant agents able to mediate ROS include vitamins E and C, N-acetylcysteine (NAC) as well as S-adenosyl methionine (SAM), and betaine.

SAM operates to synthesize glutathione, the primary cellular antioxidant[98,99]. Patients with AH and cirrhosis have decreased hepatic SAM levels[100]. In animal models, SAM supplementation can reverse liver injury and mitochondrial damage caused by alcohol[101]. However, no significant difference between SAM supplementation and placebo groups has been reported[102,103]. Betaine, a nutrient involved in the formation of glutathione, is effective in protecting against damage from chronic alcohol consumption[104,105]. Supplementation with dietary betaine in animal studies has shown to ameliorate effects of oxidative stress[106-109]. In rats, NAC was able to reduce ethanol seeking behavior by 77%[110] as well as inhibiting ethanol intake by up to 70%[111]. In 174 patients with severe AH, combination therapy with NAC and prednisolone, compared to prednisolone only increased 1-mo survival in patients with AH, although 6-mo survival did not improve[112]. There are several trials underway investigating treatment options for ALD. A current clinical trial is assessing the effects of SAM and choline treatment for 24 wk against a placebo (trial number NCT 03938662). Choline can help the liver undergo glucose metabolism as well as repairing the cell membrane[113]. As damaged livers cannot produce SAM sufficiently, administration of choline and SAM may be a beneficial treatment in patients with ALD.

Fecal bacteria transplants

Chronic alcohol consumption leads to bacterial overgrowth promoting gut dysbiosis which correlates to disease severity. In cirrhosis patients Bacteroidetes and Firmicutes phyla were found to be decreased[114], however, Proteobacteria, Fusobacteria and Actinobacteria phyla were increased[114]. Cirrhosis is also characterized by reduced beneficial autochthonous bacteria such as Lachnospiraceae, Ruminococcaceae, and Clostridiales XIV as well increased pathogenic bacteria such as *Enterococcaeae, Staphylococcaceae and Enterobacteriaceae*[115]. The significance of bacteria in liver disease has been demonstrated when ethanol-fed, germ-free mice developed severe inflammation and necrosis when supplemented with gut microbiota from AH patients[116]. Furthermore, subsequent transfer of gut microbiota from patients without ALD, led to less

inflammation and liver injury[117] implicating the importance of healthy bacteria. More recently administration of probiotics containing beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* to patients with ALD has improved liver damage and function, including reduction of ALT, AST and bilirubin[118]. Modulation of the gut microbiota with Profermin®, a disease specific food for special medical purposes, has been hypothesized to reduce disease progression (trial number NCT03863730). These studies provide strong evidence that fecal bacteria transplants/probiotic administration may prove an effective mode for the treatment of ALD.

CONCLUSION

Previous research has uncovered many elements in the pathogenesis of ALD, however, the precise triggers and biochemical alterations are yet to be fully understood. Oxidative stress can impair proliferation and alter the immune system leading to bacterial overgrowth and an increased risk of infection. Poor treatments options are available for patients with ALD which have not transformed for several years. Treatment options rely on abstinence, steroids, nutritional therapy and lastly liver transplantation. Current new therapies are aimed at reducing pro-inflammatory signals as well as treating the gut-liver axis. This highlights a need for new therapeutic intervention and advancements in the understanding of the mechanisms involved in disease pathogenesis.

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7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
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ISSN 1949-8454 (online)

World Journal of *Biological Chemistry*

World J Biol Chem 2021 November 27; 12(6): 104-130

Published by Baishideng Publishing Group Inc

WJBC

World Journal of Biological Chemistry

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INDEXING/ABSTRACTING

The *WJBC* is now abstracted and indexed in PubMed, PubMed Central, China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database.

RESPONSIBLE EDITORS FOR THIS ISSUE

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NAME OF JOURNAL
World Journal of Biological Chemistry

ISSN
ISSN 1949-8454 (online)

LAUNCH DATE
July 26, 2010

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Vsevolod Gurevich, Chunpeng Craig Wan

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1949-8454/editorialboard.htm

PUBLICATION DATE
November 27, 2021

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World Journal of Biological Chemistry

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World J Biol Chem 2021 November 27; 12(6): 104-113

DOI: 10.4331/wjbc.v12.i6.104

ISSN 1949-8454 (online)

MINIREVIEWS

Neuroprotection by dipeptidyl-peptidase-4 inhibitors and glucagon-like peptide-1 analogs *via* the modulation of AKT-signaling pathway in Alzheimer's disease

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Author contributions: Each author (Ikeda Y, Nagase N, Tsuji A, Kitagishi Y, Matsuda S) participated sufficiently in this work of drafting the article and/or revising the article for the important rational content; all authors gave final approval of the version to be submitted.

Conflict-of-interest statement: The authors declare that they have no competing financial interests.

Country/Territory of origin: Japan

Specialty type: Neurosciences

Provenance and peer review: Invited manuscript; externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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Abstract

Alzheimer's disease (AD) is the most common reason for progressive dementia in the elderly. It has been shown that disorders of the mammalian/mechanistic target of rapamycin (mTOR) signaling pathways are related to the AD. On the other hand, diabetes mellitus (DM) is a risk factor for the cognitive dysfunction. The pathogenesis of the neuronal impairment caused by diabetic hyperglycemia is intricate, which contains neuro-inflammation and/or neurodegeneration and dementia. Glucagon-like peptide-1 (GLP1) is interesting as a possible link between metabolism and brain impairment. Modulation of GLP1 activity can influence amyloid-beta peptide aggregation *via* the phosphoinositide-3 kinase/AKT/mTOR signaling pathway in AD. The GLP1 receptor agonists have been shown to have favorable actions on the brain such as the improvement of neurological deficit. They might also exert a beneficial effect with refining learning and memory on the cognitive impairment induced by diabetes. Recent experimental and clinical evidence indicates that dipeptidyl-peptidase-4 (DPP4) inhibitors, being currently used for DM therapy, may also be effective for AD treatment. The DPP-4 inhibitors have demonstrated neuroprotection and cognitive improvements in animal models. Although further studies for mTOR, GLP1, and DPP4 signaling pathways in humans would be intensively required, they seem to be a promising approach for innovative AD-treatments. We would like to review the characteristics of AD pathogenesis, the key roles of mTOR in AD and the preventive and/ or therapeutic suggestions of directing the mTOR signaling pathway.

Key Words: Alzheimer's disease; Cognitive disorder; Dementia; Glucagon-like peptide-1; Dipeptidyl peptidase-4; Mammalian/mechanistic target of rapamycin

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Received: March 26, 2021
Peer-review started: March 26, 2021
First decision: May 6, 2021
Revised: May 21, 2021
Accepted: November 28, 2021
Article in press: November 28, 2021
Published online: November 27, 2021

P-Reviewer: Byeon H
S-Editor: Chang KL
L-Editor: Filipodia
P-Editor: Chang KL

Core Tip: Disorders of mammalian/mechanistic target of rapamycin (mTOR) signaling pathways are related to Alzheimer's disease (AD). Although further studies for mTOR, glucagon-like peptide-1, and dipeptidyl-peptidase-4 signaling are needed, they seem to be a promising approach for innovative AD-treatments.

Citation: Ikeda Y, Nagase N, Tsuji A, Kitagishi Y, Matsuda S. Neuroprotection by dipeptidyl-peptidase-4 inhibitors and glucagon-like peptide-1 analogs *via* the modulation of AKT-signaling pathway in Alzheimer's disease. *World J Biol Chem* 2021; 12(6): 104-113
URL: https://www.wjgnet.com/1949-8454/full/v12/i6/104.htm
DOI: https://dx.doi.org/10.4331/wjbc.v12.i6.104

INTRODUCTION

Alzheimer's disease (AD) is a chronic neuro-degenerative disease of the central nervous system (CNS), which is described by a slow and unremitting pathology[1]. The chief clinical appearance of AD is progressive continuing dementia, which is categorized by intellectual symptoms such as diminished cognition, memorial dysfunction, and behavioral complaints[2]. The prevention of AD is a public health concern because of a lack of effective treatments. The onset of AD is associated with an increase in age and to a reduction in mitochondrial ATP synthesis in the hippocampus of the brain[3]. Estrogen has a neuro-protective effect on various nerve cells, however, estrogen also has a carcinogenic effect to non-nerve proliferating cells[4]. Pre-diabetic risk factors, obesity, and metabolic syndrome could promote cognitive dysfunction[5]. Neuro-pathological features of AD are neurofibrillary tangles, molded by hyper-phosphorylated tau protein, which may accumulate into oligomers and/or amyloid plaques[6]. There might be an association between metabolism and brain function. Insulin works as a pro-survival neurotrophic factor with its receptors at cognitive areas in the brain[7]. The commonalities have been found between AD and type 2 diabetes mellitus, which is believed as a high-risk factor for AD[8]. In addition, the animal studies have shown that GLP1 may benefit on the neuro-degeneration[9]. The GLP1 receptor agonists have also been shown as possessing neuro-protective effects in AD, which seem to improve nearly all neuro-pathological features as well as cognitive functions of AD[10]. For example, neurofibrillary tangles, amyloid plaques, and neuro-inflammations in the hippocampus have been reduced in AD model mice[11,12]. In the rat model, it has been shown that a GLP1 receptor agonist also prevents synaptic damage induced by amyloid-beta accumulation, which supports the spatial memory by affecting the phosphoinositide-3 kinase (PI3K)-AKT pathway[11]. Targeting dipeptidyl-peptidase-4 (DPP4) inhibitors that is involved in the GLP1 signaling has been considered as promising therapeutic models to AD[13]. Furthermore, mammalian/mechanistic target of rapamycin (mTOR) has been considered as a center that integrates multiple signaling cascades including the GLP1 receptor signaling, which may also be involved in the progression of AD[14]. We will review the several studies linking potential protective factors to pathogenesis of AD, focusing on the roles of GLP1 and DPP4 inhibitors in the PI3K/AKT/mTOR pathway. In addition, we will summarize the recent researches of the AD-associated biology, by which several diet factors could relate to the pathway. To overview the potential physical activities through the PI3K/AKT/mTOR signaling may contribute to the preventive and/or therapeutic strategy for AD.

