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REVIEW

Is it possible to stop nucleos(t)ide analogue treatment in chronic hepatitis B patients?

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Abstract

Chronic hepatitis B (CHB) remains a challenging global health problem, with nearly one million related deaths per year. Nucleos(t)ide analogue (NA) treatment suppresses viral replication but does not provide complete cure of the hepatitis B virus (HBV) infection. The accepted endpoint for therapy is the loss of hepatitis B surface antigen (HBsAg), but this is hardly ever achieved. Therefore, indefinite treatment is usually required. Many different studies have evaluated NA therapy discontinuation after several years of NA treatment and before HBsAg loss. The results have indicated that the majority of patients can remain off therapy, with some even reaching HBsAg seroconversion. Fortunately, this strategy has proved to be safe, but it is essential to consider the risk of liver damage and other comorbidities and to ensure a

close follow-up of the candidates before considering this strategy. Unanswered questions remain, namely in which patients could this strategy be effective and what is the optimal time point at which to perform it. To solve this enigma, we should keep in mind that the outcome will ultimately depend on the equilibrium between HBV and the host's immune system. Viral parameters that have been described as good predictors of response in HBeAg(+) cases, have proven useless in HBeAg(-) ones. Since antiviral immunity plays an essential role in the control of HBV infection, we sought to review and explain potential immunological biomarkers to predict safe NA discontinuation in both groups.

Key words: CD8; Lamivudine; Nucleos(t)ide analogues; Tenofovir; Chronic hepatitis B; Entecavir; Hepatitis B virus; Treatment cessation

Core tip: Nucleos(t)ide analogue (NA) treatment efficiently suppress hepatitis B virus replication. However, hepatitis B surface antigen loss, the optimal endpoint of NA therapy, is rarely achieved. Thus, a major unmet need in the management of chronic hepatitis B is the definition of earlier and safe treatment stopping points. There is growing clinical evidence that the majority of patients can benefit from this strategy after long-term NA therapy; yet, no criteria that distinguish which cases can safely stop treatment is established. We review here different biomarkers that could serve as a prognostic tool to safely discontinue therapy, focusing on host antiviral immunity.

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INTRODUCTION

According to recent data from the World Health Organization, about 257 million people suffer from chronic hepatitis B (CHB) worldwide. Hepatitis B virus (HBV) infection remains a major global health concern, as the disease itself and its complications, mainly hepatocellular carcinoma (HCC) and cirrhosis, caused 887000 deaths in 2015 alone. The estimated worldwide incidence of HCC in 2012 was 782000 cases, representing the fifth and the ninth most common cancer in males and females respectively. Moreover, HCC was the second cause of global cancer mortality, as it tends to have very poor prognosis with an overall ratio of mortality to incidence of 0.95[1].

Although the actual HBV vaccine is 95% effective, vaccination coverage is still suboptimal in many highly endemic areas. Besides, most of the current HBV-infected persons were born before the vaccine was widely accessible[2-4]. HBV infection chronification is not fully understood. The HBV genome assembly into a stable mini-chromosome, known as covalently closed circular (ccc)DNA, which can integrate into and persist in the hepatic cell nucleus. In addition, the immune response against HBV is profoundly impaired[5,6]. Both are, in fact, the main reasons why indefinite treatment is usually necessary.

Immune modulators were the first approach to CHB treatment. The first one, interferon (IFN)-α was approved in 1991, being afterwards substituted by its pegylated form (Peg-IFN-α) as the latter provides a safer profile. The principal mechanism of Peg-IFN-α therapy relies on the induction of long-term immune control, which occurs in almost half of the responders and with limited treatment duration. However, it poses significant drawbacks, including an adverse safety profile and a high response variableness, the reasons why a number of patients are ineligible, unsuitable or reluctant to partake in this treatment alternative[7,8].

At present, nucleos(t)ide analogues (NAs) constitute the lynchpin of CHB therapy, as they facilitate achievement of viral suppression in almost all adherent patients, while having an overall favourable safety profile[7-9]. The currently approved NAs for CHB treatment in the United States and Europe include lamivudine (LMV), telbivudine (TBV), adefovir dipivoxil (ADV), tenofovir (disoproxil fumarate, TDF; alafenamide, TAF) and entecavir (ETV). The NA mechanism of action comprises viral polymerase inhibition, which leads to decreased virion assembly and ultimately a hypothetical cccDNA downturn that would only be appreciated after an extended period of treatment[10,11].

Nonetheless, NAs are not able to stop *de novo* cccDNA synthesis in recently infected hepatocytes; thus, lingering viremia could perpetuate the viral repository. That is the reason why "complete cure" is not a realistic endpoint of NAs to date. "Functional cure", understood as HBV DNA and hepatitis B surface antigen (HBsAg) seroclearance with or without seroconversion, constitutes a more plausible goal. However, it is achieved only in a small proportion of the treated patients. Lifelong NA therapy is usually necessary, especially in hepatitis B e antigen-negative [HBeAg(-)] cases[7,12,13].

Since indefinite treatment is mandatory, development of viral resistance is a paramount concern, especially with the first- and second-generation oral NAs such as LMV, TBV and ADV. Fortunately, that problem seems to have been overcome by the new agents TDF/TAF and ETV, as they present low resistance rates and high efficacy with a very favourable safety profile[14,15].

Combination therapy has also been proposed as a strategy for HVB eradication, but results are still under evaluation and intense debate. Its rationale

comprises attacking the virus in different parts of its life cycle, and follows practical successes observed in other infectious diseases, like hepatitis C virus and human immunodeficiency virus. Potential objectives regarding this approach include viral targeting (viral entry, cccDNA, RNA interference, encapsidation, DNA replication, *etc*) as well as innate and adaptive immunomodulation (IFN, Toll-like receptor/RIG-1 agonists; and checkpoint inhibitors, T cell modification and vaccination respectively).

Other trials that have evaluated the synergies between innate immunity potentiation and NAs have already shown promising results. A preclinical phase study that combined a woodchuck hepatitis virus DNA vaccine, a programmed cell death protein 1 (PD-1) inhibitor and ETV showed restoration of the cytolytic capacities of HBV-specific T cells and better control of viral replication. Another study that associated a DNA vaccine with any NA revealed no differences in relapse after NA cessation. A third study that blended an HBsAg vaccine with LMV did not find clinical differences[16-19].

If theoretically attractive, current guidelines do not recommend combination therapy for clinical practice[9,20]. The ultimate and thus optimal target of HBV therapy for HBeAg(-) and HBeAg(+) patients comprises viral eradication. Such would involve HBsAg seroconversion or seroclearance and cccDNA elimination from hepatocytes[21]. Unfortunately, it is not a likely outcome, and recommended goals point towards sustained inhibition of replication and maintenance of alanine aminotransferase (ALT) enzyme levels within the normal range[9,20,22]. Achievement of these objectives has been shown to stop the inflammation cascade and fibrosis progression[23,24], with e consequent improvement in life-quality and survival[25]. The weight of the beneficial effects of the latest generation NAs over the risk of HCC are still controversial, as the latter develops even despite therapy[26-28].

It seems, then, reasonable to bear in mind that any strategy involving NA treatment withdrawal must guarantee the patient's safety and, therefore, the maintenance of the aforementioned objectives.

CLINICAL EVIDENCE REGARDING NA TREATMENT CESSATION

There is a growing body of evidence that helped to elucidate whether NA therapy cessation is safe and effective. A relevant study by Hadziyannis *et al*[29] must be pointed out as a point of inflexion regarding the NA cessation approach. It showed a significantly higher HBsAg clearance rate (almost reaching 40%) in the HBeAg(-) CHB patients that stopped after 5 years under ADV therapy, in comparison to that reported for their equals under NA. Since then, a set of investigations have attempted to clarify whether stopping treatment with NA may have an additional benefit in the loss of HBsAg, showing achievement of rates between 20%-24%[30,31].

Some parameters have been pointed out as possible predictors of both sustained viral response and HBsAg loss. Among these, it is worth highlighting the decrease of quantitative (q)HBsAg. Other biomarkers that could permit identification of patients in which NA cessation will be safe will be reviewed broader, later on.

The recent FINITE study[32] was the first randomized controlled trial that compared standard TDF therapy continuation against its interruption in HBeAg(-) patients that had been under treatment for at least 3.5 years. In line with the previous commented work, 13 out of 21 patients in the cessation arm remained off-therapy and 4 of them even achieved HBsAg seroclearance after 3 years of follow-up. No unexpected safety issues were reported. These and the other studies about NA interruption that have been published to date are summarised in Table 1.

Some of the current HBV management guidelines[9,22] have begun to consider treatment cessation in other selected populations of patients. The most accepted election criteria include cirrhosis absence, treatment for at least 2 or 3 years, sustained viral suppression and guaranteed patient monitoring (Table 2). Concerns regarding treatment cessation include virological and clinical relapse but also the possibility of dangerous complications, such as hepatic decompensation, liver failure and, ultimately, death. Serious complications are uncommon, and some meta-analyses have shown a decompensation rate of less than 1% in patients that presented baseline cirrhosis[33,34]. Therapy reestablishment proved to be effective in most cases, but also cases of death after liver failure have been reported.

A recent study provided alert to the risk of relapse and potentially fatal effects among Caucasian cirrhotic patients with HBeAg(+) HBV virus infection[35]. Two patients died of liver-related events: one after decompensation and sepsis, and the other one after developing a multicentric HCC 10 years after the NA treatment cessation. Nevertheless, both of those patients had presented with advanced fibrosis and cirrhosis, respectively, at the time of therapy discontinuation. Furthermore, although a few studies have claimed benefits of long-term treatment regarding HCC incidence, as previously stated, it is not doubtlessly prevented by NA therapy[26-28,36,37].

Hence, considering that the treatment withdrawal could lead to severe flares and even death in a few cases it should be avoided in patients with advanced fibrosis or cirrhosis, and a close follow-up must always be guaranteed for the rest of the cases[38]. However, severe complications are rare, and research must continue to address the optimal NA cessation point. The identification of reliable factors capable of predicting clinical, virological and biochemical relapse, or the maintenance of the viral response, would be of vital

Table 1 Summary of relevant NA treatment discontinuation studies

Study	Patients off NA, *n*						Treatment characteristics			Outcomes		
	Total	HBeAg(+)	HBeAg(-)	Cirrhosis	Age in year	Sex, male	Ethnicity	NA	Treatment duration in mo	Durable virologic response, *n*	HBsAg loss, *n*	Deaths, *n*
Fung *et al*[100] (2004)	27	0	27	7	45	40	Asian	LMV	24	15	NR	0
Enomoto *et al*[101] (2008)	22	0	22	3	49	15	Asian	LMV	NR	5	NR	0
Yeh *et al*[102] (2009)	71	71	0	11	41	55	Asian	LMV	NR	52	0	0
Fung *et al*[103] (2009)	22	22	0	NR	28	16	Asian	LMV	74	8	NR	0
Wang *et al*[104] (2010)	125	125	0	0	26/32	95	Asian	LMV	24-36	87	NR	0
Kuo *et al*[105] (2010)	124	124	0	NR	NR	NR	Asian	LMV	14	42	NR	1
Cai *et al*[106] (2010)	11	11	0	NR	29	12	Asian	TBV	24	4	NR	0
Liu *et al*[40] (2011)	61	0	61	0	32	50	Asian	LMV	27	30	8	0
Jung *et al*[107] (2011)	19	10	9	4	37	12	Asian	ADV	33	13	0	0
Chan *et al*[30] (2011)	53	0	53	18	56	43	Asian	LMV	27	16	9	NR
Chaung *et al*[108] (2012)	39	39	0	NR	34	24	Asian	LMV, ADV, ETV	21	4	0	0
Hadziyannis *et al*[29] (2012)	33	0	33	0	53	38	Caucasian	ADV	56	18	14	0
Ha *et al*[41] (2012)	145	0	145	NR	33	101	Asian	ADV	26	50	NR	0
Song *et al*[109] (2012)	48	48	0	0	42	29	Asian	ETV, CLE	26	28	NR	NR
He *et al*[110] (2013)	66	0	66	0	35	50	Asian	LMV, ADV, ETV, TBV	37	47	2	0
Kim *et al*[111] (2013)	45	0	45	9	45	33	Asian	LMV, ADV, ETV	38	12	NR	NR
Jeng *et al*[44] (2013)	95	0	95	39	52	83	Asian	ETV	24	40	0	0
Kwon *et al*[112] (2013)	16	NR	NR	NR	NR	NR	Asian	LMV	79	12	2	0
Ridruejo *et al*[113] (2014)	35	33	2	0	NR	NR	Caucasian	ETV	42	26	18	NR
Sohn *et al*[114] (2014)	95	41	54	44	47	53	Asian	LMV, ETV, CLE	22	16	0	0
Patwardhan *et al* (2014)	33	0	33	0	42	24	Mixed	LMV, ADV, ETV, TDF	64	12	0	0
He *et al*[115] (2014)	97	97	0	NR	26	53	Asian	LMV, ADV, ETV, TBV	35	89	11	0
Chen *et al*[31] (2014)	188	83	105	12	38/49	143	Asian	LAM	20-22	63	23	NR
Jiang *et al*[116] (2015)	72	33	39	8	36	53	Asian	LMV, LMV + ADV, ADV, ETV, TBV	33	25	NR	0
Seto *et al*[117] (2015)	184	0	184	34	54	125	Asian	ETV	37	15	0	0
Huang *et al*[118] (2003)	32	0	32	NR	46	29	Asian	LMV	9	14	NR	NR
Marcellin *et al*[119] (2004)	181	0	181	53	40	156	Asian	LMV	12	53	0	0
Lai *et al*[120] (2006)[1]	325/313	0	325/313	16/31	44/44	248/236	Mixed	ETV/ LMV	≥13	124/78	1/1	2[2]
Marcellin *et al*[121] (2009)[3]	181/85	0	181/85		40/39	156/74	Asian	LMV	12	52/33	0/0	1[2]
Paik *et al*[122] (2010)	50	0	50	15	39	43	Asian	LMV	24	25	NR	0
Liang *et al*[123] (2011)	84	41	43	0	37	56	Asian	LMV, ADV, ETV or LMV +ADV	33	47	5	NR
Jin *et al*[124] (2012)	138	102	36	17	39	82	Asian	LMV	35	116	82	0
Berg *et al*[32] (2017)	21	0	21	0	45	33	Caucasian	TDF	≥ 48	13	4	0
Van Hees *et al*[35] (2018)	62	62	0	11	43	45	Caucasian	LMV, TDF, ETV, LMV + ADV	70	32	6	2
Rivino *et al*[43] (2018)[4]	21/27	0/0	21/27	0/0	43/51	14/19	Caucasian/Asian	TDF, LMV	⩾ 24/⩾ 24	4/14	0/0	NR

Virologic response is considered as defined in the original study. [1]Results expressed as ETV/LMV; [2]Deaths not related with treatment discontinuation according to the authors; [3]Two follow-up durations in this study, expressed as: Initial (6 mo)/Long-term (36 mo); [4]Results obtained in two different cohorts, expressed as: cohort 1/cohort 2. ADV: Adefovir; CLE: Clevudine; ETV: Entecavir; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; LMV: Lamivudine; NA: Nucleos(t)ide analogue treatment; NR: Not reported; TBV: Telbivudine; TDF: Tenofovir.

Table 2 NA treatment cessation recommendations in the current hepatitis B virus guidelines

Society	HBeAg(+)	HBeAg(-)	Cirrhosis
EASL (2017)[9]	HBsAg clearance (safest) HBeAg seroconversion and HBV DNA undetectability with 6-12 mo of ensuing consolidation therapy	HBsAg clearance Selected patients with ⩾ 3 yr virological suppression if guaranteed close postNA monitoring for at least 1 yr	Not recommended
AASLD (2016)[20]	HBsAg clearance HBeAg seroconversion with at least 12 mo of persistently normal ALT levels and undetectable serum HBV DNA levels (close monitoring for at least 1 yr)	HBsAg clearance	Not recommended
APASL (2016)[22]	HBeAg seroconversion with undetectable HBV DNA and persistently normal ALT levels with 1-3 yr of consolidation therapy	HBsAg clearance with antiHBs seroconversion HBsAg loss with at least 12 mo of consolidation period After treatment for at least 2 yr with undetectable HBV DNA documented on 3 separate occasions, 6 mo apart	Could be considered in compensated cirrhosis with careful monitoring

AASLD: American Association for the Study of Liver Diseases; ALT: Alanine aminotransferase; APASL: Asian Pacific Association for the Study of the Liver; EASL: European Association for the study of the Liver; HBeAg: Hepatitis B e antigen; HBs: Hepatitis B surface protein; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; NA: Nucleos(t)ide analogue treatment.

importance for clinical practice.

WHY SHOULD NA TREATMENT CESSATION BE CONSIDERED?

Once the safety of NA treatment cessation has been addressed, and keeping in mind that severe complications are rare, the vast benefit may be considered. Notwithstanding that NA treatment has an overall positive safety profile in the general population, some issues arise.

Lifelong NA treatment is an unaffordable burden for healthcare systems. That is why a significant advantage of its cessation would be cost reduction[22,34]. However, increase in the incidence of some chronic conditions, such as metabolic syndrome, diabetes mellitus and renal failure, may limit NA applicability in the future. Furthermore, we are not aware of potential concerns of NA therapy in elder individuals and research must address this subject. There are some other potential concerns about long-term NA therapy[39]. The most common side effects involve nephrological and bone toxicity, which are associated with TDF, perhaps the most widely used drug. However, the new tenofovir formulation TAF seems to have a better safety profile regarding these points. Other side effects appear to be related to mitochondrion impairment derived from human DNA polymerase function alteration and resulting in bone, renal and neurologic toxicity.

NAs have a relatively benign safety profile for pregnancy, with telbivudine and tenofovir being the most favourable ones, rated B category by the Federal Drug Administration. On the other hand, ETV has shown deleterious effects for the embryo, being rated C category. Nevertheless, there is a lack of information about foetal safety in humans, so NA treatment during pregnancy should only be considered if benefits overwhelm risks.

Despite all the above-mentioned issues, NA counts on its excellent safety profile for almost the totality of the patients eligible for this therapy. To sum up, life-long therapy is usually necessary for the majority of CHB patients because the functional cure is rarely achieved. Therefore, identification of biomarkers to safely stop treatment remains an unmet need in the management of the disease.

POTENTIAL BIOMARKERS TO SAFELY STOP NA TREATMENT

Taking into account that CHB outcome relies on equilibrium between the virus and the host, in the next paragraphs it will be explained how different virological and immunological parameters could be considered or not as predictors to safely discontinue NA treatment.

Sex: Female sex was identified as an independent predictor for sustained virological response after NA discontinuation in HBeAg(-) patients[31].

Age: Older age has been correlated to higher relapse rates[31,40,41], possibly reflecting the enhanced immune response in younger individuals.

NA treatment duration: A more extended therapy time would mean more time for an exhausted immune system to recover its response efficacy. Logistic regression has revealed that sustained virologic remission is more likely in HBeAg(-) patients after long periods of treatment, at least over 2 years. Nevertheless, this

parameter has not proven to be useful in HBeAg(+) CHB[33].

ALT: The predictive role of ALT is controverted. Although it was classically accepted that ALT flares were associated with the virologic response after NA treatment cessation[42], lower ALT baseline levels have been correlated with higher rates of HBsAg loss[31]. Also, it has recently been demonstrated that patients who do not flare upon treatment withdrawal are those who remain off-therapy[43]. Given the observed disparities, more research is needed to elucidate the role of ALT as a biomarker.

DNA: Lower baseline HBV DNA titres were reported as associated independently with lower relapse rates[44], whereas elevated HBV DNA titres and its persistence after NA interruption also seem to be useful for relapse prediction[45].

Serum qHBsAg: Decrease in serum qHBsAg has been correlated to HBsAg clearance and has been spotlighted as a possible predictor of sustained response and flares after NA withdrawal[46-48]. The interest in qHBsAg has been limited, however, due to the low level required for consideration of NA cessation (100-700 IU/mL)[31,49-51], and which is rarely achieved. Taking into account that these qHBsAg levels are not adequately good predictors to safely discontinue NA therapy, because they would only represent a small portion of cases, more research has been performed to improve the prognostic accuracy.

Noncytopathic viruses, such as HBV, have developed evolutionary mechanisms to remain hidden from the immune system, which is an advantage for their persistence. HBV virus is not highly infectious but produces long-lasting disease that allows it to spread the infection over time. The host/HBV relationship is a dynamic process in which the virus tries to decrease its visibility, whereas the host attempts to prevent and eradicate infection with minimal collateral damage to itself[52].

Several viral markers have been proposed as potential biomarkers for a safe NA discontinuation, and they are discussed below.

Virological parameters

Serum HBV RNA reflects the transcriptional activity of liver cccDNA, and its decline seems to be a good predictor of HBeAg seroconversion[53]. Nevertheless, it is commonly undetectable in HBeAg(-) cases[54], making it useless as a biomarker for stoppage of NA treatment in this increasing population. Moreover, improvement of the HBV RNA assay to make it more sensitive and reproducible, as well as studies in bigger cohorts, are essential before considering it as a potential biomarker for monitoring safe discontinuation of NA therapy in HBeAg(+) patients.

Hepatitis B core (HBc) is an inner nucleocapsid surrounding the viral DNA and is the target of specific T cell response against the virus. AntiHBc is the first antibody to appear after HBV exposure and it represents a classical serological marker for HBV infection[55]. The role of antiHBc as a predictor of NA discontinuation, however, has not been fully examined, but it was recently reported that baseline antiHBc level is a strong predictor for HBeAg seroconversion during PEG-IFN-α or NA therapy[56]. Moreover, there was a trend for an inverse association between antiHBc and clinical relapse after long-term ETV treatment cessation in an Asiatic CHB cohort[57]. AntiHBc, as a predictor, needs to be further assessed and validated in non-Asiatic cohorts, to verify if it could be useful.

HBV core-related antigen (HBcrAg) includes HBcAg, HBeAg and a pre-core protein (p22cr), and its quantification closely correlates with intrahepatic cccDNA level[58,59]. In HBeAg(+) CHB patients, the dynamics of HBcrAg accurately predict spontaneous HBeAg seroconversion[60] and the combination of HBsAg together with HBcrAg quantification help to predict safe discontinuation after NA treatment cessation[61]. However, most of this research has been performed in Japan with first-generation NAs, so further validation with the currently available NAs and different areas of study is lacking.

In summary, some virological markers could be useful predictors of response in HBeAg(+) patients, but improvement of the assays together with further cohort validation is still needed for HBeAg(-) cases. The other side of the balance is the host's immune defence against the virus, presented in the next section.

Immunological parameters

To achieve control of the HBV infection, a functional adaptive immune response, in particular the cellular immune response, is essential; meanwhile, whether and how HBV triggers the components of the innate immune system remain controversial topics. Even though the humoral response is an effective line of defence against reinfection, in the setting of CHB, the virus persists despite high levels of HBV-specific antibodies[62] due to antigen overload, and only hepatitis B surface antibody is associated with disease resolution.

Primed HBV-specific CD4 T cells are crucial to allow the adequate activation of HBV-specific CD8 T cells by secretion of proinflammatory cytokines, including IFN-α[63]. Afterwards, HBV-specific CD8 T cells play a major role in the resolution of spontaneous infection because they can specifically recognise the infected hepatocytes. Moreover, they can clear the virus by inducing apoptosis of the infected cell as well as by proinflammatory cytokine production to eliminate the virus without causing cell death[64].

CD4- and CD8-specific HBV responses are vigorous, polyclonal and multispecific in acute-resolving cases, whereas are profoundly impaired in chronically infected patients[6,65-68]. During CHB, HBV-specific T cell responses gradually lose their functionality and are finally deleted[69]

due to the high and persistent antigen exposure, in order to avoid host-induced tissue damage, in a process called T cell exhaustion. T cell exhaustion is characterised by high and sustained expression of several negative pathways (*i.e.*, PD-1, immunoregulatory cytokines and so on)[70-75].

The role of HBV-specific CD4 T cell features as a predictor for NA cessation has not been intensely studied. It could be explained mainly by two reasons. First, the frequency of these cells in the chronic setting of the disease is very low[76]. Second, due to the nature of CD4 responses, *in vitro* stimulation assays are difficult because these cells are only successfully stimulated by professional antigen-presenting cells. Even though a robust HBV-specific CD4 T cell response is observed in acute resolving cases, and they are essential to support HBV-specific CD8 T cells, the difficulty of assessment makes them less useful than HBV-specific CD8 T cells or other surrogates when trying to find an easy and reproducible immunological marker to stop NA therapy safely.

CHB is one of the best models to study CD8 T cell exhaustion. In the different stages of the natural history of HBV infection, there are different virus-host interactions, reflected by different immune features of HBV-specific CD8 T cells. Bearing in mind that several studies have shown that after long-term NA treatment interruption the majority of patients remain with a viral response after long follow-up[29,32], we could infer that the host's immunity is controlling HBV replication.

After a long-term NA treatment cessation, HBV-specific CD8 T cells could be given a second chance to fight the virus. If these cells have been restored by the reduced viremia that had been induced by the antiviral therapy at that point, these cases would be able to control the infection in a similar way to chronic infection cases. Therefore, patients with viral control are likely to have a good immune response against the virus, whereas cases with virologic rebound may have a dysfunctional response.

Thus, changes in HBV-specific CD8 T cell phenotype may predict acquisition of antiviral control before HBsAg loss. Taking into account the vital role of HBV-specific CD8 T cells during the natural history of the disease, and its in-depth characterisation achieved over the last two decades, it is presumable that those different features according to viral control could give hints to answer one of the most critical questions regarding CHB management: What kind of patients could benefit from NA therapy interruption?

Boni *et al*[77] have extensively studied several immune subsets in different groups of chronically infected patients, including those under NA therapy. In the LMV treated patients, they found an initial improvement of HBV-specific T cell effector capacities against different HBV epitopes (HBcAg, HBeAg) after DNA fall[77], followed by a decline at 6 mo after the treatment has been stopped; this biphasic behaviour is irrespective of clinical outcome[78]. It appears that the first-generation NAs lack the potency needed for HBV-specific T cell restoration.

Succeeding experiments in larger cohorts under the first- and second-generation NA therapies demonstrated that HBV-specific CD8 T cell effector abilities were similar between patients after several years of antiviral treatment and acute resolving cases featured by a PD-1+ phenotype[79] (Figure 1). PD-1 up-regulation arises on HBV-specific T cells following acute and chronic infection. In the setting of acute infection, PD-1 up-regulation is transient, returning to low levels after viral clearance. However, in chronic infection, PD-1 up-regulation is sustained, and the blockade of PD-1/PD-L1 interaction has shown promising results in restoring virus-specific T cell functionality[80-83]. Therefore, a PD-1+ phenotype could mean both activation before clearance or exhaustion after persistent and high antigenemia.

The most recent work studying HBV-specific T cell response as a biomarker for HBV therapy discontinuation demonstrated that the patients who did not relapse to NA stoppage featured, during NA treatment, an increased frequency of functional PD-1+ HBV-specific T cells directed against nucleocapsid and polymerase HBV proteins[43] (Figure 1). The PD-1+ expression on functional HBV-specific T cells may reflect an activated, nonexhausted phenotype. Along these lines, patients with functional HBV-specific CD8 T cells, positive for PD-1, may no longer need NA treatment and should be considered for treatment cessation.

However, the current method is complicated to move from bench to bedside because it involves the study of rare populations by multicolour flow cytometry. Hence, the development of an assay to directly quantify PD-1+ HBV-specific CD8 T cells would be of great interest. Even though the final effectors to clear HBV are the HBV-specific CD8 T cells, it is essential to consider the interplay between them and other components of the immune system to fully understand immunity against HBV and their potential as surrogate biomarkers.

The natural enrichment of natural killer (NK) lymphocytes in the human liver underscores their potential importance in the control of hepatotropic viruses, such as HBV[84]. During CHB, NK cells express an inhibitory phenotype with altered functionality[85,86] and have predilection for apoptosis of HBV-specific T cells, resulting in HBV-specific T cell deletion after death ligand-death receptor interaction[87]. Boni *et al*[88] showed a low inflammatory profile of NKs after successful NA therapy, similar to healthy controls. In line with the previously commented work, this lower inflammatory status of NKs correlated with a better HBV-specific T cell response[88] (Figure 1). Moreover, a partial restoration of blood NK cells was shown following long-term ETV, in terms of antiviral cytokine production compared to naïve CHB[89].

So, why should study of NKs - instead of HBV-

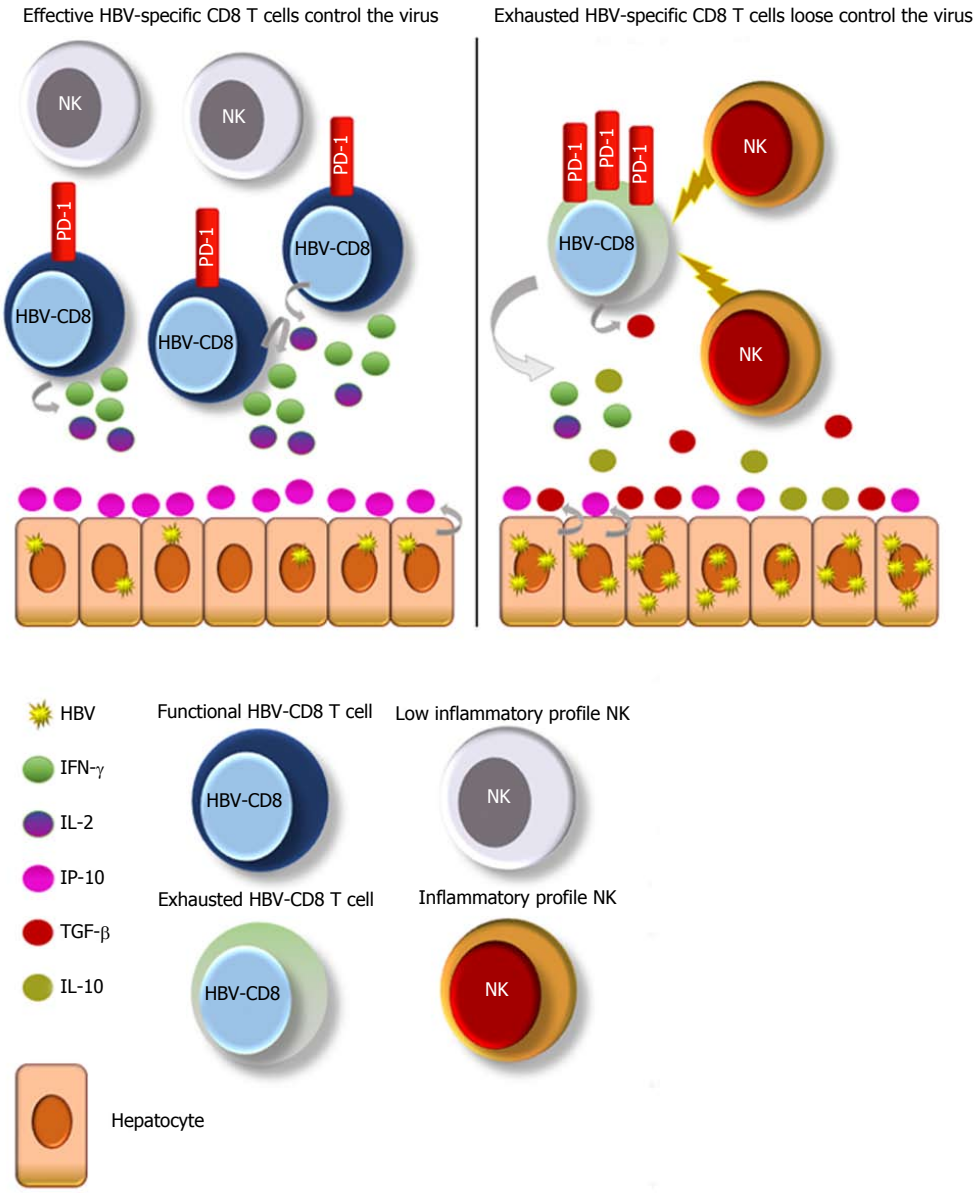

Figure 1 Potential immunological biomarkers for safe nucleos(t)ide analogue discontinuation. A possible strategy to shorten NA therapy would be to check if HBV-specific CD8 T cells have reacquired their antiviral function after long-term therapy. Effective HBV-specific CD8 T cells control the virus: PD-1+ HBV-specific CD8 T cells against different epitopes, able to mount a robust response (IFN-γ, IL-2 production) after antigen encounter, may be a good predictive tool of response. Low inflammatory profile of NK cells may likewise reflect a good point to end therapy. High levels of IP-10 also could point to anti-viral control. Exhausted HBV-specific CD8 T cells lose control of the virus: High and sustained PD-1 expression on HBV-specific CD8 T cells reflects their dysfunctionality. Lower IFN-γ and IL-2 production, along with an immunosuppressive cytokine environment (IL-10, TGF-β), renders these cells to exhaustion. An inflammatory phenotype of NK cells may reflect NK cell-mediated T cell deletion through death receptors. Low levels of IP-10 could dissuade us to stop therapy. HBV: Hepatitis B virus; IR-10: CXCL10; NA: Nucleos(t)ide analogue; NK: Natural killer; PD-1: Programmed cell death protein 1.

specific CD8 T cells - be useful? The study of NK cell inflammation does not involve multimers nor intracellular cytokine staining, as used to assess HBV-specific CD8 T cell responses, resulting in more easily reproducible experiments. A low inflammatory profile of NK cells can be evaluated by surface staining and may reflect an HBV-specific T cell restoration and subsequent control without the need of therapy. Studies in bigger cohorts after stoppage of NA treatment are needed to address if successful NA discontinuation correlates with a lower inflammation phenotype of NK cells.

The third signal of T cell activation requires an adequate cytokine profile, and long-term NA therapy has been shown to modulate it. Successful viral repression leads to antiviral response stimulation by promoting proinflammatory cytokines such as IFN-γ[90,91] and IL-2[92,93], as well as by decreasing regulatory effectors such as IL-10[91,94] and TGF-β[95]. At least theoretically, the measurement of these cytokines together with HBV-specific T cells or NK cells could also give us clues to establish a good cessation point for therapy (Figure 1).

Not only are the phenotype and functionality of the different immune subsets important components of an adequate milieu during CHB but also the trafficking of

HBV-specific T cells to the infected liver. The migration of lymphocytes to the liver is a complicated process involving adhesion, rolling, triggering and transendothelial migration. Chemokines and their receptors play an essential role in this multistep pathway[96,97].

After the analysis of several plasma chemokines, the one that appears to be a promising surrogate of HBsAg loss under NA therapy is CXCL10 (IP-10)[47]. IP-10 is a small protein, secreted by hepatocytes in response to viruses and the subsequent recruitment of proinflammatory CD4 and CD8 T cells to the infected liver[97] (Figure 1). It was previously reported that baseline serum IP-10 levels were higher in patients with HBsAg loss during NA therapy[98] and, in line with those findings, another work examined the serum IP-10 kinetics during ETV therapy. Interestingly, they found that IP-10 levels started to significantly increase after the 3rd year of treatment with ETV[99], which is in line with the timing observed to be necessary to achieve a sustained virological response in the different stopping-treatment studies[29,32].

It is likely that after a prolonged and effective viral replication suppression under NA treatment, the migration process to the liver is restored and HBV-specific T cells are functional and able to clear the remaining infected hepatocytes, thus reflecting the HBsAg decline.

CONCLUSION

The study of different immune features against HBV, especially HBV-specific CD8 T cells, is a promising strategy to characterise which patients could benefit from NA treatment cessation. Surveying HBV-specific CD8 T cells is complex, as it involves rare population assays. However, different, easier to perform surrogates of this response have been explored recently, providing a more suitable application for clinical use. NA withdrawal is still an active and attractive research field. Nevertheless, even if a considerable number of studies have tried to address this point, their methods have shown marked heterogenicity. Furthermore, although results of some randomised controlled trials are becoming available, more high-quality clinical evidence is needed. It is possible that in the future, therapies able to completely clear cccDNA will be accessible. In the meantime, advantages in the management of CHB may be achieved by using this strategy. Fortunately, the field of immunology shows how basic science can improve the health of our patients.

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REVIEW

Emergence of immunotherapy as a novel way to treat hepatocellular carcinoma

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Abstract

Tumor immunity proceeds through multiple processes, which consist of antigen presentation by antigen presenting cells (APCs) to educate effector cells and destruction by the effector cytotoxic cells. However, tumor immunity is frequently repressed at tumor sites. Malignantly transformed cells rarely survive the attack by the immune system, but cells that do survive change their phenotypes to reduce their immunogenicity. The resultant cells evade the attack by the immune system and form clinically discernible tumors. Tumor microenvironments simultaneously contain a wide variety of immune suppressive molecules and cells to dampen tumor immunity. Moreover, the liver microenvironment exhibits immune tolerance to reduce aberrant immune responses to massively-exposed antigens via the portal vein, and immune dysfunction is frequently associated with liver cirrhosis, which is widespread in hepatocellular carcinoma (HCC) patients. Immune therapy aims to reduce tumor burden, but it is also expected to prevent non-cancerous liver lesions from progressing to HCC, because HCC develops or recurs from non-cancerous liver lesions with chronic inflammatory states and/or cirrhosis and these lesions cannot be cured and/or eradicated by local and/or systemic therapies. Nevertheless, cancer immune therapy should augment specific tumor immunity by using two distinct measures: enhancing the effector cell functions such as antigen presentation capacity of APCs and tumor cell killing capacity of cytotoxic cells, and reactivating the immune system in immune-suppressive tumor micro-environments. Here, we will summarize the current status and discuss the future perspective on immune therapy for HCC.

Key words: Natural killer T cell; Natural killer cell; Chimeric antigen receptor T cell; T cell receptor; Cytokine-induced killer cell; Program death-1; Cytotoxic lymphocyte antigen-4; Regulatory T cell; Dendritic cell; Myeloid-derived suppressor cell; PD-ligand 1; Peptide vaccine; Tumor-associated antigen; Tumor infiltrating lymphocyte

Core tip: Hepatocellular carcinoma (HCC) develops or recurs from non-cancerous liver lesions with chronic inflammatory states and/or cirrhosis, and these lesions cannot be cured and/or eradicated by local and/or drug therapies. Immune therapy may be effective for HCC treatment by preventing non-cancerous liver lesions from progressing to HCC as well as reducing tumor burdens. However, tumor immunity is frequently depressed in tumor sites, particularly in liver microenvironment, which is prone to exhibit immune tolerance, to reduce aberrant immune responses to massively-exposed antigens *via* portal veins. At present, cancer immune therapy employs two distinct strategies; enhancing the effector cell functions and unleashing the immune suppressive tumor microenvironments. Here, we will summarize the current status and discuss the future perspective on immune therapy for HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is ranked as the sixth most common malignancy and is the third leading cause of cancer-related mortality worldwide[1]. Despite recent progress in prevention and diagnosis, many HCC cases are still diagnosed at an advanced stage, for which there are few effective and/or curative treatment options, and as a consequence, their prognosis remains poor. These circumstances necessitate the development of a novel therapeutic strategy for HCC, particularly for HCC at advanced stages.

HCC ensues from chronic liver diseases, particularly liver cirrhosis, arising from various risk factors including chronic hepatitis B- or C-virus infection, aflatoxin B1 exposure, excessive alcohol consumption, and occurrence of non-alcoholic fatty liver. Other independent risk factors include tobacco use[2], diabetes[3], and obesity[4]. In conjunction with the declining incidence of HBV and HCV infections, non-alcoholic fatty liver disease is becoming an important cause of HCC in the advanced economies, as the number of patients suffering from metabolic syndromes is rapidly increasing in these countries[4].

All these etiologic conditions cause sustained inflammatory reactions, consisting of persistent oxidative stress, sustained hepatocyte necrosis and regeneration, and fibrotic changes[5]. These events can lead to HCC development through the accumulation of somatic genetic alterations and epigenetic modifications in various passenger and driver genes, and these changes have been extensively clarified with the advent of next-generation sequencing technology (Figure 1)[6]. Aberrant telomerase reverse transcriptase (*TERT*) activation is observed in about 70% of HCC cases, arising from its promoter mutation and amplification, and viral genome integration[7]. Thus, *TERT* activation and subsequent telomerase reactivation can be a key event in malignant transformation, leading to unrestrained proliferation of HCC cells[8]. Inactivating mutations are also frequently observed in *CTNB1* (about 30%), which codes for β-catenin[7]. Moreover, inactivating mutations are detected in other members of the WNT pathway, such as *AXIN1* (11%), *AXIN2* (1%), *ZNRF3* (3%), or *APC* (1%). Inactivating mutations of *TP53* are also frequently observed in HCC (~30% of cases) but are rarely detected together with *CNTB1* mutations, suggesting that distinct molecular pathways are responsible for HCC evolution. Additional mutations are observed in genes involved in other pathways including chromatin remodeling, PI3K/AKT/mammalian target of rapamycin (mTOR) signaling, Ras/MAPK signaling, JAK/STAT signaling, and oxidative stress pathways[6].

DNA copy number alterations are also frequently observed with broad genomic deletions at 1p, 4p-q, 6q, 8p, 13p-q, 16p-q, 17p, 21p-q, 22q, and gains at 1q, 5p, 6p, 8q, 17q, 20q, Xq[6,7,9]. Recurrent homologous deletions involve various genes including *AXIN1*, *CDKN2A/CDKN2B*, *CFH*, *IRF2*, *MAP2K3*, *PTEN, RB1*, and *RPS6KA3*[6]. In contrast, broader DNA gains affect *JAK3*, *MET*, and *MYC*[6] while focal amplifications at 11q13 and 6p21 lead to the amplification of *FGF3/4/19/CCDN1*[10] and *VEGFA*[11], respectively. Focal amplification of *FGF19* is associated with tumor progression[10] and that of *VEGFA* confers a high sensitivity to sorafenib, the first-line treatment for advanced HCC[11].