PI3K/AKT/MTOR SIGNALING IS INVOLVED IN NEUROPROTECTION OF AD

The mTOR plays a significant role in diverse cellular processes including cell survival, cell proliferation, and cell death[15], which is a particular molecule bound to rapamycin. The rapamycin is an immune-suppressant used for the anti-rejection of tissue-transplantation[16]. Rapamycin also exhibits remarkable potential in the fields of neuro-protection, anti-aging, *etc.*[17]. It can inhibit the activity of the mTOR[18]. The mTOR is also a nutrient-sensor that mediates the signaling responses to energy status

in a cell[19]. Besides, the mTOR activity could be inhibited by nutritional signaling such as caloric restriction[20]. Inhibition of the mTOR could alter cellular responses from cell proliferation to cell quiescence with decreased protein synthesis[21]. Basically, mTOR-inhibition has been shown to increase resistance to stresses resulting in the regulation of age-related diseases, which may contribute to the extension of total life-time[22]. Modulation of the mTOR-function to inhibit cellular apoptosis is deeply involved in the protective effects of pharmacologic agents aiming against diabetes and neurodegenerative diseases[23]. The mTOR activation inhibits autophagy, which is often disrupted in age-related diseases[24]. In the mouse brain neurons, amyloid-beta oligomers have been thought to activate the JNK signaling, leading to insulin resistance[25]. Instead, activation of the PI3K/AKT signaling pathway could bring the inhibition of apoptosis cascade including caspase-signaling[26], leading to the inhibition of the induction of inflammatory cytokines[27]. Following the activation of growth-factor receptors with their ligand, PI3K/AKT gets activated directing to promotion of mitogen-associated protein kinase/extracellular signal-regulated kinases and mTOR[28]. On the other hand, adenosine monophosphate-activated protein kinase (AMPK) is an important signaling mediator of GLP1 receptor, which inhibits mTOR[29]. In fact, the AMPK-loss has resulted in hyper-proliferation and hyperactive mTOR signaling[30].

Therefore, the mTOR signaling could interact with several upstream components including PI3K/AKT and AMPK[31] (Figure 1). Increasing studies have established the involvement of the mTOR signaling in various neuro-degenerative diseases including AD[32]. In particular, activated mTOR signaling is a contributor to the progression of AD[33]. Furthermore, there is a close relationship between mTOR signaling and the presence of amyloid-beta plaques and cognitive impairment[34]. So, the development of mTOR-inhibitors may be useful for the prevention and treatment of AD and/or the other neuro-degenerative diseases. In the CNS, inhibition of the mTOR has been revealed to protect vascular functions in aging[35]. Appropriate dose of rapamycin may diminish neurofibrillary tangles and amyloid-beta plaques improving cognitive functions in AD model mice[36]. Similarly, mTOR inhibition without malnutrition is able to improve the pathology of AD[37]. Moreover, mTOR inhibition protects mitochondrial function, reduces oxidative stress, and maintains glucose homeostasis in aging[20,38]. Conversely, activation of the mTOR may shift metabolisms toward ketone-body consumption[39]. Elevated ketone-body metabolisms and/or the administration of the ketogenic diet have been shown neuro-protective against aging, neurodegeneration, and AD[40].

GLP1 AND DIPEPTIDYL PEPTIDASE-IV-INHIBITION EXHIBITS NEUROPROTECTIVE EFFECTS IN AD

GLP1 is an endogenous hormone secreted from intestinal L-cells in response to food-intake[41]. Proteolytic cleavage of the precursor GLP1 (1–37) produce two biological active forms[42]. GLP1 may stimulate insulin-secretion from beta-cells in pancreatic islets under hyperglycemic situations and may decrease glucagon secretion from alfa-cells in pancreatic islets[43]. Signal transduction of GLP1 is mediated by the GLP1 receptor, a G-protein coupled seven-pass-transmembrane domain receptor, heading to cyclic adenosine monophosphate dependent activation of protein kinase A and AMPK. In fact, it has been shown that GLP1 receptor agonists-treatment activates the AMPK signaling within myoblast C2C12 cells[44]. On the contrary, the GLP1 receptor may also operate the downstream signal transduction from the PI3K/AKT pathway so as to work against cellular apoptosis[45]. Accordingly, the GLP1 receptor could dually modulate the activity of mTOR, a key kinase regulating proliferation, survival, and protection in balance. Actually, GLP1 receptor antagonists also stimulate insulin activation by the PI3K/AKT signaling pathway, with the following activation of mTOR and inhibition of GSK3-beta, an essential kinase involved in the phosphorylation of tau protein in AD[46]. GLP1 may also be involved in the regulation of autophagy, the reduction of the oxidative stress, and in the protection of CNS with induction of anti-inflammatory signaling[47]. In addition, GLP1 plays a critical role preventing cardiovascular diseases, in which GLP1 and its analogs may contribute a great deal in the treatment of the diseases[48]. Likewise, it has been shown that GLP1 receptor agonists reduce the infarct size, inflammation, and apoptosis in a rat model of stroke[49].

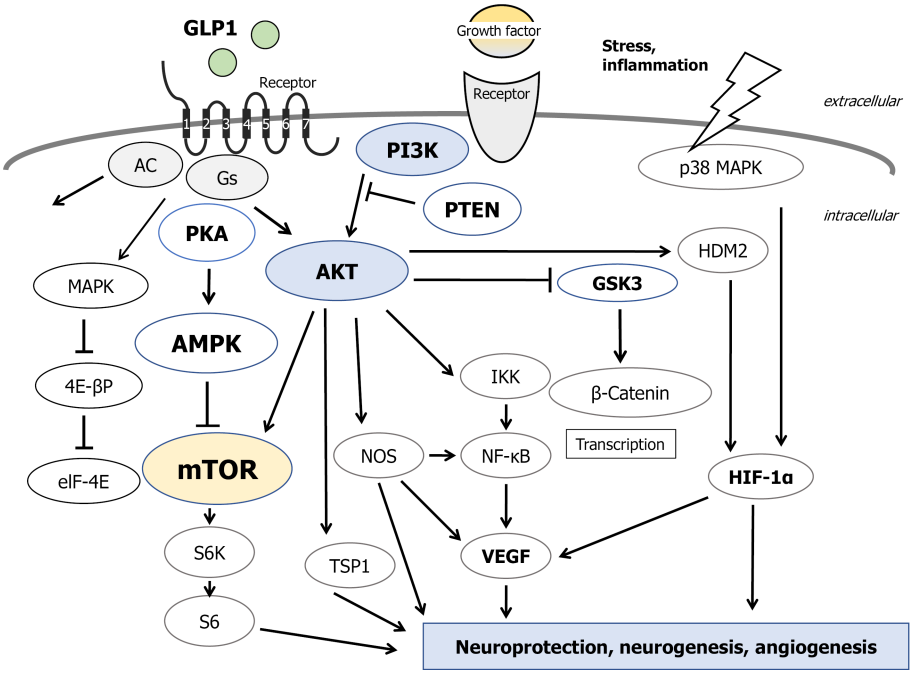

Figure 1 Several modulator molecules linked to the phosphoinositide-3 kinase/AKT/mammalian/mechanistic target of rapamycin signaling in an extracellular growth-factor response are demonstrated. Example molecules known to act on the glucagon-like peptide-1 (GLP1)-receptor/adenosine monophosphate-activated protein kinase (AMPK)/mammalian/mechanistic target of rapamycin (mTOR) signaling pathway are also shown. Note that some critical events such as immune activation and/or cytokine-induction have been omitted for clarity. Arrowhead means stimulation whereas hammerhead represents inhibition. PI3K: Phosphoinositide-3 kinase; PKA: Protein kinase A; PTEN: Phosphatase and tensin homologue deleted on chromosome 10; DPP4: Dipeptidyl-peptidase-4; GSK3: Glycogen synthase kinase 3; MAPK: Mitogen-activated protein kinase; S6K: S6 kinase; AC: Adenylate cyclase; Gs: Stimulatory G-protein; eIF-4E: Eukaryotic translation initiation factor 4E; TSP1: Thrombospondin-1; VEGF: Vascular endothelial growth factor; NOS: Nitric oxide synthase; IKK: I kappa B kinase; NF-kB: Nuclear factor-kappa B; HDM2: Human double minute 2; HIF-1a: Hypoxia inducible factor 1-alpha.

GLP1 is rapidly degraded by DPP4, a serine aminopeptidase expressed in various organs including brain, pancreas, liver, and gut[50]. Therefore, inhibitors of DPP4 may prolong the bioactive half-life of GLP1 in the circulation, which is additionally effective in amending hyperglycemia[51]. The DPP4 inhibitor, linagliptin, has been shown to protect neurons against amyloid beta-induced cytotoxicity and tau hyper-phosphorylation by restoring insulin downstream signaling in AD[52]. Furthermore, the linagliptin alleviated amyloid-beta-induced mitochondrial dysfunction and intracellular ROS generation by a mechanism involving the activation of AMPK-Sirt1 signaling pathway[53]. Chronic administration of another DPP4 inhibitor, sitagliptin, in AD model mice is associated with increased levels of brain GLP1, reductions in the inflammation-biomarkers, and reduction of amyloid-beta deposition in a dose dependent manner[54,55]. Significant reduction in amyloid-beta-42 Level has been associated with the use of linagliptin implying potential application in AD[56]. Also, linagliptin improved vascular functions by increasing creation of nitric oxide and restraining concentration of apolipoprotein B[56]. DPP4 inhibitors can block the DDP4 to diminish GLP1-degradation, prolong GLP1 active life-time, and sensitize insulin-activity for the aim of lowering hyperglycemia[57], and for neuro-protection (Figure 2).

GLP1 and various DPP4 inhibitors (linagliptin, sitagliptin, saxagliptin, *etc.*) seem to be related to their ability to rescue the insulin cascade. Brain insulin signaling has been reported to dwindle with age[58]. So, restoring insulin signaling might be advantageous to patients with AD. Amazingly, intranasal insulin administration, improves memory in healthy adults without affecting circulating levels of insulin and/or glucose[59-61]. In addition, intranasal insulin improves cognitive performance in patients with early AD[59]. It is possible that therapeutic options for AD arise from this mechanism improving for neural insulin-resistance by the DPP4 inhibitors.

DIET WITH CERTAIN KINDS OF NATURAL PRODUCTS MAY IMPROVE AD

Potential preventive factors against AD including lifestyle factors have been suggested to be neuro-protective by epidemiological research[62]. In particular, diet could play a

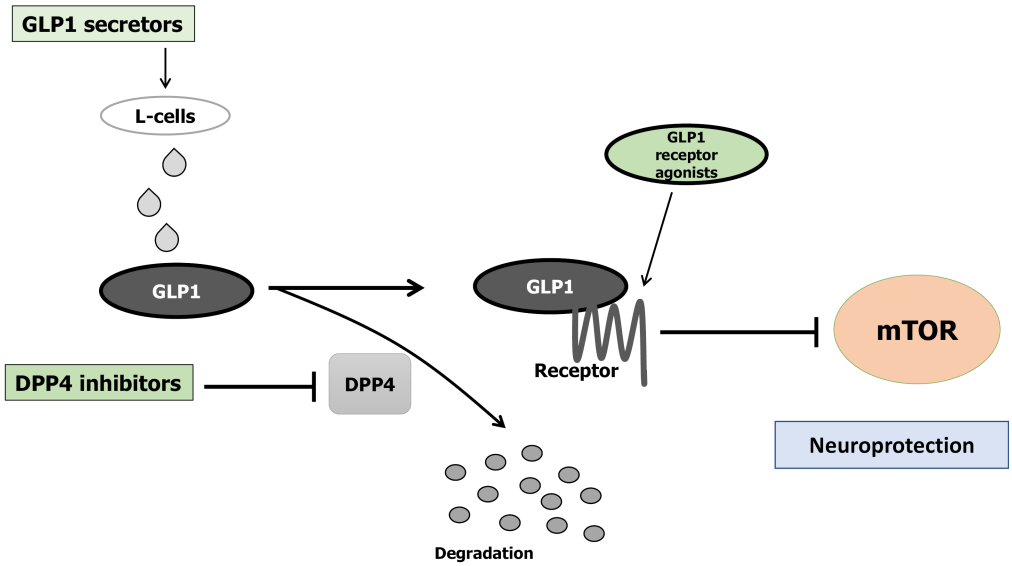

Figure 2 Implication of decreased dipeptidyl-peptidase-4 activity, increased Glucagon-like peptide-1, increased Glucagon-like peptide-1-receptor agonists, and decreased mammalian/mechanistic target of rapamycin activity for the neuroprotection. Arrowhead means stimulation whereas hammerhead represents inhibition. Note that some critical pathways have been omitted for clarity. GLP1: Glucagon-like peptide-1; mTOR: Mammalian/mechanistic target of rapamycin; DPP4: Dipeptidyl-peptidase-4.

key role in the neuro-protection of AD[63]. However, the epidemiological analysis of the relations between nutrient and neuroprotection is very intricate. In addition, we think it unlikely that a single component plays a major role in the neuro-protection. The complexity of the human diet and synergistic and/or antagonistic effects among the various nutrients and food ingredients make it more difficult to examine their distinct effects. However, natural products from several plants and animal sources have been used as good preventive factors against AD through different mechanisms and analytical techniques. Here, we partially summarize them in a view point of mTOR inhibition, GLP1 receptor agonists, GLP1 secretion, and DPP4 inhibition. (Figure 3).

First of all, dietary restriction elicits cell protective responses in nearly all cells and tissues including nerve-cells and brain, which could conduct to activation of SIRT1 and inhibition of mTOR and S6K in C57BL/6 mice[64]. Carnosic acid, a polyphenolic diterpene isolated from the herb rosemary (*Rosmarinus officinalis*) can inhibit the activity of mTOR[65].

Next, GLP1 receptor agonists could protect neurons. Currently, diabetes mellitus treatment based on GLP1 work is being developed. Geniposide, an iridoid glycoside extract from the gardenia fruit, is used in traditional Chinese medicine to alleviate symptoms of liver and inflammatory diseases[23,66]. Geniposide modulates GLP1 receptors signaling[66]. Loureirin B is a natural product derived from *Sanguis draconis*, which promotes insulin secretion of Ins-1 cells through GLP1 receptor[67]. *Lamiophlomis rotata* is an orally available Tibetan herb, which specifically reduces pain hypersensitivity states through the activation of GLP1 receptors[68]. Boschnaloside is the major iridoid glycoside in *Boschniakia rossica*, a well-known traditional Chinese medicine, which can interact with the extracellular domain of the GLP1 receptor[69].

As for compounds stimulating the GLP1 secretion, the ingredient of Hibiscus sabdariffa Linn can increase GLP1 secretion in the ileum[70] *Polygonatum cyrtonema* polysaccharide stimulates GLP1 secretion from enteroendocrine cells[71]. Polysaccharides from the stems of Dendrobium officinale can decrease fasting blood sugar levels by stimulating GLP1 secretion[72]. Spergularia marina can induce GLP1 secretion, which is a halophyte that grows in mud flats[73]. *Costus pictus* D. Don, commonly known as insulin plant, is a traditional Indian antidiabetic herbal medicine, which acutely stimulates GLP1 secretion from intestinal L-cells[74]. *Angelica dahurica* extracts can improve glucose tolerance through the GLP1 secretion[75].

Finally, the intensive search for DPP-4 inhibitors in plant materials has resulted in the identification of macrocarpal A-C from Eucalyptus globulus as a potent inhibitor of DPP4[76]. Furthermore, a variety of other plant derived compounds have been reported to be DPP4 inhibitors. For example, emodin, a natural compound from Rheum palmatum Linn, inhibits DPP4 activity in a dose-dependent manner[77].

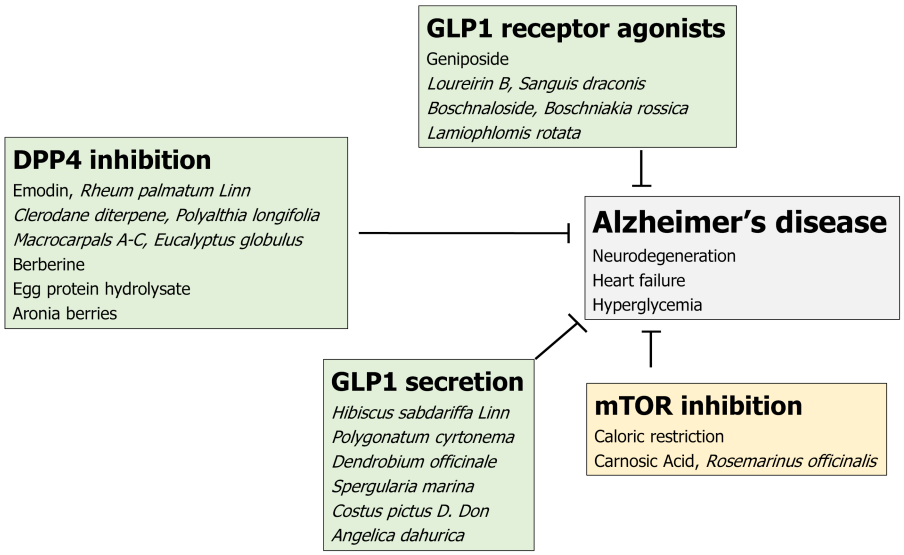

Figure 3 Simplified diagrams indicating the biochemical properties of several natural products are shown. Several herbs and/or their ingredients may contribute to the neuroprotection against the progression of Alzheimer's disease. Hammerhead represents inhibition. DPP4: Dipeptidyl-peptidase-4; GLP1: Glucagon-like peptide-1; mTOR: Mammalian/mechanistic target of rapamycin.

Clerodane diterpene can potentiate hypoglycemia *via* the inhibition of DPP4[78]. Short-term berberine administration can decrease plasma glucose levels through local inhibition of intestinal DPP4[79]. Long-term supplementation with the egg protein hydrolysate exhibits mild *in vivo* DPP4-inhibitory activities[80]. Furthermore, DPP4 is significantly inhibited by cyanidin 3,5-diglucoside present in aronia berries juice[81].

PERSPECTIVES

It is clear that AD may be a multifactorial and incurable disease. Current treatment strategies against AD are mainly directed at reducing amyloid-beta development and inhibiting amyloid-beta aggregation *via* the mechanisms including secretase-inhibition and/or impeding tau hyper-phosphorylation[82]. However, medical trials seem to have failed to demonstrate their significant efficacy without any severe side-effects in clinical situations. Since diet with natural products involved in GLP1 signaling, introduced here, are considered safe for long-term use, they could be an encouraging therapeutic approach against AD. In particular, they could exhibit a lower hypoglycemia risk in comparison to other anti-diabetic medications. On the other hand, GLP1 analogues have been found to decrease appetite. It was noticed that pyramidal neurons of the hippocampus and Purkinje cells of the cerebellum have expressed with GLP1 receptor[83]. In addition, several research reports support extra-pancreatic actions of GLP1 and its analogs by crossing the blood brain barrier (BBB), which are independent of its actions on glucose regulation[84]. AD could be considered as a brain disorder that appears to have fused features of insulin deficiency and insulin resistance. Consequently, DPP4 inhibition, GLP1 secretion, GLP1 receptor agonists, and/or mTOR inhibition may all be effective towards the treatment of AD as well as the other neurodegenerative diseases. This approach might accept new targets with simultaneously multiple molecular mechanisms with minimal side effects. Evaluation for intensive experiments should be provided to obtain further insights. Also, long-term studies are mandatory to clarify its efficacy and safety for the treatment of AD as a brain disorder.