A substantial proportion of HBV-infected patients develop HCC even when fibrotic changes are absent in the liver[12], suggesting that HBV can be directly oncogenic. A non-structural HBV protein, HBx protein, is proposed to act as an oncogene based on its *in vitro* capacity to modulate cell cycle, signaling pathways, and DNA repair in hepatocytes[13], but evidence for direct transforming activity of HBx is scarce. Like other DNA viruses, HBV can cause insertional mutagenesis[12], which can induce DNA deletions at the integration sites, thereby promoting chromosomal instability and inactivation of tumor suppressor genes. Moreover, integration of the HBV genome into loci with enhancer and promoter activities can modulate the expression and function of the genes near the integration sites, and can eventually promote clonal proliferation and malignant

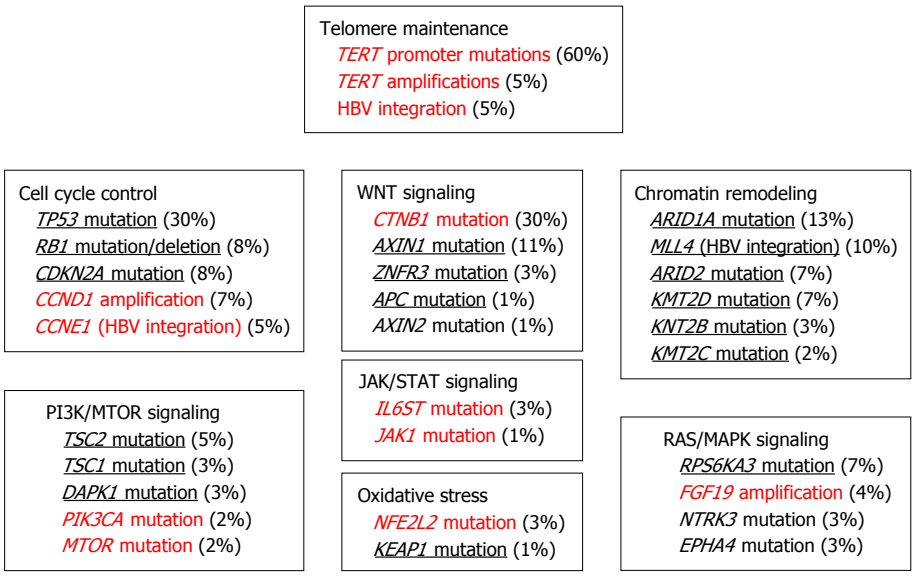

Figure 1 Mutational landscape of hepatocellular carcinoma. The figure was made by modifying the original figure in Ref. 7. Gain and loss of function events are indicated by red color and with underlines, respectively.

transformation[12]. Thus, the differences in integration sites can profoundly impact the types of the affected genes and subsequent molecular pathological changes.

Knowledge of molecular changes in HCC has expanded rapidly with the advent of gene technology, particularly next-generation sequencing technology, but has not been efficiently translated into clinical practice. A major reason is that the types of mutated driver genes and associated pathways differ considerably in each HCC case. These heterogeneities can hinder the identification and/or selection of target molecule(s) to develop molecular target drugs. Immunotherapy can overcome this problem, because it can enhance anti-tumor activity of the host cells, irrespective of the molecules and the signal pathways involved in hepatocarcinogenesis. In this review, we will discuss the present status and future perspectives on immunotherapy for HCC. The other clinical aspects of HCC including drug therapy have been reviewed in several other recent articles[1,14,15].

TUMOR IMMUNITY

Evasion of the immune system is now acknowledged as the key event necessary for the transformation of normal cells into malignant cells and their subsequent survival[16]. The immune system can sculpt cancer cells through a complicated mechanism called immunoediting (Figure 2)[17]. At the elimination phase, transformed cells are destroyed by immune cells such as cytolytic lymphocytes (CTLs) and natural killer (NK) cells, but resistant tumor cells sporadically appear and constantly change their phenotypes in the presence of the immune system. As a consequence, at the equilibrium phase, tumor cells reduce their immunogenicity and simultaneously escape the immunemediated killing mechanisms, thereby forming clinically appreciable tumor formation at the escape phase. Moreover, immune response can be dampened by immunoregulatory cells including regulatory T cells (Treg) and myeloid-derived suppressive cells (MDSCs) - cells that are abundant at tumor sites. The liver is constantly exposed to high levels of various antigens *via* the portal vein. Consequently, in order to prevent autoimmune liver injury, the liver microenvironment constantly exhibits potent immunosuppression[18]. Furthermore, immune dysfunction is frequently associated with liver cirrhosis[19], which is widespread in HCC patients. Moreover, cirrhosis can be a basis of HCC but cannot be completely removed, even after curative locoregional therapy with surgery, radiofrequency ablation (RFA), or transarterial chemoembolization (TACE)[1]. Thus, in addition to eradicating tumor mass, immunotherapy should aim to prevent the recurrence of HCC after curative locoregional therapy[20].

Immunotherapy approaches for HCC can be summarized in two ways: Activation of cytotoxic cell functions and correction of depressed immune functions inherent in HCC (Figure 3)[21]. Among the cytotoxic cells, CD8-positive CTLs are the most effective for specifically detecting and killing tumor-associated antigen (TAA)-expressing cancer cells. Antigen-presenting cells (APCs), particularly DCs, degrade exogenous and endogenous TAAs to be loaded on major histocompatibility complex (MHC) class Ⅰ and class Ⅱ, respectively (Figure 4). CD8-positive CTLs and CD4-positive helper T cells recognize the TAA-derived peptide on MHC class Ⅰ and class Ⅱ, respectively (Figure 4). In order to promote T cell survival, APCs simultaneously deliver co-stimulatory signals using several pathways including CD80/CD86-CD28 and CD40-CD40 ligand pathways (Figure 4)[22].

Antigen presentation efficiency can be improved by administering TAA-derived peptides, and/or the transfer of APCs, particularly DCs, which are loaded with or without TAA-derived peptides (Figure 3). These

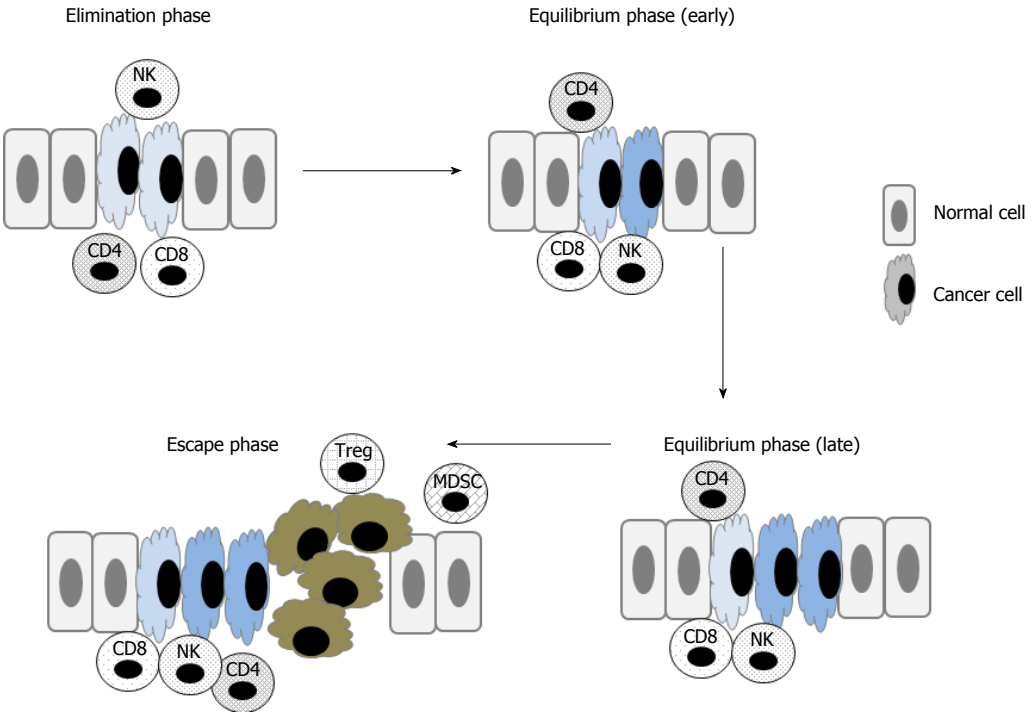

Figure 2 Cellular mechanisms underlying immunoediting. At the elimination phase, newly-appearing cancer cells can be recognized and killed by a number of immune cells, particularly natural killer (NK) cells, and $CD8^+$ and $CD4^+$ T cells. At the equilibrium phase, variant cancer cells arise that are less immunogenic, and consequently more resistant to being killed by immune cells. Over time, a variety of different cancer variants develop. At the escape phase, one variant may finally escape the killing mechanism or recruit immunosuppressive cells such as Tregs and MDSCs, and eventually form an appreciable tumor mass. MDSC: Myeloid-derived suppressive cell.

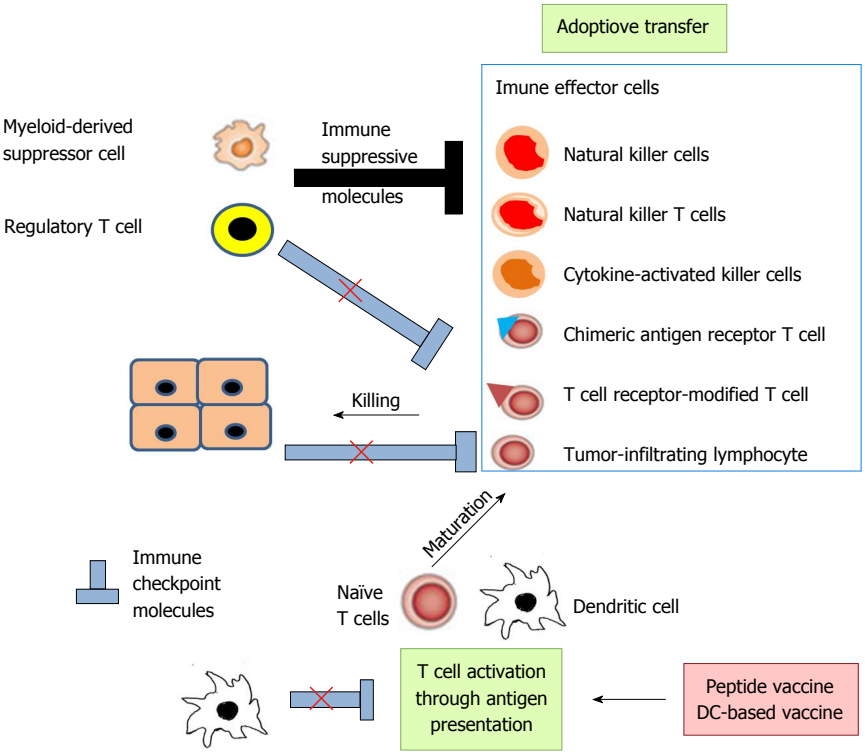

Figure 3 Strategies of immune therapy. Immune therapy can be classified to two types, promotion of immune effector cell function and reversal of depressed anti-tumor immunity. Immune effector cell function can be enhanced by peptide vaccine, DC-based vaccine, and adoptive transfer of effector cells including tumor-infiltrating lymphocytes (TILs), T cell receptor (TCR)-modified T cells, chimeric antigen receptor (CAR) T cells, natural killer (NK) cells, NKT cells, and cytokine-activated killer cells (CIKs). Depressed anti-tumor immunity can be reversed by the blockade of immune checkpoint pathways and immune suppressor cells including regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs).

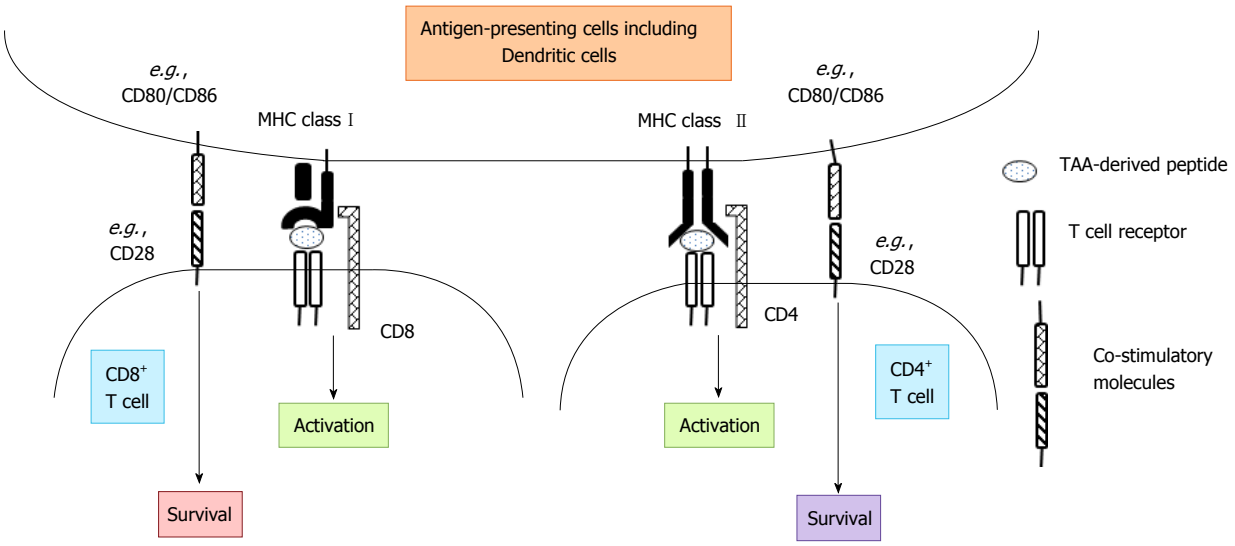

Figure 4 Tumor-associated antigen presentation of antigen-presenting cells to T cells. Endogenous antigens are degraded to peptides and loaded on MHC class Ⅱ on APCs to be presented to the $CD4^+$ T cells, while exogenous antigens are degraded to peptides and loaded on MHC class Ⅰ to be presented to the $CD8^+$ T cells. These pathways deliver activation signals to corresponding T cells. However, full activation and subsequent survival require the co-stimulatory signals delivered by several pathways including the CD80/CD86-CD28 pathway. In the absence of co-stimulatory signals, T cells become unresponsive to the antigen, a condition called anergy. TAA: Tumor-associated antigen; APCs: Antigen-presenting cells.

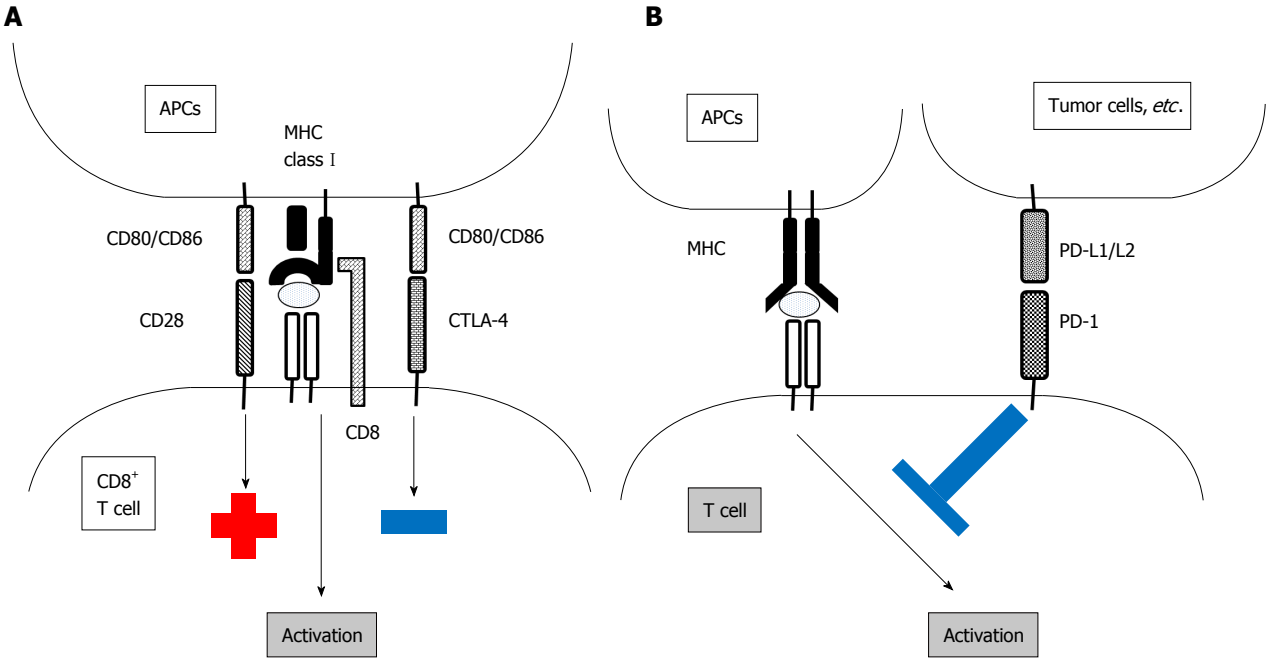

Figure 5 Mechanism underlying immune functions of CTLA-4 and PD-1-PD-L pathways. A: CTLA-4 has a higher binding affinity to CD80/CD86 than the co-stimulatory signal molecule CD28. As a consequence, CTLA-4 competitively antagonizes the stimulatory signal, which the interaction between CD80/86 and CD28 generates at the priming phase of T cells. B: The PD-L1/L2-PD-1 interaction interferes with T cell activation signals in the effector phase.

measures are named tumor vaccine therapy, as a whole. Adoptive immune therapy consists of transferring a large number of CTLs with T cell receptors recognizing specifically TAAs and/or other cytotoxic cells like NK cells into patients (Figure 3). With these maneuvers, the cells are obtained in most cases from patients and expanded *ex vivo*. The resultant cells are adoptively transferred to patients, sometimes after genetic modifications.

APCs prime T cells with the help of co-stimulatory molecules: CD80/86 on APCs and CD28 on T cells[22]. Simultaneously, a co-inhibitory molecule, CTL antigen-4 (CTLA-4) on T cells interacts with CD80/86 on APCs to dampen T cell activation (Figure 5A). Following the priming phase, CD8-positive CTLs are activated to exert cytotoxicity against foreign materials including tumor cells by using perforin, granzymes, and Fas ligand[23]. During this effector phase, T cell activation can be negatively regulated by co-inhibitory molecules expressed on APCs and other somatic cells including tumor cells[24]. One representative pathway is the programmed cell death

(PD)-1-PD ligand 1(PD-L1)/PD-L2 pathway (Figure 5B), which often works in the tumor microenvironment. Thus, immune checkpoint therapy can restore immune responses to tumors by suppressing these co-inhibitory pathways, leading to the control of tumor growth and/or its regression (Figure 3)[25]. With a main focus on the observations obtained from human clinical trials, we will discuss the immune therapy for HCC in the next chapter.

CURRENT AND EMERGING IMMUNOTHERAPY APPROACHES

Promotion of immune effector cell functions

Peptide vaccine therapy: α-fetoprotein (AFP) is a well-known TAA in HCC and is used as a tumor peptide vaccine. A phase Ⅰ clinical trial demonstrated that all six tested patients generated CD8-positive T-cell responses to the peptides as measured by direct IFN-γ enzyme-linked immunospot (ELIspot) and MHC class Ⅰ tetramer assays[26]. Specific CD8-positive T cell response may be augmented by the use of AFP conjugated with heat shock protein (HSP)70[27], HSP72[27], or glycoprotein 96[28], as revealed by studies using mouse AFP-expressing tumors. Butterfield and colleagues further examined the efficacy of AFP-pulsed DC transfer and demonstrated that six out of the ten subjects generated significant AFP-positive T cell responses to the administered peptides, although nine showed progressive disease[26]. The lack of apparent clinical responses can be attributed to the presence of an expanded pool of partially differentiated but non-functional AFP-specific CD8-positive T cells and the absence of CD4-positive T cell responses in AFP-positive HCC patients[29].

The high prevalence of TERT overexpression in HCC (Figure 1)[7] incited the use of TERT-derived peptides as a tumor vaccine for HCC patients. A phase Ⅱ clinical trial was conducted to examine the efficacy of a TERT-derived peptide vaccine in patients with advanced HCC when it was administered together with cyclophosphamide and GM-CSF[30]. The treatment increased specific T cell responses and decreased Foxp3-positive Tregs. Vaccine administration was well tolerated, and about half of the patients remained in stable condition six months after the treatment but without any complete or partial response to the treatment. Mizukoshi and colleagues also examined the efficacy of subcutaneous injection of TERT-derived peptide emulsified in incomplete Freund's adjuvant in 14 HCC patients[31]. The vaccination induced an increase in TERT-specific T cells with the effector memory phenotype and the capacity to produce multiple cytokines in ten patients. Moreover, eight out of the ten patients with TERT-specific immunity did not show relapse, whereas all patients without TERT-specific immunity recurred. Thus, vaccination with TERT-derived peptide may be effective to prevent recurrence, which is frequently observed after locoregional therapy.

Another candidate molecule for tumor vaccination is an oncofetal antigen, glypican-3 (GPC3), which is expressed in the embryonic liver but scarcely expressed in the normal adult liver, and is overexpressed in HCC[32]. A phase Ⅰ/Ⅱ clinical trial of GPC3-derived peptide vaccination was conducted on 11 patients with advanced HCC[33]. Vaccination induced GPC3-specific CTLs that infiltrated into the tumor. These CTLs were present in the tumor tissues as well as peripheral blood, as revealed by sequencing T cell receptor genes of tumor-infiltrating lymphocytes (TILs). Moreover, the frequency of GPC3-specific CTL after vaccination was correlated with overall survival. These observations imply the efficacy of GPC3-derived peptide vaccination for advanced-stage HCC. Moreover, repeated vaccination with GPC3-derived long peptide (LP) induced LP-specific and HLA class Ⅱ-restricted $CD4^+$ cell responses in 14 of 20 vaccinated HCC patients[34]. Moreover, the presence of specific helper $CD4^+$ cells was correlated with prolonged overall survival.

Additional molecules have been proposed as candidates for peptide vaccine therapy. Aspartate-β-hydroxylase (ASPH) is also overexpressed in HCC and ASPH-derived peptides induced during T cell activation *in vitro* in both an HLA class Ⅰ- and class Ⅱ-restricted manner when peripheral blood mononuclear cells from HCC patients were used[35]. Administering an adenovirus vector expressing HBx protein was effective at both protective and therapeutic antitumor immunity in hepatoma models in immune-competent mice[36], suggesting its efficacy against HBV-positive HCC. Moreover, the treatment induced infiltration of $CD8^+$ T cells, which mainly mediated its antitumor effects. Annexin A3 (ANXA3) expression is enhanced in the CD133-expressing cancer stem-like/initiating cell (CSC/CIC) population, compared with the non-CSC/CIC population of HCC[37]. Moreover, HCC CSC/CICs were preferentially killed by T cells primed with ANXA3-transfected DCs. Likewise, antigen-specific T cell responses against HCC were generated when T cells were primed with New York esophageal squamous cell carcinoma-1 (NYESO1) protein-loaded DCs[38], suggesting the potential of NYESO1-derived peptides as a tumor vaccine.

To date, vaccination with TAA-derived peptides has yielded a marginal clinical benefit in HCC patients, similar to the results reported in other types of cancer[39]. This may arise mainly from its suboptimal immunogenicity and the tolerogenic tendency of intrahepatic DCs[18]. The former can be overcome by improving antigen selection and vaccine formulation, while the latter may be solved by adoptive transfer of DCs pulsed with TAAs. Peptide vaccination alone may not be able to derepress immunosuppressive tumor microenvironments, but immune checkpoint therapy can abolish T cell dysfunction in HCC tissues and eventually can enhance specific T cell responses to tumor antigens. Hence, the combination of a peptide vaccine and immune checkpoint therapy will warrant detailed analysis in the future. Moreover, preclinical studies using mouse models demonstrated the potential efficacy of other types of

vaccines such as RNA-based adjuvants[40], DC-derived exosomes[41], or an attenuated Listeria vaccine that can express HCC-specific antigens[42].

DC-based vaccine therapy: DCs are a professional APC and can initiate and maintain T cell-mediated immune responses when they are pulsed with antigens[43]. In addition to T cells, DCs can also activate NK cells[44]. However, DC-induced immunity is frequently repressed in tumor sites, arising from multiple mechanisms including a low number of DCs in tumor sites, the low antigen-presenting capacity of DCs, and poor access of DCs to tumor antigens[43]. A low number of DCs can be overcome by administering *ex vivo* expanded DCs from peripheral blood mononuclear cells (PBMCs), which are stimulated with combinations of various cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4). Moreover, the additional stimuli such as Toll-like receptor (TLR) agonists, are required for generating mature DCs with a potent antigen-presenting capacity, and several measures are proposed to circumvent poor access of DCs to tumor antigens: Pulsing with tumor lysates, TAAs, or TAA-derived peptide; transfection of DNA constructs encoding TAAs; and fusion with tumor cells[43].

A phase Ⅱ clinical trial was conducted to investigate the safety and efficacy of intravenous vaccination with autologous DCs pulsed *ex vivo* with a liver tumor cell line lysate (HepG2) in advanced HCC patients[45]. The treatment was well tolerated, and in the patients who received at least three vaccine infusions ELIspot assay demonstrated the induction of T cell responses to vaccines and/or AFP and about 25% of patients showed a partial response or stable disease condition, as revealed by serological AFP determination or radiological examination.

Several groups reported DC-based vaccination using AFP as a TAA. A phase Ⅰ/Ⅱ clinical trial examined the effect of intradermal injection of AFP-derived peptide-pulsed DCs, which were prepared from autologous adherent PBMCs cultured with GM-CSF and IL-4[46]. The same group further reported that six of the ten tested subjects exhibited statistically significantly expanded levels of AFP-specific T cells. In addition to T cells, the transfer of AFP-derived peptide-primed DCs enhanced NK cell activation and decreased Treg frequencies in vaccinated HCC patients[47]. However, the priming of DCs with peptides was not efficient, and therefore, in order to efficiently pulse DCs, AFP gene transduction into DCs was attempted using viral vectors such as lentivirus[48] or adeno-associated virus (AAV) vectors[49]. Adoptive transfer of lentivirus-transduced DCs induced superior anti-tumor Th1 polarization in a preclinical model, compared with peptide-pulsed DCs[48]. MHC class Ⅰ and class Ⅱ and co-stimulatory molecules were expressed to a similar extent on recombinant AAV/AFP-pulsed and cancer cell lysate-pulsed DCs. However, recombinant AAV/AFP-pulsed DCs exhibited superiority over cancer cell lysate-pulsed DCs in terms of their capacity to stimulate proliferation of T cells, to induce T cells to secrete IFN-γ, and to generate an AFP-specific MHC class Ⅰ-restricted CTL response in a preclinical study[49]. Thus, the use of viral vectors may be able to prime DCs more efficiently than TAA-derived peptides to activate CTL.

Fifteen patients with advanced HCC were treated with intradermal vaccination of mature autologous DCs pulsed with cell lysates of a human HCC cell line, HepG2[50]. The treatment increased CD8-positive T cells in peripheral blood and serum IFN-γ levels. Overall survival was improved with partial radiological response in two patients, stable course in nine patients, but progressive disease in four patients. DCs transfected with HepG-2 hepatoma cell-derived RNA could induce CTLs to specifically kill HepG2 cells *in vitro*, and injection of T lymphocytes from HCC patients and transfected DCs was effective in a preclinical study using severe combined immunodeficiency mice[51]. In another clinical trial, autologous DCs were pulsed with patient-derived irradiated tumor cell lines established from surgically resected tumor tissues[52]. After one course of TACE, tumor cell-primed DCs suspended in GM-CSF were administered subcutaneously three times at one-week intervals. The treatment was well tolerated, without exacerbation of HBV infection[52].

In another clinical trial, DCs were generated from PBMCs in the presence of GM-CSF and IL-4, and pulsed with cytoplasmic transduction of peptide-attached recombinant fusion proteins consisting of three TAAs: AFP, GPC3, and MAGE-1[53]. A phase Ⅰ/Ⅱ clinical trial demonstrated that T cell response and clinical benefit were observed when subcutaneous injection of the resultant DCs near the inguinal lymph node was followed by topical application of a TLR-7 agonist. Lee and colleagues reported the results obtained from a similar phase Ⅰ/Ⅱ clinical trial using DCs pulsed with AFP, GPC3, and MAGE-1 although they did not administer a TLR-7 agonist[54]. They observed similarly enhanced anti-tumor immune responses after DC vaccination, particularly in recurrence-free patients, as evidenced by lymphocyte proliferation and IFN-γ ELIspot assays. The median time to tumor progression was 36.6 mo in the DC-vaccination group and 11.8 months in the control group. Favorable results prompted the same group to conduct a randomized phase Ⅱ trial on 156 HCC patients who were treated for HCC with no evidence of residual tumors after standard therapeutic modalities[55]. Tumor-specific immune responses were significantly enhanced in the immunotherapy group, but with a higher frequency of overall adverse events, which are mainly mild to moderate in severity. The recurrence-free survival was not significantly different between the immunotherapy and control groups. However, DC immunotherapy significantly reduced the risk of tumor recurrence in the non-RFA group patients but unexpectedly increased the risk of recurrence in the RFA group. Baseline serum IL-15 was statistically correlated with prolonged recurrence-

free survival within the immunotherapy groups[55]. Thus, DC immunotherapy may be effective for HCC patients who are treated with standard treatment modalities but not RFA.

Another TAA, heat-shock protein (HSP) 70, was used to prime DCs, based on its overexpression in HCV-related HCC. DCs transfected with HSP70 mRNA were administered intradermally in a phase Ⅰ clinical trial on 12 advanced HCC patients[56]. The trial demonstrated that the treatment was well tolerated, with complete response without any recurrence in two patients, stable disease in five, and progression of disease in five.

TACE can induce HCC cells to die and release high levels of TAAs, which can be internalized, degraded, and presented to immune cells by APCs including DCs. As a consequence, following TACE, tumor immunity can be enhanced. Supporting this notion, we observed that AFP-specific T cell frequency was further increased in HCC patients receiving TACE, and that the increment was enhanced by simultaneous transarterial administration of DCs[57]. Our subsequent clinical trial further demonstrated that the co-infusion of mature DCs into tumor sites following TACE, was well tolerated in advanced HCC patients and prolonged recurrence-free survival of patients, compared with the historical controls[58].

Nevertheless, the clinical response to adoptive DC transfer is still not satisfactory, and, as a consequence, several measures have been devised to augment the efficacy of the adoptive DC transfer. Several groups proposed the priming of DC with other antigens, such as hepatocellular carcinoma-associated antigen-519/targeting protein for Xkl-2 (HCA519/TPX2)[59], epithelial cell adhesion molecule (EpCAM)[60], or ANAXA3[37]. Since EpCAM and ANAXA3 are selectively expressed in CSCs/CICs, the priming of DCs with these antigens may be effective to kill CSCs/CICs that are rather resistant to standard therapies such as chemotherapy and/or molecular targeted therapy. Moreover, in order to enhance immunostimulating activities of DCs, other groups have tried to transfect DCs with the genes of immunostimulating cytokines, such as IL-2[61] or IL-12,[62] in preclinical or *in vitro* studies. The other measure includes the combined administration of effector cells like cytokine-activated killer cells (CIKs) with antigen-pulsed DCs, as we discuss in the following section.

Adoptive transfer of immune effector cells: Several immune effector cells are adoptively transferred cell to enhance tumor immunity; Two types of T cells are commonly used for adoptive cell therapy to enhance tumor immunity: TILs, genetically modified T cells, NK cells, natural killer T (NKT) cells, and CIKs, TILs (Figure 3).

TILs are considered to have a higher specific immunological reactivity against tumor cells than the non-infiltrating lymphocytes, and evidence is accumulating to indicate the potential role of TILs as biomarkers reflecting the immune response to the tumor[63]. TILs are obtained from surgically obtained tumor specimens and are expanded *ex vivo* with anti-CD3 antibody treatment before being transferred back to patients[64]. Adoptive cell therapy using TILs can be effective for metastatic melanoma[65], but no clinical trials are in progress to evaluate the adoptive transfer of TILs for HCC, probably due to the difficulty in obtaining a sufficient number of TILs during surgical resection of HCC.

T cells can be genetically engineered to express a T cell receptor (TCR) against a specific TAA (Figure 6)[66]. Metastatic melanoma was treated with adoptive transfer of autologous T cells with a modified TCR recognizing a melanocyte-differentiating antigen (MART-1), and the treatment resulted in long-term persistence of infused cells and tumor regression in two out of 17 patients[67]. The adoptive transfer of T cells expressing a higher-affinity TCR caused a better benefit, with tumor regressing in six out of 20 patients[68]. Subsequently, several phase Ⅰ clinical trials were conducted to evaluate the efficacy of adoptive transfer of autologous T cells, which are genetically modified to express a TAA-specific T cell receptor, and some favorable results have been reported involving melanoma, colorectal cancer, synovial cell sarcoma, and multiple myeloma to date[66]. With these results, two phase Ⅰ/Ⅱ clinical trials are now in progress to evaluate the adoptive transfer of T cells with a modified TCR, which can recognize HBV antigens, in HCC patients with HBV infection (NCT026863712, 02719782). One additional phase Ⅰ clinical trial is also recruiting participants to evaluate the safety and anti-tumor activity of autologous T cells expressing TCRs specific for AFP in advanced HCC patients (NCI03132792). Nevertheless, further progress in TCR-modified T cell therapy requires the identification of additional TAAs, the comprehensive elucidation of the structure of TCRs that specifically recognize TAAs, and improvements in genetic engineering of TCRs.

Another type of genetically modified T cell utilizes the chimeric antigen receptor (CAR) gene, which is prepared by fusing the transmembrane and cytoplasmic domains of CD3ζ with the antigen-binding portion of an antibody that can recognize a particular TAA (Figure 7)[69] The generated CAR gene is transduced to T cells, mostly with the help of a lentivirus vector. The resultant CAR T cells can deliver activating signals once they bind with a specific TAA using the antigen-binding domain of their extracellular portions. In order to enhance their *in vivo* persistence and function, CAR genes were further modified by adding one or two co-stimulator domains derived from co-stimulatory molecules such as CD28, 4-1BB, and OX-40 (Figure 7)[69]. At the end of 2017, the Food and Drug Administration (FDA) had approved two distinct CAR T cell therapies using modified CARs to treat acute lymphoblastic leukemia and large B-cell lymphoma. These groundbreaking successes have spurred research to apply CAR T cell therapy to solid tumors including HCC, beyond hematological malignancy.

GPC3 was frequently used as a target for CAR T cells, since it is expressed abundantly in HCC cells[32].

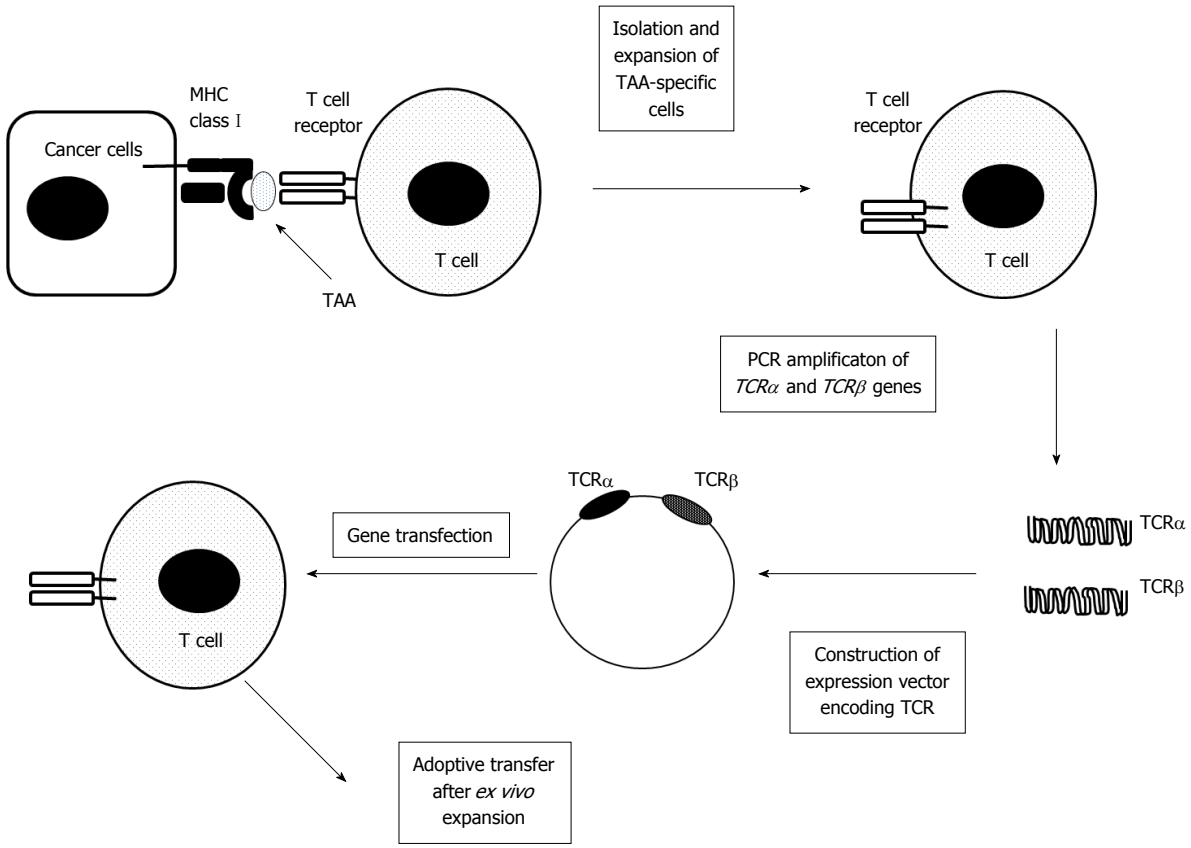

Figure 6 Preparation of genetically modified TCR-expressing T cells. *TCRα* and *TCRβ* genes are cloned and amplified with the use of PCR from TAA-specific T cells, which are isolated and expanded *ex vivo*. The obtained TCRα and TCRβ genes are cloned into an expression vector, which is used for transfection into T cells. The transfected cells are adoptively transferred after *ex vivo* expansion.TAA: Tumor-associated antigen.

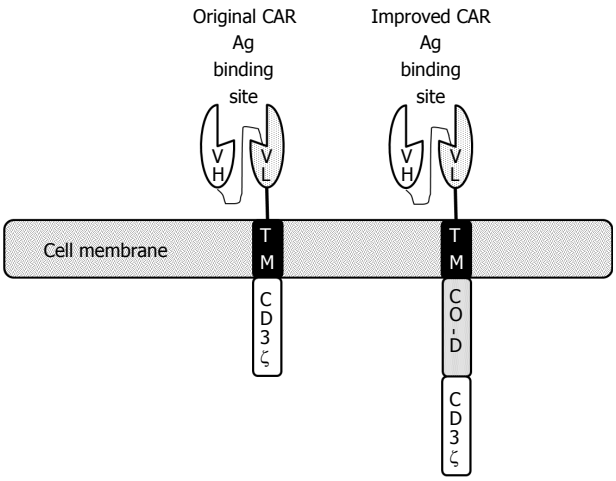

Figure 7 Schematic structure of chimeric antigen receptors. Chimeric antigen receptors (CARs) are composed of a single-chain fragment variable (scFv) containing the heavy chain variable (VH) and the light chain variable domain (VL) of a monoclonal antibody. ScFv is attached to a transmembrane (TM) domain and CD3ζ chain in the case of the first-generation CARs. Improved CARs additionally contain one or two more costimulatory domains. VH, heavy chain variable domain; VL, light chain variable domain; TM, transmembrane domain; CO-D, domain derived from co-stimulatory molecules.

The CAR gene was generated by fusing the anti-GPC3 single chain variable region (scFv), CD8α hinge, CD28 transmembrane and intracellular signaling domain, 4-1BB, and CD3ζ[70]. The resultant GPC3-targeted CAR T cells could effectively kill GPC3-positive HCC cells, but not GPC3-negative cells, *in vitro*. Moreover, GPC3-targeted CAR T cells eradicated HCC xenografts with a high level of GPC3 expression, and efficiently suppressed the growth of HCC xenografts with a low GPC3 expression level, in a preclinical mouse model. Similar observations were observed on T cells with GPC3-specific CARs that encoded CD3ζ with costimulatory domains derived from CD28, 4-1BB, or CD28 and 4-1BB[71]. These observations promoted two phase I clinical trials to examine the safety of anti-GPC3 CAR T cell transfer into HCC patients (NCT02395250, NCT02723942). These studies have been completed but the results are not yet available.

In order to reduce off-tumor toxicity, Chen and colleagues prepared dual-targeted CAR T cells coexpressing GPC3 and asialo-glycoprotein receptor 1 (ASGR1) (a liver tissue-specific protein)-targeted CARs containing both CD28 and 4-1BB signaling domains, and proposed that dual-target T cells can reduce the risk of off-tumor toxicity while maintaining relatively potent antitumor activities for $GPC3^{+}ASGR1^{+}$ HCC[72]. Moreover, CAR T cells were generated to target EpCAM[73] and mucin 1[74], and phase I clinical trials are in progress to evaluate their safety (NCT03013712 and NCT02587689).

Collectively, CAR T therapy for HCC is still in its infancy and requires further progress in many aspects: Selection of appropriate TAAs, enhancement of the binding affinity of CAR to TAAs, improvement of trafficking of CAR T cells to tumor site, and prolongation of *in vivo* survival of CAR T cells. Advances in these aspects are required for the clinical application of CAR T cells for HCC therapy.

Human NK cells express CD56 but not CD3, and are a major player in innate immunity involved in defense against both cancers and some virus-infected cells[75]. NK cells express germline-encoded activating and inhibitory receptors, and the balance between these two distinct types of receptors determines NK cell function. Activating receptors bind ligands on the target cells and induce cell lysis, whereas inhibitory receptors recognize MHC class Ⅰ molecules that normal cells abundantly express, and eventually inhibit cytotoxicity exerted by activating receptors[76]. NK cells can kill target cells by releasing cytotoxic granules or utilizing death-inducing receptors including Fas ligand and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)[75]. Moreover, antibody-dependent cell-mediated cytotoxicity (ADCC) is exerted mainly by NK cells[77]. Furthermore, although NK cells were once considered to lack memory capacity, accumulating evidence indicates that NK cells can exert immunological memory[78]. Due to these properties, NK cells can be a potent candidate cell type for immune therapy.

Autologous highly purified NK cells can be an ideal candidate, but their low number in peripheral blood precludes their use. NK cells possess killer inhibitory receptors, which can inhibit NK cell responses to the cells expressing the same MHC class Ⅰ[76]. Thus, NK cells can kill only the cells that do not express their own MHC class Ⅰ. As a consequence, allogenic NK cells can kill cancer cells expressing different MHC class Ⅰ more efficiently than autologous NK cells, which share MHC class Ⅰ with the cancer cells[79]. One clinical trial has been conducted to examine the efficacy of adoptive NK cell transfer for preventing HCC recurrence after curative therapy, but with no results available (NCT02008929).

Liver NK cells can express TRAIL more abundantly upon activation and can exhibit stronger killing activity against HCC, compared with circulating NK cells[80]. Moreover, evidence is accumulating to indicate few cytotoxic effects of TRAIL on normal cells including hepatocytes[81]. Actually, adoptive transfer of IL-2-stimulated NK cells obtained from donor livers increased an antitumor response against HCC in recipients, who were treated with a liver transplant from a live donor, without causing any injury in normal hepatocytes[82]. These promising results paved the way to initiate a phase Ⅰ clinical trial to examine the feasibility and safety of IL-2-activated NK cells obtained from cadaveric donor liver grafts when they were adoptively transferred to liver transplant recipients with HCC (NCT01147380). No severe adverse effects were observed in the 18 patients who received liver NK cells, indicating the safety of the treatment.

NKT cells are specialized CD1d-restricted T cells that recognize lipid antigens to stimulate both innate and adaptive immune cells in the tumor microenvironment, once activated[83]. In a mouse preclinical model, adoptive transfer of either NKT cells pulsed with HCC-derived antigens or NKT cells obtained from immunized donors resulted in complete disappearance of tumors within four weeks and attenuated weight loss, together with increased serum IFN-γ, IL-12, and IL-4 levels[84]. These promising results led to the initiation of a phase Ⅰ clinical trial using autologous NKT cells to treat HCC, but the results are not yet available (NCT010801852).