CONCLUSION

Current treatment strategies against AD are directed mainly at reducing amyloid-beta development and inhibiting amyloid-beta aggregation *via* the mechanisms including secretase-inhibition and/or impeding tau hyper-phosphorylation. However, medical trials seem to have failed to demonstrate their significant efficacy without any severe side-effects in clinical situations. Since diet with natural products involved in GLP1

signaling, introduced here, are considered safe for long-term use, they could be an encouraging therapeutic approach against AD. In particular, they could exhibit a lower hypoglycemia risk in comparison to other anti-diabetic medications.

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World Journal of Biological Chemistry

Submit a Manuscript: https://www.f6publishing.com

World J Biol Chem 2021 November 27; 12(6): 114-130

DOI: 10.4331/wjbc.v12.i6.114

ISSN 1949-8454 (online)

META-ANALYSIS

Remission is not maintained over 2 years with hematopoietic stem cell transplantation for rheumatoid arthritis: A systematic review with meta-analysis

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Author contributions: Muthu S and Jeyaraman M provide the conceptualization; Muthu S, Jeyaraman M, and Ranjan R contributed to the data curation, formal analysis, investigations, methodology; Muthu S and Ranjan R contributed to the administration, resources, and supervision; Muthu S contributed to the validation and visualization; Muthu S and Jeyaraman M contributed to writing the original draft and reviewing the drafts.

Conflict-of-interest statement: The authors declare no conflicts of interest for this article.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Country/Territory of origin: India

Specialty type: Rheumatology

Provenance and peer review: Invited manuscript; Externally

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Abstract

BACKGROUND

Hematopoietic stem cell (HSC) transplantation (HSCT) is being accepted as a standard of care in various inflammatory diseases. The treatment of rheumatoid arthritis (RA) has been closely evolving with the understanding of disease pathogenesis. With the rising resistance to the traditional disease-modifying anti-rheumatic drugs and targeted biological therapy, researchers are in pursuit of other methods for disease management. Since the ultimate goal of the ideal treatment of RA is to restore immune tolerance, HSCT attracts much attention considering its reparative, paracrine, and anti-inflammatory effects. However, a systematic review of studies on HSCT in RA is lacking.

AIM

To investigate the role of HSCT in the management of RA.

METHODS

A detailed search of PubMed, Scopus, EMBASE, Cochrane, and the Web of Science databases was made to identify the relevant articles till September 2020 following Cochrane and PRISMA guidelines. We extracted data including the number of patients, source of hematopoietic stem cells, their mobilization and conditioning regimens, results, and complications from the eligible studies.

peer reviewed.

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): E

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Received: March 30, 2021
Peer-review started: March 30, 2021
First decision: May 12, 2021
Revised: May 21, 2021
Accepted: November 26, 2021
Article in press: November 26, 2021
Published online: November 27, 2021

P-Reviewer: Tung TH, Wang G, Xiao T
S-Editor: Ma YJ
L-Editor: Wang TQ
P-Editor: Ma YJ

Results were dichotomized into success (ACR 50/70) and failure (ACR 20) based on the improvement from baseline characteristics. The methodological quality of the included studies was also assessed. Analysis was performed using OpenMeta[Analysis] software.

RESULTS

We included 17 studies (1 randomized controlled trial, 11 prospective, and 5 retrospective studies) with 233 patients for analysis. HSCT provided a significantly beneficial overall improvement in the clinical grades of ACR criteria ($Z = 11.309$, $P < 0.001$). However, the remission was noted only till 24 mo and later on the significance of the result was lost ($Z = 1.737$, $P = 0.082$). A less than 1% treatment-related mortality was noted from the included studies. No major drug-related toxicities were noted in any of the included studies. All patients who underwent allogeneic HSCT received immunosuppression in the conditioning regimen to counteract the graft-*vs*-host reaction which made them vulnerable to infections. It is noted that the source of hematopoietic stem cells did not play a role in altering the functional outcome and both autologous ($Z = 9.972$, $P < 0.001$) and allogenic ($Z = 6.978$, $P < 0.001$) sources produced significant improvement in the outcome compared to the pre-operative state despite having a significant heterogeneity among the studies reporting them ($I^2 = 99.4$, $P < 0.001$).

CONCLUSION

Although the available literature is encouraging towards the use of HSCT in refractory cases with significant improvement from baseline till 2 years, the inclusion of HSCT into the standard of care of RA needs further exploration.

Key Words: Hematopoietic stem cell; Rheumatoid arthritis; Disease-modifying anti-rheumatic drug; Biological therapy; Systematic review; Meta-analysis

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Core Tip: With the rising resistance to the traditional disease-modifying anti-rheumatic drugs and targeted biological therapy, we performed this systematic review and meta-analysis to evaluate the role of hematopoietic stem cell therapy in the management of rheumatoid arthritis. Literature on the effectiveness of the intervention is encouraging with significant improvement till 2 years post-therapy. We have explored the ambiguity in the current treatment methods in hematopoietic stem cell therapy that needs further exploration to optimize the results out of this treatment modality.

Citation: Muthu S, Jeyaraman M, Ranjan R, Jha SK. Remission is not maintained over 2 years with hematopoietic stem cell transplantation for rheumatoid arthritis: A systematic review with meta-analysis. *World J Biol Chem* 2021; 12(6): 114-130
URL: https://www.wjgnet.com/1949-8454/full/v12/i6/114.htm
DOI: https://dx.doi.org/10.4331/wjbc.v12.i6.114

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disorder essentially triggered by the activation of fibroblast like synoviocytes which in turn triggers a series of inflammatory reactions leading to the disease process[1,2]. The treatment of this disease has been closely evolving with an understanding of its pathogenesis. The key principle guidelines recommended in their routine management include: Disease-modifying anti-rheumatic drugs (DMARDs) is started as soon as possible after diagnosis, methotrexate remains the best drug of choice to start with, serial monitoring of disease activity is adopted, use of biologics is limited to patients with persistently active disease refractory to methotrexate, and the treatment target aims for remission or low disease activity[3]. Hematopoietic stem cell (HSC) transplantation (HSCT) is a misnomer because the procedure involves the infusion of the patient's stem cells. HSCT is being accepted as a standard of care in various inflammatory diseases such as

multiple sclerosis, systemic sclerosis, aplastic anaemia, and various immune-mediated cytopenias[4-6]. It is now being widely used for rheumatological diseases such as systemic lupus erythematosus and vasculitic conditions[7,8].

HSCT as a treatment option in the management of RA has been tried with contrasting results[9-13]. With the introduction of biologic therapy for RA, HSCT was resorted only to refractory cases not responding to DMARD[14]. Since the ultimate goal of the ideal treatment of RA is to restore immune tolerance, HSCT attracts much attention considering its reparative, paracrine, and anti-inflammatory effects. However, information on the implications of this therapy including their clinical response rate and complications is limited from the sources like European Group for Bone Marrow Transplantation (EMBT) data registry and Autologous Blood and Marrow Transplant Registry[15].

While many reviews are available evaluating the role of HSCT in various inflammatory disorders[6-8,16], this is the first systematic review article to analyze the effectiveness of HSCT in RA. In this review, we intend to summarize the available evidence on the role of HSCT in the management of RA and analyze whether it holds a future in the treatment spectrum, and discuss some of the potential queries that need further exploration for the applicability in the current scenario of disease management.

MATERIALS AND METHODS

We followed Cochrane guidelines and Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) guidelines[17,18] for the conduction and reporting of this systematic review. We included studies that satisfied the below mentioned PICOTS criteria: (1) Population: Patients with RA; (2) Intervention: HSCT; (3) Comparator: Placebo; (4) Outcome: American College of Rheumatology (ACR) 20/50/70 criteria of improvement from baseline; (5) Timeline: Minimum 6 mo follow-up period; and (6) Study design: Any study design satisfying PICOT.

Search strategy

In September 2020, two reviewers (SM and MJ) performed an extensive independent search of electronic databases - PubMed, Scopus, Embase, Cochrane, and the Web of Science - to identify all the relevant studies using keywords: “Rheumatoid arthritis”, “RA”, “Inflammatory Arthritis”, “Stem Cell”, and “Hematopoietic Stem Cell”. The search strategy used for PubMed has been provided in Supplementary File 1. The reference list of the selected articles was also searched to identify studies not identified in the primary search. As per the inclusion and exclusion criteria, eligible studies were included in the systematic review. We utilised kappa statistics to analyse the level of agreement of the reviewers for the inclusion of studies in the review and any discrepancy between the authors was resolved through discussion until a consensus was obtained.

Data extraction

Two reviewers (SM and MJ) retrieved independently relevant data from articles included for analysis. The following data were extracted: (1) Study characteristics: Year of publication, authors, nature of the study, and number of patients involved; (2) Baseline characteristics: Age, source of HSC (autologous/allogenic), HSC mobilization regimen, HSC manipulation methods utilized, HSC characterization, HSC conditioning regimen, and follow-up period; (3) Main outcome: ACR 20/50/70 criteria of improvement from baseline; and (4) Secondary outcome: Complications.

We expected heterogeneity in the scales and scores utilized for reporting the functional outcome of HSCT in the included studies. Hence, we utilized the standard ACR 20/50/70 criteria to categorize the outcome of the patients undergoing HSCT for RA which was commonly used in the studies[19]. In case of studies not reporting their outcome based on the ACR criteria, we utilized the description of recovery of the patient to categorize them under the ACR 20/50/70 criteria and if sufficient information was not available from the study, the corresponding authors were contacted for further information to categorize the patient into appropriate categories.

For ease of analysis, we dichotomized the results of HSCT into treatment success if the patients achieved a minimum of ACR50 criteria of improvement from the baseline as used by Nikolov *et al*[20]. Moreover, we also expected the included studies to have a variable follow-up period. Hence, we grouped the studies based on their follow-up period to analyze the results of the studies on HSCT for RA at various time points following the procedure. We utilised kappa statistics to analyse the level of agreement

of the reviewers in data extraction and any disagreements were resolved by discussion until a consensus was achieved.

Risk of bias and quality assessment

The methodological quality of the included studies was assessed independently by two reviewers using the risk of bias tool for case series and case reports given by Murad *et al*[21]. Risk of bias of the randomized controlled trials was estimated using the RoB 2 tool of Cochrane Collaboration[22]. To evaluate the methodological index of the prospective non-randomized studies, we utilized MINORS criteria[23].

Statistical analysis

Meta-analysis of the pooled data was performed in the R platform using the OpenMeta[Analyst] software[24]. For dichotomous variables, we utilized proportions with 95% confidence intervals (CIs). We evaluated the heterogeneity of the pooled data using I^2 statistics. If $I^2 < 50\%$ and $P > 0.1$, a fixed-effects model was employed in meta-analysis and if $I^2 > 50\%$ and $P < 0.1$, a random-effects model was utilised. A P value < 0.01 was considered significant. We performed sensitivity analysis and subgroup analysis to explore the source of heterogeneity when it existed.

RESULTS

Search results

The electronic database search resulted in 919 articles which after initial screening for duplicate removal gave a total of 714 articles. Title and abstract screening were done in those articles and 195 of them were excluded. Nineteen articles were qualified for full-text review. We noted that none of the studies utilized a dual-arm study design to compare the effectiveness of the therapy against control as intended. Instead, we found 17 single-arm studies which analyzed the results of HSCT for RA. Hence, we included those 17 studies into the systematic review and performed a single-arm meta-analysis of the reported results stratified based on their study design. PRISMA flow diagram of the study selection is given in Figure 1. The list of studies excluded from full-text screening with the reason for their exclusion is provided in Supplementary File 2. The inter-reviewer kappa agreement was strong in both study selection and data extraction process with kappa values 0.84 and 0.89, respectively.

Quality assessment

The methodological quality of the included studies was given in Table 1. The included studies did not show a high risk of bias to warrant exclusion. The included case reports satisfied all the criteria laid down by Murad *et al*[21] to be eligible for consideration in systematic review and analysis. The range of MINORS score achieved by the prospective studies was from 12-15, which is acceptable for analysis. The randomised controlled trial (RCT) by Moore *et al*[13] showed a low risk of bias among all five domains of assessment for inclusion into the analysis based on the RoB2 tool of Cochrane Collaboration.

General characteristics

Seventeen studies including one RCT[13], eleven prospective studies[9,11,15,25-32], and five retrospective studies[12,33-36] involving 233 patients were qualified for this systematic review. The baseline characteristics of the included studies are shown in Table 2. Although the publication timeline showed a steady increase in the total number of publications since 1997 to 2005, it was followed by an abrupt cessation of studies owing to the introduction of biological therapy in the management spectrum of RA. However, the common indication for HSCT in RA in the included studies was patients who failed to respond to the traditional lines of management with classical DMARDs or biological therapy. The age of the population included for analysis ranged from 18–65 years. Of the 17 included studies, 14 utilized autologous HSCT, and 3 utilized allogeneic HSCT from compatible donors.

Mobilization regimen

Of the 14 studies that utilized autologous HSCT for RA, all utilized granulocyte colony stimulating factor (G-CSF) for progenitor cell mobilization at a dosage ranging from 5–10 μg/kg and cyclophosphamide (CYC) at a variable non-myeloablative dosage ranging from 1.5 g/m^2 to 4 g/m^2. Etoposide was also used along with CYC by Durez *et*

Table 1 Methodological quality and risk of bias assessment of the included studies (*n* = 17)

Randomized controlled trial

Ref.	Randomization process	Deviation from the intended interventions	Missing outcome data	Measurement of the outcome	Selection of the reported result	Overall Bias
Moore *et al*[13]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk

Prospective studies

Ref.	A clearly stated aim	Inclusion of consecutive patients	Prospective collection of data	Endpoints appropriate to the aim of the study	Unbiased assessment of the study endpoint	Follow-up period appropriate to the aim of the study	Loss to follow up less than 5%	Prospective calculation of the study size
Tyndall *et al*[29]	1	1	2	2	2	1	2	1
Burt *et al* [26]	2	2	2	1	2	1	2	1
Burt *et al* [25]	2	2	2	1	2	1	2	1
Verburg *et al*[30]	2	2	2	2	2	2	2	1
Snowden *et al*[27]	2	2	2	1	2	1	2	1
van Laar *et al*[28]	2	2	2	2	2	2	2	1
Snowden *et al*[15]	1	1	2	1	2	1	2	1
Bingham *et al*[11]	2	2	2	2	2	2	2	1
Teng *et al* [9]	2	2	2	2	2	2	2	1
Pavletic *et al*[31]	2	2	2	2	2	2	2	1
Verburg *et al*[32]	2	2	2	2	2	2	2	1

Case reports

Ref.	Selection score	Ascertainment score	Causality score	Reporting score	Total score
Silva *et al* [12]	1	2	1	1	5
Joske *et al*[33]	1	1	1	1	4
Kim *et al* [35]	1	2	1	1	5
Durez *et al*[34]	1	1	1	1	4
Burt *et al* [36]	1	2	1	1	5

al[34] Similarly, all the studies utilized leukapheresis to remove the autoimmune inflammatory cells from the circulation. All the included studies manipulated the cells mobilized by selective isolation of CD 34+ cells. Two of them compared the effect of this selective manipulation in their study and did not find any substantial benefit out of the process[10,15]. The complete HSCT protocol including the mobilization protocol followed by the individual studies is given in Table 3.

Table 2 General characteristics of the included studies (*n* = 17)

Sl. No	Ref.	Year	Study design	Indication	Sample size	Age (yr)	Source (Autologous/allogenic)	Mean follow-up (mo)
1	Joske *et al* [33]	1997	Case report	Failed DMARDs	1	46	Autologous	6
2	Durez *et al* [34]	1998	Case report	Failed DMARDs	1	22	Autologous	10
3	Burt *et al* [26]	1998	Prospective study	Failed DMARDs	2	44	Autologous	12
4	Snowden *et al*[27]	1999	Prospective study	Failed DMARDs	8	18-65	Autologous	18
5	Burt *et al* [25]	1999	Prospective study	Failed DMARDs	4	46.2	Autologous	12
6	Kim *et al* [35]	2002	Case report	Failed DMARDs	1	54	Autologous	6
7	Tyndall *et al*[29]	2001	Prospective study	Primary treatment	43	NR	Autologous	11
8	van Laar *et al*[28]	2001	Prospective study	Failed DMARDs	8	18-60	Autologous	18
9	Verburg *et al*[30]	2001	Prospective study	Failed DMARDs	14	43	Autologous	12
10	Bingham *et al*[11]	2001	Prospective study	Failed DMARDs	6	37.33	Autologous	20
11	Pavletic *et al*[31]	2001	Prospective study	Failed DMARDs	6	42.5	Autologous	26.5
12	Moore *et al* [13]	2001	RCT	Failed DMARDs	33	18-65	Autologous	12
13	Burt *et al* [36]	2004	Case report	Failed DMARDs	1	52	Allogenic	12
14	Snowden *et al*[15]	2004	Prospective study	Failed DMARDs	73	42	Autologous	18
15	Verburg *et al*[32]	2005	Prospective study	Failed DMARDs	8	35-55 years	Autologous	24
16	Teng *et al* [9]	2005	Prospective study	Failed DMARDs	8	43	Allogenic	60
17	Silva *et al* [12]	2018	Retrospective study	Failed DMARDs (10), failed autologous HSCT (1), secondary haemophagocytic lymphohistiocytosis (5)	16	12	Allogenic	29

DMARDs: Disease modifying anti-rheumatic drugs; HSCT: Haematopoietic stem cell transplant; NR: Not reported; RCT: Randomised controlled trial.

Conditioning regimen

The commonly employed drug in the conditioning regimen of the included studies to avoid rejection of HSCT in the RA patients was CYC at a dosage ranging from 100-200 mg/kg. In addition to CYC, anti-thymocyte globulin (ATG) was used in 5/17 included studies at a constant dose of 90 mg/kg[25,26,29,35], and busulfan in one of them at 4 mg/d dosage[34]. Two studies utilized fludarabine and alemtuzumab in their conditioning regimen. The detailed list of drugs used by the individual studies in their conditioning regimen is given in Table 3.

Functional outcome

We noted significant heterogeneity among the scales used for the assessment of the functional improvement in the included studies such as ACR outcome improvement criteria, Visual Analog Scale, Health Assessment Questionnaire, Disease Activity Score, Larsen Score, C-reactive protein level, Erythrocyte Sedimentation Rate, and Rheumatoid Factor. However, ACR was the most commonly employed outcome measure in HSCT to assess the functional outcome post-procedure. Hence, we

Table 3 Hematopoietic stem cell transplant protocol in the included studies (*n* = 17)