CIKs are non-MHC-restricted cytotoxic cells, which are expanded *ex vivo* from PBMCs stimulated with anti-CD3 antibody, IL-2, and IFN-γ, and can even exhibit potent *in vivo* anti-tumor effects[85]. CIKs are T cells that have acquired the natural cytotoxic potential of NK cells[86]. Thus, the cells can recognize tumor cells by using mainly the natural killer group 2 member D (NKG2D) receptor, and eventually kill them without a prior exposure or priming[87,88]. CIKs have typical phenotypes, characteristic of terminally differentiated $CD8^+$ effector memory cells, and simultaneously recognize target cells in a MHC class Ⅰ-restricted manner[86]. A meta-analysis was conducted on 11 clinical trials with CIK cells for solid tumors including HCC and gastric cancer[89]. The treatment was well tolerated, with a low incidence of severe adverse effects. Of the 384 patients where a clinical response was reported, 24 patients showed a complete response, 27 patients showed a partial response, 40 patients showed a minor response, 161 patients had stable disease, and 129 patients had progressive disease. Disease-free survival rates were significantly higher in patients treated with CIK cells than those in the control group without CIK treatment. A decrease in tumor volume was only described in three patients. Interestingly, a reduction of hepatitis B virus load was described in patients undergoing treatment with CIK cells. These promising results spurred the application of CIK-based immunotherapy to HCC treatment. To date, eight randomized clinical trials (RCTs), six prospective studies, and three retrospective studies have been reported[90]. A meta-analysis of these studies revealed that CIK treatment increased survival rate as a whole, but without any significant prolongation of progression free-survival. Moreover, patients in the CIK cell-treatment group had lower rates of relapse even in RCTs. To date, two phase Ⅲ clinical trials using CIKs have been completed (NCI00769106, 01749865) but the results are not yet deposited in the database. In order to enhance the efficacy of adoptive transfer of CIKs, patients with HCC were treated with RFA and three courses of immunotherapy, which consisted of the co-injection of CIKs with immature or tumor cell lysate-pulsed DCs[91]. The treatment was well-tolerated, while $CD4^+CD25^{high}$ Tregs decreased with a reciprocal increase in $CD8^+CD28^-$ effector cells one month after the treatment, but no differences were observed six months after treatment.

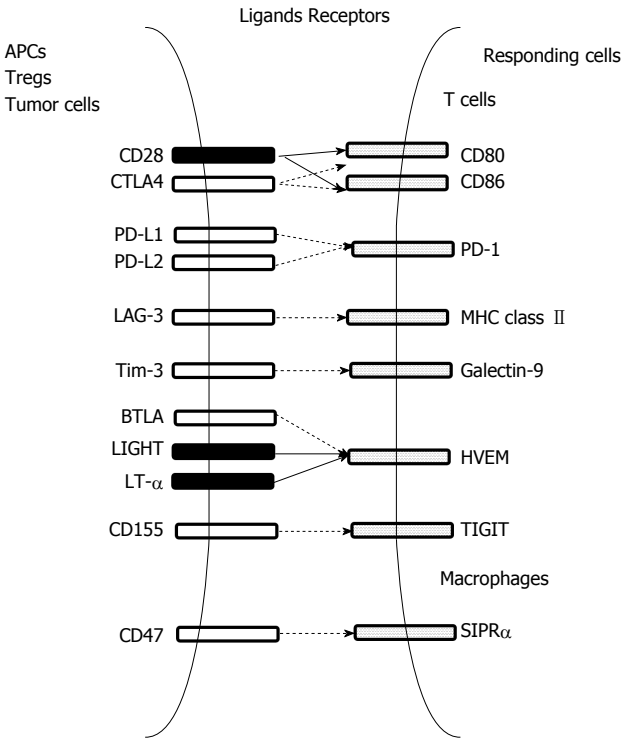

Figure 8 Interaction of major immune co-stimulatory and inhibitory molecules, and their cognate receptors. Co-stimulatory and co-inhibitory molecules are indicated by closed and open boxes, respectively. Co-stimulatory and inhibitory signals are indicated by filled and hatched arrows, respectively.

A phase Ⅰ/Ⅱ clinical trial is now in progress to evaluate the combination of CIKs, DCs, and anti-PD-1 antibody for HCC treatment (NCT02886897).

Reversal of T cell dysfunction

T cells can induce tumor regression upon recognizing TAAs expressed by tumor cells[92], but tumors frequently progress even in the presence of abundant TAA-specific CTLs in tumor tissues[93]. This paradoxical tumor growth can arise from multiple immune suppressive pathways that impair the function of CTLs present in tumor tissues[94]. The most notable immune suppressive mechanisms are immune checkpoint pathways, which include CTLA-4, PD-1-PD-L1/PD-L2, CD47-singal regulatory protein-α (SIRPα), lymphocyte activation gene 3 (LAG-3), T-cell immunoglobulin mucin-3 (Tim-3), T-cell tyrosine-based B and T lymphocyte attenuator (BTLA), and inhibitory motif domain (TIGHT) (Figure 8)[24]. These pathways can dampen T cell activation through ligand-receptor interactions. Moreover, T cell response can also be negatively regulated by several types of resident cells present in the tumor microenvironment, such as Tregs and myeloid-derived suppressor cells (MDSCs)[94].

The concept of tumor immunotherapy has been drastically changed by the clinical success of CTLA-4 and/or PD-1-PD-L1/PD-L2 blockade in treating several types of advanced solid tumors. As a consequence, unleashing the immunosuppressive tumor microenvironment becomes a potential therapeutic measure to enhance tumor immunity. In the next sections, we discuss immune checkpoint therapy and the potential of immune suppressive cell blockade as a novel type of immunotherapy.

Immune checkpoint therapy: Cancer cells or other resident cells in the tumor microenvironment express various ligands that inhibit or stimulate immune activity, and these ligands bind their corresponding receptors on immune cells, thereby modulating immune responses[94]. The ligand-receptor pairs are denoted as immune checkpoints (Figure 8), which control effector T cell- and NK cell-responses at multiple steps from priming by APCs to activation[24]. Based on accumulating evidence to indicate the presence of T cell dysfunction in the tumor microenvironment, a novel type of immunotherapy, immune checkpoint therapy, has been proposed to reverse T cell dysfunction through unleashing immune suppression mediated by inhibitory immune checkpoint pathways. Due to their remarkable effectiveness observed on several types of cancers, the FDA has already approved the antagonistic antibodies targeting two immune checkpoint pathways - CTLA-4 and PD-1-PD-L1/PD-L2 - for cancer treatment[95].

CTLA-4 is expressed on T cells and has greater affinity for CD80 and CD86, the molecules that are expressed on APCs and can bind the co-stimulatory molecule CD28 (Figure 5A)[96]. The interaction between CD28 and CD80/86 is indispensable for T cell activation, particularly at the priming phase. CTLA-4 can interfere with the interaction between CD80/CD86 and CD28, thereby rendering T cells unresponsive to an antigen. Moreover, Tregs can inhibit immune responses using CTLA-4 expressed on their surface[97]. Thus, an antagonistic anti-CTLA-4 antibody was clinically evaluated in advanced melanoma patients, and it elicited enhanced immune responses, with a clinical response in a substantial proportion of patients[95]. This promising observation spurred a phase Ⅰ clinical trial using an anti-CTLA-4 monoclonal antibody (tremelimumab) for HCC patients with chronic HCV infection (NCT01008358)[98]. Tremelimumab was well tolerated, without any severe adverse effects except an intense, but transient, elevation of transaminases after the first dose in some patients. Specific anti-HCV immunity was enhanced with a significant drop in viral load, but new emerging variants of the hypervariable region 1 of HCV replaced the predominant variants present before therapy. The partial response rate was 17.6% and the disease control rate was 76.4% with time to progression of 6.48 mo. Another phase Ⅰ clinical trial evaluated the efficacy of tremelimumab for advanced HCC patients when combined with TACE or RFA (NCT01853618)[99]. No dose-limiting toxicities were reported. Of the 19 evaluable patients, five achieved a confirmed partial response. After the treatment, viral load was reduced markedly in 12 of 14 patients with HCV infection. Moreover, at six months after the treatment, tumor biopsies showed an apparent increase in $CD8^+$ T cells restricted to the patients showing

a clinical benefit. Six and 12-mo probabilities of tumor progression-free survival were estimated to be 57.1% and 33.1%, respectively, with median time to tumor progression of 7.4 mo and median overall survival of 12.3 mo. Additionally, one phase Ⅰ/Ⅱ clinical trial is in the process of recruitment to examine the efficacy of anti-CTLA-4 antibody in combination with ablative therapy (NCT02821754). Nevertheless, a large-scale phase Ⅲ clinical trial is required to validate these observations.

In contrast to CTLA-4, the PD-1-PD-L1/PD-L2 pathway dampens T cell activation mainly at its effector phase (Figure 5B)[100]. PD-1 is expressed on a wide variety of immune cells, including activated $CD4^+$ and $CD8^+$ T cells, B cells, NK cells, monocytes, and DCs. PD-L1 is expressed on a wide variety of cells, including non-hematopoietic cells such as endothelial cells, mesenchymal stem cells, and corneal cells, as well as hematopoietic cells such as T and B cells, DCs, macrophages, and mast cells. On the contrary, PD-L2 expression is restricted to activated DCs, macrophages, and mast cells. Moreover, PD-L1, as well as PD-L2, is expressed on various tumor cells. As a consequence, in the tumor microenvironment, the interaction between PD-1 and PD-L1/PD-L2 can dampen T cell receptor-mediated signaling pathways to inhibit T cell activation and subsequent antitumor immunity[100].

Immunohistochemical analysis demonstrated an increased expression of PD-1 and PD-Ls in HCC tissues, with PD-1 expression in liver-infiltration lymphocytes and PD-L1 and PD-L2 expression in non-parenchymal liver cells and tumor cells[101]. Moreover, PD-L1 expression was significantly correlated with hepatitis B virus infection and with HCC stage. Consistently, the expression of PD-Ls positively correlates with $FoxP3^+$ Treg infiltration but not granzyme B-expressing CTL infiltration, suggesting that PD-L expression contributes to immunosuppression in HCC tissues[102]. Moreover, a higher expression of PD-L1 and PD-L2 in HCC tissues has been associated with poorer prognosis. Together with a good safety and substantial clinical responses to the treatment with anti-PD-1 or anti-PD-L antibodies in patients with several types of solid tumors, particularly non-small cell lung carcinoma[103,104], these observations provide a rationale for initiating a clinical trial using anti-PD-1 or anti-PD-L1/PD-L2 antibody for HCC treatment.

A Phase Ⅰ/Ⅱ clinical trial was conducted with the support from Bristol-Myers Squibb to evaluate the safety and efficacy of anti-PD-1 monoclonal antibody (nivolumab) for histologically confirmed advanced HCC patients, who were included regardless of complicated HCV or HBV infection, and previous sorafenib treatment[105]. A total of 262 eligible patients were treated with 48 patients in the dose-escalation phase and 214 in the dose-expansion phase, and 202 (77%) of 262 patients have completed treatment. During dose escalation, nivolumab showed a manageable safety profile, including acceptable tolerability and 3 mg/kg every two weeks was chosen as a dosage for dose expansion. The objective response rate in the dose-expansion phase was 20%, at similar levels when a single administration of nivolumab was given for other types of solid tumors[100]. A phase Ⅰ/Ⅱ clinical trial has just started to evaluate another anti-PD-1 monoclonal antibody, pembrolizumab, for HCC (NCT 02702414).

The promising results have encouraged the initiation of several phase Ⅲ clinical trials for HCC patients (Table 1). A phase Ⅲ clinical trial was conducted to compare the efficacy of nivolumab with that of sorafenib as a first-line therapy (NCT 02576509), but the results are not yet available. Recently, another phase Ⅲ clinical trial was started to investigate if nivolumab would improve recurrence-free survival, compared with placebo in HCC patients who have undergone complete resection or have achieved a complete response after local ablation, and who are at high risk of recurrence (NCT03383458). Additionally, a phase Ⅲ trial of pembrolizumab (MK-3475) was conducted in patients with advanced HCC who were systemically treated previously (NCT02702401). The primary objectives of this study were to determine progression-free survival and overall survival of pembrolizumab plus best supportive care (BSC) compared with placebo plus BSC. The following phase Ⅲ trial was planned to determine the efficacy and safety of pembrolizumab or placebo given with BSC in Asian patients with HCC (NCT03062358). In Japan, a phase Ⅲ, randomized, open-label, multicenter, global study was designed to compare the efficacy and safety of tislelizumab (BGB-A317) versus sorafenib as a first-line systemic treatment in patients with unresectable HCC (NCT03412773). This study also includes a substudy investigating the safety, tolerability, pharmacokinetics, and preliminary efficacy in HCC in Japanese patients.

Other immune checkpoint pathways are proposed to be candidates for immune checkpoint therapy (Figure 8). SIPRα is a unique immune checkpoint molecule expressed on myeloid cells, particularly on macrophages but not lymphoid cells, and binds CD47, which is expressed abundantly on various types of cancer cells[106]. The CD47-SIRPα interaction can inhibit macrophage function, including its phagocytosis capacity, and therefore, CD47 blockade promotes macrophage phagocytosis of cancer cells[107]. Moreover, several preclinical studies demonstrated that CD47 blockade reduces tumor growth by enhancing macrophage phagocytosis and inducing macrophage phenotype change from pro-tumorigenic M2 to pro-inflammatory and anti-tumorigenic M1 states[108-110]. These promising results spurred the development of various agents targeting the CD47-SIRPα axis, including humanized anti-CD47 monoclonal antibody, SIRPα fused with and human IgG_1 Fc portion, and SIRPα variant protein, and the clinical trials using these agents have been initiated[106]. However, these clinical trials are still in the process of patient enrollment.

Treg and anergic T cells abundantly express LAG-3, which binds a nonholomorphic region of MHC class Ⅱ with greater affinity than CD4 and thereby can negatively regulate $CD4^+$ cell proliferation and cytokine production[111]. Phase Ⅰ clinical trials were conducted

Table 1 Current phase Ⅲ clinical trials of immune checkpoint inhibitors for hepatocellular carcinoma patients

NCT number	Targets	Experimental arm	Comparator arm	Outcome measures	Enrollment	Start date	Completion date	Locations
NCT02576509	PD-1	Nivolumab	Sorafenib	OS;PFS/PD-L1 expression/ ORR	726	25-Nov-15	22-Jun-19	Australia, Austria, Belgium, Canada, China, Czechia, France, Germany, Hong Kong, Israel, Italy, Japan, South Korea, Poland, Russian Federation, Singapore, Spain, Sweden, Switzerland, Taiwan, United Kingdom, United States
NCT02702401	PD-1	Pembrolizumab + BSC	Placebo + BSC	PFS/OS; ORR/ DCR/TTP/ DOR	408	26-May-16	1-Feb-19	
NCT03062358	PD-1	Pembrolizumab + BSC	Placebo + BSC	OS; PFS/ORR/DOR/ DCR/ TTP/AE/Discontinuation	330	27-Apr-17	23-Dec-19	China, Hong Kong, South Korea, Malaysia, Taiwan
NCT03298451	PDL-1	Durvalumab + tremelimumab	Sorafenib	OS; PFS/ORR/DOR/DCR/ TTP/PK	1200	11-Oct-17	27-Mar-20	United States, Brazil, Canada, China, France, Germany, Hong Kong, India, Italy, Japan, Russia, Spain, Taiwan, Thailand, Ukraine, Vietnam
NCT03383458	PD-1	Nivolumab	Placebo	RFS; OS/TTR	530	18-Dec-17	2-May-25	Japan, South Korea, Taiwan, United States
NCT03412773	PD-1	Tislelizumab (BGB-A317)	Sorafenib	OS; Safety/AE/DLT/Cmax/ Cmin/AUC/ADA/Vital signs/physical examination/ clinical laboratory results/ electrocardiogram/ORR/PFS/ DOR/TTP/HRQoL DCR/ CBR/Anti-BGB-A317 antibody	660	28-Dec-17	May-22	United States

ADA: Anti-drug antibodies; AE: Adverse events; AUC: Area under the curve; BSC: Best supportive care; CBR: Clinical benefit rate; Cmax: Maximum concentration; Cmin: Trough serum concentration; DCR: Disease control rate; DLT: Dose-limiting toxicities; DOR: Duration of response; HRQoL: Health-related quality of life; ORR: Overall response rate; OS: Overall survival; PFS: Progression-free survival; TTP: Time to progression; TTR: Time to recurrence.

to examine an LAG-3 antagonist or anti-LAG-3 antibody for treating several solid tumors but not HCC[112]. Tim-3 is expressed on IFN-γ-producing T cells, Tregs, DCs, and macrophages, and can suppress their function upon binding its ligand, galectin-9[113]. Tumor outgrowth can be linked to the exhaustion of TAA-specific $CD8^+$ T cells, which frequently express Tim-3 and PD-1 simultaneously[114]. Moreover, the combined targeting of the Tim-3 and PD-1 pathways is more effective in controlling tumor growth in mouse preclinical models than targeting either pathway alone[115]. This observation incited the initiation of several phase Ⅰ/Ⅱ clinical trials on the combined administration of anti-Tim-3 and anti-PD-1 antibodies to patients with various solid tumors (NCT02608268, 02817633, 03099109), but the results are not yet available. Another immune checkpoint molecule, BTLA, is expressed on T cells, resting B cells, macrophages, and DCs, and binds herpesvirus entry mediator (HVEM), a member of the tumor necrosis factor (TNF) receptor family, which binds with LIGHT and lymphotoxin-α, members of TNF family[116]. The BTLA-HVEM interaction delivers co-inhibitory signals, whereas the LIGHT-HVEM interaction delivers co-stimulatory signals[116]. Aberrant expression of the BTLA-HVEM axis in tumor tissues[117] and BTLA-mediated inhibition of human $CD8^+$ tumor-specific T cell functions[118] suggest that this axis may be able to be used for cancer immunotherapy. Additionally, TIGIT is expressed on activated T cells, memory T cells, Tregs, and NK cells and can dampen T and NK cell functions through interacting with CD155 expressed on APCs and tumor cells[119]. However, their roles in tumor immunity still remain enigmatic.

Immune checkpoint therapy can confer cancer patients with a remarkable clinical efficacy and durable response, even at advanced disease stages, but many patients do not respond to the therapy. Several measures have been proposed to increase the efficacy of the treatment. One is the identification of a biomarker to select patients who are sensitive to checkpoint blockades[120,121]. PD-L1 overexpression was proposed to be a predictive biomarker for the response to PD-1/PD-L1 antibodies, but PD-L1 staining

has low prediction accuracy. Other candidate biomarkers include intratumoral lymphocyte infiltrates and genetic markers such as oncogenic mutations, mismatch repair deficiency, and mutation loads[120,121]. Most HCC cases develop in the presence of chronic inflammation, which can cause innumerable genetic mutations (Figure 1). Thus, genome-wide analysis on HCC genetics may be helpful to determine which patients can respond well to immune checkpoint therapy. Furthermore, recent clinical trials revealed that patient HLA class Ⅰ genotype influences the response to the treatment with anti-CTLA-4 and ani-PD-1 antibodies in melanoma and lung cancer patients[122]. Maximal heterozygosity at HLA class Ⅰ and the HLA-B44 supertype was associated with a favorable response, whereas the HLA-B62 supertype or somatic loss of HLA class Ⅰ heterozygosity was associated with poor outcome. A good response in patients with the HLA-B44 supertype suggests the possibility of improving the efficacy of immune checkpoint therapy by introducing a neoantigen-based therapeutic vaccine.

Another way to enhance the efficacy of immune checkpoint therapy is the combined administration with other treatment modalities, such as radiotherapy, chemotherapy, or molecular targeted therapies[123]. Especially, radiotherapy can cause the abscopal effect, where localized radiation-induced tumor cell death can induce anti-tumor responses against tumors at other sites[124]. Immune checkpoint therapy may be able to augment radiotherapy-induced abscopal effects, and several clinical trials were initiated to evaluate the combined treatment of anti-PD-1 antibody with β irradiation in HCC patients(NCT03033446, 02837029, 03099564). Moreover, phase Ⅰ/Ⅱ clinical trials are now evaluating the combined treatment of the anti-PD-1 antibody with anti-angiogenic agents (NCT02572687, 03006926, 02856425, 02942329, 02988440) or molecular targeted therapies (NCT02423343, 02859324, 03095781, 02474537, 02325739) in HCC patients. However, the results are not yet available.

Each immune checkpoint therapy acts at a distinct phase of the immune response to the tumor[123] and therefore, the combination of different immune checkpoint therapies are proposed or being evaluated to treat various types of cancers. However, at present, four phase Ⅰ/Ⅱ (NCT01658878, 02519348, 02821754, 03222076) and one phase Ⅲ clinical trial (NCT03298451) are in progress to evaluate the combined administration of anti-CTLA-4 with either anti-PD-1 or anti-PD-L1 antibody in patients with HCC.

Immune checkpoint therapy can reverse tumor-induced T cell exhaustion, but impaired DC function can depress T cell priming and activation, thereby reducing T cell trafficking to tumor cells[125]. Thus, the supplementation of DC vaccine therapy may be able to enhance the effectiveness of immune checkpoint therapy.

Collectively, immune checkpoint therapy can be a promising therapeutic modality for HCC treatment and/or prevention of its recurrence after curative local and regional therapy, but its clinical application may require an additional thorough analysis to select optimal patients and determine efficient co-administration methods.

Blockade of immune suppressor cells: Tregs and MDSCs are two distinct types of hematopoietic cell-derived immunosuppressive cells present in tumor tissues. Tregs express a transcription factor, FoxP3, and can suppress aberrant T cell-mediated immune responses against TAAs as well as self-antigens through several mechanisms[126]. Tregs display abundantly high-affinity IL-2 receptor α chain (CD25), which can bind IL-2 to limit its amount available to effector T cells, thereby attenuating effector T cell activation and proliferation. Tregs constitutively express CTLA-4 to depress CD80/CD86-mediated co-stimulatory signals and secrete immune suppressive mediators including IL-10 and transforming growth factor (TGF)-β. A detrimental role of Tregs was suggested by an inverse correlation of intratumoral Tregs with overall survival in patients with various types of cancers including HCC[127,128]. Thus, reducing the number of intratumoral Tregs and/or dampening their function may be effective to enhance tumor immunity.

The reduction of intratumoral Tregs was achieved in several mouse models by treating with an anti-CD25 antibody, and this reduction was associated with depressed tumor growth[129]. Moreover, anti-tumor effects were synergistically enhanced by co-administration with an anti-PD-1 antibody. However, the efficacy of the anti-CD25 antibody awaits validation in clinical trials. In other mouse models, intratumoral Tregs and tumor growth were reduced also by treating with an antibody for the chemokine receptor CCR4, which is abundantly expressed on Tregs[130]. The observations were translated into a phase Ⅰ clinical trial which is in progress to evaluate the combination of anti-CCR4 antibody and anti-PD-1 antibody for various solid tumors except HCC (NCT02946671). Tregs and $CD8^+$ effector cells express glucocorticoid-induced TNF receptor (GITR), and its triggering can abrogate the suppressive activity of Treg cells but co-stimulate responder T cells[131]. Consistently, GITR activation can eradicate established tumors in several mouse preclinical models[132,133]. Consequently, several phase Ⅰ/Ⅱ clinical trials are now in progress to evaluate the combined treatment of an agonistic anti-GITR antibody or a GITR agonist with other immune checkpoint inhibitors, such as an anti-PD-1 antibody, for several types of solid tumors, but not HCC[134].

Another immunosuppressive cell type present abundantly in tumor tissues is MDSCs, which are a heterogeneous population of myeloid cells with potent immune regulatory activity that are generated during cancer and chronic inflammation[135]. MDSCs consist of two large groups of cells: polymorphonuclear (PMN)-MDSCs and monocytic (M)-MDSCs, which represent immature neutrophils and a pathological state of activation of monocytes, respectively. In humans, PMN-MDSCs share many surface phenotypes with neutrophils,

but exhibit a lower density than neutrophils. M-MDSCs exhibit similar surface phenotypes as monocytes do, but do not express MHC class Ⅱ and CD11c, in contrast with monocytes[135]. Evidence is accumulating to indicate the association of a high frequency of intratumoral MDSCs with poor clinical outcomes in patients with various types of cancers[136,137]. We also observed that the frequency of MDSCs in HCC patients was significantly increased, and was correlated with tumor progression, but not with the degree of liver fibrosis and inflammation[138]. Moreover, the frequency of MDSCs after treatment was inversely correlated with recurrence-free survival time in HCC patients who received curative RFA therapy. These observations promoted the evaluation of treatments targeting MDSCs.

Indeed, treatment with several chemotherapeutic drugs including gemcitabine[139], 5-fluorouracil[140], and anthracyclines[141], decreased intratumoral MDSCs and attenuated tumor growth in several preclinical mouse models. Similar observations were obtained from preclinical models when administered with selective PI3-kinase δ/γ inhibitors[142] or a JAK2/STAT3 inhibitor[143]. Moreover, an antibody against the chemokine receptor CXCR2 inhibited MDSC trafficking to tumors and enhanced anti-PD-1-mediated anti-tumor effects, also in a mouse preclinical model[144]. These promising results spurred the initiation of more than 40 phase Ⅰ/Ⅱ clinical trials to evaluate therapies targeting MDSCs in various types of cancers, including one trial on HCC (NCT03203005).

To date, various maneuvers have been proposed to target Tregs and MDSCs as tumor immunotherapies, but their efficacy requires validation through human clinical trials.

FUTURE PERSPECTIVES

Various immune therapeutic modalities have been proposed to eradicate or reduce tumor burden and/or to prevent recurrence after successfully removing a primary tumor in HCC patients. Promising results have been obtained from preclinical and/or phase Ⅰ clinical trials to evaluate various types of immune therapies for HCC patients, as discussed here. However, to date, only immune checkpoint therapy using an anti-PD-1 antibody has produced favorable outcomes in phase Ⅱ clinical trials, and these outcomes need validation in large-scale RCTs.

Adoptive immune cell therapy has several hurdles to overcome before its clinical application to HCC treatment. The first one deals with the preparation of cell populations used for adoptive transfer. At present, cell preparation has not been standardized, and, therefore, it is difficult to compare the results reported by different research teams. Moreover, several papers described the results obtained from cell populations prepared under conditions not in compliance with the good manufacturing practice (GMP) conditions. Thus, the cells should be prepared in a standardized manner and under GMP conditions to be used in large-scale RCTs.

The problem inherent in immune therapy is that it can stabilize disease status for a long period without reducing tumor burden, in contrast with the effects exerted by chemotherapy and/or radiotherapy. Moreover, one object of immune therapy for HCC is the prevention of tumor recurrence after a successful local and regional therapy. Thus, it is absolutely necessary to contrive a measure to evaluate immune therapy for HCC from a standpoint distinct from that used to assess chemotherapeutics.

Immune dysfunction can arise in cancer patients at multiple levels including depressed antigen presentation, reduced effector T cell function, and immunosuppressive tumor microenvironments, and therefore, these results suggest distinct mechanisms responsible for immune suppression present in individual cancer patients. These heterogeneities may account for the efficacy of a single type of immune therapy in a limited proportion of patients. Thus, the combination of several distinct modalities may synergistically augment the effectiveness of immune therapy and future studies should explore this. Alternatively, this finding may arise from the presence of several different patient cohorts who respond differentially to a specific immune therapy. If so, it is necessary to detect the good-responder cohort by identifying a biomarker to predict the responsiveness to each immune therapeutic modality.

Collectively, immune therapy for HCC is still in its infancy. However, most HCC can develop repetitively from chronic inflammatory lesions and/or cirrhosis in non-cancerous liver portions, and recurrence has a great impact on the long-term prognosis of patients with HCC[1]. However, these lesions cannot be eliminated by other therapies at all, and only immune therapy can prevent these non-cancerous tissues from progressing into HCC. Thus, it is absolutely necessary to expand immune therapies for HCC to prevent HCC recurrence, and to eventually improve prognosis in patients with HCC.

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MINIREVIEWS

Endoscopic management of Crohn's strictures

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Abstract

Symptomatic intestinal strictures develop in more than one third of patients with Crohn's disease (CD) within 10 years of disease onset. Strictures can be inflammatory, fibrotic or mixed and result in a significant decline in quality of life, frequently requiring surgery for palliation of symptoms. Patients under the age of 40 with perianal disease are more likely to suffer from disabling ileocolonic disease thus may have a greater risk for fibrostenotic strictures. Treatment options for fibrostenotic strictures are limited to endoscopic and surgical therapy. Endoscopic balloon dilatation (EBD) appears to be a safe, less invasive and effective alternative modality to replace or defer surgery. Serious complications are rare and occur in less than 3% of procedures. For non-complex strictures without adjacent fistulizaation or perforation that are less than 5 cm in length, EBD should be considered as first-line therapy. The aim of this review is to present the current literature on the endoscopic management of small bowel and colonic strictures in CD, which includes balloon dilatation, adjuvant techniques of intralesional injection of steroids and anti-tumor necrosis factor, and metal stent insertion. Short and long-term outcomes, complications and safety of EBD will be discussed.

Key words: Endoscopy; Crohn's disease; Stricture; Stenosis; Inflammatory bowel disease; Endoscopic balloon dilation

Core tip: Endoscopic balloon dilation (EBD) for Crohn's disease-related fibrostenotic strictures has been recognized as a safe, and less invasive intervention with rare

complications that occur in less than 3% of procedures. EBD can replace or defer surgery and help avoid frequent intestinal resections, which result in short bowel syndrome and impair quality of life. For non-complex strictures without adjacent fistulization or perforation that are less than 5 cm in length, EBD should be considered as first-line therapy. In this review we discuss safety, short and long-term outcomes, as well as adjuvant techniques of intralesional injection of steroids, anti-tumor necrosis factor, and metal stent insertion.

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INTRODUCTION

Intestinal strictures are a common complication of Crohn's disease (CD) affecting one-third of the patient population within 10 years of disease onset. This number, however, is likely under-reported[1,2]. In general, CD strictures are classified into inflammatory, fibrotic or mixed, although all symptomatic inflammatory strictures likely have some component of fibrosis and vice-versa[2,3]. Risk factors and predictors of intestinal strictures to date are clinical, environmental, genetic or endoscopic parameters[4] (Table 1). Although no clinical factors exist which can accurately predict the stricturing phenotype of CD, there do exist factors which may predict the likelihood of small bowel disease and a disabling disease course thus indirectly may suggest an increased risk for the development of fibrostenotic disease. These factors include the presence of perianal disease, age of CD diagnosis less than 40 years old and the need for steroids during the first flare[4,5]. Patients frequently complain of progressive post-prandial abdominal pain, bloating, nausea, vomiting and weight loss. The diagnosis of intestinal strictures usually coincides with a spiraling decline in quality of life and results in surgery in 75% of patients at least once during their lifetime[1]. CD patients will frequently undergo multiple bowel resections over their lifetime that repeatedly exposes them to immediate and long-term post-operative complications such as anastomotic leaks with intra-abdominal sepsis, short bowel syndrome, and adhesions with recurrent bowel obstructions[2,6].

The pathogenesis of CD complications develops from chronic accumulation of inflammatory bowel damage variably leading to stricture, fistula and/or abscess formation[2]. Stricture development, although not fully understood, involves the progressive deposition of extracellular matrix protein (ECM) produced by myofibroblasts at variable sites of the bowel being injured by chronically uncontrolled relapsing and remitting transmural inflammation[7]. During chronic intestinal inflammation, the baseline release of profibrotic cytokines (*e.g.*, IL-4 and IL-13) increases over time further accelerating the process of excessive matrix deposition[7,8]. There may also exist a point where inflammation is no longer required to trigger fibrosis. As ECM is deposited during chronic inflammation, the bowel wall becomes stiffer. Bowel wall stiffness acts independently as a mesenchymal cell activator, resulting in ongoing myofibroblast stimulation, thus progressive fibrotic stenosis[9].

Treatment options for fibrostenotic strictures are limited to endoscopic and surgical therapy (*i.e.*, strictureplasty and small bowel resection)[10]. Fortunately, most *de novo* strictures form in the ileum and ileocolic regions, which are accessible by ileocolonoscopy or balloon-assisted enteroscopy[11]. Although pharmacotherapy may delay the time before operative management, it has not been shown to prevent it[12]. Approximately 80% of patients will have their first bowel resection 10 years following their diagnosis of CD[2]. To date no specific intestinal anti-fibrotic therapy exists, nor has any immunosuppressant or biologic therapy been shown to prevent stricture formation.

The following review presents the current data on the endoscopic management of small bowel and colonic strictures in CD. Short and long-term outcomes, complications and a description of the procedure will be discussed.

EFFICACY OF ENDOSCOPIC BALLOON DILATION

Endoscopic balloon dilation (EBD) is a minimally invasive bowel-length preserving mean of managing symptomatic CD patients with short fibrotic strictures (Figure 1). EBD has become an established modality of therapy and often plays an important role in delaying or acting as a bridge to surgery[10,13]. The most common location of the small bowel to undergo EBD using a colonoscope is the distal ileum or at the ileocolonic anastomosis of a patient following a small bowel resection[14]. Strictures located in the distal duodenum to proximal jejunum or distal jejunum to proximal ileum may be accessed with ante- or retrograde enteroscopy, respectively[15].

Short- and long-term efficacy has been inconsistently defined in studies[13]. In general, short-term efficacy has been described as the technical success of the procedure or the ability to traverse the dilated area freely with the endoscope immediately after dilatation[13,16]. Long-term efficacy, in most studies, has been described as the time elapsed until another intervention (either surgical or endoscopic) is required[2,13,16]. Despite the lack of a formal definition, excellent short- and moderate long-term efficacy of EBD for CD strictures has been documented in many studies[14,16,17]. Table 2 shows a summary of published studies on EBD using conventional colonoscopy in CD patients. In a systematic review and descriptive

Table 1 Risk factors and predictors of fibrostenosing Crohn's disease

Clinical[4]	Age at diagnosis < 40 yr
	Perianal disease at diagnosis
	Need for steroids during first flare
	Small bowel disease location
	Prior appendectomy
Environmental[4]	Smoking
Endoscopic[4]	Deep mucosal ulcerations
Genetic[4]	Nucleotide oligomerisation domain 2 (*NOD2*) variants
	Janus-associated kinase 2 (*JAK2*)
	Caspase-recruitment domain 15 (*CARD15*)
	NOD2/*CARD15* mutations on both chromosomes
	TNF superfamily 15 (*TNFSF15*) in Asians
	5T5T in the *MMP3* gene
	rs1363670
Serological[4]	Antimicrobial antibodies
	anti-*Saccharomyces cerevisiae* antibodies (ASCA) IgA in Asians

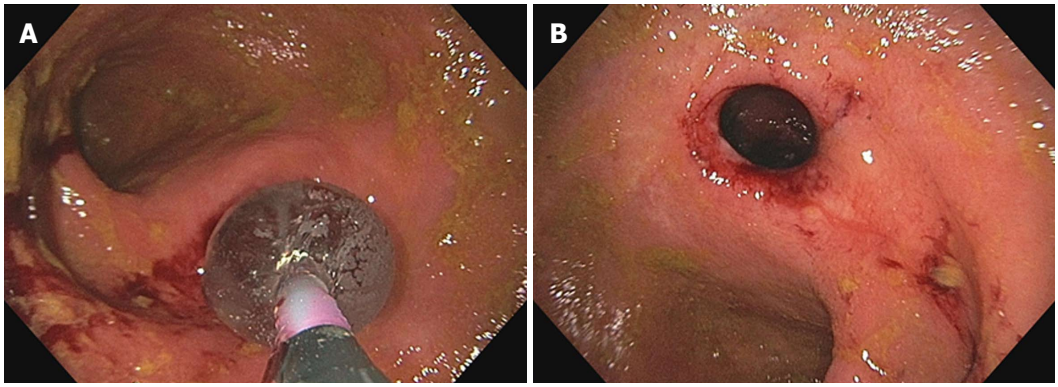

Figure 1 Endoscopic balloon dilatation of ileocolonic anastomosis (A) and endoscopic appearance post endoscopic dilatation (B).

pooled analysis of 12 studies conducted between 1991 to 2013 evaluating 1463 CD patients who underwent 3213 EBD procedures, the technical success rate was 89% with an associated relief of clinical symptoms in 81% of patients[14]. The majority of strictures were ileal (98.6%) at anastomotic sites (62%), which were 2 *cm* or less. However, the recurrence rate of strictures was high. At the 36.6 mo median follow-up, 47.5% of patients had symptomatic recurrence and 28.6% of all patients had required surgical intervention. This study concluded that the chance of requiring repeat EBD or surgical intervention at 2 years was 73.5% and 42.9%, respectively[14]. Another large recent systematic review with meta-analysis involving 1089 patients (2664 EBDs) across 25 studies revealed similar results[17]. The technical success rate was 92.3% with a reported symptomatic response rate of 70.4%. The proportion of patients requiring a repeat dilation after 1 and 2 years was 31.6% (160/506) and 25.9% (117/451), respectively. Most patients within 5 years required recurrent dilations (80%) and/or surgical interventions (75%)[17]. Of interest is the lower symptomatic success rate as compared to the technical success rate across studies. This likely occurred due to a lack of a standardized means of reporting technical and clinical efficacy and/or a superimposed process existing that contributed to the patient's symptoms (*e.g.*, ongoing inflammation, intestinal bacterial overgrowth, IBS, *etc.*)[10]. Despite this discrepancy, the short-term clinical success rate remains high.

In the setting of small bowel strictures not in reach of the enteroscope or colonoscope, the double balloon enteroscope can be used in an antegrade or retrograde fashion for diagnostic and/or therapeutic intervention[15]. Although there are only a few small studies which have evaluated its use in dilating small bowel CD strictures, the results were positive[18,19]. Nishida *et al*[20] performed a retrospective review on their center's experience with dilating small bowel strictures between 2006 to 2015. Overall, small bowel dilation using the double balloon enteroscope was found to be successful but there was a greater risk for requiring surgery in patients with multiple strictures as compared to those with a single stricture (adjusted hazard ratio, 14.94; 95%CI: 1.91-117.12; P = 0.010)[20]. As such, a single stricture but not necessarily multiple strictures may be a good indication for considering dilation using the double balloon enteroscope.

Table 2 Summary of published studies on endoscopic balloon for Crohn's disease strictures

Authors	Pubished year	No. of patients	Anastomotic strictures (%)	Maximum balloon caliber (mm)	Technical success (%)	Clinical efficacy (%)	Major complication (%)
Blomberg *et al*[52]	1991	27	100	25	100	67	0
Williams *et al*[53]	1991	7	71	20	71	71	0
Breysem *et al*[54]	1992	18	78	18	89	50	0
Cockuyt *et al*[55]	1995	55	67	20	85	62	8
Ramboer *et al*[56]	1995	13	69	18	100	100	0
Matsui *et al*[57]	2000	55	43	20	86	78	2
Dear *et al*[58]	2001	22	95	18	100	73	0
Brooker *et al*[59]	2003	14	79	20	100	79	0
Morini *et al*[60]	2003	43	67	18	79	42	0
Sabate *et al*[61]	2003	38	68	25	84	53	3
Thomas-Gibson *et al*[62]	2003	59	90	18	73	41	3
Singh *et al*[63]	2005	17	35	20	100	76	18
Aljouni *et al*[64]	2006	37	37	20	90	87	3
Ferlitsch *et al*[65]	2006	46	59	20	85	66	4
Nomura *et al*[66]	2006	16	35	20	94	65	6
Foster *et al*[67]	2008	24	41	20	92	NA	13
Hoffman *et al*[68]	2008	25	57	20	100	52	16
Stienecker *et al*[69]	2009	25	42	18	97	94	3
Mueller *et al*[70]	2010	55	23	18	95	76	2
Thienpont *et al*[71]	2010`	138	84	18	97	76	3
Scimeca *et al*[72]	2011	37	90	20	84	89	0
Gustavsson *et al*[51]	2012	178	80	25	89	64	11
Karstensen *et al*[73]	2012	23	24	15	83	74	1.9
De'Angelis *et al*[74]	2013	26	52	18	100	93	2
Endo *et al*[75]	2013	30	36	20	94	64	10
Honzawa *et al*[76]	2013	25	21	20	88	62	12
Nanda *et al*[77]	2013	31	100	18	100	45	0
Atreja *et al*[78]	2014	128	48	20	83	67	3
Bhalme *et al*[79]	2014	79	61	20	95	77	0
Hagel *et al*[80]	2014	77	57	20	55	65	10
Krauss *et al*[81]	2014	20	25	18	100	NA	14
Ding *et al*[82]	2016	54	100	20	89	82	2

Clinical efficacy was defined according to each study (*i.e.*, resolution of obstructive symptoms after dilation with the avoidance of surgery or additional intervention). Technical success was defined by successful passage of the endoscope or colonoscope immediately after dilation. Clinical efficacy was defined as the resolution of obstructive symptoms after dilation with the avoidance of surgery. Major complications (calculated per number of dilations) included were perforations, bleeding, intra-abdominal abscesses or fistulas. NA: Not available.

PREDICTORS OF SUCCESSFUL ENDOSCOPIC DILATATION

Factors that are predictive of a successful EBD include short straight strictures in-line with the bowel lumen distal to the duodenum, which are non-ulcerated in a location without any adjacent abscess and at least 5 cm from a fistula orifice[21,22]. Strictures located in the duodenum were found to have a 5 fold increased hazard for time to shorter surgery as compared to strictures located in the jejunum/ileum or colon (HR = 4.7, P = 0.038; HR = 5.6, P = 0.03; respectively)[23]. Additionally, a stricture length $\leqslant$ 5 cm was associated with a lower chance of requiring surgical intervention following EBD (HR = 2.5, 95%CI: 1.4-4.4; P = 0.002). For every 1 *cm* increase in stricture length, the risk for surgery increased by 8% (P = 0.005)[23]. In contrast to popular belief, anastomotic strictures have been associated with poorer short-term outcomes than *de novo* strictures[23,24]. This was highlighted in the aforementioned review by Bettenworth *et al*[14] which documented a lower technical success rate for post-surgical strictures as compared to native strictures (OR = 2.3, P < 0.001). Similarly, a recent study published by the Cleveland Clinic group after performing a retrospective review on 307 patients who had undergone either EBD or surgical resection for an ileocolonic anastomotic stricture had worse short-term outcomes (*i.e.*, technical success) but similar long-term outcomes as compared to the aforementioned studies evaluating EBD of *de novo* strictures[24]. Of the 176 patients who had undergone EBD, the technical success rate was 86% (range 71% to 100%) with a long-term clinical efficacy, defined as an avoidance of surgery, of 58% over a follow-up period of 33 mo[24]. The presence of active inflammation identified on endoscopy, elevated CRP, medical treatment after dilation, cigarette smoking and intralesional steroid injection have demonstrated conflicting results with respect to the need for surgery and successful EBD[2,17,23,24].