Sl. No	Ref.	Mobilization regimen	Graft manipulation	HSC selection	Conditioning regimen
1	Joske *et al* [33]	CYC 4 g/m^2, G-CSF 10 μg/kg	Leukapheresis	CD 34 +ve selection	CYC 200mg/kg
2	Durez *et al* [34]	CYC 1.5 g/m^2, etoposide 300 mg/m^2, G-CSF 5 μg/kg	Leukapheresis	CD 34 +ve selection	CYC 60 mg daily and busulfan 4 mg daily
3	Burt *et al* [26]	CYC, G-CSF	Leukapheresis	CD 34 +ve selection	CYC 200 mg/kg, ATG 90 mg/kg
4	Snowden *et al*[27]	CYC 100-200 mg/kg, G-CSF 5 μg/kg	Leukapheresis	CD34 +ve selection	CYC 100 mg/kg or 200 mg/kg
5	Burt *et al* [25]	CYC 2 g/m^2, G-CSF	Leukapheresis	CD34 +ve selection	CYC 200 mg/kg, ATG 90 mg/kg
6	Kim *et al* [35]	CYC 4 g/m², G-CSF 5 μg/kg	Leukapheresis	CD 34 +ve selection	CYC 200 mg/kg, ATG 90 mg/kg
7	Tyndall *et al*[29]	CYC, G-CSF	Leukapheresis	NR	CYC 200 mg/kg, ± ATG 90 mg/kg, ± Busulfan
8	van Laar *et al*[28]	CYC 4 g/m², G-CSF 10 μg/kg	Leukapheresis	CD34 +ve selection	CYC 200 mg/kg
9	Verburg *et al*[30]	CYC 4 g/m², G-CSF 10 μg/kg	Leukapheresis	CD 34 +ve selection	CYC 200 mg/kg
10	Bingham *et al*[11]	CYC 2 g/m^2, G-CSF	Leukapheresis	CD 34 +ve selection	CYC 200 mg/kg
11	Pavletic *et al*[31]	CYC 2 g/m^2, G-CSF	Leukapheresis	CD34 +ve selection	CYC 200 mg/kg, ATG 90 mg/kg
12	Moore *et al* [13]	CYC 200 mg/kg, G-CSF 10 μg/kg	Leukapheresis	CD34 +ve selection (18) / No selection (15)	CYC 200 mg/kg
13	Burt *et al* [36]	NA	NA	CD 34 +ve selection	CYC 150 mg/kg, fludarabine 125 mg/m^2, alemtuzumab 20 mg
14	Snowden *et al*[15]	CYC 200 mg/kg, G-CSF 5-10 μg/kg	Leukapheresis	CD 34 +ve selection (45) / No selection (28)	CYC 200 mg/kg
15	Verburg *et al*[32]	CYC 200 mg/kg, G-CSF	Leukapheresis	CD 34 +ve selection	CYC 200 mg/kg
16	Teng *et al*[9]	NA	NA	CD 34 +ve selection	CYC 200 mg/kg
17	Silva *et al* [12]	NA	NA	CD 34 +ve selection	Fludarabine 30 mg/m²/d, melphalan 140 mg/m²/d, alemtuzumab 0.2 mg/kg/d or fludarabine 30 mg/m²/d, treosulfan 14 mg/m²/d, alemtuzumab 0.2 mg/kg/d

ATG: Anti-thymocyte globulin; CD: Cluster differentiation: CYC: Cyclophosphamide; G-CSF: Granulocyte colony stimulating factor; HSC: Hematopoietic stem cell; NA: Not applicable; NR: Not reported.

converted the outcome of all the studies included under ACR criteria based on the outcome characteristics reported. Significant heterogeneity existed in the ACR results among the included studies (I^2 = 81.86%, P < 0.001). Hence, a random-effects model was utilized for analysis.

We also found the follow-up period of the included studies to range from 6-60 mo. Hence, we grouped the studies based on their follow-up period to analyze the results at various time points following the procedure. Figure 2 shows the change in the grades of ACR criteria at various time points among the included studies using HSCT for RA. Figure 3A shows the forest plot of analysis of results of studies at various time points following HSCT in comparison to their pre-operative status of RA using a random binary effects model. HSCT provided a significantly beneficial overall improvement in the clinical grades of ACR criteria (Z = 11.309, P < 0.001). A significant difference in the preoperative state of ACR was noted till 24 mo and later on the significance of the result was lost (Z = 1.737, P = 0.082) as shown in Figure 3A.

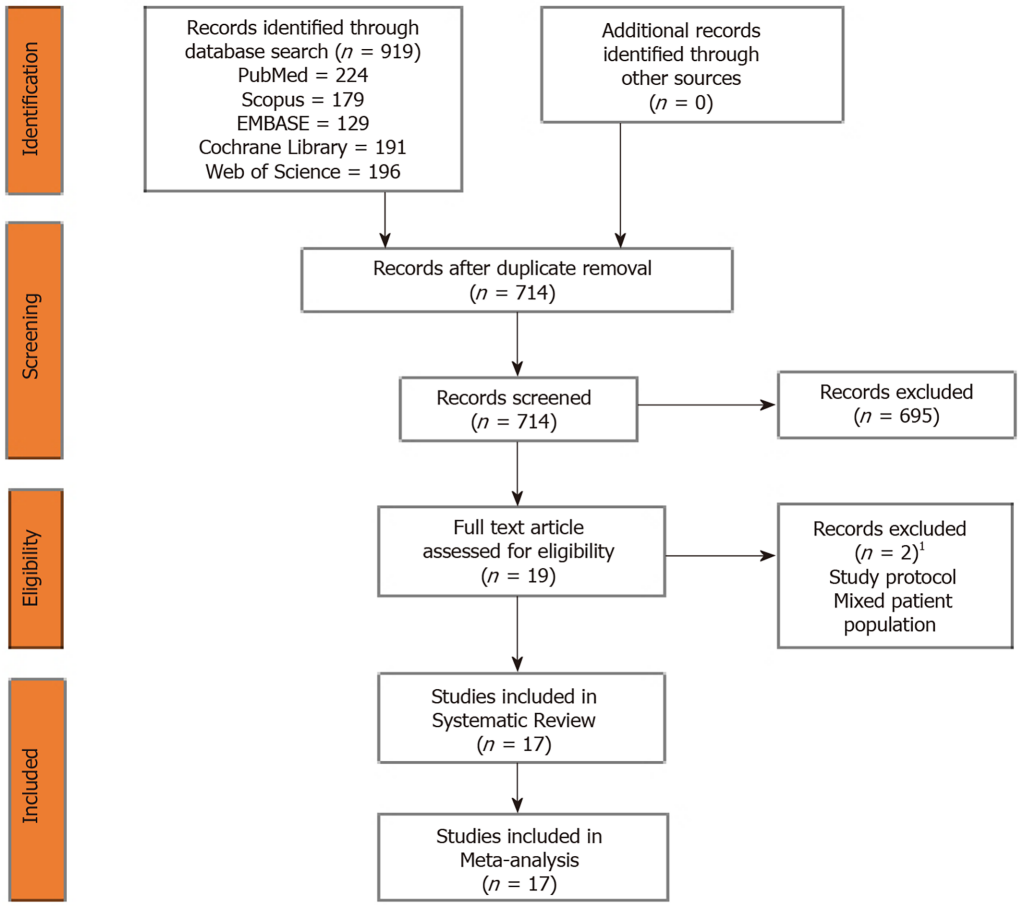

Figure 1 Preferred reporting items for systematic reviews and meta-analyses flow diagram of the included studies. [1]List of excluded studies given in Supplementary File 2.

We explored the heterogeneity among the included studies through subgroup analysis of the results based on the nature of HSCT (*i.e.*, autologous and allogeneic types) and presented the results in Figure 3B. It was noted that the source of HSCT did not play a role in altering the functional outcome and both autologous ($Z = 9.972$, $P < 0.001$) and allogenic ($Z = 6.978$, $P < 0.001$) sources produced significant improvement in the outcome compared to the pre-operative state despite having a significant heterogeneity among the studies reporting them ($I^2 = 99.4$, $P < 0.001$). On exploring the heterogeneity, variability was noted in the follow-up period of the included studies despite maintaining the significance of the outcome results.

Complications

Despite using a non-myeloablative regimen in the HSCT protocol, the patients tended to undergo a spectrum of side effects. The routine side effects of chemotherapy such as nausea, vomiting, hair loss, skin rash, and fever were noted in most of the patients. We took into account the procedure-related mortality, drug-related major toxicities, and grade III/IV graft-*vs*-host reaction (GVHD) as significant complications due to the procedure and analyzed their prevalence among the included studies. One transplant-related death was noted by Tyndall *et al*[29] and death due to sepsis was noted in a study by Snowden *et al*[15]. We noted a < 1% (2/233) procedure-related mortality from the included studies. No major drug-related toxicities were noted in any of the included studies.

All patients who underwent allogeneic HSCT received immunosuppression in the conditioning regimen to counteract the GVHD which made them vulnerable to infections. High-grade GVHD was noted in patients undergoing allogeneic HSCT by Silva *et al*[12] along with a higher prevalence of viral infections noted in them. It was noted from the forest plot that HSCT was not associated with a significant increase in the listed major complications ($P = 0.015$, 95% CI: 0.005-0.041) as shown in Figure 3C. However, it should be prudent to consider on a case-by-case basis whether these risks outweigh the benefits from the therapy.

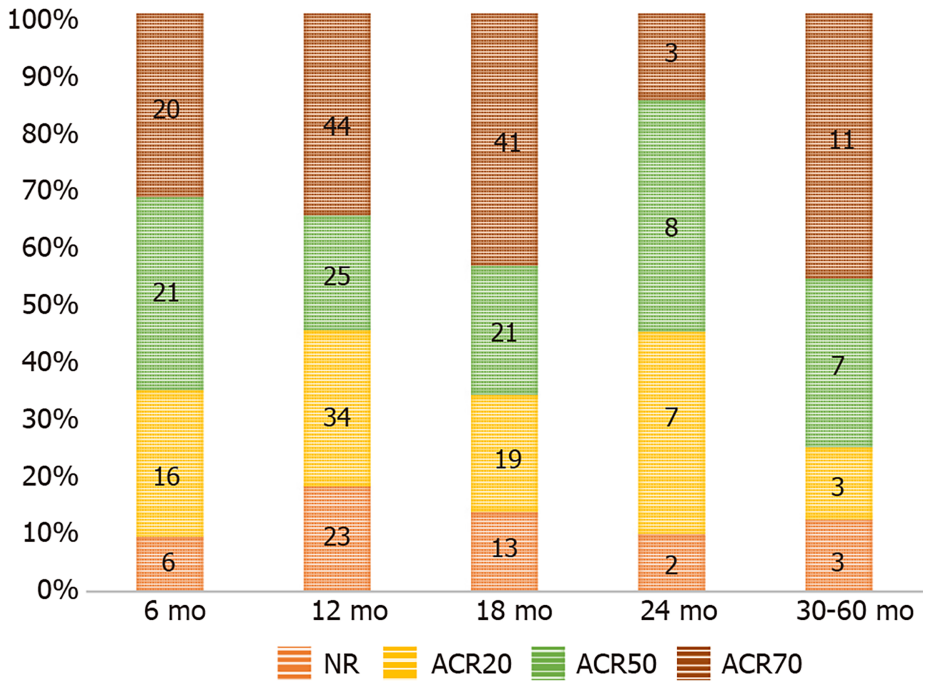

Figure 2 Transition trend of American College of Rheumatology criteria in the included studies across various time points.

Sensitivity analysis

A sensitivity analysis was performed in each analysis. The results of the outcomes analysed were not significantly altered by sequentially omitting each study in the meta-analysis within each study design. On the other hand, the consistency of the results was maintained after reanalysis by changing the random-effects model.

DISCUSSION

Despite the usage of both conventional DMARDs and newer biologicals, 40% of patients with RA continue to have frequent relapses with active and progressive disease[20,37]. Autologous HSCT has been considered as an alternative modality of management of such resistant candidates[38]. Although HSCs are multipotent stem cells with the potential to give rise to blood, endothelial cells, and immune cells, in the context of their role in autoimmune diseases they are viewed as immune stem cells [39]. The major complication from the HSCT arises not from the HSC transfer itself but from the immunosuppressive conditioning regimens utilized to inhibit the autoreactive immune cells before the transfer[8]. The rationale of using the immunosuppressive conditioning regimens is not to myeloablate the host immune system but to lymphoablate the autoimmune cells so that immune regeneration starts from the transferred HSCs[20]. These non-myeloablative regimens used in the included studies commonly employed CYC as shown in Table 2. Special attention should be given to the regimen-related side effects particularly from the high dose CYC which forms the backbone of these regimens[40].

Response to the HSCT was shown by the reduction in the serum auto-antibody titers noted in the included studies[10-12]. This shows a temporal relationship between the immune balance restoration and clinical response outcomes as a precondition to get immune tolerance in RA patients[41]. However, to obtain optimal results from the HSCT patient selection is of key importance. Although HSCT is recommended for patients who failed conventional spectrum of management, good results from HSCT are obtained from patients presenting with an early aggressive disease with poor prognostic factors who also have enough residual functional capacity to benefit out of the procedure[14,42].

HSCT is also associated with considerable morbidity and treatment-related mortality (TRM). Based on the registry data, 1-year transplant-related mortality due to autologous HSCT for haematological malignancies was 2%-5%[43]. However, from the included studies we noted a < 1% TRM from the HSCT procedure for RA. The most

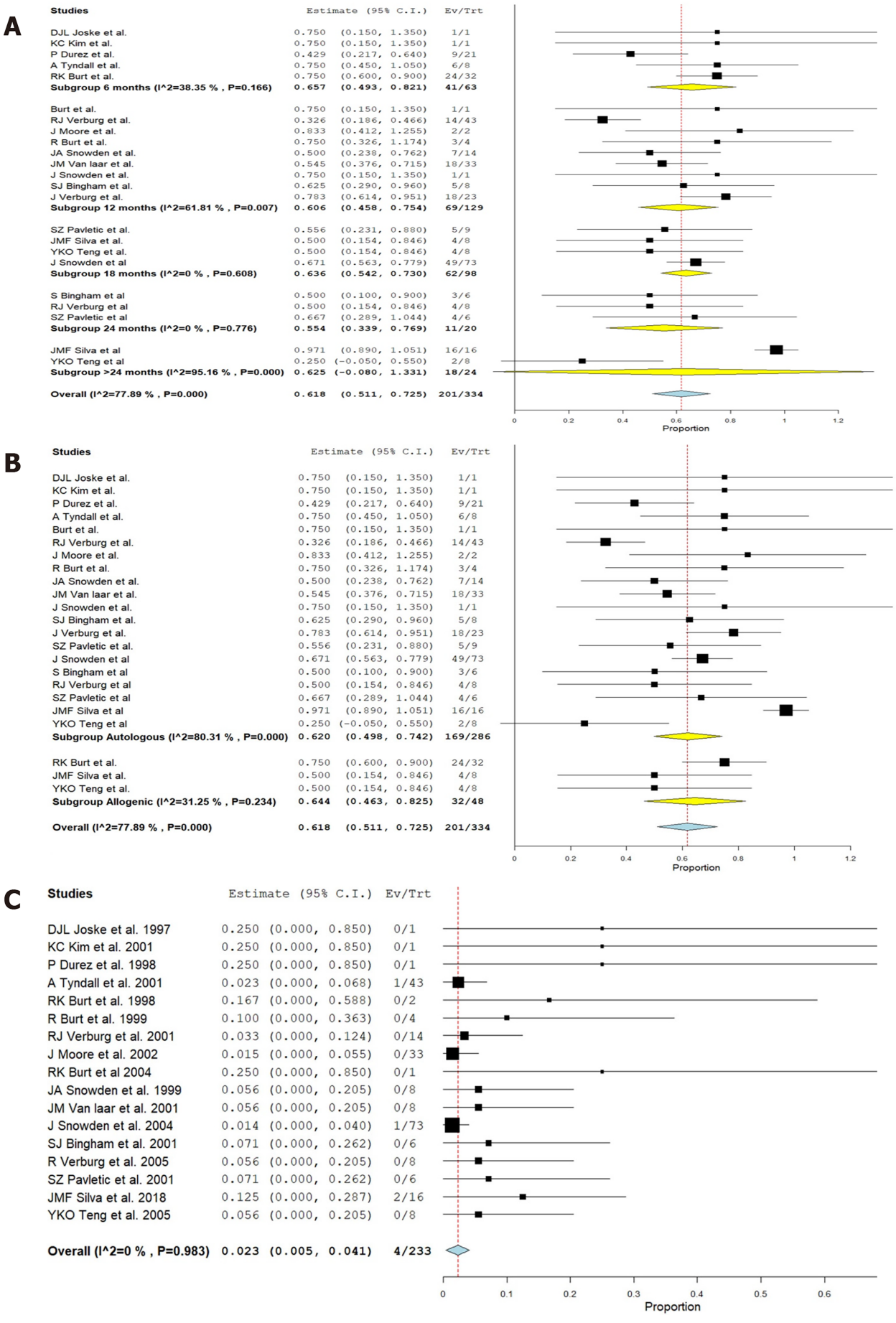

Figure 3 Forest plot. A: Analysis of results of included studies at various time points following hematopoietic stem cell transplantation (HSCT) in comparison to

their pre-operative status of rheumatoid arthritis using a random binary effects model; B: Sub-group analysis of the results based on the nature of HSCT (autologous and allogeneic types); C: Major complications noted in the included studies.

common complication encountered with HSCT from all the included studies is infection due to the immunosuppression that accompanies the conditioning protocol followed[44]. Owing to the neutropenia, bacterial or fungal infections may occur and lymphopenia may lead to latent viral and opportunistic infections[45]. This has been counteracted by the empirical use of broad-spectrum antibacterial, antiviral, and antifungal medication in this high-risk period post-transplantation.

The risk of TRM and toxicity depends not only on the HSCT protocol used but also on the source of the donor cells[46]. Allogenic HSCT is associated with a higher risk of complications especially due to the GVHD associated with them. Most of the adverse events are associated with the conditioning regimens utilized following allogeneic HSCT[45]. To clear the autoreactive inflammatory cells causing GVHD an array of conditioning regimens including drugs such as fludarabine, melphalan, alemtuzumab, and treosulfan along with CYC have been utilized in the included studies[9,12]. To optimize the safety of the procedure, the treatment must be offered after preliminary screening for comorbidities and cardiopulmonary ailments and administration of the regimens in dedicated centres with appropriate supportive care to make the procedure successful and safe.

Cost-effectiveness

With due consideration to the selected group of patients who is eligible for HSCT, the impact of the disease on society is far from negligible[47]. Although they are small in proportion, consumption of the health care services by these seriously ill patients remains significant[48]. Compared to the lifetime costs incurred in the management of such resistant cases of RA utilizing biologically targeted therapies which are required in the long term without any guaranteed universal effectiveness[49], HSCT appears a promising cost-effective strategy although it is also an expensive treatment by itself. A complete remission out of HSCT would lead to significant cost savings in the long run [50,51]. Apart from the economic benefits, complications of chronic immunosuppressive therapies with targeted biologicals could be avoided with the use of HSCT [50]. So far, no cost-effectiveness analysis has been made for HCST in RA.

Future recommendations

Before the inclusion of HSCT into the routine management protocol for RA, certain questions need further exploration to standardize the treatment protocol to harness maximum benefits out of the procedure. The potential questions that need answering in the various stages of HSCT are enumerated in Figure 4.

Question 1: Is stem cell rescue necessary after high dose immune ablation?

With the studies reporting complete remission of severe cases of RA after a myeloablative dose of CYC without being followed by HSCT[52], a question arises as to whether the procedure needs firsthand. Regeneration of the marrow function similar to HSCT was noted but at a slower pace. Introducing the auto-immune lymphocytes into the host following a high dose of CYC may be a reason for noted failure in some of the cases of autologous HSCT[52]. Although the concept appears appealing, whether it could be qualified to be investigated under a clinical trial poses ethical considerations. However, one could plan for a trial with and without immediate stem cell rescue following high dose CYC therapy for RA patients[53].

Question 2: What is the ideal source of HSCs?