ENDOSCOPIC ADJUVANT TECHNIQUES

Intralesional injection of steroids has been demon-

Table 3 Practical considerations

Predictors favoring successful dilation[11,22-25]	Symptomatic predominantly fibrotic stricture
	Short (≤ 5 cm) stricture
	Single straight stricture
	Stricture distal to the duodenum
	Anastomotic stricture more favorable than de novo stricture
	First dilation
	Lack of a superimposed process contributing to symptoms (*e.g.*, SIBO or IBS)
Risk factors for complications[22-25]	Predominantly inflammatory stricture without medical optimization
	Stricture greater than 5 cm
	Multiple small bowel strictures
	Strictures caused by extrinsic compression (*e.g.*, adhesions)
	Fistulization within 5 cm of the area to be dilated
	Adjacent perforation or intra-abdominal collection
	Complete small bowel obstruction
	Tortuous or tethered small bowel or significant stricture angulation
	Duodenal stricture
[1]Short term outcome[15,18]	85%-95% (technical success), 70%-80% (clinical response)
[2]Long term outcome[15,18]	32% (year 1 post dilation), 80% (year 5 post dilation)
[3]Complication rate[25,45]	1%-4%

[1]Short term outcome refers to the time elapsed immediately after the dilation takes place; technical success refers to the ability to successfully complete the dilation; clinical response refers to the symptomatic improvement of the patient immediately following the dilation; [2]Long term outcome refers to the percentage of patients requiring a repeat intervention; [3]Complication rate encompasses only major complications requiring urgent intervention such as bleeding, perforation and infection.

strated to be effective for peptic, corrosive, anastomotic or post-radiotherapy fibrotic strictures[25]. However, strong evidence for the use of intralesional injection of steroids in CD is lacking[25-28]. Studies that have evaluated its use in CD have used the formulation triamcinolone due to its rapid onset of action and long-lasting duration of effectiveness of 3-4 wk[29]. Only two small randomized placebo controlled studies have been performed evaluating the use of intralesional steroids versus saline injection after failing medical therapy and EBD. The first study conducted in 2007, included 13 adult patients with short (≤ 5 cm) ileocolonic anastomotic strictures[30]. Five of the seven patients in the intervention group required re-dilation after the procedure and one patient had a complication versus one of six in the placebo group required re-dilation. There was no significant difference with respect to success of the procedure between groups[30]. This trial was stopped early due to the trend toward harm and remains the influential study behind the current American College of Gastroenterology and British Society of Gastroenterology position statements against the routine use of intralesional steroids[31,32]. The second study published in 2010 included 29 pediatric patients with short ileal or colonic strictures (12 anastomotic, 17 *de novo*)[33]. In contrast, this study did demonstrate a reduction in time to re-dilation and surgery in the intervention group. Within the sub-group of patients evaluated in a recent large systematic review evaluating the management of CD strictures, intralesional steroid injection did not improve outcomes[33]. Similarly, a review conducted in 2013 summarizing the findings from five retrospective case-series evaluating the use of intralesional steroids in CD patients concluded the data to be contradictory and limited[34].

Although controversial, intralesional injection of anti-tumor necrosis factor has been evaluated in patients with small bowel and colonic CD strictures with promising results, but concerns related to immunization may limit its potential as a therapeutic option[35,36]. One small case series evaluated the effect of a 90-120 mg intralesional injection of infliximab in three symptomatic patients with colonic CD strictures. All three patients had an improved endoscopic appearance of the stricture as well as relief of their obstructive symptoms for at least four months following the injection[35]. Similarly, another small case series evaluating intralesional injections of 40 mg of infliximab into small bowel CD strictures combined with EBD in six patients was associated with improved symptoms and a reduction in their modified simple endoscopic score for Crohn's disease (SES-CD)[37]. The results of a larger randomized controlled trial evaluating the efficacy of performing intralesional injections of adalimumab into intestinal CD strictures are awaited[38].

Endoscopic metal stent insertion has been attempted in few patients with CD strictures. Although the technical success rate has been reportedly high, major complications such as bowel perforation, stent migration and fistulization was reported in 67% of patients[39]. Additionally, in order to avoid stent impaction, most studies suggest removing the stent after one month[40-42]. One small prospective cohort study concluded the risk for complications was too high to suggest the use of endoscopic metal stents as a treatment option for CD strictures after evaluating the data from 11 patients at their center[40]. The use of biodegradable instead of metal stents has been evaluated recently in a case-series last year involving six patients with intestinal and colonic CD strictures. Although technical success was good,

premature stent failure occurred in all of the patients[43].

SAFETY OF ENDOSCOPIC BALLOON DILATION

Although EBD is a minimally invasive procedure, bowel perforation and severe bleeding has been reported in most large studies[17,23,24]. In the aforementioned review by Bettenworth *et al*[14], major complications requiring hospitalization occurred in 2.8% of patients. Similarly, another large systematic review evaluating 24 non-randomized studies including 1163 patients found the rate of iatrogenic perforation to be 3%[44]. The rate for major complications including infection and hemorrhage in this study was 4%[44]. In a study directly comparing EBD to surgical intervention for the management of intestinal CD strictures, perforation occurred in 1.1% of the patients in the EBD group whereas the post-operative complication rate (*e.g.*, intra-abdominal sepsis) was 8.8%[24]. Despite these significant complications, no deaths have been reported to date. Since benign or inflammatory intestinal strictures are indistinguishable from early adenocarcinoma on imaging, there exists a risk that malignancy may be missed when EBD is performed instead of surgical excision[3]. Population based studies have suggested a greater risk for small bowel malignancy in patients with longstanding CD. Several case reports exist documenting the development of small bowel malignancy following stricturoplasty and bypassed loops[45-51]. As such, biopsies of the stricture should occur prior to dilation[22]. There has been no evidence to suggest obtaining biopsies prior to EBD increases the risk for perforation.

CONCLUSION

EBD remains a safe and effective modality of treating CD strictures in appropriately selected patients. Although it may not be able to prevent operative management in all patients, it can significantly delay it. For an isolated intestinal fibrostenotic CD stricture less than or equal to 5 cm in length without adjacent fistulization or perforation, EBD should be considered as first-line therapy (Table 3).

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MINIREVIEWS

Anti-integrin therapy for inflammatory bowel disease

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Abstract

In inflammatory bowel disease (IBD), tumor necrosis factor plays an important role in mediating inflammation, but several other pathways are also involved in eliciting an inflammatory response. One such pathway is the invasion of the intestinal mucosa by leukocytes. Leukocytes within the systemic circulation move to sites of inflammation, and blocking this pathway could be an important treatment strategy for IBD. Anti-integrin therapy blocks the action of integrin on the surface of circulating immune cells and endothelial cell adhesion molecules, thereby inhibiting the interactions between leukocytes and intestinal blood vessels. Natalizumab, which acts on α4-integrin, was the first such drug to be approved for Crohn's disease, but its use is limited due to the risk of progressive multifocal leukoencephalopathy. Vedolizumab produces few systemic adverse effects because it acts on gut-trophic α4β7 integrin, and has been approved and is being used to treat IBD. Currently, several anti-integrin drugs, including etrolizumab, which acts on β7-integrin, and PF-00547569, which targets mucosal addressin cell adhesion molecule-1, are undergoing clinical trials and the results are being closely watched.

Key words: Integrin; Ulcerative colitis; Crohn's disease; Natalizumab; Abrilumab; Etrolizumab; PF-00547659; Inflammatory bowel disease; AJM300; Vedolizumab

Core tip: Anti-integrin therapies have attracted attention as new therapeutic agents in inflammatory bowel disease. They inhibit the extravasation of leukocytes by blocking the interaction between integrins on immune cells and endothelial cell adhesion molecules. The use of the first developed anti-integrin agent, natalizumab is now limited due to the risk of progressive multifocal leukoencephalopathy. However, vedolizumab which acts

selectively on the gut has shown few adverse events and is currently used in clinical practice. Newer anti-integrin drugs that act on different integrins-related targets, such as AJM300, abrilumab, etrolizumab, and PF-00547659 have also been developed and are in clinical trials.

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INTRODUCTION

Causes of inflammatory bowel disease (IBD) have not yet been clearly elucidated, but it is known that genetic susceptibility, altered gut microbiota, and environmental factors are all involved. It has also been reported that a combination of these factors causes an inappropriate immune response, resulting in impaired intestinal barrier function[1-3].

As continual research further reveals the immunopathogenesis of IBD, the treatment of IBD has shifted from conventional treatments, such as aminosalicylates, glucocorticoids, and immunomodulators (thiopurines and methotrexate), toward the biological drugs that target inflammation-related pathways[4]. Anti-tumor necrosis factor (TNF) agents were the first biologics used to treat IBD, and the objective of IBD treatment has shifted from controlling symptoms to changing the progression of disease and preserving the intestinal function. However, anti-TNF agents are not effective in all IBD patients, and a considerable number of patients experience relapse after stopping medication. The pathophysiology of IBD is very complex. This means that the most appropriate treatment method may vary for each patient, and therefore, constant efforts are being made to develop effective drugs[4]. In particular, new biologics that inhibit leukocyte trafficking to the site of inflammation have been developed and used. These drugs are called anti-integrin or anti-adhesion agents, or leukocyte-trafficking inhibitors because they block the actions of integrin, a cell surface protein expressed by circulating immune cells and endothelial cell adhesion molecules (CAMs), thereby selectively preventing the intestinal recruitment of lymphocytes to the site of inflammation[5]. Thus, unlike anti-TNF drugs, anti-integrin agents inhibit the interactions between leukocytes and the intestinal vasculature, and selectively prevent the influx of inflammatory cells, which mediate the inflammatory process in IBD, into intestinal lesions. In this report, we aim to discuss anti-integrin therapy, which is currently being highlighted as a new drug therapy for the treatment of IBD.

NEED FOR NEW DRUGS

A variety of inflammatory and anti-inflammatory cytokines define and regulate various aspects of the inflammatory response and play an important role in the pathogenesis of IBD including Crohn's disease (CD) and ulcerative colitis (UC), with the former mediated by type 1 T helper cells (TH1) and TH17, and the latter reportedly caused by an abnormal TH2 response. The immunopathogenesis of IBD is made more complex by imbalances in different T cell subsets, such as regulatory T cells, natural killer T cells, and TH9, as well as the interactions between these cell populations. Ultimately, the production of numerous cytokines is disturbed. These cytokines include the well-known TNF-α as well as IL-1β, IL-6, IL-8, IL-10, IL-12, IL-17, IL-23, and transforming growth factor-α[3,6].

The use of TNF antagonists showed that just blocking a single cytokine could be sufficient to induce significant clinical remission. Until recently, in moderate-to-severe active IBD patients, especially if initial treatment with systemic corticosteroids or immunomodulators failed, anti-TNF agents were the only remaining treatment option.

Inspired by the treatment outcomes of the first generation anti-TNF agent infliximab, next-generation TNF antagonists, such as adalimumab, golimumab, and certolizumab pegol, were introduced for the treatment of IBD, drastically changing this treatment field; however, even these drugs did not show an effect in all IBD patients. Specifically, although reports differ slightly, anti-TNF agents produce primary non-response (PNR) in approximately 10%-30% of patients[7]. Several factors have been suggested as causes of PNR. One known cause of PNR is that TNF is not a major factor in the development of inflammation in some patients, and therefore, there is an increased need for drugs with new mechanisms[8].

Although anti-TNF agents show an initial effect, secondary non-response or loss of response (LOR) is seen in 23%-46%[7,9]. LOR is known to occur due to pharmacokinetic issues or the production of antibodies against the drug; however, it can also be caused by a shift in the inflammatory response pathway from TNF signaling to non-TNF signaling. Moreover, due to their comprehensive immunosuppressive effects, the use of anti-TNF agents can cause severe adverse reactions, including tuberculosis (TB), hepatitis B, pneumonia, herpes zoster, and other infections, as well as skin cancer, malignant lymphoma, psoriasis, lupus-like syndrome, demyelinating disease, congestive heart failure, and hepatotoxicity.

Although anti-TNF therapy has reduced the rate of surgery in IBD patients, a considerable number of patients experience a relapse of inflammation after as they stop anti-TNF[10]. After stopping TNF antagonist, the 12-mo relapse rate is 40% for CD and 28% for UC[9,11]. Therefore, there is an urgent need for drugs with novel mechanisms that are more effective and safer than anti-TNF agents, or in particular, that can be used when anti-TNF therapy is ineffective or causes an adverse reaction.

Since biological drugs have a high molecular weight, they are inevitably delivered by injection, and their

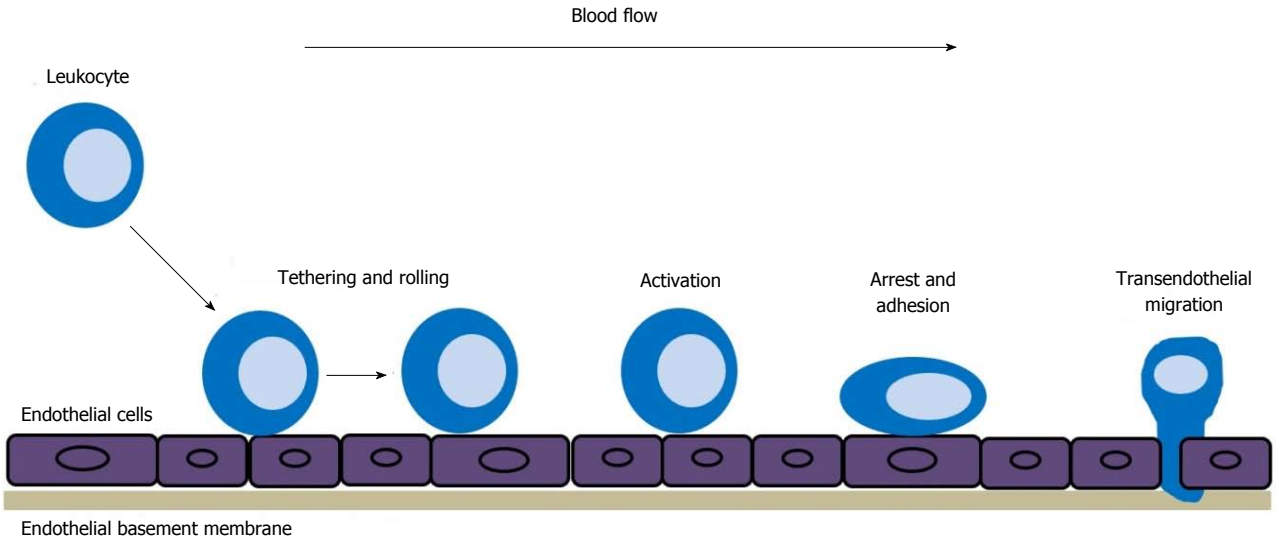

Figure 1 Process of leukocyte migration through the endothelium. Leukocytes moving in the blood begin to tether and roll at a specific site of the vessel wall, undergo activation, arrest and adhesion to the vascular endothelial cells, eventually migrate between the endothelial cells.

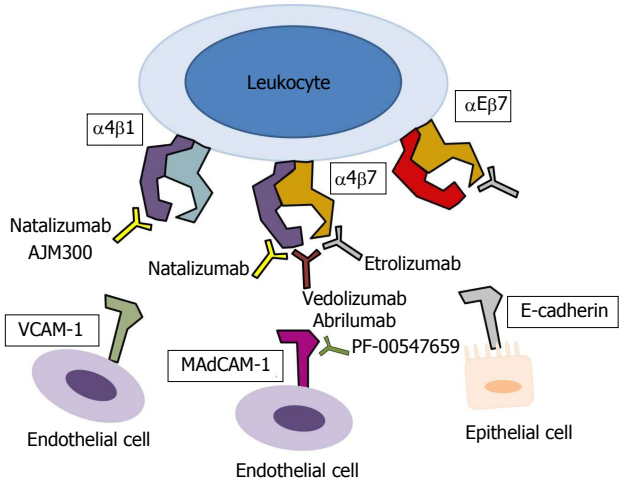

Figure 2 Therapeutic targets of anti-integrin agents[14]. VCAM-1: Vascular cellular adhesion molecule-1; MadCAM-1: Mucosal addressin cellular adhesion molecule-1.

immunogenicity leads to infusion reactions or LOR associated with the antidrug antibody. Therefore, one aspect of new drug development is to focus on small molecules of less than 1 kDa that could be taken orally, thereby increasing compliance, relatively inexpensive, and have almost no immunogenicity, allowing them to be taken safely on a long-term basis.

IMMUNE CELL TRAFFICKING

Innate and adaptive immune responses depend on the trafficking of immune cells to the organ targeted by the disease. During an inflammatory response, circulating leukocytes migrate to the target tissues through a homing process that takes place in several stages. Migrating leukocytes in the bloodstream begin tethering (capture) and rolling to a specific place, through the activation process, arrest and adhere to vascular endothelial cells, and finally undergo transendothelial migration (Figure 1). This process of leukocytes migration is mediated by interactions between leukocytes and adhesion molecules expressed by endothelial cells, which enables circulating leukocytes to migrate to the target tissues[12].

Leukocytes also express CAMs on the surface, called integrins which allow them to interact with the vascular endothelial cells or other cells. Integrin is a heterodimeric receptor formed from α and β subunits and is divided into several groups depending on the structure of the α and β subunit, and different populations of leukocytes express different integrins. These integrins include α4β1 (found on most leukocytes), α4β7 [found specifically on lymphocytes in the gastrointestinal (GI) tract], and αEβ7 (found on intraepithelial T cells, dendritic cells, mast cells or regulatory T cells)[13]. Integrins react with CAMs in the immunoglobulin (Ig) superfamily expressed by other cells to induce cell adhesion; α4β1, α4β7, and αEβ7 integrins bind to vascular cell adhesion molecule-1 (VCAM-1) on vascular endothelial cells, mucosal addressin cell adhesion molecule-1 (MAdCAM-1) on intestinal endothelial cells, and E-cadherin on mucosal epithelial cells (Figure 2)[14].

The migration of leukocytes to the intestinal mucosa and the recruitment of immune cells to the site of inflammation due to increased expression of CAMs are essential to the development and maintenance of intestinal inflammation. Therefore, leukocyte trafficking to the gut is central to the immunopathogenesis of IBD, and its inhibition is recognized as an important goal in the development of anti-IBD drugs[5].

ANTI-INTEGRIN THERAPIES

Anti-integrin therapies block the action of integrins, expressed by circulating immune cells, on endothelial CAMs, thereby decreasing the trafficking of immune

Table 1 Anti-integrin therapies for inflammatory bowel disease

Drug	Formula	Target	Route	Clinical studies	Summary
Natalizumab	Humanized IgG_4 mAb	α4-integrin	*i.v.*	ENCORE	Induction and maintenance in CD
AJM300	Small molecule	α4-integrin	Oral	Phase Ⅱa	Induction in UC
Vedolizumab	Humanized IgG_1 mAb	α4β7-integrin	*i.v.*	GEMINI 1	Induction and maintenance in UC
				GEMINI 2	Induction and maintenance in CD
				GEMINI 3	Induction in CD
Abrilumab (AMG 181/MEDI 7183)	Fully human IgG_2 mAb	α4β7-integrin	*s.c.*	Phase Ⅱb	Induction in UC
				Phase Ⅱb	Induction in CD
Etrolizumab	Humanized IgG_1 mAb	β7-integrin	*i.v./s.c.*	EUCALYPTUS	Induction in UC
				BERGAMOT	Induction in CD
				HICKORY	Induction in CD
PF-00547659 (SHP647)	Fully human $IgG_{2\kappa}$ mAb	MAdCAM-1	*i.v./s.c.*	TURANDOT	Induction in UC
				OPERA	Induction in CD

IgG: Immunoglobulin; mAb: Monoclonal antibody; *i.v.*: Intravenous; CD: Crohn's disease; UC: Ulcerative colitis; *s.c.*: Subcutaneous; MAdCAM: Mucosal addressin cell adhesion molecule.

cells to the endothelium and suppressing the recruitment of inflammatory cells such as lymphocytes to intestinal lesions. Table 1 shows the anti-integrin agents currently approved and in use or in clinical trials.

Natalizumab

Natalizumab is a chimeric recombinant human IgG4 antibody that targets the α4 subunit in α4β7 and α4β1 integrins on leukocytes. α4β1 integrin interacts with VCAM-1. Natalizumab was first approved by the U.S. Food and Drug Administration (FDA) as a treatment for multiple sclerosis, which is an autoimmune disease of the central nervous system (CNS), and clinical trials were conducted to test its efficacy against CD.

In the phase Ⅲ Efficacy of Natalizumab in Crohn's disease Response and Remission (ENCORE) trial, 509 patients with moderate-to-severe activity and elevated C-reactive protein (CRP) (> 0.287 mg/dL) were allocated, in a 1:1 ratio, into groups receiving either 300 mg of natalizumab or placebo by intravenous injection at weeks 0, 4, and 8. The primary end point, which was the percentage of patients showing a clinical response [defined as a decrease of at least 70 points in CD activity index (CDAI) score] at week 8 and sustaining this response until week 12, was higher in the natalizumab group, at 48%, than in the placebo group, at 32% ($P < 0.001$)[15]. The percentage of patients showing sustained clinical remission (defined as a CDAI score under 150 points) at both week 8 and week 12 was also higher in the natalizumab group, at 26%, than in the placebo group, at 16% ($P = 0.002$). However, natalizumab prevents α4β1 integrin on leukocytes from binding VCAM-1 on vascular endothelial cells in the CNS as well as in the intestines; it has been reported that by reducing T cell trafficking to the brain, natalizumab can affect cerebral antiviral immunity, and in some cases, can cause a fatal brain infection called progressive multifocal leukoencephalopathy (PML) due to the reactivation of the John Cunningham (JC) virus[16,17]. Based on clinical trial data, the risk of PML after a mean of 17.9 mo of natalizumab treatment is approximately 1 case per 1000 patients[18]. The use of immunomodulators before natalizumab administration, a positive test for anti-JC virus antibody, and longer duration of natalizumab treatment are risk factors for PML[19]. Thus, natalizumab has been approved by the United States. FDA only in moderate-to-severe CD patients who did not respond to or were intolerant of conventional treatment or TNF inhibitor therapy; it has not been approved for use in Europe.

AJM300

Despite safety issues for natalizumab, the oral α4 integrin antagonist AJM300 was developed and evaluated for use in UC. A phase Ⅱa clinical trial was conducted in Japan on 102 patients with moderately active UC, who were intolerant or showed an inappropriate response to mesalamine or corticosteroids; when AJM300 960 mg or placebo was administered 3 times per day, the primary end point, which was the rate of clinical response (defined as a decrease of at least 3 points, and at least 30% compared to baseline, in the complete Mayo score, as well as a decrease of at least 1 point for the rectal bleeding or an absolute rectal bleeding subscore of 1 point or less) at week 8, was significantly higher in the AJM300 group, at 62.7%, than in the placebo group, at 25.5% ($P = 0.0002$)[20]. Meanwhile, the clinical remission (defined as a complete Mayo score of 0-2 points and no subscore higher than 1 point) rate was 23.5% in the AJM300 group and 3.9% in the placebo group ($P = 0.0099$), and the mucosal healing rate was 58.8% in the AJM300 group and 29.4% in the placebo group ($P = 0.0014$), both of which were significantly different. In this clinical study, serious adverse events did not occur, and adverse events were mild and self-limiting. However, considering that AJM300 shares the mechanism of natalizumab, and the number of subjects in this trial was small, and the study period was short, there are concerns about its practicality as a therapeutic drug. Nevertheless, the duration of effect for AJM300 is very short compared to that of natalizumab, and since it is an oral formulation, there is some expectation that it may cause fewer systemic adverse events.

Vedolizumab

Vedolizumab (VDZ; MLN0002) is a humanized monoclonal IgG1 antibody against α4β7-integrin that inhibits the adhesion of leukocytes to the endothelium by blocking the interaction between α4β7-integrin and MAdCAM-1 expressed on blood vessels and lymph nodes associated with the GI tract. The main difference between natalizumab and VDZ is that natalizumab inhibits leukocyte trafficking in multiple organs, including the brain, whereas VDZ acts specifically only on gut-trophic α4β7 heterodimers, and therefore, inhibits lymphocyte trafficking selectively in the intestine. Although MAdCAM-1 exists rarely at the blood-brain barrier, VDZ is known to have no effect on CNS immunity[21]. In a study in support of this idea, healthy volunteers were injected VDZ and when the cerebrospinal fluid (CSF) was tested 5 wk later, no change was observed in CSF lymphocyte counts or CD4:CD8 ratio following VDZ administration[22]. In another randomized controlled trial comparing VDZ with a placebo, the serum antibody response to a parenteral hepatitis B vaccine did not differ between the 2 groups, but the response to an oral cholera vaccine showed less antibody formation in the VDZ group compared to the placebo group, demonstrating that while VDZ has no effect on systemic immunity, it decreases immune surveillance in the GI tract[23].

The phase Ⅲ GEMINI 1 trial, consisting of 2 cohorts, analyzed the efficacy of VDZ in 895 moderate-to-severe UC patients who had previously received steroid, immunomodulator, or anti-TNF therapy[24]. The 374 patients in cohort 1 were randomly allocated in a ratio of 3:2, with each group receiving 2 intravenous injections of VDZ 300 mg or placebo at week 0 and 2, and evaluated at week 6. The primary endpoint in the induction phase, which was the clinical response rate at week 6, was significantly higher in the VDZ group, at 47.1%, than in the placebo group, at 25.5% ($P < 0.001$). The clinical response rate at week 6 was also significantly higher in the VDZ group than in the placebo group among patients who had previously experienced treatment failure with anti-TNF agents (39.0% *vs* 20.6%, $P = 0.01$) or steroids (59.5% *vs* 20.0%, $P < 0.001$). Moreover, the clinical remission rate at week 6 was 16.9% in the VDZ group and 5.4% in the placebo group ($P = 0.001$), whereas the mucosal healing rate at week 6 was 40.9% in the VDZ group and 24.8% in the placebo group ($P = 0.001$), and these differences were statistically significant. To meet the required sample size for the maintenance phase, an additional 521 patients (cohort 2) were recruited for an open-label trial, and administered VDZ by the same method. In the maintenance phase, the 373 patients who achieved a clinical response with VDZ at week 6 were randomized in a 1:1:1 ratio, with each group receiving either a placebo, or VDZ 300 mg every four weeks, or every eight weeks. The trial lasted for a total of 52 wk. The primary endpoint in the maintenance phase, which was the clinical remission rate at week 52, was 15.9% in the placebo group, 41.8% in the VDZ every eight weeks group, and 44.8% in the VDZ every four weeks group, which showed that the effect was 2-fold higher in the VDZ groups than in the placebo group ($P < 0.001$). The durable clinical response (response at both week 6 and 52) was 23.8% in the placebo group, 56.6% in the VDZ every eight weeks group, and 52.0% in the VDZ every four weeks group, which was significantly different ($P < 0.001$). Similarly, mucosal healing at week 52 was 19.8% in the placebo group, 51.6% in the VDZ every eight weeks group, and 56.0% in the VDZ every four weeks group, which was also significantly different ($P < 0.001$). There was no significant difference in the efficacy of VDZ between the four-week and eight-week interval groups. Among patients who had experienced failure with anti-TNF therapy, the clinical remission rate was much lower in the placebo group, at 5.3%, than in the VDZ every eight weeks group, at 37.2%, and the VDZ every four weeks group, at 35.0% ($P < 0.001$). Therefore, VDZ demonstrated an effect against moderate-to-severe UC at week 6 and at week 52, irrespective of previous anti-TNF therapy. In the post-hoc analysis for the GEMINI Ⅰ trial, patients were divided into those who were naïve to TNF antagonist (464 patients) and failed to TNF antagonist (367 patients)[25]. The treatment effect measured by the clinical response at week 6 was stronger in patients who were naïve to anti-TNF therapy [absolute difference (AD) between VDZ and placebo 26.4%] than in those who failed to anti-TNF therapy (AD 18.1%). In the maintenance phase, the ADs in week 52 clinical remission rates were 28.0% in patients who were naïve to anti-TNF therapy and 29.5% in patients who failed to anti-TNF therapy, respectively. Even among patients who had previously experienced failure with anti-TNF therapy, those who experienced LOR showed a lesser effect of VDZ than those who experienced PNR or intolerance.

The GEMINI 2 trial, consisting of 2 cohorts, analyzed the efficacy of VDZ in active CD patients[26]. The 368 patients in cohort 1 were randomly allocated in a 3:2 ratio, with each group receiving intravenous VDZ 300 mg or placebo at weeks 0 and 2, and evaluated at week 6. The primary endpoint, which was the clinical remission rate at week 6, was significantly higher in the VDZ group, at 14.5%, than in the placebo group, at 6.8% ($P = 0.02$). The other primary endpoint, the CDAI-100 response rate (defined as a decrease of at least 100 points in the CDAI score relative to baseline), was higher in the VDZ group, at 31.4%, than in the placebo group, at 25.7%; however, this difference was not statistically significant ($P = 0.23$). To meet the required sample size for the maintenance phase, an additional 747 patients (cohort 2) were recruited for an open-label trial, and administered VDZ by the same method. In the maintenance phase, 461 patients who had shown a clinical response to VDZ at week 6 which administered either placebo, or VDZ 300 mg every four weeks or every eight weeks. The primary endpoint in

the maintenance phase, which was the clinical remission rate at week 52, was 21.6% in the placebo group, 39.0% in the VDZ every eight weeks group, and 36.4% in the VDZ every four weeks group, indicating that both the VDZ every eight weeks ($P < 0.001$) and VDZ every four weeks ($P = 0.004$) groups showed significantly higher clinical remission rates than the placebo group. Similarly, the CDAI-100 response rate at week 52 was 30.1% in the placebo group, 43.5% in the VDZ every eight weeks group, and 45.5% in the VDZ every four weeks group, indicating that the response rate was significantly higher in the VDZ every eight weeks group ($P = 0.01$) and the VDZ every four weeks group ($P = 0.005$) than in the placebo group. Among patients who had previously experienced failure with anti-TNF therapy, the remission rates at week 52 were 28.0%, 27.3%, and 12.8% for the VDZ every eight weeks, VDZ every four weeks, and placebo groups, respectively. This was significantly higher in the VDZ every eight weeks group ($P = 0.01$) and the VDZ every four weeks group ($P = 0.02$) than in the placebo group.

The GEMINI 3 trial was a phase Ⅲ randomized controlled trial examining the efficacy and safety of VDZ in 416 moderate-to-severe CD patients[27]. Most of the participants (315 patients) had previously experienced failure with anti-TNF therapy (PNR, LOR, or intolerance). After the injection of VDZ 300 mg at weeks 0, 2, and 6, unlike the GEMINI Ⅰ and Ⅱ trials, the effects of VDZ were evaluated at week 10 as well as week 6. Among anti-TNF-naïve patients, the clinical remission rate at week 6 was 12.0% in the placebo group and 31.4% in the VDZ group, which was significantly different ($P = 0.012$). However, among patients with previous anti-TNF therapy failure, the clinical remission rate at week 6 was 12.1% in the placebo group and 15.2% in the VDZ group, which was not a statistically significant difference ($P = 0.433$), whereas the clinical remission rate at week 10 was significantly higher in the VDZ group, at 26.6%, than in the placebo group, at 12.1% ($P = 0.001$). Meanwhile, in patients with previous anti-TNF therapy failure, the CDAI-100 response rates at weeks 6 and 10 were 22.3% and 24.8%, respectively, in the placebo group, but were significantly higher in the VDZ group, at 39.2% and 46.8% ($P = 0.001$ and $P < 0.001$, respectively). These results show that patients who experience anti-TNF therapy failure take longer to show an effect from VDZ than anti-TNF-naïve patients. Notably, among the subjects in this trial, patients who had experienced anti-TNF therapy failure had a longer disease duration and more structural damage than anti-TNF-naïve patients, which could have affected the clinical effects of VDZ. In the post-hoc analyses for the GEMINI 2 and 3 trials, for patients in the VDZ group, the clinical remission rate at week 52 was 48.9% in patients who were naïve to anti-TNF therapy and 27.7% in patients who had experienced anti-TNF therapy failure, whereas the remission rates in the placebo group were 26.8% and 12.8%, respectively[28]. This shows that the clinical remission rates are higher in the VDZ group than in the placebo group, and that this effect is larger when patients have not previously been exposed to anti-TNF therapy.

Vedolizumab was approved by the FDA and the European Medicines Agency, for the treatment of moderate to severe ulcerative colitis and CD adult patients which are not responding to one or more conventional treatment such as steroids, immunosuppressive agents, or TNF antagonists. The results of the VDZ clinical trials showed different treatment effects in UC and CD. There are several theories to explain why the clinical effect of inhibiting leukocyte trafficking in CD appeared later than that in UC. CD can show systemic manifestations and affect the whole GI tract from the oral cavity to the anus, showing inflammation in all layers of the intestine; conversely, UC is limited to the colonic mucosa, which could explain the discrepancy in the treatment response. Recently, a study on IBD patients and a humanized mouse model found that VDZ treatment in CD reduced the expression of $\alpha4\beta1$ in the peripheral blood and increased the expression of $\alpha4\beta1$ in the intestine, suggesting that in CD, the VDZ-mediated inhibition of $\alpha4\beta7$ could have been circumvented by homing to the ileum *via* $\alpha4\beta1$ on effector T cells[29]. Thus, further in-depth research is required to better understand the pharmacokinetics and pharmacodynamics of VDZ in CD.

The GEMINI long-term safety (LTS) study examined the long-term safety and efficacy of VDZ[30,31]. Among patients in the phase Ⅱ trial C13004, the GEMINI 1 trial, and VDZ-naïve UC patients who showed a response to VDZ at week 6 were switched to an open-label study and administered VDZ 300 mg continually at four-week intervals for 152 wk[30]. In an interim report on the efficacy of VDZ, the remission rates after 104 and 152 wk were 88% (120/136) and 96% (70/73), respectively, demonstrating a high maintenance of remission. Among patients who dropped out of the VDZ maintenance treatment at eight-week intervals before 52 wk in GEMINI Ⅰ trial ($n = 32$), increased dosing frequency to every four weeks in GEMINI LTS improved clinical responses and remission rates from 19% and 6% to 41% and 28%, after 52 wk of GEMINI LTS, respectively. Similarly, among CD patients who had participated in the C13004, GEMINI 2, or GEMINI 3 trial, or were VDZ-naïve, those who showed a response to VDZ at week 6, when switched to an open-label study and monitored for 152 wk while receiving VDZ every four weeks, showed remission rates after 104 and 152 wk of 83% (100/120) and 89% (62/70), respectively[31]. Among patients who dropped out of the VDZ maintenance treatment at eight-week intervals before 52 wk in GEMINI 2 trial ($n = 57$), increased dosing frequency to every four weeks in GEMINI LTS improved clinical responses and remission rates from 39% and 4% to 47% and 32%, after 52 wk of GEMINI LTS, respectively. Therefore, for patients who show a response to VDZ every eight weeks in the induction phase, but show LOR in the maintenance phase, increasing the dosing frequency to every four weeks could produce a response again.

To examine mucosal and histological healing when VDZ was administered, prospective surveillance colonoscopy was performed in patients registered for the GEMINI LTS trial[32]. The follow-up period was over 1 year (1.1-6.1 years, median 3.2 years), the rate of mucosal healing with a Mayo score of 1 or less was 50% (17/34) for UC and 29% (7/24) for ulcer-free mucosal healing in CD patients. Histological healing with mucosal healing in UC and CD patients was 32% (11/34) and 21% (5/24), respectively.

The VERSIFY study was examined endoscopic mucosal healing at week 26 after VDZ treatment in 101 moderate-to-severe CD patients who had previously experienced failure with corticosteroids, immunomodulators, and/or anti-TNF agents. The endoscopic remission [simple endoscopic score for CD (SES-CD) $\leqslant$ 4] rate was 12% overall, 20% for patients who were naïve to anti-TNF therapy (n = 46), and 6% for patients who had previously experienced anti-TNF therapy failure (n = 55)[33]. The endoscopic response (SES-CD decrease of at least 50%) and complete endoscopic healing (no ulcerations) rates were, respectively, 25% and 15% overall, 28% and 24% for patients who were naïve to anti-TNF therapy, and 22% and 7% for patients who had failed at anti-TNF therapy. Thus, VDZ is effective at inducing endoscopic remission and healing in refractory CD patients, and the rates of endoscopic remission and healing are higher in anti-TNF-naïve patients than in those who have experienced anti-TNF therapy failure.

The US VICTORY Consortium provides data relating to VDZ from real-world experience; among 212 moderate-to-severe CD patients, 90% had exposed to anti-TNF therapy, and the median follow-up duration was 39 wk[34]. In responders, the median time to respond to VDZ was 19 wk. After 6, 12, and 18 mo of VDZ therapy, patients showed clinical remission rates of 18%, 35%, and 54%, respectively, and after 6 and 12 mo of treatment, showed cumulative mucosal healing rates of 20% and 63%, respectively, and cumulative deep remission (clinical remission and mucosal healing) rates of 14% and 26%, respectively. Higher disease activity, active perianal disease, smoking history, and prior TNF antagonist exposure were all factors that decreased the effectiveness of VDZ.

In a German cohort study including 115 active UC patients and 97 active CD patients, only 24.3% of UC patients and 5.2% of CD patients were naïve to TNF antagonist[35]. When these patients were treated with VDZ and monitored for 14 wk, at week 14, 23.5% of UC patients and 23.7% of CD patients achieved clinical remission, 57.4% of UC patients and 60.8% of CD patients showed a clinical response, and steroid-free remission was observed in 19.1% of UC patients and 19.6% of CD patients. Serum CRP and calprotectin levels were measured at weeks 0, 6, and 14; patients are showed decreased CRP levels, but this was not statistically significant, whereas calprotectin levels decreased significantly.

In the GETAID Cohort Data from France, the effects of VDZ treatment were analyzed in 121 UC patients and 173 CD patients who had failed with anti-TNF therapy. At week 6, the clinical remission rates were 32% and 31%, the steroid-free clinical remission rates were 21% and 19%, and the clinical response rates were 41% and 57% in the UC and CD patients, respectively[36]. At week 14, the clinical remission rates for UC and CD patients were respectively 39% and 36%, the steroid-free remission rates were 36% and 31%, and the clinical response rates were 57% and 64%, demonstrating that VDZ is effective for both UC and CD. The fact that a superior treatment response was observed at week 14 compared to week 6 re-confirms that it takes time for the effects of treatment to become apparent. When patients were monitored for 1 year, steroid-free remission at week 22 was 40% for UC patients and 34% for CD patients, indicating that remission rates gradually increased for both diseases, and that UC patients achieved steroid-free remission sooner than CD patients.

In summary, real-world data for VDZ treatment were similar to results of randomized controlled studies. In particular, it takes considerable time before the maximal effects of VDZ therapy can be observed, and corticosteroid treatment may be required during this period. The results of a network meta-analysis show that VDZ is more effective overall than anti-TNF therapy in the maintenance phase[37]. Thus, the effect of VDZ, once it becomes apparent, is maintained more strongly, and this sustained effect is considered its greatest advantage. In addition, for patients showing PNR, LOR, or intolerance to anti-TNF therapy, it is worth considering VDZ as a secondary treatment (Table 2).

Because VDZ acts selectively on the intestine, it causes relatively little systemic immunosuppression, and this is expected to result in fewer adverse events. In the GEMINI 1 and 2 trials, the most commonly reported adverse reactions to VDZ (incidence $\geqslant$ 5%) were nausea, nasopharyngitis, upper respiratory tract infection, arthralgia, fever, fatigue, headache, and cough[24,26]. In safety data from the 6 VDZ clinical trials (placebo-controlled trials C13002, GEMINI 1, 2, and 3, and open-label trials C13004 and GEMINI LTS), VDZ showed no significant difference from the placebo in overall adverse reactions[38]. In particular, the exposure-adjusted incidence rates of infections and serious infections, which is a problem in anti-TNF therapy, were 63.5/100 person-years (PYs) and 4.3/100 PYs in patients receiving VDZ, respectively, and 82.9/100 PYs and 3.8/100 PYs in the placebo group, respectively. However, the rates of gastroenteritis and *Clostridium difficile* infection were low but higher in VDZ-treated patients (4.0/100 PYs and 0.4/100 PYs, respectively) than those in the placebo group (1.4/100 PYs and 0.0/100 PYs, respectively), and further studies will be required to determine whether these results are due to gut-selective immune suppression by VDZ. In safety data from the 6 VDZ clinical trials, 18 patients developed malignancy, including GI cancer (6 patients), skin cancer (5 patients), lung cancer (2 patients), genitourinary

Table 2 Comparison of properties of anti-tumor necrosis factor and gut-specific anti-integrin therapy

	Anti-TNF therapy	Gut-specific anti-integrin therapy
Mechanism of action	TNF-α inhibitor	α4β7-integrin inhibitor
Available agents	Infliximab (UC, CD) Adalimumab (UC, CD) Certolizumab pegol (CD) Golimumab (UC)	Vedolizumab (UC, CD)
Therapeutic efficacy	Frequent loss of response during maintenance therapy	Modest effect on induction therapy for CD
Side effects	Infections, reactivation of latent tuberculosis, potential risk of lymphoma	Nasopharyngitis, arthralgia, headache, nausea
Immunogenicity	Measure the ADA if available Add immunomodulator (infliximab)	No significant immunogenicity

TNF: Tumor necrosis factor; UC: Ulcerative colitis; CD: Crohn's disease; ADA: Antidrug antibodies.

cancer (2 patients), breast cancer (2 patients), and B cell lymphoma (1 patient). Colon cancer (0.1/100 PYs) was the most common type of GI cancer, but its incidence was lower than that observed in IBD patients in the HealthCore Integrated Research Database (2.1/1000 PYs; 95%CI: 1.3-3.2)[38,39]. Infusion-related reactions were reported with a low incidence of less than 5% in patients who received VDZ[38]. VDZ does not affect α4β1-related nervous system leukocyte trafficking, and no cases of PML were observed in the clinical trials. Therefore, VDZ can be considered as a primary biological drug in elderly patients with a high risk of opportunistic infections or cancer and in young male patients at risk of hepatosplenic T cell lymphoma. Especially in countries with a high prevalence of TB, such as Korea, China, and India, the risk of TB needs to be considered when selecting a therapeutic drug. VDZ is expected to be a very low-risk drug in this regard, with only 4 TB cases out of approximately 3000 patients who received VDZ (0.1%). Another advantage of VDZ is that it can be used even in the presence of comorbidities that contraindicate anti-TNF therapy, such as demyelinating disease, congestive heart failure, and lymphoma.

Nevertheless, due to the gut selectivity of VDZ, it may not be expected to be effective in patients with extraintestinal symptoms. Recently, a case of CD involving the pleura and lungs after 3 doses of VDZ has been reported[40]. After isolating peripheral blood mononuclear cells from the patient, flow cytometry revealed an upregulation of β1 integrin, which is required for homing of lymphocytes to the lungs, and the condition of the patient improved after prednisolone treatment. This shows that the shift in integrin expression triggered by VDZ can cause immune cells to migrate to organs other than the gut, thereby increasing the risk of extraintestinal autoimmune manifestations in CD.