There has been a shift in the source of autologous HSCT from bone marrow (BM) to peripheral blood stem cells (PBSC) because of the rapid haematological recovery especially platelet and neutrophil counts following reinfusion when PBSC are used as a source of HSCT[54]. It also makes the procedure more cost-effective[55]. But it is also noted that, when PBSC is used as a source of HSCT, an 11-fold increase in T cells and an 8-fold increase in B-cells were noted, thereby making them less likely to provide any sustained benefit compared to the BM source which has a less cellular load on reinfusion[56]. It is also not evident whether the peripheral T cell counts have any temporal association with the damage caused by the disease. One other finding in allogenic HSCT is that patients who undergone HCST with PBSC source did not document any proportional increase in GVHD compared to BM source[57]. Hence,

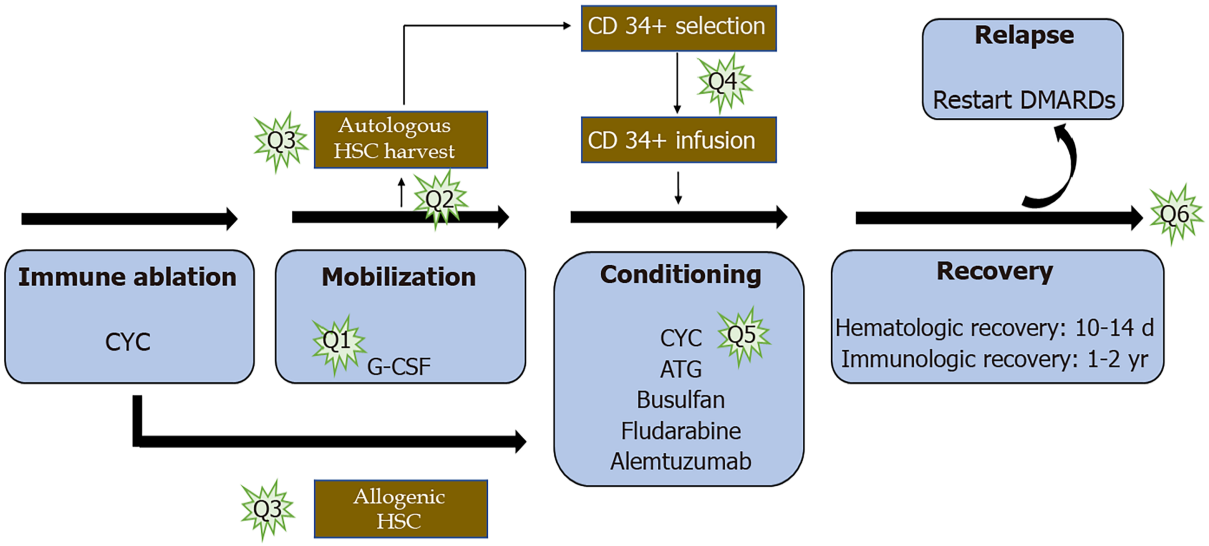

Figure 4 Potential areas of future research to optimize hematopoietic stem cell transplantation treatment for rheumatoid arthritis. Q1 is to evaluate whether stem cell rescue is necessary after high dose immune ablation; Q2 is to assess the ideal source of hematopoietic stem cells (HSCs); Q3 deals with either autologous or allogeneic source; Q4 deals with the need for T cell depletion from the harvested material; Q5 probes into the ideal conditioning regimen; and Q6 evaluates the ideal timing of HSC transplantation in the course of the disease. HSC: Hematopoietic stem cell.

comparative long-term clinical trials to explore the ideal source of HSCs are needed to further explore this issue.

Question 3: Autologous or allogeneic HSCT?

There is a theoretical concern in allogenic HSCT that the patient's immune cells could not be able to continue the disease process following intensive immunosuppressive therapy since the reconstituted immune progenitors belong to the donor. Moreover, the donor T cell elicits a GVHD which enables elimination or suppression of the residual autoimmune clones in the body. We do not have any evidence on this "graft-versus-autoimmune disease" effect, to state a correlation between the degree of GVHD and the resolution of the disease process to weigh one over the other.

Question 4: Should T cells be depleted from the harvested material?

Although phase I and phase II clinical studies have established the therapeutic potential, clinical safety, and efficacy of HSCT therapy[20], there is a paucity of literature to provide a consensus on whether the lymphocytes from the collected peripheral HSCs should be depleted before re-infusion. Although T-cell depletion (TCD) prevents the re-entry of autoimmune cells in the system, the procedure bears the risk of late opportunistic infections especially those by cytomegalovirus and Ebstein-Barr virus[44]. There is a current understanding that complete lymphoablation may not be needed since the immune reset happens with the development of the immune regulatory networks and immune tolerance. Moreover, Joske *et al*[33] in their study did not find any significant difference due to CD34+ selection either. Hence, the need for TCD has to be further explored with randomized controlled trials to arrive at a definite conclusion on this aspect of HSCT.

Question 5: What is an ideal conditioning regimen?

Most of the included studies utilized CYC based regimens to minimize the effect of residual autoreactive clones in the body. The effect of the conditioning regimen used on the results of the transplant remains unexplored although the intensity of the conditioning might play a role. Most of the included studies utilized CYC at a high dose of 200 mg/kg administered for 4 d. Such high dose chemotherapy has its side effects such as hemorrhagic cystitis. Further research to identify alternative conditioning regimens that are immunosuppressive without being myelosuppressive and also prolong the remission achieved in patients is needed. Other combinations such as regimens containing ATG were also tried in some of the included studies as shown in Table 2. Administration of G-CSF in the post-transplant state would shorten the recovery period but in some cases, G-CSF has been shown to trigger an exacerbation of arthritis which needs further exploration[58]. Moreover, clinical trials are needed to

evaluate the difference between regimens with enhanced immunoablative capacity where the greater toxicity is justified with prolonged remission and conventional regimens with added post-transplant immune suppression to prolong the remission.

Question 6: What is the ideal timing of HSCT?

The major challenge in utilizing HSCT for RA is the timing of initiation of the treatment in the course of the disease. If the patient is considered for HSCT after a trial of response to immunosuppressive therapy with DMARDs, the disease could have evolved beyond the point of maximum benefit from HSCT since they are less effective in patients with advanced organ damage and immune dysregulation. Since there are no specific guidelines to the timing and patient profile selection for enrolling into HSCT, the decision largely lies in the hands of the patients and their treating physicians. Clinicians should help the patients choose the right treatment by weighing their pros and cons together and provide clear information to aid in the decision-making process considering the prognostic factors associated with the disease process in the individual patients[59]. Although the treatment seems promising, the guidelines drafted by the European League against Rheumatism (EULAR) and EMBT for patient selection for optimal response need further improvement on the above-mentioned areas.

The small sample size of the included studies with heterogeneity in their patient selection methods, HSCT protocols utilized, the reported results, and their definition of remission limits their utility in decision making. In the absence of large clinical trials, a Markov clinical decision analysis to compare the conventional therapy with HSCT could be utilized. The model predicted HSCT to be superior to conventional therapy if the TRM could be maintained < 3.3% or if the treatment results are sustainable for 5 years. Having done in the early era of biological therapy, these analyses emphasize that a subset of RA patients could also benefit from HSCT. The differences in the Quality Adjusted Life Years between the two groups involved in the model reinstate the role of the patients in the decision-making process[9]. With the improvement in the treatment methods, the safety of the procedure has largely been improved. In selected cases, HSCT may remain the only effective method available making these risks acceptable. Yet, the decision lies in the hands of the patient, hence it needs careful discussion before making the treatment choice. With the rise in the resistance to traditional therapy for RA, earlier identification of those non-responders based on clinic-serological profile and prognostic markers remains a key element to reap the maximum benefit out of this modality.

CONCLUSION

Although the available literature is encouraging towards the use of HSCT in refractory cases with significant improvement from baseline till 2 years, the inclusion of HSCT therapy into the standard of care of RA needs further exploration. With the rising proportion of non-responders to conventional DMARDs and biologic therapy, HSCT therapy would find a place in the treatment spectrum of RA provided that large clinical trials with longer follow-up are conducted to establish the ideal treatment strategy to get optimal results out of this treatment modality.

ARTICLE HIGHLIGHTS

Research background

Hematopoietic stem cell (HSC) transplantation (HSCT) has been accepted as a treatment method in the management of various inflammatory diseases. With the evolution in the management of rheumatoid arthritis (RA), and the rising resistance to the traditional disease-modifying anti-rheumatic drugs, researchers are in pursuit of alternate methods for disease management. Having the ultimate goal of achieving systemic immune tolerance, HSCT has now been considered in the management of RA with respect to its reparative, paracrine, and anti-inflammatory properties.

Research motivation

Despite the understanding of the potential of HSCT towards immune reconstitution, considering RA to be an auto-immune disease, a systematic review of studies on utilization of HSCs in RA is lacking. If HSCT proves to be useful in refractory cases of

RA, future studies to strengthen the evidence on the same could be recommended.

Research objectives

To investigate the role of HSCT in the management of RA.

Research methods

A detailed search of PubMed, Scopus, EMBASE, Cochrane, and the Web of Science databases was made to identify the relevant articles till September 2020 following Cochrane and PRISMA guidelines. All the studies included were analyzed to evaluate the role of HSCT in RA by dichotomizing their outcome based on American College of Rheumatology (ACR) criteria for success (ACR 50/70) and failure (ACR 20) based on the improvement from baseline characteristics. The methodological quality of the included studies was also assessed. Analysis was performed using OpenMeta [Analysis] software.

Research results

Upon meta-analysis of the 17 included studies on the use of HSCT for refractory cases of RA, it was noted that remission was maintained for 2 years. However, for the implementation of the intervention into routine clinical practice, further studies are needed to shed some light on the ideal source of the HSCs for transplantation, the ideal conditioning regimen to be utilized, and the ideal timing of transplantation to reap the maximum benefit it.

Research conclusions

Utilization of HSCT in RA cases that are refractory to the conventional line of management maintained remission to a maximum of 2 years. With the rise in the resistance to traditional therapy for RA, earlier identification of those non-responders based on clinic-serological profile and prognostic markers remains a key element to reap the maximum benefit out of this modality.

Research perspectives

Before the inclusion of HSCT into the routine management protocol for RA, certain questions need further exploration to standardize the treatment protocol to harness maximum benefits out of the procedure.

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