Anti-VDZ antibodies (AVAs) were detected in 56 out of 1434 patients (4%) who were treated with VDZ up to week 52 in the GEMINI 1 and 2 trials, but of these, only 9 patients (0.6%) continued to show AVA positivity, and 33 patients (2.6%) developed neutralizing antibodies[38]. In the GEMINI LTS trial, the immunogenicity rate did not increase over time. When VDZ was administered in combination with immunosuppressants at baseline, the AVA positivity rate was 3%, which was 1% lower than the AVA positivity rate of 4%. However, these measurements were taken when the patients had a high serum drug concentration, which could have interfered with the assay. Therefore, VDZ seems to have low immunogenicity and could be used without immunosuppressants; however, further research is required.

VDZ may be expected to have a positive effect on fistula closure rate in CD. The phase IV ENTERPRISE trial (NCT02630966), which is currently underway, focuses on fistula healing at week 30 after 22 wk of VDZ medication in patients with fistulizing CD.

Research on combination therapy has so far been limited to case reports. One report found that VDZ + etanercept, the soluble TNF receptor, combination therapy is effective at controlling severe pouchitis and spondylarthritis that developed in a patient with UC; one UC patient who showed no response to treatment with methotrexate, adalimumab, infliximab, azathioprine, cyclosporine A, or golimumab showed clinical remission and mucosal healing when treated with a combination of VDZ + certolizumab pegol and monitored for 21 mo[41,42]. These reports indicate that combination therapy using VDZ and an anti-TNF agent can provide additional clinical benefits, and an open-label study is currently underway to examine the effects of three-drug combination therapy using VDZ, adalimumab, and methotrexate in high-risk CD patients (NCT02764762).

Recently, a study was published on biomarkers that can predict response to VDZ[43]. Using VDZ labeled with fluorescein isothiocyanate, α4β7-expressing cells were detected by confocal laser endomicroscopy; clinical response and endoscopic remission to VDZ were observed in patients who showed pericryptal α4β7+ cells in the mucosa, whereas patients without α4β7+ cells did not respond to VDZ.

Abrilumab

Abrilumab (AMG 181/MEDI 7183) is a fully human monoclonal IgG2 antibody against α4β7 integrin that has recently been used in several clinical trials.

In a phase Ⅱb study to evaluate the efficacy and safety of abrilumab in 354 moderate-to-severe UC patients who showed an inappropriate response or LOR to anti-TNFs, immunomodulators, or corticosteroid

therapy, patients were divided into a placebo group, groups receiving subcutaneous abrilumab 7, 21, or 70 mg at weeks 0, 2, and 4, followed by its administration once every four weeks, and a group receiving a single subcutaneous 210 mg dose of abrilumab[44]. The primary endpoint, which was remission rate at week 8, was 1.6%, 2.9%, 13.5%, and 13.4% in the abrilumab 7 mg, 21 mg, 70 mg, and 210 mg groups, respectively, and was 4.4% in the placebo group; the abrilumab 70 mg group (P = 0.021) and 210 mg group (P = 0.030) both showed a significantly higher remission rate than the placebo group. Abrilumab increased α4β7-high central memory CD4+ T cell counts in the peripheral blood, and high trough abrilumab concentrations were associated with increased remission rate. No PML or severe adverse events were observed in the abrilumab groups through week 24 and no patients developed neutralizing antibodies to abrilumab. Thus, abrilumab showed advantageous pharmacokinetics, pharmacodynamics, very low immunogenicity, and an acceptable safety profile; further results are expected in the future.

A phase Ⅱb trial was conducted to evaluate the efficacy and safety of abrilumab in 249 patients with moderate-to-severe CD who showed evidence of active inflammation and an inappropriate response, LOR, or intolerance to immunosuppressants, anti-TNFs, or corticosteroid therapy[45]. Patients were divided into a placebo group and groups receiving abrilumab 21 mg or 70 mg at weeks 0, 2, and 4, followed by once every four weeks, and a group receiving a single 210 mg dose of abrilumab. The primary endpoint, which was CDAI remission (CDAI score of < 150 points) rate at week 8, was 23.1%, 14.4%, and 21.9% in the abrilumab 21 mg, 70 mg, and 210 mg groups, respectively, and 12.8% in the placebo group; there were no statistically significant differences between the abrilumab groups and the placebo group. However, among patients who had previously experienced anti-TNF treatment failure, CDAI remission rates at week 12 were 22.9%, 17.4%, and 24.8% in the abrilumab 21 mg, 70 mg, and 210 mg groups, respectively, which were all significantly higher than the remission rate of 8.2% in the placebo group (P < 0.01). Also, in patients with prior anti-TNF failure, the CDAI response (decrease of at least 100 points in CDAI score compared to baseline) rates at week 12 in the abrilumab 21 mg, 70 mg, and 210 mg groups were 30.0%, 39.4%, and 37.4%, respectively, and these values in the abrilumab 70 mg and 210 mg groups were significantly higher than the response rate of 14.2% in the placebo group (P < 0.01). Adverse events up to week 24 were the same in the abrilumab groups and the placebo group, and there were no cases of PML or death in any of the abrilumab groups. Thus, in CD, although abrilumab did not show a significant improvement in the primary endpoint, it could show useful effects.

Etrolizumab

Etrolizumab (rhuMAb β7) is a humanized monoclonal IgG1 antibody against the β7 subunit of α4β7 and αEβ7 that blocks not only the interaction between α4β7 and MAdCAM-1, but also the interaction between αEβ7 and E-cadherin expressed mostly by epithelial cells. Thus, etrolizumab suppresses the trafficking of lymphocytes into the gut and the retention of lymphocytes in the intraepithelial compartment.

The phase Ⅱ EUCALYPTUS induction study was conducted on 124 moderate-to-severe UC patients who showed no response to conventional therapy[46]. Patients were randomly allocated in a 1:1:1 ratio into a placebo group, a group administered subcutaneous etrolizumab 100 mg at weeks 0, 4, and 8 (and placebo at week 2), and a group administered a loading dose (LD) of subcutaneous etrolizumab 420 mg, followed by subcutaneous doses of 300 mg at weeks 2, 4, and 8. The primary endpoint, which was clinical remission rate at week 10, was 0% in the placebo group, 20.5% in the etrolizumab 100 mg group (P = 0.004), and 10.3% in the etrolizumab 300 mg plus LD group (P = 0.048); the clinical remission rate was higher in the etrolizumab groups than in the placebo group. In a subgroup analysis, among anti-TNF-naïve patients, the clinical remission rates in the etrolizumab 100 mg group and the etrolizumab 300 mg plus LD group were 44% and 25%, respectively; however, among patients who had not responded to anti-TNF therapy, the clinical remission rates were 5% and 4%. Although there were no cases of severe infection in the etrolizumab-treated groups, and there was no significant difference in the rate of adverse reactions sufficient to stop medication in the three groups, influenza-like illness (7% *vs* 0% and 2%) arthralgia (15% *vs* 5% and 9%), and rash (7% *vs* 3% and 2%) were observed more frequently in the etrolizumab 100 mg group than the etrolizumab 300 mg plus LD group or the placebo group. However, these adverse events were all mild or moderate, demonstrating that etrolizumab is safe and tolerable.

One notable aspect of this study is that when quantitative PCR and immunohistochemistry were used to measure the number of αE gene (*ITGAE*)-expressing and αE-positive cells in the colonic mucosa, higher αE expression was associated with a higher rate of clinical remission at week 10 in patients treated with etrolizumab, suggesting that αE expression could be used as a biomarker in etrolizumab treatment[46]. The subsequent study was conducted on colon tissues taken by biopsies from the UC patients in this phase Ⅱ trial, as well as the patients with UC and a control group without IBD in an observational study[47]. Here, the mRNA for granzyme A (*GZMA*), a serine protease that promotes cell migration and is associated with the secretion of inflammatory cytokines such as IL-1β and TNF-α, showed high expression in colonic CD4+ integrin αE+ cells; higher levels of *GZMA* mRNA or *ITGAE* mRNA were associated with a higher likelihood of responding to etrolizumab, and their expression after etrolizumab treatment decreased significantly by 40%-80%.

Currently, there are 5 ongoing phase Ⅲ randomized

controlled trials (HIBISCUS Ⅰ, HIBISCUS Ⅱ, GARDENIA, LAUREL, and HICKORY) and 1 rollover open-label extension trial (COTTONWOOD) on UC.

The phase Ⅲ BERGAMOT trial aimed to evaluate the safety and efficacy of etrolizumab in 300 moderate-to-severe CD patients who were previously refractory or intolerant to anti-TNFs, immunomodulators, and/or corticosteroid therapy[48]. The patients were randomly allocated in a ratio of 1:2:2 into a placebo group, a group receiving subcutaneous etrolizumab 105 mg every four weeks, and a group receiving subcutaneous etrolizumab 210 mg at weeks 0, 2, 4, 8, and 12. The symptomatic remission (abdominal pain $\leqslant$ 1 and unweighted stool frequency $\leqslant$ 3) rates at week 6 were 15.0% and 25.6% in the etrolizumab 105 mg and etrolizumab 210 mg groups, respectively, which were higher than the rate of 8.5% in the placebo group. Similarly, the symptomatic remission rates at week 10 were higher in the etrolizumab 105 mg and etrolizumab 210 mg groups, at 15.8% and 27.3%, than the placebo group, at 8.5%, and the symptomatic remission rates at week 14 were still higher in the etrolizumab 105 mg and 210 mg groups, at 20.8% and 24.8%, than in the placebo group, at 11.9%. The endoscopic improvement (decrease of at least 50% in SES-CD compared to baseline) rates at week 14 were also higher in the etrolizumab 105 mg and 210 mg groups, at 21.0% and 17.4%, than in the placebo group, at 3.4%. There were no significant differences between the placebo group and the etrolizumab groups in adverse events. Thus, etrolizumab showed a rapid effect at week 6 in the treatment of moderate-to-severe CD, and research is underway investigating the maintenance phase.

The phase Ⅲ HICKORY open-label induction trial aimed to investigate the efficacy of etrolizumab in 130 moderate-to-severe UC patients who showed intolerance or no response to anti-TNFs[49]. After patients were administered etrolizumab 105 mg by subcutaneous injections for 14 wk, at four-week intervals, the clinical response and remission rates at week 14 were 50.8% and 12.3%, respectively, and 43.9% of patients receiving etrolizumab showed an endoscopic improvement, represented by a decrease of at least 1 point in endoscopy score compared to baseline. HICKORY including double blind induction phase and maintenance phase is currently ongoing (NCT02100696).

αEβ7 and α4β7 are differentially expressed in T lymphocyte effector subsets in the peripheral blood and intestines of IBD patients; T cell receptor stimulation and transforming growth factor-β treatment increased the expression of αEβ7, especially in CD8+ lymphocytes[50]. When used in a humanized mouse model of colitis, etrolizumab surrogate antibody decreased the accumulation of CD8+ and CD4+ Th9 cells in the intestine more strongly than VDZ; this seems to be because etrolizumab had an additional inhibitory effect on the αEβ7-mediated retention of lymphocytes[50].

If β7 integrin is blocked, it could reduce gut specificity; this is because αEβ7 is expressed by T cells in other tissues as well as in the intestines; therefore, problems can arise with the control of local infection[51]. Therefore, in the ongoing phase Ⅲ trials, it is important to determine whether latent infection is a significant adverse effect of etrolizumab.

PF-00547659

PF-00547659 (SHP647) is a fully human monoclonal IgG2κ antibody targeting MAdCAM-1, an intestinal endothelial CAM that binds α4β7 integrin on lymphocytes. This is another strategy for inhibiting leukocyte adhesion by blocking the endothelial CAM from binding to the integrin ligand.

The phase Ⅱ TURANDOT trial analyzed 357 moderate-to-severe UC patients who had either shown failure or intolerance for at least one conventional therapy[52]. PF-00547569 was administered every four weeks by subcutaneous injection at either one of the 4 different doses (7.5, 22.5, 75, or 225 mg) and the outcomes were compared with a placebo. The primary endpoint, which was clinical remission rate at week 12, was 2.7% in the placebo group, 11.3% in the PF-00547569 7.5 mg group (P = 0.0425), 16.7% in the 22.5 mg group (P = 0.0099), 15.5% in the 75 mg group (P = 0.0119), and 5.7% in the 225 mg group (P = 0.1803), indicating that the remission rate was significantly higher in the PF-00547569 7.5 mg, 22.5 mg, and 75 mg groups than in the placebo group, and the efficacy was the highest in the 22.5 mg and 75 groups. The mucosal healing rate at week 12 was 8.2% in the placebo group, 15.5% in the 7.5 mg group (P = 0.0099), 27.8% in the 22.5 mg group (P = 0.0038), 25.4% in the 75 mg group (P = 0.0080), and 14.3% in the 225 mg group (P = 0.0099), showing the highest value in the PF-00547569 22.5 mg and 75 mg groups. In a subgroup analysis, among patients experiencing anti-TNF therapy failure, the remission rate at week 12 was 0% in the placebo group, 7.3% in the 7.5 mg group (P = 0.0425), 9.8% in the 22.5 mg group (P = 0.0099), 9.8% in the 75 mg group (P = 0.0119), and 2.5% in the 225 mg group (P = 0.1803), showing significantly higher values than the placebo group in the PF-00547569 7.5 mg, 22.5 mg, and 75 mg groups. The reason that the clinical effect of PF-00547659 at the highest dose decreased may be because of study design or the depletion of the anti-inflammatory regulatory T cells to the intestine, and further research is needed[53]. There were no significant differences between the placebo group and the PF-00547569 groups in the frequency of adverse events, and there were no cases of severe infection or PML. When GI side effects were investigated considering the gut selectivity of PF-00547659, *Clostridium difficile* infection, anal abscess and anal fistula were observed in patients treated with PF-00547659 and one patient was diagnosed with adenocarcinoma of colon during the study period. Therefore, special attention should be paid to GI complications in the treatment of PF-00547659, and additional data is necessary to establish its safety. A large-scale phase Ⅲ clinical trial is currently underway in

patients with UC (NCT03259334).

The phase Ⅱ OPERA trial aimed to evaluate the efficacy and safety of PF-00547569 in 265 moderate-to-severe CD patients who had previously shown no response or intolerance for anti-TNFs and/or immunosuppressants[54]. Patients were randomly allocated, in a 1:1:1:1 ratio, to a placebo group and groups receiving PF-00547569 at either one of the 3 doses (22.5, 75, or 225 mg). The CDAI-70 response rates at week 8 showed no significant differences, at 47.7% in the placebo group and 52.7%, 60.1%, and 62.7% in the PF-00547569 22.5, 75, and 225 mg groups, respectively. Similarly, the CDAI-70 response rates at week 12 also showed no significant differences, at 58.6% in the placebo group and 62.8%, 64.7%, and 57.5% in the PF-00547569 22.5, 75, and 225 mg groups, respectively. However, among patients with high baseline CRP levels (> 5 mg/dL or > 18.8 mg/dL), the CDAI remission rates at week 8 or 12 were higher in the PF-00547569 groups than in the placebo group. Moreover, in the PF-00547569 groups, soluble MAdCAM level decreased significantly at week 2 in a dose-dependent manner and circulating β7+ CD4+ central memory T lymphocytes increased at weeks 8 and 12. Therefore, although the high clinical response rate in the placebo group indicated that there was no significant difference between the PF-00547569 groups and the placebo group, PF-00547569 seems to be effective in patients with active inflammation.

Given the clinical success of drugs that block α4β7 integrin, antibodies against MAdCAM-1 should produce a similar clinical effect. However, this is not reflected in the study results because α4β7 not only binds MAdCAM-1, but also has epitopes for binding VCAM-1 and fibronectin, though it is known that VDZ does not affect the adhesion of α4β7 to VCAM-1[55].

CONCLUSION

The introduction of anti-TNF drugs in IBD treatment demonstrated superior therapeutic effects compared to conventional treatment. However, the development of new drugs is required for several reasons, including inadequate response, LOR or intolerance. The aim of developing new treatments for UC and CD is to produce targeted drugs that can enhance the clinical effect while reducing systemic adverse events. In this regard, anti-integrin agents are one of the most promising drug classes for IBD after anti-TNF agents. Anti-integrin agents inhibit the extravasation of lymphocytes by blocking the interactions between integrin and CAMs. The use of the initially developed natalizumab is limited use due to the risk of PML; however, VDZ developed later acts selectively on the intestine and shows few systemic adverse effects. VDZ has been approved and is currently used in clinical practice. Newer anti-integrin drugs that act on different targets associated with integrin, such as AJM300, abrilumab, etrolizumab, and PF-00547659 are also being developed and currently undergoing clinical trials.

In the future, clinical trials of anti-integrin drugs are expected to demonstrate their clinical efficacy, their place in the treatment of IBD, and their associated adverse effects. This will widen the range of drugs available to physicians and patients for treating IBD, and is an important step toward truly personalized treatment.

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MINIREVIEWS

Olfactomedin-4 in digestive diseases: A mini-review

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Abstract

Olfactomedin-4 (OLFM4, GW112, hGC-1) is a glycoprotein belonging to the olfactomedin family. The expression of OLFM4 is strong in the small intestine, colon and prostate, and moderate in the stomach and bone marrow. Previous studies have revealed that OLFM4 is closely associated with many digestive diseases. Up-regulation of OLFM4 has been detected in the *Helicobacter pylori* (*H. pylori*)-infected gastric mucosa, inflammatory bowel disease tissue and gastrointestinal malignancies, including gastric cancer, colorectal cancer, pancreatic cancer and gallbladder cancer. Down-regulation of OLFM4 has also been detected in some cases, such as in poorly differentiated, advanced-stage and metastatic tumors. Studies using OLFM4-deficient mouse models have revealed that OLFM4 acts as a negative regulator of *H. pylori*-specific immune responses and plays an important role in mucosal defense in inflammatory bowel disease. Patients with OLFM4-positive gastric cancer or colorectal cancer have a better survival rate than OLFM4-negative patients. However, the prognosis is worse in pancreatic cancer patients with high levels of expression of OLFM4. The NF-κB, Notch and Wnt signaling pathways are involved in the regulation of OLFM4 expression in digestive diseases, and its role in pathogenesis is associated with anti-inflammation, apoptosis, cell adhesion and proliferation. OLFM4 may serve as a potential specific diagnostic marker and a therapeutic target in digestive diseases. Further studies are required to explore the clinical value of OLFM4.

Key words: Olfactomedin-4; Inflammation; Cancer; *Helicobacter pylori* infection

Core tip: This review is based on the currently available literature about olfactomedin-4 (OLFM4) and is intended to reveal the link between OLFM4 and digestive diseases, including *Helicobacter pylori* infection, inflammatory bowel disease and gastrointestinal malignancies. The data on the expression, function and regulatory pathways of OLFM4 in digestive diseases are summarized. The potential clinical value of OLFM4 in digestive diseases is also discussed.

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INTRODUCTION

Olfactomedin-4 (OLFM4, also called GW112 or hGC-1) is a 72-kDa glycoprotein belonging to the olfactomedin family and is characterized by the presence of an olfactomedin domain with approximately 250 amino acids, which is located in the C-terminal region[1]. OLFM4 was initially cloned from human hematopoietic myeloid cells treated with granulocyte colony-stimulating factor[1]. The *OLFM4* gene, located on chromosome 13q14.3, encodes a 510-amino acid N-linked glycoprotein with the olfactomedin domain[1,2]. OLMF4 can be expressed in the membrane, cytoplasm, nucleus, mitochondria and mature neutrophil granules[1,3-5]. OLFM4 is strongly expressed in the small intestine, colon and prostate, moderately expressed in the stomach and bone marrow, and weakly expressed or not expressed in other tissues[1].

Compared with that in normal tissues, aberrant expression of *OLFM4* has been detected in many pathological tissues, such as the gastric mucosa infected with *Helicobacter pylori* (*H. pylori*)[6,7], inflamed intestinal tissue in inflammatory bowel disease[8,9] and many types of gastrointestinal malignancies[10-14] (Figure 1). The primary function of *OLFM4* in gastrointestinal malignancies is associated with its role as an antiapoptotic factor that promotes the tumor growth[4]. In addition, *OLFM4* down-regulates innate immunity against *H. pylori* infection[7] and affects the anti-inflammatory function in inflammatory bowel disease[15]. In this review, we summarize the data on the expression, function and regulatory pathways of OLFM4 in digestive diseases.

OLFM4 IN *H. PYLORI* INFECTION

Expression

H. pylori infection is a well-recognized risk factor for gastric diseases as well as extra-gastric diseases[16-18]. The host immune response plays a key role in the course and outcome of *H. pylori* infection[19,20]. The innate immune system serves as the first line of defense against *H. pylori* infection[21]. An adaptive immune response to *H. pylori* is also elicited in nearly all *H. pylori*-infected individuals[22]. OLFM4 is a novel glycoprotein that negatively regulates the host defense system against bacterial infection[23].

An early microarray study found that OLFM4 expression is significantly up-regulated in the gastric mucosa of *H. pylori*-infected patients compared with that in uninfected controls[6]. OLFM4 expression was also found to be significantly up-regulated in the gastric mucosa of *H. pylori*-infected mice. However, further study is warranted to determine whether eradication of *H. pylori* leads to the normalization of OLFM4 levels. The expression of OLFM4 is up-regulated in neutrophils, macrophages and epithelial cells after *H. pylori* infection, which suggests that overexpression of OLFM4 upon *H. pylori* infection is due to its direct action on epithelial cells as well as to activation of neutrophil and macrophage infiltration[7], thus suggesting a potential role for OLFM4 in the host immune response against *H. pylori* infection.

Function

The exact function of OLFM4 in *H. pylori* infection has been demonstrated by generating an OLFM4-deficient mouse model. Colonization of *H. pylori* in the gastric mucosa is significantly reduced after knocking out the *OLFM4* gene, as compared with that in wild-type mice[7]. In addition, in response to *H. pylori* infection, infiltration of inflammatory cells was significantly enhanced, the production of proinflammatory cytokines and chemokines was increased, and the bacterial load was reduced in OLFM4-deficient mice[7]. Therefore, OLFM4 acts as a negative regulator of the *H. pylori*-specific immune responses[7].

Regulation

OLFM4 is a target gene of the NF-κB pathway and expression of the *OLFM4* gene can be regulated by the transcription factor NF-κB[7,24]. The regulation is achieved by binding of NF-κB to the 5'-upstream region of the *OLFM4* gene[24]. Moreover, OLFM4 exerts a negative feedback effect on the NF-κB pathway[7].

Mouse experiments have revealed that *H. pylori* infection up-regulates the OLFM4 expression in an NF-κB-dependent manner, and then, due to the negative feedback effect of OLFM4, the *H. pylori*-induced NF-κB activation is down-regulated[7]. Furthermore, OLFM4 inhibits the nucleotide oligomerization domain (NOD)-1/2-mediated NF-κB activation and subsequent cytokine and chemokine production through direct association with NOD1 and NOD2[7]. The reduced cytokine and chemokine production results in a weak inflammatory response and a high level of colonization of *H. pylori* in the gastric mucosa[7].

Experiments in a MyD88 and OLFM4 double-

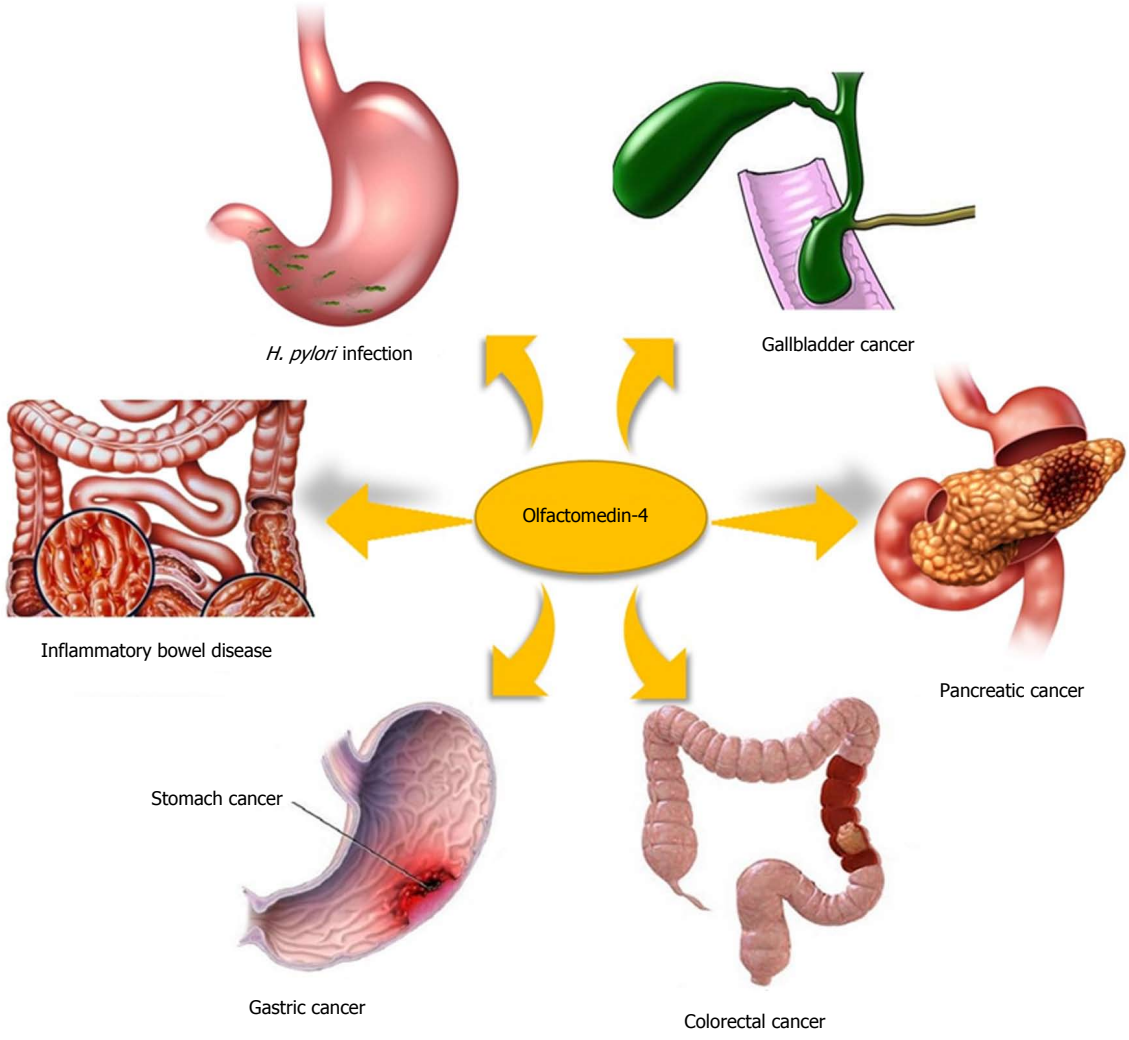

Figure 1 Relationship between olfactomedin-4 and digestive diseases. Olfactomedin-4 is related to *Helicobacter pylori* infection, inflammatory bowel disease and gastrointestinal malignancies, including gastric cancer, colorectal cancer, pancreatic cancer and gallbladder cancer.

knockout mouse model have demonstrated that the *H. pylori* colonization level in the model is similar to that in wild-type mice[25]. Even though the immune and inflammatory responses are enhanced compared with those in wild-type mice, infiltration of inflammatory cells in the gastric mucosa of double-knockout mice is lower than that in OLFM4 knockout mice[25]. Additionally, knocking out OLFM4 significantly up-regulates the MyD88 expression. It has been shown that deletion of OLFM4 indirectly increases the MyD88 expression by enhancing NOD2 expression, whereas the deficiency of MyD88 leads to a loss of the feedback inhibition of the NF-κB pathway and of the resulting response[25,26].

OLFM4 IN INFLAMMATORY BOWEL DISEASE

Expression

OLFM4 is a robust marker for murine intestinal stem cells as well as human intestinal stem cells[27]. Both OLFM4 mRNA and protein expression levels are significantly up-regulated in the intestinal epithelium in Crohn's disease and ulcerative colitis[8,9]. Compared with that in inflamed tissue from Crohn's disease patients, the OLFM4 expression is more obviously increased in inflamed tissue from patients with active ulcerative colitis[8,9]. Moreover, in active ulcerative colitis, the expression of OLFM4 expands to the surface of epithelial cells as well as to the crypt lumen, and OLFM4 seems to be secreted into the mucus[8,9]. In contrast, the *OLFM4* gene expression is almost absent in luminal surface cells and mesenchymal cells and is confined to the lower third of the crypt in normal tissues[8,9].

Function

OLFM4 plays an important role in the mucosal defense of the stomach and colon[9]. Experiments using OLFM4-deficient mice have revealed severe inflammation and proliferation in intestinal crypts in small intestines[15]. Serious inflammation and mucosa damage have also been found in the colon of OLFM4-deficient mice[15].

The anti-inflammatory function of OLFM4 in inflammatory bowel disease is consistent with that in the stomach. The function against inflammatory bowel disease may be related to the tissue-specific human beta-defensins (HBD)1, HBD2 and HBD3. As mucus

components with different electric charges, OLFM4 and HBD1–3 can interact, and the binding ability of OLFM4 was ranked, from high to low, as HBD3 > HBD2 > HBD1[9]. Furthermore, OLFM4 binding leads to a decrease in the antimicrobial activities of HBD1-3[9].

Regulation

OLFM4 is a target gene for the Notch signaling pathway, which regulates intestinal cell proliferation and differentiation[28]. The expression of OLFM4 increases after activation of Notch signaling[28]. Conversely, the expression of OLFM4 rapidly decreases after treatment with the Notch blocker dibenzazepine[9,28]. Researchers have found that after mesenchymal stem cell transplantation, the expression of OLFM4 is down-regulated, while that of Atoh1 is up-regulated[29]. This result suggests that the suppression of Notch signaling leads to decreased OLFM4 expression.

Although some studies have shown that cell incubation with TNF-α alone does not influence the OLFM4 expression, some other studies have found that TNF-α and components of the Notch pathways synergistically up-regulate the OLFM4 expression[9,30,31]. TNF-α is one of the most important proinflammatory cytokines promoting inflammatory bowel disease[30]. Microarray analysis has revealed that up to 21 genes are involved in the synergistic up-regulation of TNF-α and the Notch intracellular domain[30]. Further studies have suggested a markedly increased expression of OLFM4, reaching up to a 2500-fold increase in LS174T cells, when overexpression of Notch intracellular domain-1 (NICD1) or hairy and enhancer of split-1 (HES1) is combined with TNF-α stimulation[30,31]. Such a synergistic effect is mediated through transcriptional regulation, which is dependent on a proximal NF-κB binding site[31].

OLFM4 IN GASTROINTESTINAL CANCER

Increased *OLFM4* expression has been reported in some gastrointestinal cancers, such as gastric cancer[10,11,32,33], pancreatic cancer[12] and early-stage colon cancer[13,14]. In addition, the expression of OLFM4 is correlated with the histological type of cancer, differentiation, lymphatic metastasis and prognosis[10,11,34]. Furthermore, OLFM4 is relevant to many cellular processes, including cell adhesion, apoptosis and proliferation[2,11,35]. Therefore, OLFM4 may serve as a candidate biomarker for these gastrointestinal cancers[36]. Here, we briefly summarize the recent advances in the expression, function and regulation of OLFM4 in gastrointestinal cancers.

Gastric cancer

Up-regulated OLFM4 expression is a frequent event in the gastric mucosa in gastric cancer[10,11,32,33]. Highly expressed OLFM4 is found in intestinal-type adenocarcinoma, while OLFM4 expression does not occur in diffuse-type adenocarcinoma[10]. Moreover, enhanced expression of OLFM4 occurs in well- or moderately differentiated and early-stage adenocarcinomas, and the expression is remarkably decreased or even lost in poorly differentiated and advanced-stage gastric cancer[10]. Furthermore, the OLFM4 expression is higher in patients without lymphatic metastasis than in those with lymphatic invasion[11,37]. OFLM4 expression is also related to the prognosis. OLFM4-positive gastric cancer patients have a better survival rate than do OLFM4-negative patients[34,37]. Using serum OLFM4 alone or in combination with human regenerating protein IV as biomarkers for gastric cancer patients is more sensitive than using CA199[32]. Down-regulation of OLFM4 suppresses the tumor proliferation, migration and invasion of gastric cancer cells *in vitro*[33,38].

The *OLFM4* gene was found to be up-regulated *via* the NF-κB signaling pathway and to exert an antiapoptotic effect in gastric cancer[39]. The anti-apoptotic effect caused by OLFM4 can be induced by reducing H_2O_2 or TNF-α[38]. Moreover, the antiapoptotic factor OLFM4 is a direct target of miR-486, which is a frequently lost microRNA (miRNA) in gastric cancer patients and may act as a tumor suppressor miRNA in gastric cancer[40]. miR-486 directly targets and inhibits OLFM4 and thereby induces antioncogenic effects against gastric cancer[40].

Colorectal cancer

OLFM4 is enriched in human colon crypts, although it is not expressed in the murine colon[15,27,41,42]. It has been universally accepted that OLFM4 is a useful marker of intestinal stem cells (ISCs) in humans, similar to LGR5, which is a confirmed ISC marker[27,43,44]. Up-regulation of OLFM4 is detected more frequently in highly differentiated and early-stage colon cancers than in the normal colon mucosa, whereas it is often down-regulated or not expressed in poorly differentiated, late tumor-node-metastasis stage, and metastatic cancers[35]. OLFM4-positive colorectal cancer patients have a better survival rate than do OLFM4-negative patients[45]. In addition, precancerous colorectal lesions also show aberrant OLFM4 expression. For example, OLFM4 is expressed in a diffuse manner in traditional serrated adenomas, while other ISC markers such as LGR5 and ASCL2 are localized as in normal tissue[44]. OLFM4 silencing enhances the proliferation in intestinal crypts and inflammation initiated by azoxymethane/dextran sodium sulfate[15]. Moreover, systemic OLFM4 deletion promotes colon tumorigenesis, which may be associated with the loss of mucosal neutrophils[15].

There is an intimate connection between OLFM4, Wnt/β-catenin signaling, crypt biology[15,46-48] and colon cancer[27,49,50]. OLFM4 is a target gene that acts as a negative regulator of the Wnt/β-catenin signaling pathway and inhibits colon cancer progression by down-regulating the Wnt signaling pathway[15].

Pancreatic cancer

OLFM4 mRNA is expressed at higher levels in pancreatic cancer tissues than in noncancerous pancreatic tissue samples[12]. In addition, OLFM4 was found to be

Table 1 Effects of olfactomedin-4 in digestive diseases

Disease	Expression	Function	Regulation
H. pylori infection	Up-regulated in the *H. pylori*–infected gastric mucosa	Negative regulator of *H. pylori*-specific immune responses	NF-κB, NOD-1/2, MyD88
Inflammatory bowel disease	Up-regulated in the intestinal epithelium in Crohn's disease and ulcerative colitis	Mucosal defense, anti-inflammatory effects	Notch, TNF-α
Gastrointestinal malignancies	Up-regulated in well/moderately differentiated, early-stage gastrointestinal malignancies without lymphatic metastasis	Biomarker, candidate therapeutic target	NF-κB, TNF-α, miR-486, Wnt/β-catenin

H. pylori: *Helicobacter pylori*.

significantly over-expressed in peripheral blood mononuclear cells in pancreatic cancer patients compared with its expression in a control group[51]. Furthermore, OLFM4 has also been detected in pancreatic juice and ascites[52]. Pancreatic cancer may occur in a background of chronic pancreatitis. Whether OLFM4 is associated with chronic pancreatitis or acute pancreatitis flares is worth further investigation. In the PANC-1 cell line, OLFM4 is especially increased during the early S phase of the cell cycle and promotes proliferation by supporting the S to G2/M phase transition[12]. OLFM4 binds to the apoptosis-promoting factor GRIM-19 to induce antiapoptosis[4]. Pancreatic cancer patients with high levels of OLFM4 expression have a worse prognosis[53].

Gallbladder cancer

Similar to the above findings, expression of the *OLFM4* gene has been found to be increased in gallbladder cancer tissues[54]. In addition, the expression level of OLFM4 is significantly related to the age of gallbladder cancer patients[54]. However, further studies are needed to clarify the precise role of OLFM4 in gallbladder cancer.

CONCLUSION

Since the initial discovery of OLFM4, researchers have explored many aspects of OLFM4, including its aberrant expression, biological functions and related mechanisms (Table 1). The expression of OLFM4 has been relatively well studied in normal tissues as well as in numerous diseases. The anti-inflammatory and antiapoptotic roles of OLFM4 are generally accepted. However, the exact mechanism for its effects in gastrointestinal diseases remains to be determined. Moreover, the clinical applications of OLFM4 as a specific detection marker or a therapeutic target need to be defined in the future.

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ORIGINAL ARTICLE

Basic Study

Oral treatment with plecanatide or dolcanatide attenuates visceral hypersensitivity *via* activation of guanylate cyclase-C in rat models

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Abstract

AIM

To investigate the effects of plecanatide and dolcanatide on maintenance of paracellular permeability, integrity of tight junctions and on suppression of visceral hypersensitivity.

METHODS

Transport of fluorescein isothiocyanate (FITC)-dextran was measured to assess permeability across cell monolayers and rat colon tissues. Effects of plecanatide and dolcanatide on the integrity of tight junctions in Caco-2 and T84 monolayers and on the expression and localization of occludin and zonula occludens-1 (ZO-1) were examined by immunofluorescence microscopy. Anti-nociceptive activity of these agonists was evaluated in trinitrobenzene sulfonic acid (TNBS)-induced inflammatory as well as in non-inflammatory partial restraint stress (PRS) rat models. Statistical significance between the treatment groups in the permeability studies were evaluated using unpaired *t*-tests.

RESULTS

Treatment of T84 and Caco-2 monolayers with lipopolysaccharide (LPS) rapidly increased permeability, which was effectively suppressed when monolayers were also treated with plecanatide or dolcanatide. Similarly, when T84 and Caco-2 monolayers were treated with LPS, cell surface localization of tight junction proteins occludin and ZO-1 was severely disrupted. When cell monolayers were treated with LPS in the presence of plecanatide or dolcanatide, occludin and ZO-1 were localized at the cell surface of adjoining cells, similar to that observed for vehicle treated cells. Treatment of cell monolayers with plecanatide or dolcanatide without LPS did not alter permeability, integrity of tight junctions and cell surface localization of either of the tight junction proteins. In rat visceral hypersensitivity models, both agonists suppressed the TNBS-induced increase in abdominal contractions in response to colorectal distension without affecting the colonic wall elasticity, and both agonists also reduced colonic hypersensitivity in the PRS model.

CONCLUSION

Our results suggest that activation of GC-C signaling might be involved in maintenance of barrier function, possibly through regulating normal localization of tight junction proteins. Consistent with these findings, plecanatide and dolcanatide showed potent anti-nociceptive activity in rat visceral hypersensitivity models. These results imply that activation of GC-C signaling may be an attractive therapeutic approach to treat functional constipation disorders and inflammatory gastrointestinal conditions.

Key words: Plecanatide; Guanylyl cyclase-C agonists; Dolcanatide; Uroguanylin; Preclinical; Cyclic guanosine monophosphate; Constipation; Inflammatory bowel diseases

Core tip: Our results indicate that plecanatide and dolcanatide, guanylate cyclase-C receptor agonists designed to replicate the activity of the human intestinal peptide uroguanylin, maintain intestinal barrier function and exhibit potent anti-nociceptive activity in animal models of visceral hypersensitivity, suggesting a novel mechanism, beyond the well described secretory function, for these agonists in the treatment of functional constipation disorders and inflammatory bowel disease.

Boulete IM, Thadi A, Beaufrand C, Patwa V, Joshi A, Foss JA, Eddy EP, Eutamene H, Palejwala VA, Theodorou V, Shailubhai K. Oral treatment with plecanatide or dolcanatide attenuates visceral hypersensitivity *via* activation of guanylate cyclase-C in rat models. *World J Gastroenterol* 2018; 24(17): 1888-1900 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i17/1888.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i17.1888

INTRODUCTION

Chronic idiopathic constipation (CIC) and irritable bowel syndrome with constipation (IBS-C), affecting approximately 20% of the United States population[1,2], are characterized by abnormalities in motility and visceral hypersensitivity with overlapping symptoms such as abdominal pain and discomfort, bloating, incomplete bowel movements, straining, or hard and lumpy stools[3,4].

Therapeutic targets for CIC and IBS-C have focused primarily on promotion of gastrointestinal (GI) fluid secretion through activation of chloride channels such as chloride channel type 2 and the cystic fibrosis transmembrane conductance regulator (CFTR)[5]. Additionally, inhibition of sodium-hydrogen exchanger 3 is being explored for treating IBS-C[6]. Drugs approved by the United States. Food and Drug Administration (FDA) for treating IBS-C include Amitiza® (lubiprostone) for adult women and Linzess® (linaclotide)[7,8] for adults. Lubiprostone, a bicyclic fatty acid metabolite of prostaglandin E1, specifically stimulates chloride channel type 2 causing an efflux of chloride into the lumen of the GI tract, which promotes fluid secretion, facilitating bowel movement[9]. Linaclotide, an analog of the heat-stable enterotoxin of *Escherichia coli* (*E. coli*), binds and activates guanylate cyclase-C (GC-C) to stimulate production of cyclic guanosine monophosphate (cGMP), which enhances secretion of electrolytes and fluid into the GI lumen to promote bowel movement and ameliorate abdominal pain[10]. Plecanatide (Trulance®) is an FDA-approved drug for treatment of adults with CIC[11,12] and the drug was recently approved for treatment of adults with IBS-C.

Recent studies suggest that the immune system is

dysregulated in irritable bowel syndrome (IBS), leading to increased total counts of innate immune cells, including mast cells, monocytes and macrophages in IBS patients[13-15]. Mast cell mediators and cytokines, including tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and IL-1α, modulate colorectal afferent excitability and disrupt intestinal barrier function. The paracellular permeability of the intestinal epithelial barrier is regulated by a tight junction (TJ) protein complex composed of transmembrane proteins such as occludin, claudin and zonula occludens (ZO), which bind to the actin cytoskeleton[16]. Alterations in the structure and/or function of the TJ protein complexes are associated with epithelial barrier disruption and increased permeability of the mucosa, allowing entry of inflammatory mediators to promote low-grade inflammation and visceral hypersensitivity[16-19]. Studies have correlated down-regulation of TJ proteins with the severity of visceral hypersensitivity in IBS[19,20].

There is considerable overlap in clinical symptoms between IBS and inflammatory bowel disease (IBD)[21-23]. Pro-inflammatory cytokines, such as TNF-α and interferon-γ released during GI inflammation, activate myosin light chain kinase (MLCK) responsible for phosphorylation of the myosin Ⅱ regulatory light chain, resulting in contraction of actomyosin and dysfunction of the intestinal barrier[20]. Activation of GC-C signaling protects intestinal barrier function by regulating MLCK activity. In this regard, loss of GC-C signaling in GC-C$^{-/-}$ mice leads to a dysfunctional intestinal barrier and increased paracellular permeability[24]. Coincidently, the loss in expression of uroguanylin, the endogenous agonist of GC-C receptors, is also associated with colon cancer[25] and IBD[26,27]. Thus, activation of GC-C signaling is an attractive strategy for the treatment of GI disorders and inflammatory diseases.

Plecanatide is structurally identical to uroguanylin, differing only in the substitution of Asp with Glu at the 3-position at the N-terminus for greater binding affinity. Dolcanatide is similar to plecanatide in structure except that L-Asn^1 and L-Leu^{16} are replaced by D-Asn^1 and D-Leu^{16} at the N- and C-termini, respectively, which is thought to provide enhanced biostability. In this study, we provide the first evidence that plecanatide and dolcanatide, both analogs of uroguanylin, suppress lipopolysaccharide (LPS)-mediated increase in permeability in epithelial cell models and reduce visceral hypersensitivity in trinitrobenzene sulfonic acid (TNBS) and partial restraint stress (PRS) animal models.

MATERIALS AND METHODS

Ethical approval

Animal care and handling procedures for *ex vivo* studies performed in the United States were as per the approved protocol by the Institutional Animal Care and Use Committee of Lampire Biologicals (Pipersville, PA, United States). Animal handling procedures for *in vivo* studies conducted in France were approved by the Institutional Animal Care and Use Local Committee (Toulouse, France). The investigators affirm that all appropriate measures were taken to minimize pain or discomfort of the animals used in this study.

Test peptides, chemicals and reagents

Plecanatide (CAS: 467426-54-6) and dolcanatide (CAS: 1092457-65-2) were synthesized by AmbioPharm, Inc. (Augusta, SC, United States). For all *in vitro* experiments with cell lines and colon tissues, optimal concentrations derived from dose response curves were used.

Fluorescein isothiocyanate (FITC)-dextran (approximate molecular weight, 4 kD) and *E. coli* LPS were purchased from Sigma (St Louis, MO, United States). Trypsin, GlutaMax, and Pen Strep were procured from Life Technologies (Grand Island, NY, United States). Rabbit anti-occludin antibody, rabbit anti-ZO-1 antibody, DAPI (4′, 6′-diamidino-2-phenylindole, dihydrochloride), and Alexa Fluor 488 conjugated goat anti-rabbit secondary antibodies were from Thermo Fisher Scientific (Waltham, MA, United States). Ussing chamber and its accessories were purchased from Physiologic Instruments (San Diego, CA, United States). All other chemicals and reagents were obtained from Sigma-Aldrich Corp. (St Louis, MO, United States) or Fisher Scientific (Pittsburgh, PA, United States).

Measurement of epithelial cell paracellular permeability

Human colon carcinoma cell lines T84 and Caco-2, obtained from ATCC (Manassas, VA, United States), were cultured by procedures as previously described[25]. Paracellular permeability was determined by calculating the flux of FITC-dextran across epithelial cell monolayers. T84 (1.5×10^5) and Caco-2 (8×10^3) cells were cultured on 12 mm Transwell® permeable polyester membrane inserts (pore size, 0.4 μm) until the transepithelial resistance reached > 1000 Ω cm^2 for T84 cells or > 400 Ω.cm^2 for Caco-2. Cell monolayers were treated overnight with 100 μg/mL LPS in the presence or absence of 1 μmol/L plecanatide or 1 μmol/L dolcanatide. Subsequently, the media was aspirated and 1 mg/mL FITC-dextran dissolved in Krebs Ringer buffer solution was added to the apical chamber and cells were incubated for an additional one h at 37 ℃. Fluorescence in 100 μL of basolateral buffer solution was measured in a Tecan M-1000 plate reader. The excitation and emission wavelengths for FITC were 494 nm and 518 nm respectively. Data represent mean relative fluorescence ± standard error of the mean from at least two biological replicates, each analyzed in triplicate.

Measurement of permeability across colon tissues from rats

Adult Sprague Dawley male and female rats [Crl: CD(SD)], aged seven to eight wk, weighing 170-210 g were purchased from Charles River Laboratories (Shrewsbury, MA, United States) and allowed to acclimate for a minimum of one wk. Animals were

maintained on a 12 h light-dark cycle and were fasted overnight with free access to water prior to tissue harvest. The next morning the animals were euthanized by CO_2 inhalation. Following a midline abdominal incision, the entire colon segment was removed for permeability studies.

Freshly harvested tissue from proximal to mid-colon (approximately 2 cm pieces) were randomly selected and transferred to RPMI media in 24-well tissue culture plates containing vehicle, 10 μmol/L plecanatide or 10 μmol/L dolcanatide in the presence or absence of 100 μg/mL LPS. The plates were placed in a humidified incubator (5% CO_2) at 37 ℃. The next day, each tissue piece was mounted on an Ussing chamber slider (0.5 cm^2). Apical and basolateral chambers were bathed in Krebs Ringer buffer solution and gassed with 95% O_2 and 5% CO_2. The temperature was maintained at 37 ℃ with a water-jacketed system. To measure permeability, 2 mg/mL of FITC-dextran dissolved in Krebs Ringer buffer solution (pH 7.4) was added to the apical chamber. Fluorescence was measured every 15 min (for two h) in samples (100 μL of buffer) from the basolateral chamber using a Tecan M-1000 plate reader. The excitation and emission wavelengths used were 494 nm and 518 nm respectively. Data values represent mean relative fluorescence ± standard error of the mean recorded 75 min after the addition of FITC-dextran from multiple independent treatments.

Immunofluorescence microscopy

T84 and Caco-2 monolayers in 24-well plates were treated overnight with 100 μg/mL of LPS in the presence or absence of 1 μmol/L plecanatide or 1 μmol/L dolcanatide. Following the treatment, cells were washed three times with chilled phosphate buffer saline (PBS), fixed with 4% formaldehyde in PBS for 15 min, blocked and permeabilized in PBS containing 3% bovine serum albumin (BSA) and 0.3% Triton X-100 at room temperature for 30 min. Subsequently, monolayers were incubated with PBS containing 0.1% Tween-20, 2% BSA, rabbit anti-occludin (1:150) or rabbit anti-ZO-1 (1:25) antibodies and incubated overnight at 4 ℃ followed by three washes in chilled PBS and incubation for one h at room temperature in Alexa Fluor 488 labeled secondary antibody (1:500) and counterstained with DAPI. Occludin and ZO-1 were visualized with an Olympus IX81 microscope and images were obtained using SlideBook 5.0 software. Two independent experiments were conducted and approxiamately 30 fields examined for each treatment. Images were acquired at 40 × resolution.

Surgical procedures in rats used in the visceral hypersensitivity studies

Wistar male rats (n = 8/dosage group) weighing 220 -250 g (Janvier SA, Le Genest St Isle, France) were used in the visceral hypersensitivity experiments. Rats were housed individually in a temperature controlled (approxiamately 25 ℃) room with relative humidity (50%) with food and water *ad libitum*. The evening prior to the procedure, food and water were removed from the cages. Rats were sedated with acepromazine (0.5 mg/kg *i.p.*) and ketamine (100 mg/kg *i.m.*) (Imalgène; Rhône Mérieux, Toulouse, France). Pairs of nichrome wire electrodes (60 cm in length and 80 μM in diameter) were implanted bilaterally in the abdominal external oblique musculature, just superior to the inguinal ligament, 2 cm laterally from the midline. The free ends of electrodes were exteriorized on the back of the neck and protected by a plastic tube attached to the skin. Baseline electromyographic recordings began eight to nine days after surgical implantation of electrodes. Electrical activity of abdominal striated muscle was recorded with an electroencephalograph (Mini Ⅷ, Alvar, Paris, France) using a short time constant (0.03 s) to remove low frequency signals (< 3 Hz) and to selectively record spike bursts corresponding to abdominal contractions**.**

Colorectal distention

The colorectal distension (CRD) procedure was based on methods previously described[28]. During the acclimation sessions, rats were placed in plastic tunnels where they could move but not escape. Prior to the CRD procedure, a balloon (latex condom) was inserted into the rectum of conscious rats until the base of the balloon was at the anus (4 cm insertion). The tube was fixed at the base of the tail and animals were allowed to recover for 30 min. The balloon was then connected to a barostat and inflated progressively from 0-60 mmHg in 15 mmHg steps. Each step of inflation lasted five min. Responses to applied CRD pressure levels were measured with electromyographic recordings during the five-min interval and data are expressed as contractions/five min. Colonic volume adaptation to increasing pressures (compliance) was also measured using a potentiometric recorder (Linseis, Germany).

TNBS-induced visceral hypersensitivity in rats

The effect of plecanatide or dolcanatide on CRD was evaluated in a basal condition prior to TNBS exposure, as well as after treatment with TNBS in rats (n = 8/dose group). Plecanatide or dolcanatide were formulated in PBS to deliver via oral gavage doses of 0.01 or 0.05 mg/kg in 1.5 mL. Following the basal test, after a 12 h fasting period, rats were treated with 0.3 mL of TNBS (80 mg/kg in 50% ethanol), intrarectally through a silicone rubber catheter introduced to a depth of 6 cm into the anus under light anesthesia as previously described[29]. Following administration of TNBS, the animals were routinely evaluated for changes in physical appearance or behavior. Four days after the TNBS treatment, the oral administration of plecanatide or dolcanatide and the CRD testing was repeated.

Partial restraint stress-induced colorectal hypersensitivity

PRS was performed as described by Williams *et al*[30].

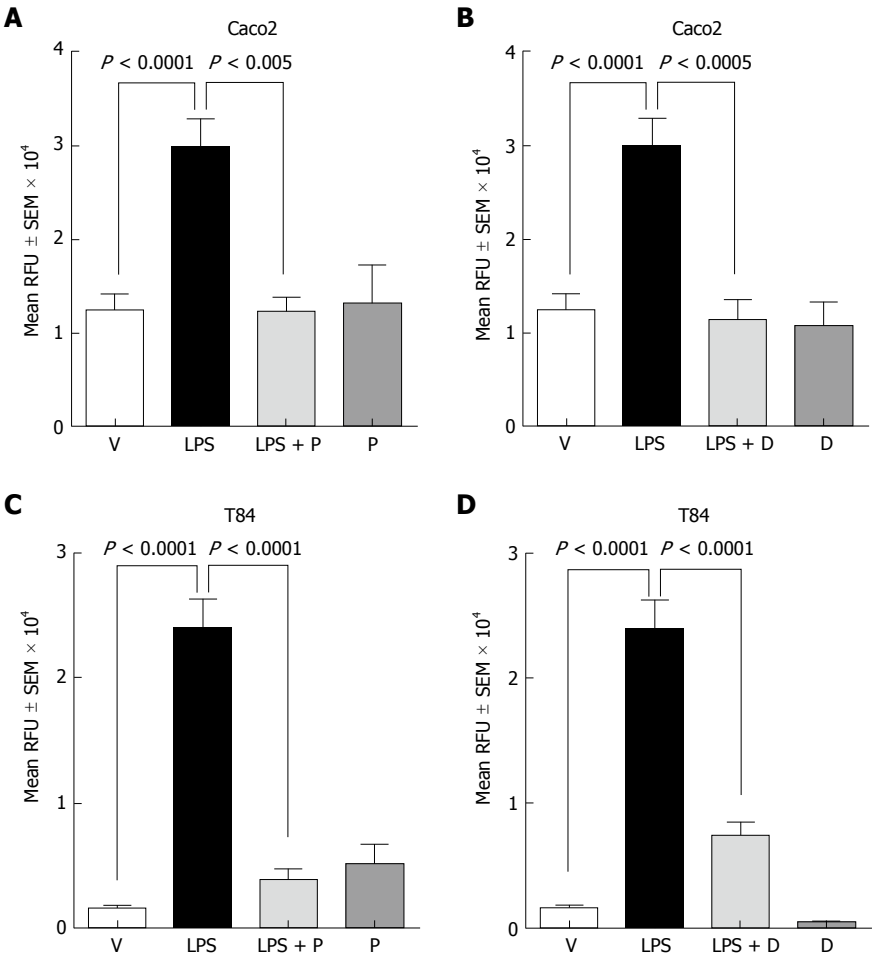

Figure 1 Effect of plecanatide and dolcanatide on lipopolysaccharide-induced increase in permeability of 4 kDa fluorescein isothiocyanate-dextran across Caco-2 and T84 cell monolayers. Caco-2 (A and B) and T84 (C and D) cells cultured on snap well inserts were treated with vehicle or 1 μmol/L of plecanatide (A and C) or dolcanatide (B and D) in the presence or absence of 100 μg/mL of LPS for 16 h. Subsequently, 1 mg/mL of FITC-dextran was added to the apical compartment. Paracellular permeability was determined by measuring the amount of FITC-dextran present in the basal compartment. Data represent mean ± SEM analyzed in triplicates. D: Dolcanatide; FITC: Fluorescein isothiocyanate; LPS: Lipopolysaccharide; P: Plecanatide; RFU: Relative fluorescence units; SEM: Standard error of the mean; V: Vehicle.

Rats were lightly anesthetized with ethyl-ether and foreshoulders, upper forelimbs and thoracic trunk were wrapped in a confining harness of paper tape to restrict, but not prevent, body movements. Rats (n = 8/dose group) were then placed in their home cage for two h. For the control condition, rats were anesthetized but not wrapped. Subsequently the rats were administered 1.5 mL of vehicle (phosphate-buffered saline), plecanatide or dolcanatide formulated to deliver doses of 0.01 and 0.05 mg/kg by oral gavage 30 min before the end of the PRS session. Thirty min following the stress procedure, rats underwent the CRD testing.

Statistical analysis

GraphPad Prism (Version 6.05) was used to calculate descriptive statistics and inferential tests. Differences between the treatment groups in the permeability studies were evaluated using unpaired *t*-tests. Two-way analyses of variance were used to evaluate differences between the vehicle control and the plecanatide or dolcanatide dose groups followed by comparisons at each pressure level using Dunnett's or Sidak's multiple comparison tests.

RESULTS

Plecanatide and dolcanatide suppressed LPS-induced paracellular permeability

Treatment with LPS reportedly can disrupt the TJ complex by down-regulating junctional protein expression. Additionally, LPS is known to augment mucosal hypersensitivity through secretion of inflammatory cytokines and other mediators[31]. Treatment with LPS (100 μg/mL) resulted in a statistically significant increase in paracellular permeability of FITC-dextran across Caco-2 (Figure 1A and B) and T84 (Figure 1C and D) cell monolayers. Importantly, the LPS-induced increase in the permeability of FITC-dextran was completely suppressed in both cell monolayers treated with plecanatide (Figure 1A and C) or dolcanatide (Figure 1B and D). No appreciable effect on paracellular permeability was observed when monolayers were

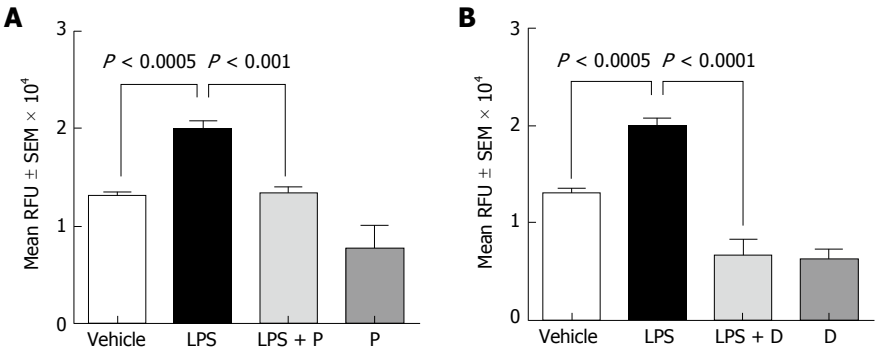

Figure 2 Effect of plecanatide (A) and dolcanatide (B) on lipopolysaccharide-induced increased permeability of 4 kD fluorescein isothiocyanate-dextran across rat colon tissues. Rat colon tissues (2 cm pieces) were incubated overnight with vehicle, 10 μM plecanatide or dolcanatide in the presence or absence of 100 μg/mL LPS. Data represent mean fluorescence ± SEM recorded 75 min after the addition of FITC-dextran. D: Dolcanatide; FITC: Fluorescein isothiocyanate; LPS: Lipopolysaccharide; P: Plecanatide; RFU: Relative fluorescence units; SEM: Standard error of the mean.

treated with either GC-C agonist alone.

Next, we examined the effects of plecanatide or dolcanatide on the LPS-mediated increase in permeability across freshly harvested rat colon tissues. Consistent with the results presented in Figure 1, LPS treatment resulted in a statistically significant increase in the paracellular permeability of FITC-dextran across rat colon tissues, which was effectively suppressed by treatment with plecanatide or dolcanatide (Figure 2). These results indicate that activation of GC-C signaling may be suppressing the deleterious permeability effects of caused by LPS treatment.

Plecanatide and dolcanatide maintain TJ integrity

Since LPS treatment consistently increased paracellular permeability in both cell lines and colonic tissue, we decided to examine the effects of LPS on the expression and localization of TJ proteins in these epithelial cell monolayers by immunofluorescence microscopy. Treatment with LPS severely disrupted localization of occludin and ZO-1 proteins at TJs in Caco-2 (Figures 3A and 4A) and T84 cells (Figures 3B and 4B), respectively. Importantly, when cell monolayers were treated with LPS in the presence of plecanatide or dolcanatide, expression of occludin and ZO-1 were normalized and localized at the cell surface of adjoining cells, similar to that observed for vehicle treated cells (Figures 3 and 4). Notably, treatment of cell monolayers with plecanatide or dolcanatide without LPS did not alter expression and localization of either of the TJ proteins. These results indicate that treatment with plecanatide or dolcanatide suppress LPS-mediated disruption in expression/localization of TJs in Caco-2 and T84 monolayers.

Basal colorectal sensitivity in rats

Initially, we conducted several pilot experiments to optimize experimental conditions and dose range of plecanatide and dolcanatide for evaluation. Under basal conditions, with no CRD pressure in vehicle treated rats, abdominal contractions occurred at approximately 4.1 contractions/5 min. As expected, increasing CRD pressure (0-60 mmHg) led to a linear increase in the number of abdominal contractions reaching approximately 6-fold higher than without any pressure. Oral treatment with plecanatide or dolcanatide without CRD pressure did not alter the rate of abdominal contractions (data not shown).

Effect of plecanatide and dolcanatide in TNBS-induced rectal allodynia in rats

Consistent with our prior experience in this model, the number of abdominal contractions, as an index of inflammation-induced visceral pain, four days after TNBS treatment (Figure 5), gradually increased in a pressure-dependent manner as compared to the number of abdominal contractions under basal conditions without TNBS treatment[32]. As expected, TNBS treatment resulted in increased abdominal contractions even in the absence of distending pressure. Oral treatment with plecanatide or dolcanatide at the lower doses (0.01 and 0.05 mg/kg) considerably attenuated ($P \leqslant 0.001$) the TNBS-induced increase in the number of abdominal contractions with increasing distending pressures up to 60 mmHg (Figure 5B and C). However, higher doses (> 0.1 mg/kg) had no significant effect on reduction in abdominal contractions at any pressure of distention (data not shown).

Effect of plecanatide and dolcanatide on stress-induced colorectal hypersensitivity

To investigate the anti-nociceptive effect of plecanatide or dolcanatide under non-inflammatory conditions, we utilized a wrap restraint model of stress-induced visceral hypersensitivity in Wistar rats, a strain with high stress responsiveness (Figure 6A). In vehicle treated rats, the number of abdominal contractions increased after the PRS session with increasing CRD pressures up to 60 mmHg. Oral treatment with plecanatide (Figure 6B and C) or dolcanatide (Figure 6D and E) resulted in a significant reduction in the rate of PRS-induced abdominal contractions with increasing CRD pressures. Both GC-C agonists exhibited no effect on colorectal

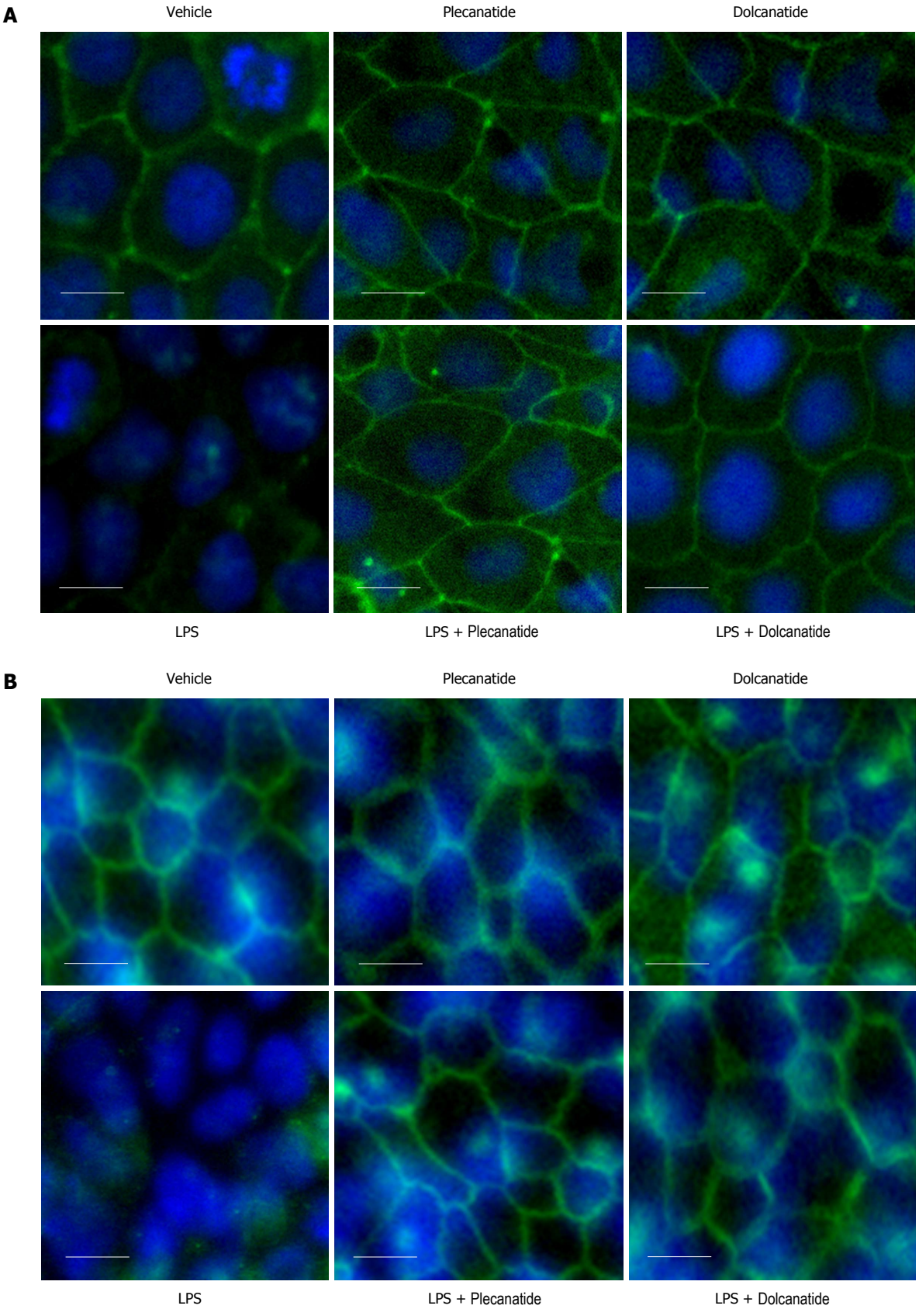

Figure 3 Effect of plecanatide and dolcanatide on localization of occludin in epithelial cells. Caco-2 (A) and T84 (B) cell monolayers were treated with 1 μmol/L plecanatide or dolcanatide in the presence or absence of 100 μg/mL of LPS for 16 h followed by immunofluorescence imaging for occludin. Representative microscopic fields demonstrate disruption of occludin localization by LPS. Co-treatment of LPS with plecanatide or dolcanatide preserved occludin localization around the cell membrane, as was observed for vehicle treated cells. Images taken at 40 × resolution. Blue fluorescence corresponds to DAPI stained nucleus. DAPI: 4', 6'-diamidino-2-phenylindole; LPS: lipopolysaccharide.

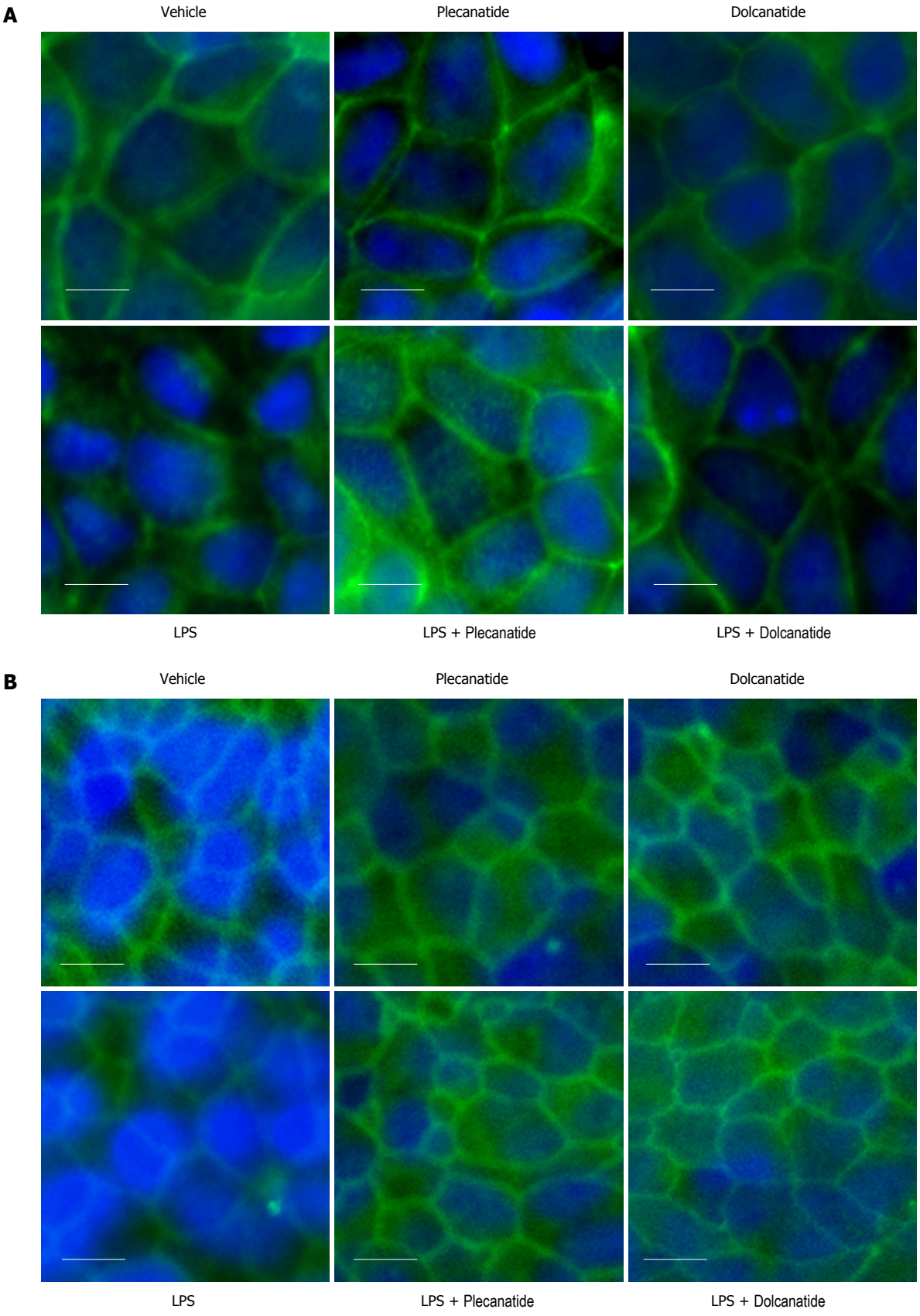

Figure 4 Effect of plecanatide and dolcanatide on localization of ZO-1 in epithelial cells Caco-2 (A) and T84 (B) cell monolayers were treated with 1 μmol/L plecanatide or dolcanatide in the presence of 100 μg/mL of LPS for 16 h followed by immunofluorescence imaging for ZO-1. Representative microscopic fields depicted above demonstrate disruption of ZO-1 localization by LPS. In Caco-2 cells, LPS treatment appears to cause accumulation of ZO-1 in the cytoplasm. Co-treatment of LPS with plecanatide or dolcanatide preserved ZO-1 localization around the cell membrane as observed for vehicle treated cells. Images taken at 40 × resolution. Blue fluorescence corresponds to DAPI stained nucleus. DAPI: 4', 6'-Diamidino-2-phenylindole; LPS: Lipopolysaccharide; ZO-1: Zonula occludens-1.

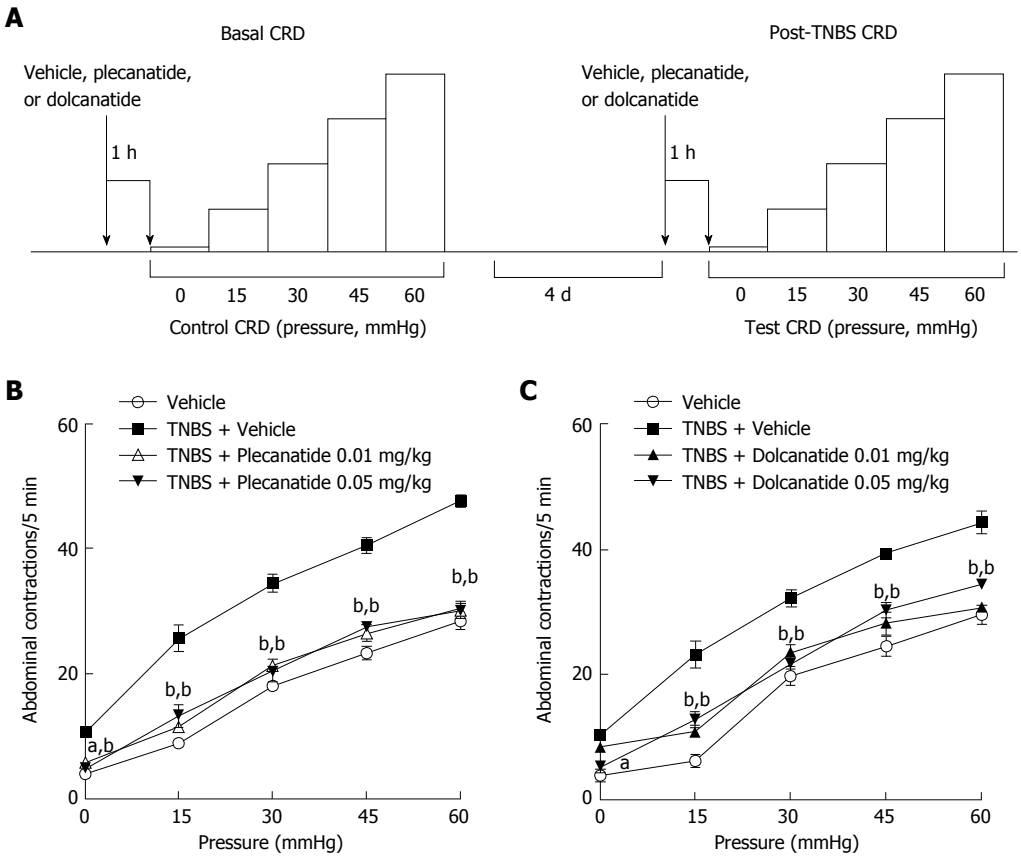

Figure 5 Design and results of the TNBS-induced visceral hypersensitivity models. A: Schematic depicting the sequence of test sessions and treatments to evaluate visceral hypersensitivity induced by TNBS rat models. Effects of oral administration of plecanatide or dolcanatide as compared with vehicle on the increase in abdominal contractions to colorectal distention (CRD) during testing conducted four days after intrarectal administration of TNBS. Doses of 0.01 and 0.05 mg/kg of plecanatide (B) or dolcanatide (C) reduced the rate of muscular contractions toward levels observed in the vehicle group prior to TNBS administration. Data are the mean ± SEM (n = 8 rats/group). $^{a}P < 0.05$, $^{b}P < 0.01$ as compared to the values for the post-trinitrobenzene sulfonic acid vehicle group. SEM: Standard error of the mean; TNBS: Trinitrobenzene sulfonic acid.

volume at the doses tested (data not shown).

DISCUSSION

The role of GC-C signaling in the regulation of ion and fluid homeostasis in the GI tract is well established[33,34] and affirmed clinically with the approval of plecanatide for the treatment of adults with CIC in the United States. However, recent advances have expanded our understanding of the involvement of GC-C signaling cascade in additional physiological activities such as in the maintenance of intestinal barrier function[33,35] and in the protection against GI inflammation and colorectal carcinogenesis[25,26,34]. Dysregulation of GC-C signaling either due to familial mutations in GC-C gene or loss of its endogenous ligands has further underscored the pathophysiological importance of GC-C signaling in GI indications[36]. In this study, we present *in vitro* and *in vivo* data with plecanatide and dolcanatide, two GC-C agonists, to demonstrate the physiological role GC-C signaling plays in the maintenance of intestinal barrier function and in suppression of visceral hypersensitivity in inflammatory and non-inflammatory rat models.

Dysregulation of the intestinal epithelial barrier function, known to be associated with several gut disorders, can be elicited by a number of agents, including luminal bacterial antigens eliciting activation of immune system and pro-inflammatory cytokines[20,37,38]. Our results demonstrate that LPS treatment considerably increased paracellular permeability in cell monolayers (Caco-2 and T84) and in rat colon tissues. The concentrations of plecanatide and dolcanatide used in these experiments were based on the dose-response curves established with these cell lines and rat tissues as reported earlier[25,39-41]. These deleterious effects of LPS were completely suppressed by treatment with plecanatide or dolcanatide. The fluorescence microscopy data with monolayers of Caco-2 and T84 indicate that LPS treatment also severely disrupted the localization of TJ proteins such as occludin and ZO-1. Importantly, treatment with either agonist effectively suppressed LPS-mediated disruption in localization of occludin and ZO-1 at the TJ surrounding the cells. These data are consistent with the recent findings that GC-C signaling plays a critical role in the maintenance of intestinal barrier function[24,35]. As expected from analogs of uroguanylin, plecanatide and dolcanatide are likely to exert their pharmacological activities through activation of GC-C signaling in the GI tract. In this context, we recently reported that oral treatment with plecanatide or dolcanatide ameliorated GI inflammation through activation of GC-C signaling in the distal

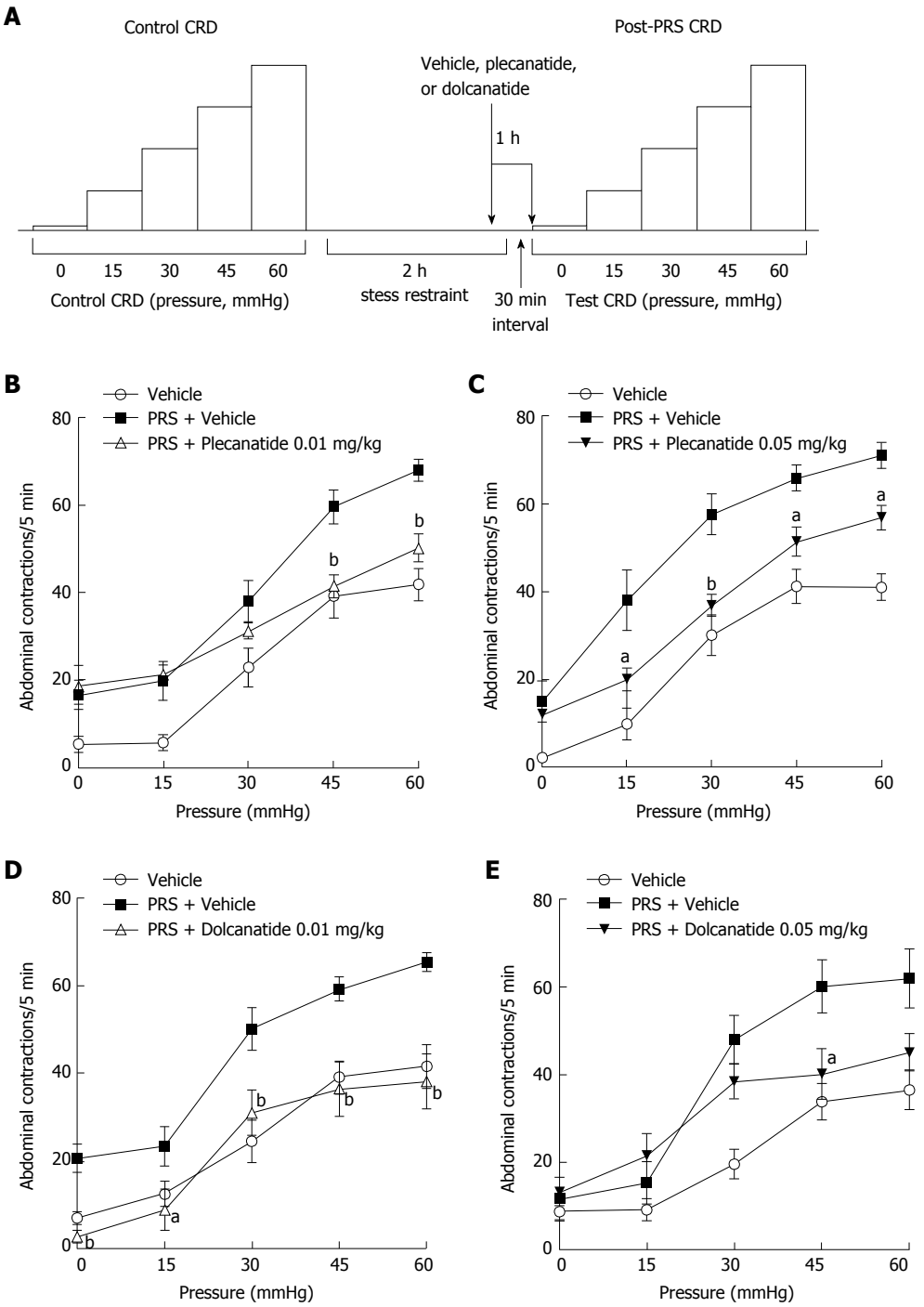

Figure 6 Design and results of the partial restraint stress-induced visceral hypersensitivity models. A: Schematic depicting the sequence of test sessions and treatments to evaluate visceral hypersensitivity induced by PRS in rat models. Effects of oral administration of plecanatide, dolcanatide or vehicle on the increase in abdominal contractions to CRD when tested 30 min after a two h period of partial restraint. Doses of 0.01 and 0.05 mg/kg of plecanatide (B and C) or dolcanatide (D and E) 30 min before completion of the restraint session reduced the rate of muscular contractions toward the levels observed in a previous test session without exposure to partial restraint. Data are the mean ± SEM (n = 8 rats/group). $^{a}P < 0.05$, $^{b}P < 0.01$ as compared to the values for PRS + vehicle control. CRD: Colorectal distention; PRS: Partial restraint stress; SEM: Standard error of the mean.

large intestine[39]. Subsequently, we reported that oral treatment with plecanatide delayed the onset of inflammation-driven colitis to colorectal carcinogenesis in $Apc^{Min/+-FCCC}$ mice[40]. The emerging paradigm suggests that normal function of GC-C signaling may include host defense by limiting systemic dissemination of luminal antigens through maintenance of mucosal barrier function.

Results presented in this study further demonstrate that oral treatment with plecanatide or dolcanatide reduced TNBS- and PRS-induced visceral hypersensitivity, as assessed by reductions in CRD-induced abdominal contractions in rats. However, treatment with either of the GC-C agonists did not alter the colonic compliance

produced by either of the methods to induce visceral hypersensitivity. Only the lower doses (0.01 and 0.05 mg/kg) showed the most significant inhibition of visceral hypersensitivity in both models and that higher doses (> 0.5 mg/kg) of either GC-C agonist were not effective in these models. These results are consistent with the results obtained with orally administered linaclotide, also a GC-C agonist, in the same rat models showing that only the lower doses of linaclotide (0.01 to 0.3 mg/kg) were effective in the TNBS model, whereas the higher doses (3 and 30 mg/kg) were completely ineffective[32]. Although the precise explanation for the discrepancy in dose response remains to be determined, it is possible that the loss of anti-hyperalgesic effect at higher doses is associated with the loss of pharmacological specificity of the treatment. It is known that high levels of cGMP also down-regulate cAMP-specific phosphodiesterases resulting in increased levels of cAMP and activation of cAMP-dependent signaling pathways[42,43]. In this context, elevated levels of cAMP are known to be associated with hyperalgesia. We have also observed a similar bell-shaped response in animal models of colitis, inflammation-driven colorectal carcinogenesis, and polyp formation in $Apc^{Min/+}$ mice[39,40].

Although the molecular mechanisms by which plecanatide or dolcanatide reduce visceral hypersensitivity still remain to be fully elucidated, these drug candidates seem to mimic the physiological function of uroguanylin in activating GC-C, resulting in increased fluid secretion to promote bowel movement. Consistent with this notion, we reported earlier that oral treatment with plecanatide promotes normal bowel movement in adult patients with CIC[11,12]. Based on the data presented herein, it is conceivable that activation of GC-C signaling and the initiation of downstream events may ameliorate the low-grade inflammation and thereby suppress activation of visceral nociceptive sensory pathways in the gut. The unique combination of regulation of ion/fluid secretion and anti-inflammatory activities of plecanatide makes this drug suitable to treat CIC and IBS-C. Oral treatment with plecanatide also demonstrated efficacy and a well-tolerated safety profile in two phase Ⅲ clinical trials in patients with CIC[11,12]. Notably, the efficacy of plecanatide in reducing visceral hypersensitivity in animal models is consistent with the reduction in abdominal pain observed in two large phase Ⅲ IBS-C clinical studies[44]. Thus, GC-C agonists are emerging as promising drug candidates for the treatment of functional GI disorders and IBD[8,11,12,44].

ARTICLE HIGHLIGHTS

Research background

Activation of guanylate cyclase-C (GC-C) signaling is an emerging therapeutic target for the treatment of gastrointestinal disorders and inflammatory diseases. Loss of GC-C signaling may disrupt intestinal water and ion secretion, resulting in chronic idiopathic constipation or irritable bowel syndrome with constipation (IBS-C). In addition, reduced GC-C signaling may also disrupt intestinal barrier function due to increased paracellular permeability, allowing entry of inflammatory mediators to promote low-grade inflammation and visceral hypersensitivity associated with abdominal pain in IBS-C patients.

Research motivation

Plecanatide and dolcanatide are analogs of human uroguanylin, the endogenous agonist of GC-C receptors, and are targeted at the treatment of functional constipation disorders and inflammatory bowel disease (IBD), respectively; therefore we sought to further characterize the mechanisms of these peptides using *in vitro* and *in vivo* models of these diseases.

Research objectives

To discern the role of plecanatide and dolcanatide in the maintenance of mucosal membrane integrity and in the reduction of visceral hypersensitivity in inflammatory and non-inflammatory animal models.

Research methods

Maintenance of epithelial cell integrity by plecanatide or dolcanatide in response to chemical challenge by lipopolysaccharide (LPS) was assessed using cell lines, as well as tissue harvested from rat intestines. Paracellular permeability was determined by calculating the flux of fluorescein isothiocyanate (FITC)-dextran using immunofluorescence microscopy. Electromyographic recordings were used to assess suppression of visceral hypersensitivity by plecanatide or dolcanatide in rat models of inflammatory and non-inflammatory visceral pain.

Research results

Plecanatide or dolcanatide effectively suppressed LPS-induced paracellular permeability. Oral treatment with plecanatide or dolcanatide considerably attenuated visceral hypersensitivity in inflammatory and non-inflammatory models of visceral pain.

Research conclusions

The data presented suggest further mechanisms, in addition to their better known secretory effects, whereby plecanatide or dolcanatide treatment, through activation of the GC-C receptor, may protect the epithelial barrier from increased paracellular permeability and provide anti-nociceptive activity, which may ultimately benefit patients with functional constipation disorders and IBD.

Research perspectives

Plecanatide is a secretagogue approved in the US for the treatment of adults with chronic idiopathic constipation or IBS-C. Dolcanatide is under evaluation for the treatment of opioid-induced constipation and ulcerative colitis. This study provides preclinical evidence that plecanatide and dolcanatide may act to preserve the integrity of the intestinal epithelium and to provide anti-nociceptive activity, supporting ongoing investigations of these peptides in functional constipation disorders and IBD.

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ORIGINAL ARTICLE

Basic Study

Mitochondrial pathway mediated by reactive oxygen species involvement in α-hederin-induced apoptosis in hepatocellular carcinoma cells

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Abstract

AIM

To investigate the antitumor activity of α-hederin in hepatocellular carcinoma (HCC) cells and its underlying mechanisms *in vitro* and *in vivo*.

METHODS

SMMC-7721, HepG-2 and Huh-7 HCC cells were cultured *in vitro* and treated with α-hederin (0, 5 μmol/L, 10 μmol/L, 15 μmol/L, 20 μmol/L, 25 μmol/L, 30 μmol/L, 35 μmol/L, 40 μmol/L, 45 μmol/L, 50 μmol/L, 55 μmol/L, or 60 μmol/L) for 12 h, 24 h, or 36 h, and cell viability was then detected by the Cell Counting Kit-8. SMMC-7721

cells were treated with 0, 5 μmol/L, 10 μmol/L, or 20 μmol/L α-hederin for 24 h with or without DL-buthionine-*S*,*R*-sulfoximine (2 mmol/L) or *N*-acetylcysteine (5 mmol/L) pretreatment for 2 h, and additional assays were subsequently performed. Apoptosis was observed after Hoechst staining. Glutathione (GSH) and adenosine triphosphate (ATP) levels were measured using GSH and ATP Assay Kits. Intracellular reactive oxygen species (ROS) levels were determined by measuring the oxidative conversion of 2′,7′-dichlorofluorescin diacetate. Disruption of the mitochondrial membrane potential was evaluated using JC-1 staining. The protein levels of Bax, Bcl-2, cleaved caspase-3, cleaved caspase-9, apoptosis-inducing factor and cytochrome C were detected by western blotting. The antitumor efficacy of α-hederin *in vivo* was evaluated in a xenograft tumor model.

RESULTS

The α-hederin treatment induced apoptosis of HCC cells. The apoptosis rates in the control, low-dose α-hederin (5 μmol/L), mid-dose α-hederin (10 μmol/L) and high-dose α-hederin (20 μmol/L) groups were 0.90% ± 0.26%, 12% ± 2.0%, 21% ± 2.1% and 37% ± 3.8%, respectively ($P < 0.05$). The α-hederin treatment reduced intracellular GSH and ATP levels, induced ROS, disrupted the mitochondrial membrane potential, increased the protein levels of Bax, cleaved caspase-3, cleaved caspase-9, apoptosis-inducing factor and cytochrome C, and decreased Bcl-2 expression. The α-hederin treatment also inhibited xenograft tumor growth *in vivo*.

CONCLUSION

The α-hederin saponin induces apoptosis of HCC cells *via* the mitochondrial pathway mediated by increased intracellular ROS and may be an effective treatment for human HCC.

Key words: Hepatic carcinoma; α-hederin; Apoptosis; Reactive oxygen species; Mitochondria

Core tip: The α-hederin saponin induces apoptosis of hepatocellular carcinoma cells *in vitro* and *in vivo*. We found that reactive oxygen species and the mitochondrial pathway play a vital role in α-hederin-induced apoptosis.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a highly prevalent disease worldwide, particularly in many Asian countries, with a very high incidence of over 20 cases/100000 individuals[1]. It is the fifth most common malignancy and the second most common cause of cancer-related death, and related deaths increased from 600000 in 2008 to 746000 in 2012[1,2]. It is also recognized as the main cause of death in patients with cirrhosis[3]. HCC treatment mainly includes systemic chemotherapy, radiofrequency ablation, transarterial chemoembolization, ethanol or acetic acid injection, surgical resection, and, in rare cases, liver transplantation[4]. Although resection is the most common therapy, most patients are not eligible for this treatment because of tumor extent or poor hepatic condition[4,5]. Systemic chemotherapy is another possible treatment option, but it often has a low response rate and severe side effects. Multidrug resistance occurs frequently in patients treated with chemotherapy, leading to recurrence and poor survival[6]. The poor general prognosis is related to a low overall survival rate after 5 years, ranging from 24% to 41%[7]. Therefore, it is important to develop highly effective natural treatments with limited toxicity for HCC.

Triterpene saponins are natural amphiphilic compounds that have the potential to induce cancer cell death and increase the activity of chemotherapeutic agents or radiotherapy[8,9]. The α-hederin is a secondary saponin isolated from *Hedera* or *Nigella* species. It is the major active component of various traditional medicinal herbs and shows promising activity against colon and lung cancers. The α-hederin also has biological activities, such as antioxidant activity, antiinflammatory activity, and effects on smooth muscle contraction[10-14]. It is thought to promote cell apoptosis and/or membrane alterations[15], and excess reactive oxygen species (ROS) have been reported to be involved in these processes[16]. Excess ROS can cause oxidative damage to the mitochondrial membrane and trigger apoptosis through downstream signal transduction[17,18].

Reports on the anti-HCC activity of α-hederin are limited. In this study, we evaluated the effects of α-hederin on HCC cells both *in vitro* and *in vivo* and explored the underlying mechanisms.

MATERIALS AND METHODS

Cell lines and culture

The human SMMC-7721, HepG-2 and Huh-7 HCC cell lines were purchased from the Shanghai Cell Collection (Shanghai, China). HCC cells were cultured in DMEM (Gibco, Grand Island, NY, United States) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin. All cells were cultured in a 5% CO_2 humidified incubator at 37 ℃. The α-hederin was purchased from Sigma-Aldrich (St. Louis, MO, United States), dissolved in 100% dimethyl sulfoxide and stored at 5 ℃.

Cell proliferation assays

Cells were seeded at a density of 5×10^3 cells per well

in 96-well plates and then treated with 0, 5 μmol/L, 10 μmol/L, 15 μmol/L, 20 μmol/L, 25 μmol/L, 30 μmol/L, 35 μmol/L, 40 μmol/L, 45 μmol/L, 50 μmol/L, 55 μmol/L, or 60 μmol/L α-hederin for 12 h, 24 h, or 36 h. Cell proliferation was assessed at different times using Cell Counting Kit-8 (Beyotime, Shanghai, China) according to the manufacturer's protocol. Ten microliters of CCK-8 solution was added to each well for 1 h; the absorbance was then measured at 450 nm with a microplate reader (Victor31420 Multilabel Counter; PerkinElmer, Waltham, MA, United States) to calculate the cell viability in different groups.

Cell apoptosis assays

Apoptotic cells were examined using the Hoechst 33258 staining kit (Beyotime). SMMC-7721 cells were treated with 0, 5 μmol/L, 10 μmol/L, or 20 μmol/L α-hederin for 24 h with or without pretreatment with 2 mmol/L DL-buthionine-*S*,*R*-sulfoximine (BSO) (Sigma-Aldrich) or 5 mmol/L *N*-acetylcysteine (NAC) (Sigma-Aldrich) for 2 h and then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. After staining with 20 μmol/L Hoechst 33258 for 20 min, the cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan), and apoptotic cells were identified by fragmented and condensed nuclei.

Measurement of intracellular glutathione and adenosine triphosphate

Glutathione (GSH) and adenosine triphosphate (ATP) levels were measured using a GSH Assay Kit (Beyotime) and an ATP Assay Kit (Beyotime). SMMC-7721 cells were treated with 0, 5 μmol/L, 10 μmol/L, or 20 μmol/L α-hederin for 24 h with or without pretreatment with BSO (2 mmol/L) or NAC (5 mmol/L) for 2 h, and the subsequent procedures were performed according to the manufacturers' instructions. The experimental data were obtained with a microplate reader.

ROS detection

Intracellular ROS levels were determined by measuring the oxidative conversion of 2′,7′-dichlorofluorescin diacetate (DCFH-DA) to the fluorescent compound dichlorofluorescin (DCF) using a ROS Assay Kit (Beyotime). After treatment with 0, 5 μmol/L, 10 μmol/L, or 20 μmol/L α-hederin for 24 h with or without pretreatment with BSO (2 mmol/L) or NAC (5 mmol/L) for 2 h, SMMC-7721 cells cultured in 6- and 96-well plates were incubated with 10 μmol/L DCF-DA for 20 min at 37 ℃. Cells cultured in 6-well plates were observed under an upright fluorescence microscope, while cells in 96-well plates were evaluated with a microplate reader.

Mitochondrial membrane potential ($\Delta\Psi_m$)

Changes in the $\Delta\Psi_m$ were identified using JC-1 dye according to the manufacturer's specifications. SMMC-7721 cells were pretreated with BSO (2 mmol/L) or NAC (5 mmol/L) for 2 h, treated with 0 or 10 μmol/L α-hederin for 24 h, and then incubated with 1 mL of the JC-1 dye for 30 min in a 37 ℃ incubator. The cells were washed twice with PBS and then evaluated with a confocal laser scanning microscope (Olympus). JC-1 forms a red fluorescent aggregate at hyperpolarized membrane potentials, whereas it remains in the green fluorescent monomeric form at depolarized membrane potentials.

Western blot analysis

Total cellular protein was extracted on ice using RIPA lysis buffer containing protease inhibitors (Beyotime). Proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% nonfat dry milk and incubated overnight with various primary antibodies at 4 ℃. Next, antirabbit secondary antibodies were added for 1 h at room temperature. Band intensity was measured using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, United States).

Xenograft tumor model

All animal experiments were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Wuhan University. The animal protocol was designed to minimize animal pain and discomfort. The animals were acclimatized to laboratory conditions (23 ℃, 12 h/12 h light/dark cycle, 50% humidity, and *ad libitum* access to food and water) for 1 wk prior to experimentation. All animals were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) after being fasted overnight, and tissues were collected.

The antitumor efficacy of α-hederin *in vivo* was evaluated using a xenograft tumor model. Male BALB/c-nu/nu nude mice (4-6 wk old) were purchased from HFK Experimental Animal Center (Beijing, China). HCC cells (5.0×10^6) suspended in 100 μL of PBS were subcutaneously inoculated into the right dorsal flank of nude mice. When the tumors reached 100-150 mm^3, the mice were randomly divided into four groups (n = 6 per group): control group, low-dose group (2.5 mg/kg), mid-dose group (5 mg/kg), and high-dose group (10 mg/kg). The α-hederin was administered *via* intraperitoneal injection every 3 d.

To create the tumor growth curve, the diameter of each xenograft tumor was measured with a caliper. The mice were weighed every 3 d. At the end of the experiment, xenotransplanted tumors, livers, lungs and brains were harvested for additional analysis. Mouse blood was collected for hepatic and renal function tests.

Hematoxylin and eosin and TUNEL staining

To further evaluate treatment efficiency, the tumors were dissected and fixed in 4% formaldehyde. Next, tumors were sectioned into slices and stained with hematoxylin and eosin (HE) for histological analysis.

We performed TUNEL staining to detect apoptotic cells. Positive cells were identified, counted (eight random fields per slide), and analyzed by light microscopy (Olympus).

Statistical analysis

All data were collected from at least three independent experiments. One-way analysis of variance (ANOVA) and *t*-tests were performed to analyze all the data (SPSS 20.0 software; IBM Corp., Armonk, NY, United States). $P < 0.05$ indicated statistical significance.

RESULTS

α-hederin reduces HCC cell viability and induces apoptosis of HCC cells via GSH depletion and ROS accumulation

To investigate the effects of α-hederin on HCC cell growth, we treated HCC cells with different concentrations of α-hederin for 0, 12 h, 24 h, and 36 h. As shown in Figure 1A, α-hederin significantly reduced HCC cell viability in a dose- and time-dependent manner, with IC_{50} values at 24 h for SMMC-7721, HepG-2 and Huh-7 cells being 13.880 μmol/L, 18.450 μmol/L and 25.520 μmol/L, respectively. We further use the one-way ANOVA to analyze the IC_{50} values for each time period with SMMC-7721, HepG-2 and Huh-7 cells; there was statistical significance among the IC_{50} value of three time periods ($P < 0.05$).

The Hoechst 33258 staining results are shown in Figure 1B; α-hederin induced the apoptosis of HCC cells in a dose-dependent manner. The apoptosis rates in the control, low-dose α-hederin (5 μmol/L), mid-dose α-hederin (10 μmol/L) and high-dose α-hederin (20 μmol/L) groups were 0.90% ± 0.26%, 12% ± 2.0%, 21% ± 2.1% and 37% ± 3.8%, respectively ($P < 0.05$).

To determine whether α-hederin affected the intracellular ROS generation, SMMC cells were treated with α-hederin for 24 h. As shown in Figure 1C, the relative DCFH-DA fluorescence significantly increased in a dose-dependent manner. The α-hederin significantly reduced cellular GSH (Figure 1D) and ATP levels (Figure 1E) ($P < 0.05$). These results show that α-hederin may reduce HCC cell viability and induce the apoptosis of HCC cells *via* GSH depletion and ROS accumulation.

BSO and NAC influence α-hederin-induced apoptosis of SMMC-7721 cells

To further determine whether α-hederin induces the apoptosis of HCC cells *via* GSH depletion and ROS accumulation, SMMC cells were treated with 10 μmol/L α-hederin for 24 h with or without BSO (2 mmol/L) or NAC (5 mmol/L) pretreatment for 2 h. As shown in Figure 2A, the apoptosis rate varied as expected: 0.94% ± 0.25% in the control group, 22% ± 2.4% in the α-hederin group, 27% ± 3.5% in the α-hederin and BSO group, and 13% ± 3.3% in the α-hederin and NAC group ($P < 0.05$). Intracellular ROS levels are shown in Figure 2B.

Relative DCFH-DA fluorescence was significantly increased in the α-hederin (10 μmol/L) group compared to the control group ($P < 0.05$), and this increase was enhanced in the α-hederin and BSO group but reduced in the α-hederin and NAC group ($P < 0.05$). As shown in Figure 2C and D, intracellular GSH and ATP levels were significantly decreased in the α-hederin (10 μmol/L) group compared to the control group ($P < 0.05$), and these decreases were enhanced in the α-hederin and BSO group but reduced in the α-hederin and NAC group ($P < 0.05$). This result suggested that α-hederin induced apoptosis of HCC cells in an indirect way which is closely related to GSH and ROS.

α-hederin induces apoptosis through activation of the mitochondria-mediated pathway

To investigate the underlying mechanism of apoptosis induced by α-hederin, we ascertained the effect of α-hederin on mitochondrial membrane depolarization using the JC-1 cationic dye. Compared to the control group, the ratio of aggregate-to-monomer fluorescence in the α-hederin (10 μmol/L) group was decreased ($P < 0.05$), as JC-1 fluorescence changed from red (aggregate) to green (monomer) (Figure 3A). Compared to that in the α-hederin group, the aggregate-to-monomer fluorescence ratio was decreased in the α-hederin and BSO group and increased in the α-hederin and NAC group ($P < 0.05$).

Then, we conducted western blotting to examine the effect of α-hederin on the levels of mitochondrial pathway-related proteins. As shown in Figure 3B, α-hederin increased the levels of Bax, cleaved caspase-3 and cleaved caspase-9, and decreased Bcl-2 expression levels. Meanwhile, the mitochondria-mediated apoptosis-related proteins apoptosis-inducing factor (AIF) and cytochrome C (Cyt C) in cytoplasm were increased by α-hederin, but AIF and Cyt C in mitochondria were decreased (Figure 3C). Pretreatment with BSO augmented the α-hederin-induced changes in protein levels, whereas pretreatment with NAC weakened these effects of α-hederin.

α-hederin inhibits tumor growth in vivo

The anticancer effects of α-hederin *in vivo* were analyzed in a human xenograft tumor model. As shown in Figure 4A, the transplanted tumor volume increased more slowly with increasing α-hederin concentration, and the final tumor weight was lower in the α-hederin-treated groups. At the end of the experiment, the tumor weights in the control and 2.5 mg/kg, 5 mg/kg and 10 mg/kg α-hederin groups were 1217 mg ± 177 mg, 917 mg ± 84 mg, 778 mg ± 105 mg and 539 mg ± 96 mg, respectively. Tumor growth was significantly suppressed in the α-hederin groups in a dose-dependent manner ($P < 0.05$). TUNEL staining of the tumors is shown in Figure 4B, and cells stained brown are apoptotic. Compared to the control group, the α-hederin groups showed a gradual increase in the proportion of apoptotic cells with increasing drug concentration ($P < 0.05$).

Liver, lung and brain tissue from each group was

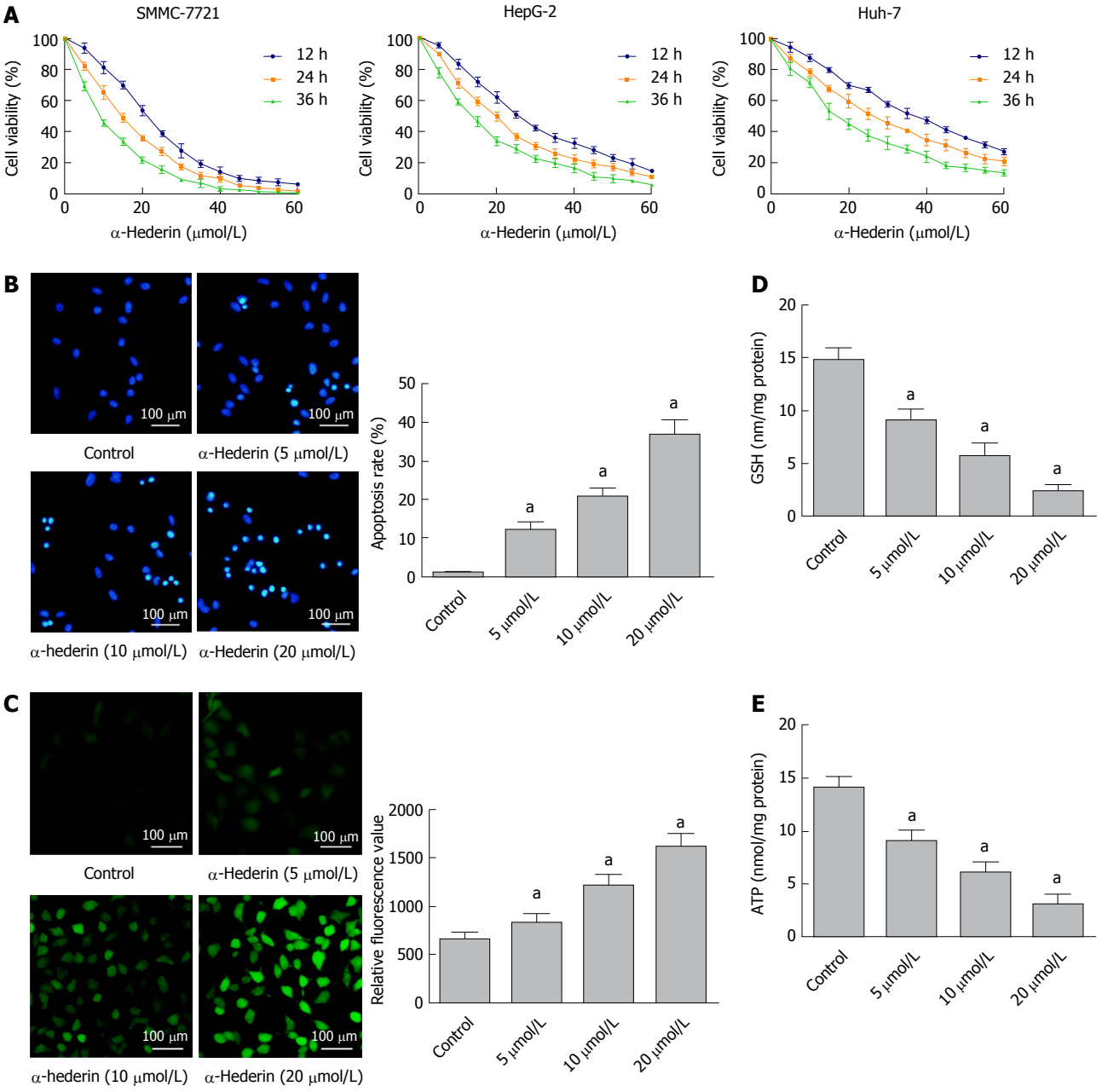

Figure 1 α-hederin reduces hepatocellular carcinoma cell viability and induces the apoptosis of hepatocellular carcinoma cells through GSH depletion and reactive oxygen species accumulation. A: Cell Counting Kit-8 assays showed that α-hederin inhibits the viability of hepatocellular carcinoma cells (SMMC-7721, HepG-2, and Huh-7) in a dose- and time-dependent manner; B: SMMC-7721 cells were incubated with α-hederin (0, 5 μmol/L, 10 μmol/L, or 20 μmol/L) and stained with Hoechst 33258. Apoptotic cells were identified by fragmented and condensed nuclei under a fluorescence microscope. The percentage of apoptotic cells was calculated, *P* for trend < 0.01; C: SMMC-7721 cells were incubated with α-hederin (0, 5 μmol/L, 10 μmol/L, or 20 μmol/L), followed by incubation with DCFH-DA and observation under a fluorescence microscope or measurement using a microplate reader, *P* for trend < 0.01; D and E: SMMC-7721 cells were treated with α-hederin (0, 5 μmol/L, 10 μmol/L, or 20 μmol/L). GSH and ATP levels were measured using GSH and ATP Assay Kits and a microplate reader, *P* for trend < 0.01. $^{a}P < 0.05$ *vs* control. ATP: adenosine triphosphate; GSH: Glutathione; ROS: Reactive oxygen species.

stained with HE, and no tumor metastases were observed. We assayed the hepatic and renal functions of nude mice treated with control or α-hederin and found that alanine aminotransferase, aspartate aminotransferase, urea and creatine levels were not significantly different.

DISCUSSION

The α-hederin saponin has various biological activities, including anticancer activity in some cancer cells. However, its effects on HCC have not been clarified. In the present study, to investigate the effects of α-hederin on HCC cells, we performed the following: Cell proliferation and apoptosis assays; detected ROS, GSH and ATP levels and the mitochondrial membrane potential; conducted Western blotting analysis to examine related proteins; and generated a xenograft tumor model to evaluate the antitumor efficacy of α-hederin *in vivo*. Our results show that α-hederin

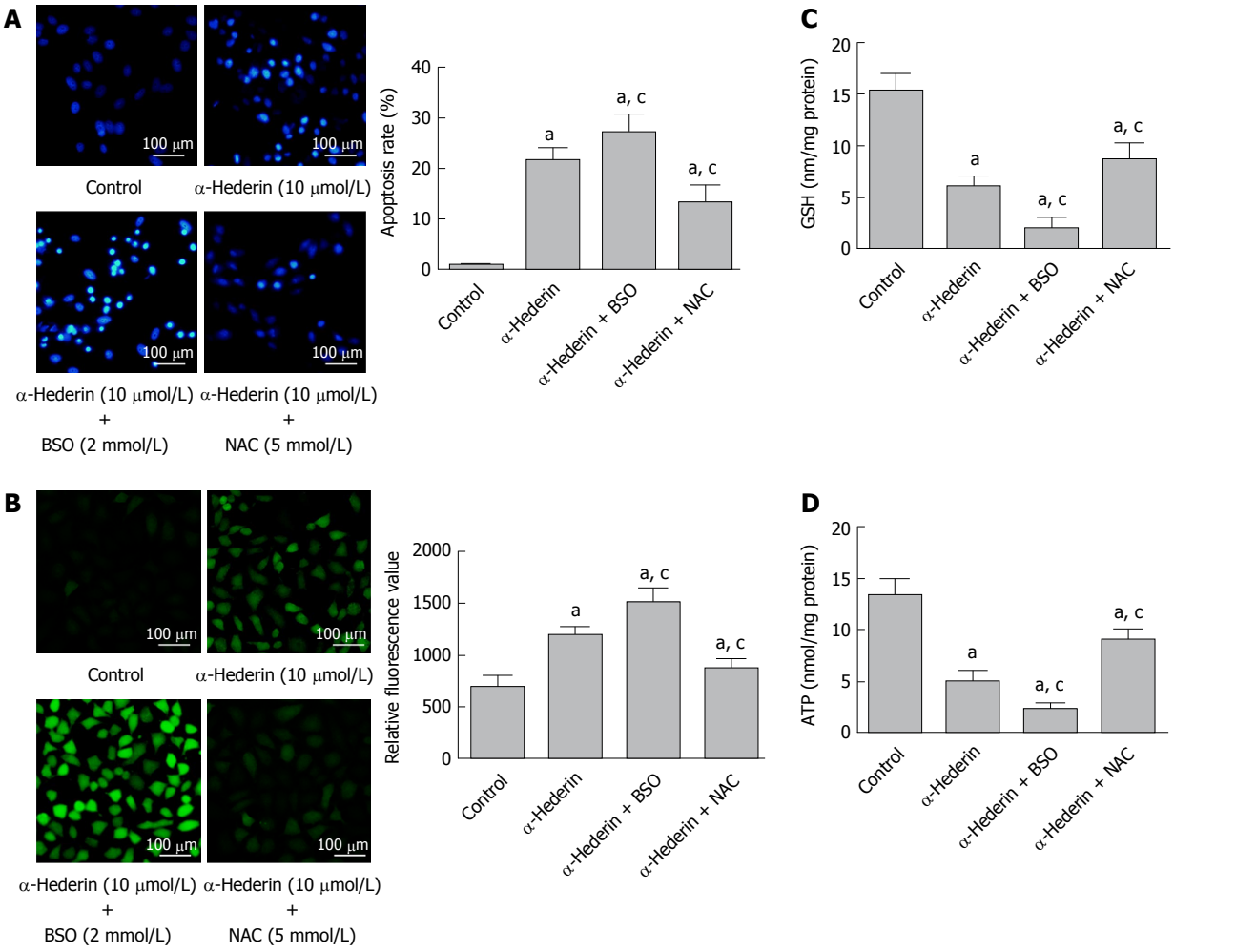

Figure 2 BSO and NAC influence the α-hederin-induced apoptosis of SMMC-7721 cells. SMMC-7721 cells were incubated with α-hederin (10 μmol/L) with or without BSO (2 mmol/L) or NAC (5 mmol/L) pretreatment. A: Cell apoptosis was determined by Hoechst 33258 staining; B: ROS levels in SMMC-7721 cells; C and D: Effect of α-hederin on intracellular GSH and ATP levels. $^{a}P < 0.05$ *vs* control; $^{c}P < 0.05$ *vs* α-hederin (10 μmol/L). ATP: adenosine triphosphate; BSO: DL-buthionine-*S*,*R*-sulfoximine; GSH: Glutathione; NAC: *N*-acetylcysteine.

induces the apoptosis of HCC cells *in vitro* and *in vivo* and suggest that the mechanism involves the mitochondrial pathway mediated by increased intracellular ROS.

In this study, we found that α-hederin significantly inhibited the proliferation of HCC cells and induced their apoptosis in a dose- and time-dependent manner. We also found that α-hederin decreased GSH and ATP levels and increased ROS levels in a concentration-dependent manner. These results are consistent with those of Swamy *et al*[16], who reported that α-hederin increased the apoptosis of murine P388 leukemia cells and increased the production of ROS in a dose- and time-dependent manner. It has been reported that cancer cells have increased ROS production compared to normal cells. ROS is generated through a variety of extracellular and intracellular actions. Severe accumulation of cellular ROS may induce lethal damage in cells.

GSH is one of the most common intracellular compounds that plays a vital role in the cellular defense against ROS damage. GSH clears intracellular ROS by nonenzymatic and enzymatic catalysis. The non-enzymatic process involves GSH acting directly. The enzyme catalyzed process is based on GSH as the substrate, and induces the clearance of ROS in cells under the catalysis of GSH-peroxidase or GSH S transferase[19,20]. During intracellular GSH synthesis, two ATP-dependent enzyme catalyzes are required: Glutamate cysteine ligase and glutathione synthetase[21].

Our study shows α-hederin significantly reduced cellular ATP levels. Therefore, a reduction in intracellular ATP contributes to a decrease in GSH, leading to ROS accumulation and cellular damage. To determine whether the apoptotic effect of α-hederin on HCC cells is associated with the generation of intracellular ROS, we pretreated SMMC-7721 cells with BSO or NAC, which improved/decreased the levels of intracellular GSH and ROS. The results showed that the apoptotic effect of α-hederin was greater after pretreatment with BSO but was ameliorated by NAC. These data indicate that the apoptosis-inducing potential of α-hederin is related to intracellular ROS production.

Mitochondria play an important role in cancer

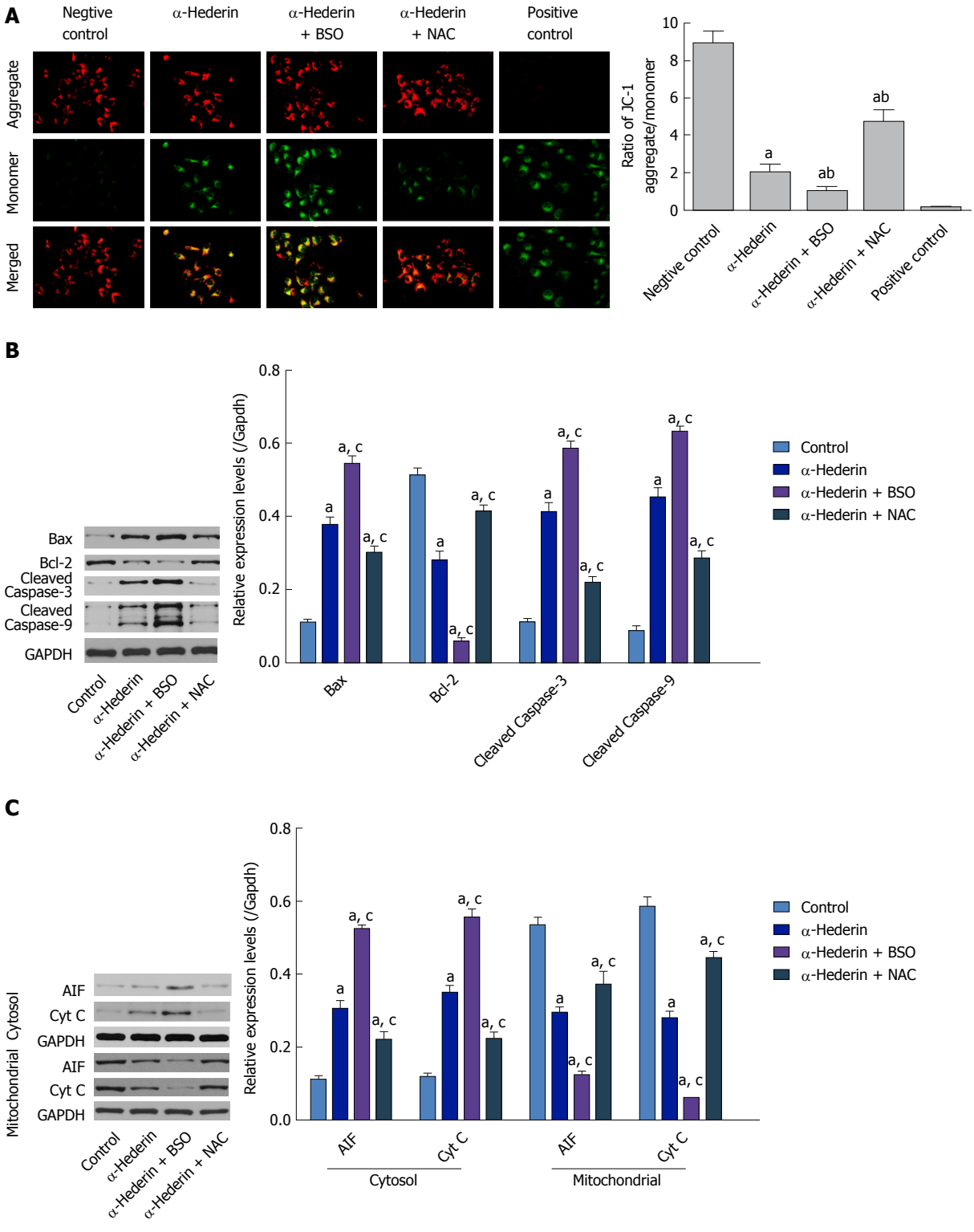

Figure 3 α-hederin induces apoptosis through activation of the mitochondria-mediated pathway. A: Mitochondrial membrane potential was detected with JC-1. JC-1 aggregates (red fluorescence) under conditions of a normal mitochondrial membrane and forms a monomer (green fluorescence) under depolarizing conditions. Fluorescence was detected by a confocal laser scanning microscope (400 ×); B and C: Western blots showing the expression of mitochondrial pathway-related proteins *in vitro*. SMMC-7721 cells were treated with α-hederin (0 or 10 μmol/L) with or without BSO (2 mmol/L) or NAC (5 mmol/L) pretreatment, and the protein levels of Bcl-2, Bax, caspase-9, caspase-3, AIF, and Cyt C in SMMC-7721 cells were then detected by western blotting. GAPDH expression was used as an internal control. The relative expression levels of these proteins in SMMC-7721 cells in different groups were compared. $^{a}P < 0.05$ *vs* control; $^{c}P < 0.05$ *vs* α-hederin (10 μmol/L). AIF: Apoptosis-inducing factor; ATP: adenosine triphosphate; BSO: DL-buthionine-*S*,*R*-sulfoximine; Cyt C: Cytochrome C; NAC: *N*-acetylcysteine.

cell survival[22], as they are major sources of cellular bioenergetics and the target of ROS. ROS can induce oxidative damage that affects mitochondrial function, and a decrease in $\Delta\Psi_m$ indicates damage to

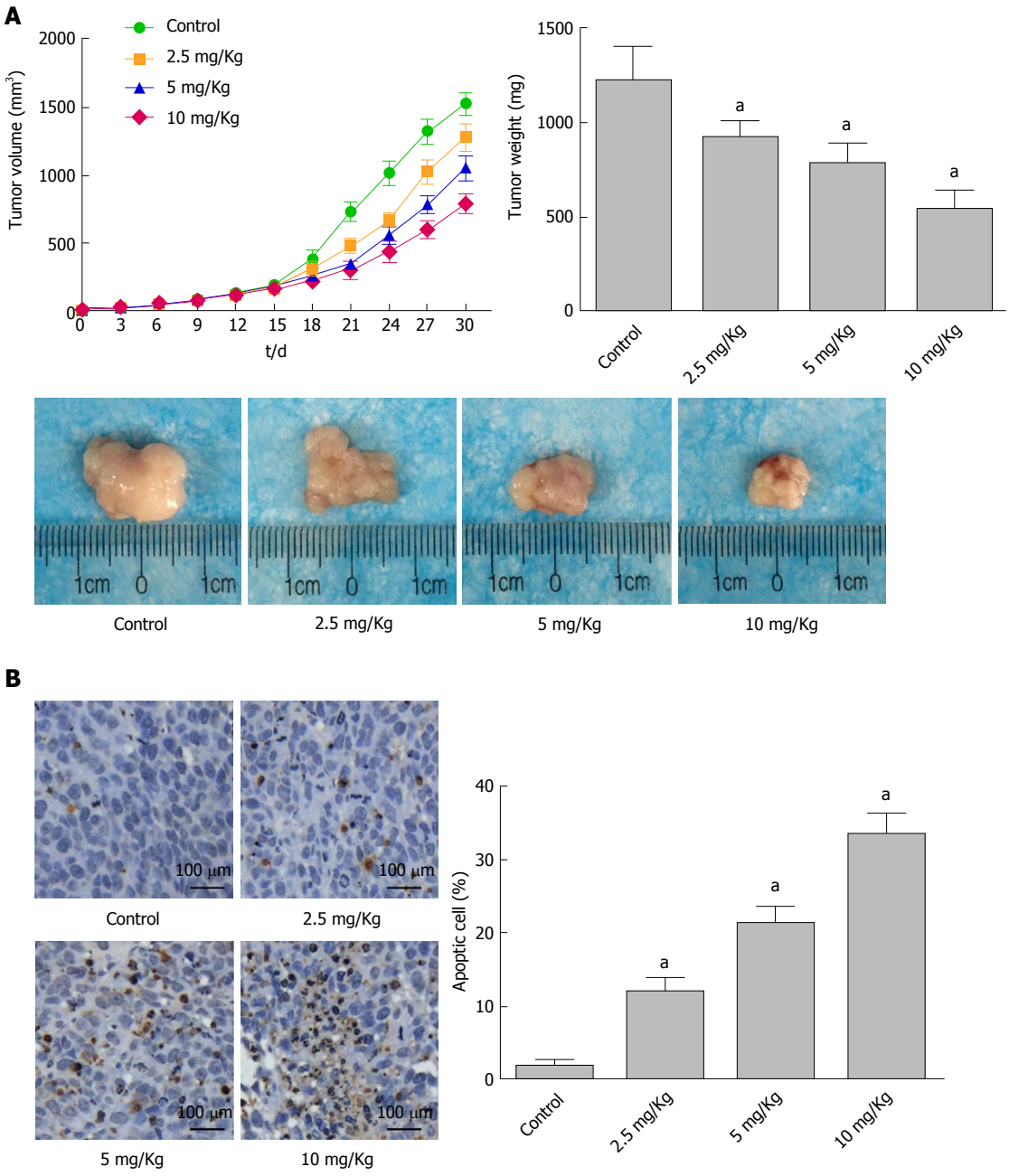

Figure 4 **α-Hederin inhibits tumor growth *in vivo*.** Mice with xenograft tumors were divided into four groups (Control and 2.5 mg/kg, 5 mg/kg and 10 mg/kg α-Hederin, n = 6 mice per group). A: Mean tumor volume at each time point and final tumor weight, P for trend < 0.05; B: TUNEL assays detected apoptotic cells in xenograft tumor tissue, as evidenced by the presence of nut-brown nuclei under a fluorescence microscope. The percentage of apoptotic cells was calculated, P for trend < 0.05. ^{a}P < 0.05 *vs* control.

mitochondrial function. Cheng *et al*[23] reported on the mitochondrial apoptotic activity of α-hederin in breast cancer cells. A previous study showed that ROS causes the mitochondrial permeability transition pore (mPTP) to open in HepG-2 cells[24]. We next evaluated whether ROS induced this mitochondria-mediated apoptotic mechanism in HCC cells treated with α-hederin. Similar to breast cancer cells, SMMC-7721 cells treated with α-hederin showed a clear decrease in $\Delta\Psi_m$ compared to untreated cells. Additionally, the $\Delta\Psi_m$ decrease was aggravated by BSO but relieved by NAC.

To further investigate whether the ROS increase and $\Delta\Psi_m$ loss induced by α-hederin led to HCC cell apoptosis, we detected the levels of related proteins. We found that α-hederin increased the protein levels of Bax, cleaved caspase-3 and cleaved caspase-9 but decreased Bcl-2 levels. Thus, the antiapoptotic/proapoptotic (Bcl-2/Bax) protein ratio decreased. AIF and Cyt C protein levels were increased by α-hederin. Although the α-hederin-induced changes in the above proteins were enhanced by pretreatment with BSO, they were weakened by NAC pretreatment. Bcl-2 family proteins are reported to be key factors in regulating the mitochondrial apoptosis pathway[25]. Disruption of the Bcl-2/Bax protein balance

induces apoptosis.

Bcl-2 family proteins are also components of the mPTP. A decrease in Bcl-2 levels alters the mPTP structure and the $\Delta\Psi_m$, increasing mitochondrial membrane permeability[26]. Additionally, excess ROS can trigger opening of the mPTP[27]. As a result, AIF and Cyt C proteins are released to activate procaspase-9, which activates the caspase cascade that ultimately generates caspase-3 to induce apoptosis. On the other hand, AIF can mediate apoptosis directly in a caspase-independent way[28]. These data indicate that the mechanism by which α-hederin induces HCC cell apoptosis involves the mitochondrial pathway mediated by increased intracellular ROS.

In human xenograft tumor models in nude mice, α-hederin significantly inhibited tumor growth without causing liver and kidney damage, indicating the efficacy and safety of α-hederin for the treatment of HCC *in vivo*.

In conclusion, we show herein that α-hederin induces the apoptosis of HCC cells *via* the mitochondrial pathway mediated by increased intracellular ROS *in vitro* and *in vivo*. These findings identify α-hederin as a potential highly effective natural medicine with limited toxicity for HCC treatment. However, α-hederin has been reported to have other effects, such as membrane permeabilizing activity, which can directly induce cell death[29]. This study is not sufficient to clarify the antitumor effects of α-hederin. Further studies should focus on the detailed mechanism.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is a highly prevalent disease worldwide, with poor general prognosis. To develop highly effective natural treatments with limited toxicity for HCC is important. The α-hederin saponin is reported to have antitumor activity. However, the effect of α-hederin on HCC remains to be examined. We evaluated the effect and possible mechanism of α-hederin on HCC cells both *in vitro* and *in vivo*.

Research motivation

Developing new, effective and nontoxic chemotherapeutic drugs will contribute to the treatment and prognosis for HCC patients in clinic.

Research objectives

To investigate the antitumor activity of α-hederin in HCC cells and its underlying mechanisms *in vitro* and *in vivo*.

Research methods

Three HCC cells lines (SMMC-7721, HepG-2 and Huh-7 HCC cells) were used to detect the effect of α-hederin on HCC. Cell viability was detected by Cell Counting Kit-8 assay after cells were treated with α-hederin. (BSO) *N*-acetylcysteine (NAC) and DL-buthionine-*S*,*R*-sulfoximine (BSO) were used to interfere with the synthesis of glutathione (GSH) in the SMMC-7721 cells, then, the effects of α-hederin on cell proliferation, cell apoptosis, adenosine triphosphate (ATP) and reactive oxygen species (ROS) and mitochondrial membrane potential were detected. The protein levels of Bax, Bcl-2, cleaved caspase-3, cleaved caspase-9, apoptosis-inducing factor (AIF) and cytochrome C (Cyt C) were detected by western blotting. The antitumor efficacy of α-hederin on HCC was also evaluated in nude mice with xenograft tumor. The apoptosis of cancer cells in xenograft tumor were examined by TUNEL staining. In this research, as we used NAC and BSO to interfere with the synthesis of GSH, the mechanism we explored was more persuasive.

Research results

The α-hederin treatment inhibited cell growth of the three cell lines in a dose- and time-dependent manner. The IC_{50} values at 24 h for SMMC-7721, HepG-2 and Huh-7 cells were 13.88, 18.45 and 25.52 μmol/L, respectively, so we used SMMC-7721 cells for the on-going experiments. The results showed that the apoptosis rates in the control, low-dose α-hederin (5 μmol/L), mid-dose α-hederin (10 μmol/L) and high-dose α-hederin (20 μmol/L) groups were 0.90% ± 0.26%, 12% ± 2.05, 21% ± 2.15 and 37% ± 3.8%, respectively. In comparison to the control, after treatment with α-hederin, ROS increased significantly, while the ATP levels decreased. When SMMC-7721 cells were pretreated with BSO (2 mmol/L), compared with the mid-dose α-hederin group, the apoptosis rate increased to 27% ± 3.5% ($P < 0.05$); what's more, the increase of ROS and the decrease of ATP were both enhanced. However, NAC pretreatment had a protective effect on SMMC-7721 cells and could alleviate the change of ROS and ATP. The proteins involving in the mitochondria-mediated pathway were detected by western blotting. The results showed α-hederin increased the levels of Bax, cleaved caspase-3 and cleaved caspase-9, and decreased Bcl-2 expression levels. Meanwhile, AIF and Cyt C in cytoplasm were up-regulated, but AIF and Cyt C in mitochondria were down-regulated. Subcutaneous xenografts were successfully constructed in 24 nude mice. After treatment with α-hederin for 3 wk, the weight of xenograft tumor was significantly reduced ($P < 0.05$). Compared to the control group, TUNEL staining showed a gradual increase in the proportion of apoptotic cells with the increase of α-hederin concentration ($P < 0.05$). There was no difference between the control mice and α-hederin-treated mic for the hepatic and renal functions. This research indicated that α-hederin could induce HCC cell apoptosis *via* mitochondria-mediated pathway by depleting GSH and accumulating ROS. But it did not explain how α-hederin changed the expression of GSH and ROS, and the effect of α-hederin on HCC cell invasion was not studied either. In addition, apoptosis involves multiple factors and multiple links, making it necessary to conduct in-depth research to clarify the specific mechanism.

Research conclusions

The α-hederin saponin induces apoptosis of HCC cells *via* the mitochondrial pathway mediated by increased intracellular ROS and may be an effective treatment for human HCC.

Research perspectives

It is of great value to discover natural anticancer compounds which have high efficacy and low toxicity in the treatment of HCC. In our study, we show that α-hederin could induce HCC cell apoptosis *via* mitochondria-mediated pathway by depleting GSH and accumulating ROS, which identifies α-hederin as a potential highly effective natural medicine with limited toxicity for HCC treatment. But some points remain unclear. How does α-hederin change the expression of ATP? The effect of α-hederin on HCC cell migration and invasion was not studied, either. In addition, apoptosis involves multiple factors and multiple links, and it's necessary to conduct in-depth research to clarify specific mechanism. These results will facilitate the development of treatment for HCC.

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ORIGINAL ARTICLE

Retrospective Study

Usefulness of three-dimensional visualization technology in minimally invasive treatment for infected necrotizing pancreatitis

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Author contributions: Wang PF, Liu ZW and Cai SW carried out the studies, participated in collecting the data, and drafted the manuscript; He L and Feng J performed the statistical analysis and participated in its design; Su JJ, Xin XL and Lu SC helped to draft the manuscript; all authors read and approved the final manuscript.

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Abstract

AIM

To explore the value of three-dimensional (3D) visualization technology in the minimally invasive treatment for infected necrotizing pancreatitis (INP).

METHODS

Clinical data of 18 patients with INP, who were admitted to the PLA General Hospital in 2017, were retrospectively analyzed. Two-dimensional images of computed tomography were converted into 3D images based on 3D visualization technology. The size, number, shape and position of lesions and their relationship with major abdominal vasculature were well displayed. Also, percutaneous catheter drainage (PCD) number and puncture paths were designed through virtual surgery (percutaneous nephroscopic necrosectomy) based on the principle of maximum removal of infected necrosis conveniently.

RESULTS
Abdominal 3D visualization images of all the patients were well reconstructed, and the optimal PCD puncture paths were well designed. Infected necrosis was conveniently removed in abundance using a nephroscope during the following surgery, and the median operation time was 102 (102 ± 20.7) min. Only 1 patient underwent endoscopic necrosectomy because of residual necrosis.

CONCLUSION
The 3D visualization technology could optimize the PCD puncture paths, improving the drainage effect in patients with INP. Moreover, it significantly increased the efficiency of necrosectomy through the rigid nephroscope. As a result, it decreased operation times and improved the prognosis.

Key words: Infected necrotizing pancreatitis; Three-dimensional visualization; Percutaneous catheter drainage; Percutaneous nephroscopic necrosectomy

Core tip: As a lethal disease, infected necrotizing pancreatitis is gradually treated by minimally invasive surgery. Percutaneous catheter drainage (PCD) is the prerequisite of various minimally invasive treatment, which has been of great significance for prognosis of the disease. In this study, three-dimensional (3D) visualization technology was used preoperatively to optimize the puncture position and direction of PCD path. As a result, it improved the drainage effect and increased the efficiency of subsequent necrosectomy. So, the 3D visualization technology was great help for the prognosis of infected necrotizing pancreatitis.

Wang PF, Liu ZW, Cai SW, Su JJ, He L, Feng J, Xin XL, Lu SC. Usefulness of three-dimensional visualization technology in minimally invasive treatment for infected necrotizing pancreatitis. *World J Gastroenterol* 2018; 24(17): 1911-1918 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i17/1911.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i17.1911

INTRODUCTION

Infected necrotizing pancreatitis (INP), which often leads to sepsis and multiple organ failure, is one of the most severe complications of acute pancreatitis[1,2]. In recent years, various kinds of minimally invasive treatments have achieved good results in treating INP and improved prognosis of the patients[3,4]. However, irrespective of the kind of minimally invasive surgeries applied, the prerequisite has been to establish a convenient surgical approach through preoperative percutaneous catheter drainage (PCD)[5,6]. Whether the puncture paths of PCD were ultimately appropriate was related to not only the effect of drainage but also the efficiency of subsequent minimally invasive removal of infected necrotic tissues.

In recent years, three-dimensional (3D) visualization technology has been widely applied in hepatobiliary and pancreatic surgeries, helping surgeons to intuitively identify the relationship between the shape of lesions and the important anatomical structures around[7,8]. Therefore, this technique was applied in our center on patients with INP to improve the quality of PCD since January 2017. This study retrospectively analyzed the clinical data of enrolled patients to investigate the value of the 3D visualization technique in guiding PCD of INP.

MATERIALS AND METHODS

General data

Eighteen patients [12 males and 6 females, with an average age of 46 (51 ± 12.9) years] were enrolled in this study. The inclusion criteria were as follows: (1) acute pancreatitis with pancreatic or peripancreatic necrosis accompanied by infection but without any invasive treatment; and (2) patients or their families accepting the evaluation of 3D visual reconstruction. The exclusion criteria were as follows: patients with acute pancreatitis without local complications or liquefied necrotic lesions without infection in a stable condition. According to the 2012 Atlanta classification of acute pancreatitis[9], 9 patients had moderately severe acute pancreatitis and the other 9 patients had severe acute pancreatitis. This study was approved by the ethics committee of the PLA General Hospital.

Methods

Data acquisition and 3D reconstruction: The 128-slice spiral computed tomography (CT) scanner (GE Corporation, Stamford, CT, United States) was used for obtaining abdominal enhanced CT scans of arterial and portal venous phases. The layer thickness was 1.5 mm, and the layer distance was 1.5 mm. Lipiodol was injected as the contrast medium (Beilu Pharmaceutical Co., Beijing, China); the concentration was 350 mg/mL. A high-pressure syringe was used for elbow vein injection. The dose was 60 mL, and the injection rate was 4 mL/s. The collected image data were stored in the form of Digital Imaging and Communications in Medicine and introduced into 3D visualization system (Mimics 17.0; Materialise Co., Leuven, Belgium) for reconstruction.

Anatomical evaluation and determination of position and paths of optimal puncture points: The reconstructed model could be viewed from any direction (magnified, contracted, rotated, or transparent) through the 3D visualization system. Therefore, the size, shape, position and number of the lesions and surrounding structure were intuitively demonstrated. Moreover, virtual operations could be done on the 3D image to observe different debridement ranges through various puncture points. At last, the optimal puncture number and paths were determined based on the following

principles. First, retroperitoneal access was preferred to transabdominal access, which meant less intraperitoneal contamination. Second, the paths were established along the longitudinal axis of the necrotic cavity, and the puncture point should be as close to the necrotic cavity as possible, facilitating the maximum removal of necrosis. Third, multiple drains should be placed during the same procedure, if necessary, to avoid the visual blind area or operational blind area, which were also estimated using the 3D virtual system.

PCD and percutaneous nephroscopic necrosectomy for removing necrotic tissues: PCD was performed under the guidance of CT in strict accordance with the position and direction of puncture points designed by the 3D visualization system. Subsequently, an anti-inflammatory drug was administered according to the drainage culture and the results of drug sensitivity test. The changes in the disease were assessed with a reexamination of abdominal CT scan weekly. If the condition did not improve obviously or continued to aggravate, the percutaneous nephroscopic necrosectomy was performed under general anesthesia. Briefly, a 1.2-cm skin incision was made that was centered on the PCD. Amplatz renal dilators (Cook Urological Incorporated, Bloomington, IN, United States) were used to serially dilate to create a 30F tract, following which a 12-mm trocar was inserted.

An operating nephroscope (Hopkins Telescopes; Karl Storz-Endoskope, Tuttingen, Germany) with an 8-mm working channel was then passed through the trocar into the necrotic cavity. Subsequently, the piecemeal removal of solid necrosis was performed repeatedly using a fenestrated grasper through the working channel. Finally, a 10F catheter sutured to a tube drain of 28F was placed into the distal end of the necrotic cavity to allow continuous lavage after surgery. All patients underwent CT scanning reexamination weekly to evaluate the results of necrosectomy and drain placement. The operation might be performed again, if necessary according to the changes in the condition. The position and range of the lesion, the time and number of surgery, postoperative complications, and the time of hospitalization of each patient were recorded.

Statistical analysis

The SPSS 17.0 software (SPSS Inc., Chicago, IL, United States) was used for statistical analysis. Measurement data were expressed as $\chi \pm s$.

RESULTS

Results of 3D reconstruction

The 3D reconstruction was successfully completed for all of the 18 patients. The results clearly showed the shape, size and number of necrotic lesions, as well as the anatomical relationship with surrounding blood vessels and organs. Thus, the stereoscopic visual observation of the lesions from any angle and virtual surgery using the aforementioned software system were performed. Further, the removal range and residual blind area of different puncture points were defined. Also, the individualized optimal position, direction and number of puncture paths suitable for patients were determined.

A typical case is given here to illustrate the use of 3D visualization technology: Patient No. 4, a 43-year-old male with alcoholic pancreatitis. The patient suffered from a high fever and abdominal pain for more than 2 wk till he was transferred to the hospital. The CT examination showed extensive peripancreatic necrosis in the retroperitoneal space. The results of 3D reconstruction clearly showed horseshoe-shaped necrosis, as the purple lesion shown in Figure 1A, involving the head of pancreas, uncinate process, root of mesentery, body and tail of pancreas, splenic hilus and left paracolic sulcus, which was closely related to surrounding organs and blood vessels. Virtual percutaneous nephroscopic necrosectomy was performed through the 3D virtual system to determine the three optimal puncture paths. As shown in Figure 1B, the necrosis in the blue area could be debrided from the right puncture path. As shown in Figure 1C, the necrosis in the green area could be debrided from the upper left puncture path. As shown in Figure 1D, the necrosis in the orange area could be debrided from the lower left puncture path. Residual necrosis was found without any single puncture path. Therefore, the three puncture paths were used simultaneously to avoid the operational blind area or residual necrosis. Figures 2–4 are the cross-section, coronal-section and 3D reconstruction images of puncture points on the right side, upper left side, and lower left side, respectively. These were critical to ensure the accuracy of PCD under the guidance of CT.

Clinical results

The CT-guided PCD of the 18 patients was successfully performed strictly according to 3D visualization design, and subsequent percutaneous nephroscopic necrosectomy was carried out according to the changes in the condition, debriding maximum necrosis. Twelve patients were cured by conducting one-time surgery. Five patients were cured after two-time surgery and only one patient underwent the surgery three times. Patient No. 14 underwent additionally endoscopic necrosectomy for removing residual small lesions due to the winding sinus. The median operation time was 102 (102 ± 20.7) min, and the postoperative hospitalization time was 35 (35 ± 8.1) d. No major surgical complications occurred. All the patients were cured and discharged from the hospital eventually (Table 1).

The patient in the aforementioned typical case underwent percutaneous nephroscopic necrosectomy for removing necrotic tissues 14 d after PCD. A large number of infected necrotic tissues were removed from the three puncture points. The clinical condition of the patient improved obviously after surgery. Figure 5 shows the results of abdominal CT re-examination on the 15th day after surgery. Finally, he was discharged from the

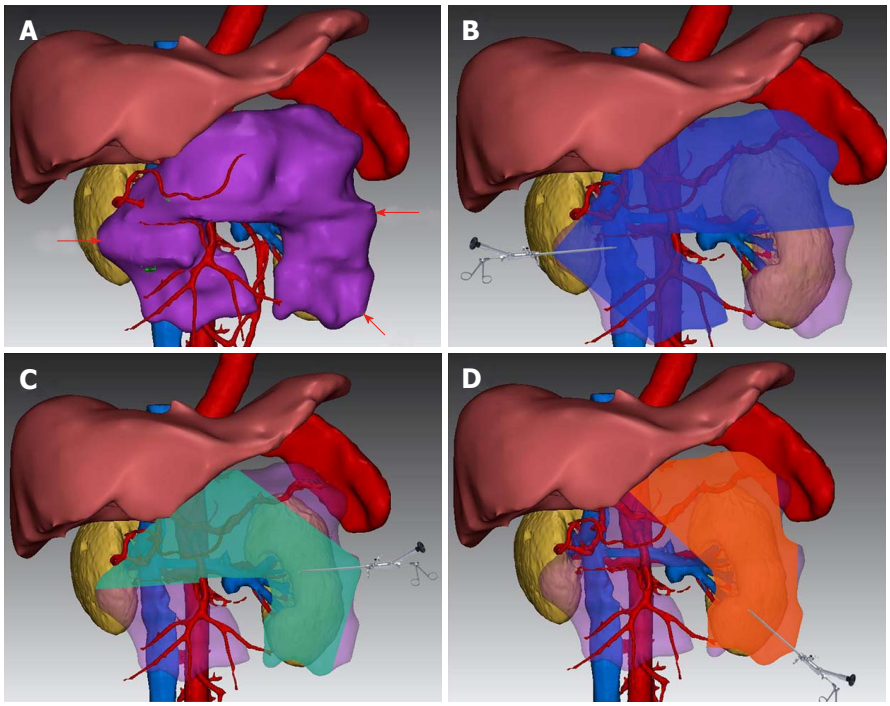

Figure 1 Three-dimensional visualized reconstruction image and virtual surgery for Patient No. 4. A: Horseshoe-shaped infected necrotic lesions (purple) adjacent to important organs and blood vessels; B: Debrided area of percutaneous nephroscopic necrosectomy through the point of puncture on the right puncture point (blue area); C: Debrided area of percutaneous nephroscopic necrosectomy through the point of puncture on the upper left puncture point (green area); D: Debrided area of percutaneous nephroscopic necrosectomy through the point of puncture on the lower left puncture point (orange area).

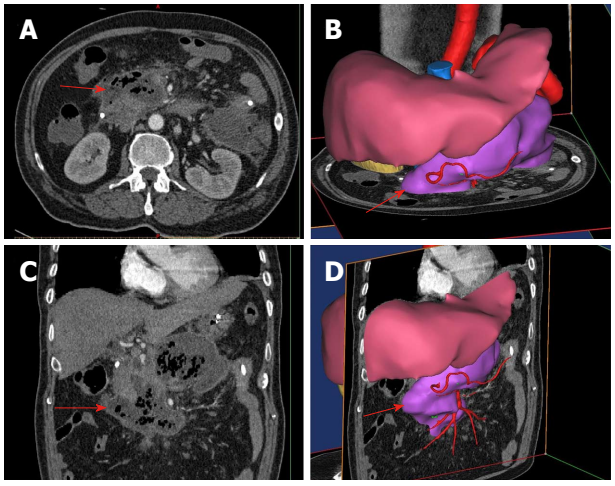

Figure 2 Cross-section, coronal-section and three-dimensional reconstruction images of the right-side puncture path for percutaneous catheter drainage in Patient No. 4. The red arrow represent the fictional direction and path of puncture.

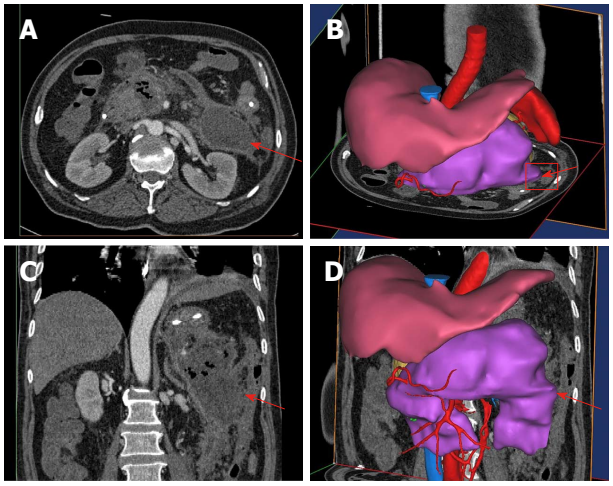

Figure 3 Cross-section, coronal section and three-dimensional reconstruction images of the upper left-side puncture path for percutaneous catheter drainage in Patient No. 4. The red arrow represents the fictional direction and path of puncture.

hospital on the 29th day postoperatively.

DISCUSSION

INP is a serious disease with a mortality of approximately 30% and up to 80% of cases having multiple organ failure[10,11]. Open necrosectomy has been considered the gold standard treatment for decades. However, the morbidity and mortality rates of the surgery were high[12,13]. In 2000, Carter *et al*[14] reported a new treatment method called minimal access retroperitoneal pancreatic necrosectomy, which yielded good results. Since then, various kinds of minimally invasive approaches have been increasingly used worldwide[14-17].

The first randomized controlled trial compared a minimally invasive necrosectomy with traditional laparotomy necrosectomy and showed a significant decrease in the incidence of complications[18]. Recently, another clinical study that summed up 394 cases in the last 17 years found that the mortality and incidence of

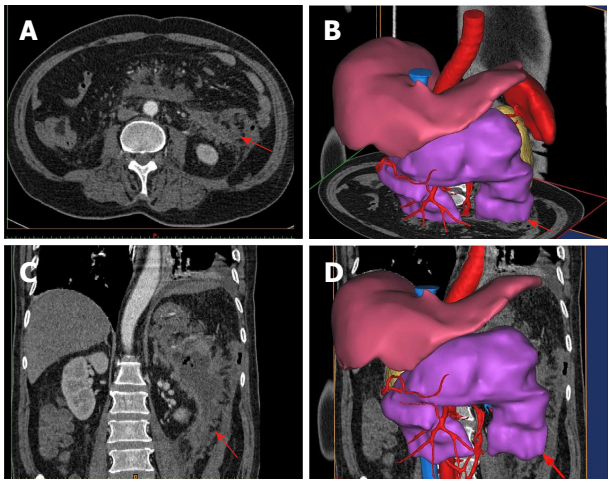

Figure 4 Cross-section, coronal section and three-dimensional reconstruction images of the lower left-side puncture path for percutaneous catheter drainage in Patient No. 4. The red arrow represents the fictional direction and path of puncture.

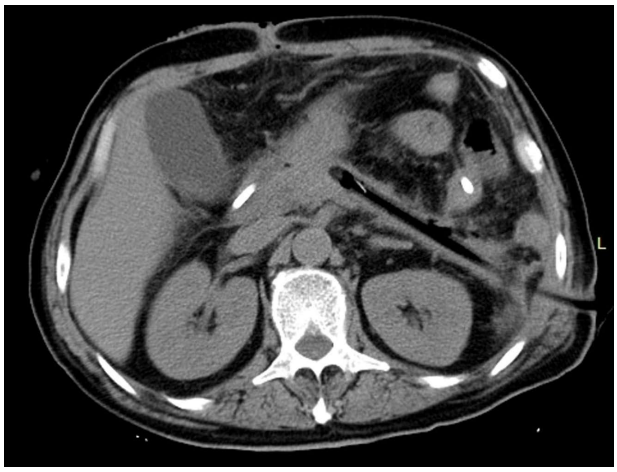

Figure 5 Image of abdominal CT reexamination of Patient No. 4. Abdominal CT reexamination 15 d after percutaneous nephroscopic necrosectomy showed that the abscess cavity disappeared and the drainage tube was unobstructed. CT: Computed tomography.

complications in the minimally invasive nephroscope group were 15.3% and 63.5%, respectively, which were significantly lower than those in the laparotomy group (23.3% and 81.7%, respectively)[19]. All these studies were based on the belief that the necessary precondition of the minimally invasive surgery was correct PCD.

As the initial step of minimally invasive treatment, the purpose of PCD was to attenuate sepsis and establish an access track for further necrosectomy. Some patients with infected necrotic pancreatitis could even be cured only using PCD[20,21]. More importantly, PCD established a guide channel for subsequent minimally invasive removal of necrotic tissues. The blind area in percutaneous nephroscopic necrosectomy could be avoided through a reasonable and correct PCD path, which was critical for prognosis. In our hospital, the minimally invasive method has been used since 2008[22] to treat more than 200 patients with INP until now. A number of them were referrals from other hospitals, and some had undertaken PCD by interventional doctors without considering the convenience for subsequent surgical operation. A few of the patients even undertook a wrong PCD due to the doctors' lack of experience; the catheter passed through the colon, stomach or other hollow organs, which was a disaster for patients with severe pancreatitis. Therefore, it was believed that preoperatively reliable and visualized imaging guidance was crucial for correct PCD.

With the development of digital medicine, 3D visualization software has been gradually applied to clinical practice and proved useful in many diseases. Compared with the traditional two-dimensional ultrasound, CT or magnetic resonance imaging, the 3D visualization image has shown huge advantages in the objective, direct and visual image display of lesions and surrounding anatomical structures. Therefore, it could reduce doctor "mistakes" due to lack of experience.

In this study, the technique was first adopted to treat patients with INP. The site, shape and number of infected necrotic lesions, as well as their relationship with peripheral vessels and important organs, could be identified through image reconstruction based on CT before surgery, which instructed doctors on how to select the optimal puncture position and direction to establish an ideal PCD path. The infected necrotic tissue was fully drained using multiple ideal PCD paths, which was critically important to attenuate sepsis. Moreover, it helped avoid the blind area and maximally debride the necrosis conveniently in the subsequent surgery. This was the most important reason why two-thirds of patients were cured through a single operation, no patient was switched to laparotomy, and all were cured finally. Consequently, the operation-related complications and time of hospitalization decreased significantly.

The disadvantage of this study was the limited number of patients. Therefore, analysis of more cases is needed to support the findings. However, the initial good results of the study indicated that the 3D visualization technology was meaningful for this terrible disease.

In conclusion, 3D visualization technique could help clinicians in selecting the optimal puncture path of catheterization, which could not only maximize the degree of drainage of infected lesions in pancreatitis patients but also significantly improve the efficiency of subsequent percutaneous nephroscopic necrosectomy and therefore improve the prognosis.

ARTICLE HIGHLIGHTS

Research background

Infected necrotizing pancreatitis (INP) is a severe disease with high mortality, which generally requires percutaneous catheter drainage (PCD) and following surgical debridement if necessary. Whether the puncture paths of PCD are appropriate or not is related to not only the effect of drainage but also the efficiency of subsequent minimally invasive removal of infected necrosis. However, a number of patients' PCDs were insufficient or even the catheter passed through the hollow organs due to the doctors' lack of experience, which was a disaster for INP patients.

Table 1 Clinical data of the 6 patients with infected necrotizing pancreatitis after three-dimensional reconstruction and surgery

No.	Sex	Age	Severity	Infected necrosis position	No. of PCD	Operation time in minute	No. of operation times	Surgical complications	Postoperative hospitalization time in day
1	M	58	Moderate to severe	Body and tail of pancreas	1	85	1	None	34
2	F	38	Severe	Around the pancreas	2	145	2	None	48
3	M	39	Moderate to severe	Lesser peritoneal sac, and body and tail of pancreas	1	90	1	None	23
4	M	43	Severe	Head of pancreas, uncinate process, root of mesentery, neck of pancreas, body and tail of pancreas, porta lienis, and left paracolic sulcus	3	150	1	None	29
5	M	58	Moderate to severe	Body and tail of pancreas	1	90	1	None	28
6	M	44	Moderate to severe	Lesser peritoneal sac, and body and tail of pancreas	2	105	1	None	36
7	F	62	Severe	Body and tail of pancreas, paracolic sulcus, and head of pancreas	3	100	2	None	32
8	F	36	Severe	Head of pancreas, uncinate process, body and tail of pancreas, porta lienis, and left paracolic sulcus	3	120	3	None	48
9	M	32	Severe	Lesser peritoneal sac, and body and tail of pancreas	2	105	1	None	39
10	M	67	Severe	Head of pancreas, uncinate process, body and tail of pancreas, porta lienis, and left paracolic sulcus	3	120	1	None	34
11	M	51	Moderate to severe	Body and tail of pancreas, paracolic sulcus, and head of pancreas	2	95	1	None	30
12	F	27	Moderate to severe	Lesser peritoneal sac, and body and tail of pancreas	1	80	1	None	28
13	M	33	Severe	Body and tail of pancreas, paracolic sulcus, and head of pancreas	3	95	2	None	29
14	F	62	Severe	Body and tail of pancreas, paracolic sulcus, and head of pancreas	2	95	1	Residual uncinate process lesions; endoscopic necrosectomy was used for removal	41
15	M	44	Moderate to severe	Lesser peritoneal sac, and body and tail of pancreas	1	90	1	None	45
16	F	39	Severe	Body and tail of pancreas, paracolic sulcus, and head of pancreas	3	105	2	None	48
17	M	67	Moderate to severe	Lesser peritoneal sac, and body and tail of pancreas	1	70	2	None	25
18	M	35	Moderate to severe	Lesser peritoneal sac, and body and tail of pancreas	2	80	1	None	31

PCD: Percutaneous catheter drainage.

Research motivation
Three-dimensional (3D) visualization technology has been proved to be of great help for precise intervention or surgery, which also might be useful to optimize the puncture paths of multiple PCDs for INP patients.

Research objectives
To explore the value of 3D visualization technology for PCDs in INP patients.

Research methods
Preoperative computed tomography images were converted into 3D modellings through a software and the lesions were well displayed. PCD number and puncture paths were designed through virtual surgery (percutaneous nephroscopic necrosectomy) based on the principle of maximum removal of infected necrosis conveniently. We retrospectively analyzed 18 INP patients' clinical data and present a typical case in detail.

Research results
All the patients' 3D modellings was well reconstructed, through which the optimal PCD paths were designed. As a result, infected necrosis was conveniently removed in abundance using a nephroscope during the following surgery and two-thirds of the patients were cured after only one-time operation. Postoperative hospitalization time was 35 d on average, no major surgical complications occurred, and no one died.

Research conclusions
3D visualization technology was useful for INP patients to maximize the PCD effect. Moreover, it significantly improved the efficiency of subsequent percutaneous nephroscopic necrosectomy, which was critically important for improving the prognosis.

Research perspectives
Although the case number of this study was limited, the initial result indicated the value of 3D visualization technology for this terrible disease. Of course, analysis of more cases from multiple centers is needed to support the findings.

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CASE REPORT

Development of tenofovir disoproxil fumarate resistance after complete viral suppression in a patient with treatment-naïve chronic hepatitis B: A case report and review of the literature

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Author contributions: Cho WH, Lee HJ and Bang KB collected the patient's clinical data; Cho WH, Kim SB and Song IH designed the report and wrote the paper.

Informed consent statement: The patient was not required to give informed consent for this study because this study used clinical data that were obtained after this patient agreed to treatment but before treatment initiation.

Conflict-of-interest statement: All authors declare no conflicts of interest related to this article.

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Abstract

Tenofovir disoproxil fumarate (TDF) is a potent nucleotide analogue that is recommended as first-line therapy for patients with chronic hepatitis B. The results of a longitudinal study of TDF treatment demonstrated no development of resistance. We observed one treatment-naïve chronic hepatitis B (CHB) patient who developed TDF resistance after complete viral suppression during long-term TDF treatment. A 37-year-old HBeAg-positive man received TDF 300 mg/d for 43 mo. The hepatitis B virus (HBV) DNA titer was 8 $\log_{10}$ copies/mL at baseline and became undetectable at 16 mo after treatment. However, the HBV DNA titer rebounded to 7.5 $\log_{10}$ copies/mL at 43 mo after treatment. We performed full sequencing to find mutation sites associated with virologic breakthrough. The results showed 9 mutation sites, most of which had not been well-known as mutation sites. We changed the therapy from tenofovir to entecavir with a regimen of 0.5 mg once daily. After 4 mo, the HBV DNA titer decreased to 267 copies/mL, and the liver enzyme levels were normalized.

Key words: Chronic hepatitis B; Tenofovir; Resistance; Mutation

Core tip: The results of many clinical longitudinal studies of Tenofovir disoproxil fumarate (TDF) treatment have demonstrated no development of resistance until now.

Recently, a few cases of resistance to TDF have been reported. However, the mutation site had not been clearly revealed and confirmed because of the rarity of resistant cases. In the present case, TDF resistance developed following the complete suppression of HBV DNA in a treatment-naïve patient. We detected 9 mutation sites, including some that have been unknown until now. We believe that the present study is helpful in revealing the exact mutation sites associated with TDF resistance.

Cho WH, Lee HJ, Bang KB, Kim SB, Song IH. Development of tenofovir disoproxil fumarate resistance after complete viral suppression in a patient with treatment-naïve chronic hepatitis B: A case report and review of the literature. *World J Gastroenterol* 2018; 24(17): 1919-1924 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i17/1919.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i17.1919

INTRODUCTION

Chronic hepatitis B (CHB) affects approximately 250 million people worldwide and can lead to liver cirrhosis, liver failure, hepatocellular carcinoma (HCC), and death[1-3]. Globally, approximately 30% of cirrhosis cases and 53% of HCC cases have been attributed to CHB[2]. Antiviral therapy for hepatitis B virus (HBV) infection can suppress viral replication and halt disease progression[4,5]. The reduction of HBV DNA concentrations to very low or undetectable levels through antiviral therapy is associated with a reduced risk of mortality and HCC[6-8]. However, the therapeutic benefits are diminished because of the emergence of drug-resistant viruses.

Entecavir (ETC) and tenofovir disoproxil fumarate (TDF) are the two first-line therapies recommended for the treatment of CHB because they have a more potent antiviral effect and higher genetic barriers against resistance than other antiviral agents. The rate of antiviral resistance in previously untreated patients has been reported for ETC, *i.e.*, 1.2% of patients develop resistance in 5 years[9,10]. The development of resistance to TDF has not been reported in treatment-naïve patients until now[11].

The rtA194T polymerase mutation combined with the rtL180M and rtM204V polymerase mutations is reported to be associated with TDF resistance in HIV/HBV co-infected patients[12]. However, TDF resistance-related mutations in patients infected only with HBV were unknown until now. Recently, Lee *et al*[13] reported two TDF resistance mutations in CHB patients during "The Liver Week 2017" symposium in the United States. The patients harbored CHB mutants with three new substitutions, namely, rtS106C, rtH126Y and rtD134E. These patients had previously been treated with various therapies, including lamivudine (LAM), adefovir (ADV) and ETC. We observed the development of TDF resistance in a patient who had no treatment history. This patient showed virologic and biochemical breakthroughs after he achieved a complete virologic response. In this study, we report the first case of TDF resistance in a treatment-naïve patient and review the pertinent literature.

CASE REPORT

The patient was a 37-year-old Korean male who visited the clinic because of elevated liver enzymes. He was first diagnosed as having chronic hepatitis B at the age of 20 and was followed up regularly in the family medicine department of Dankook University Hospital. Until his visit to the clinic, he had no history of liver enzyme elevation. His mother was also diagnosed with chronic hepatitis B but did not receive antiviral treatment. The patient's laboratory examination showed that he was positive for HBsAg and HBeAg. His aspartate transaminase (AST) and alanine aminotransferase (ALT) levels were high, at 51 IU/L and 80 IU/L, respectively. His HBV DNA titer was greater than 8.99 $\log_{10}$ copies/mL, as measured by the Amplicor™ Monitor PCR assay (lower limit of detection, 116 copies/mL; Roche Diagnostics, Basel, Switzerland). Abdominal sonography revealed a diffuse mild fatty liver. No evidence of cirrhosis, such as splenomegaly, thrombocytopenia, or esophageal varices, was observed. The patient was started on Tenofovir disoproxil fumarate (TDF) 300 mg, one tablet daily. After 16 mo, the HBV DNA level was undetectable. The AST and ALT levels had also normalized to 27 IU/L and 35 IU/L, respectively. The patient continued the same treatment with complete adherence, but HBeAg was not converted. However, after 43 mo of continuous treatment, HBV DNA had increased to 7.5 $\log_{10}$ copies/mL. The levels of AST and ALT were also increased to 61 IU/L and 109 IU/L, respectively. The patient's history showed that he took TDF regularly every day, and there was no history of the use of any other medicine that could either decrease the efficacy of TDF or increase its rate of metabolism. Tests for HIV and anti-HCV antibodies were negative. We performed mutation testing on the rtL80, rtI169, rtL180, rtA181, rtT184, rtA194, rtS202, rtM204, rtL220, rtN236, and rtM250 sites, all of which were previously known to be mutation sites, but all of these sites were identified as wild type. We performed further examinations on the rtS106, rtH126, rtD134, and rtL269 sites, which have been revealed as mutation sites associated with tenofovir resistance, and only the rtS106C mutation was detected. We performed full genome sequencing to find other mutation sites associated with virologic breakthrough because the rtS106C mutation alone was not sufficient to cause tenofovir resistance (Figure 1). The results showed mutations at 9 sites, namely, rtY9H, rtL91I, rtS106C, rtS106G, rtT118C, rtT118G, rtQ267L, rtI269L, rtA317S, rtK333Q, and rtN337H. Both the rtS106 and rtT118 sites demonstrated a variable nucleotide substitution of 50% C and 50% G on a chromatogram (Figure 2). Although we observed the patient for a few additional weeks, the AST and ALT levels increased to 202 IU/L

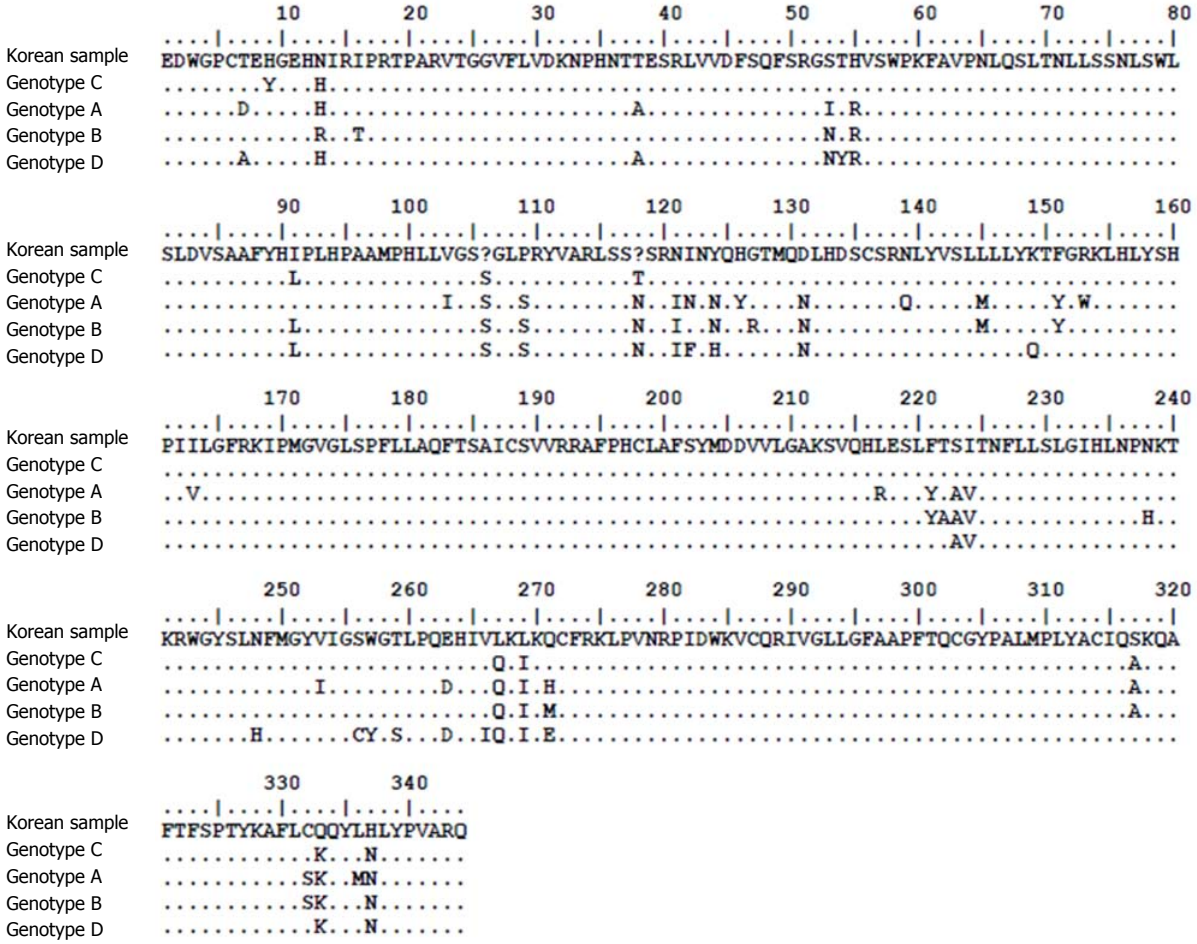

Figure 1 Full sequencing analysis of the hepatitis B virus reverse transcriptase gene from the patient (Korean sample). The sequence analysis shows that mutations occurred at 9 sites compared to the wild-type genotype C (the patient was infected with genotype C). The rt106 and rt118 sites are expressed as "?" because the sites contained a substitution by 2 different nucleotides.

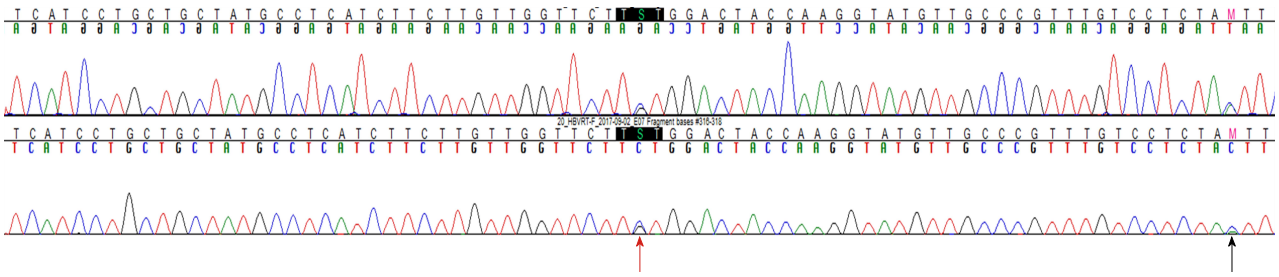

Figure 2 Chromatogram of the hepatitis B virus reverse transcriptase gene from the patient. The rt101 (red arrow) and rt118 (arrow) sites are shown as a double line because the site contained a substitution by 2 different nucleotides (cytosine and guanine).

and 539 IU/L, respectively. We changed the therapy from tenofovir to entecavir with a regimen of 0.5 mg once daily. After 4 mo, the HBV DNA titer decreased to 267 copies/mL. The AST and ALT levels normalized to 32 IU/L and 26 IU/L, respectively (Figure 3). HBeAg seroconversion had not yet occurred.

DISCUSSION

The treatment of chronic hepatitis B has improved in the last decade primarily because of the availability of oral nucleos(t)ide analogue antiviral agents, such as LAM, telbivudine (LdT), ADV, ETC, and TDF. These agents are well tolerated and very effective in suppressing viral replication, and they appear to be safe, to the best of our knowledge and experience. The major limitation of long-term antiviral therapy for chronic hepatitis B is the emergence of drug resistance followed initially by an increase in HBV DNA level (virologic breakthrough) and then by an increase in serum aminotransferase level (biochemical breakthrough)[14].

Antiviral resistance is likely to develop primarily because the mutation rate during HBV replication is high and viral replication is increased in response to selection

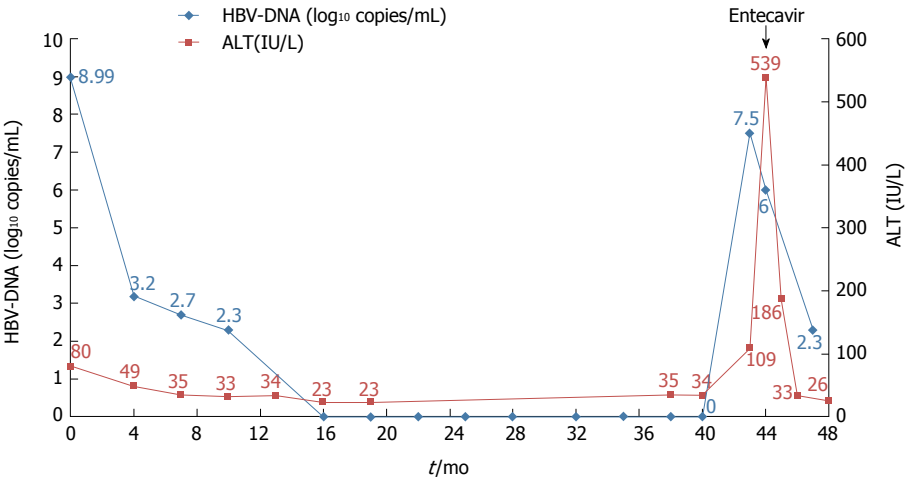

Figure 3 Clinical course of the patient. Hepatitis B virus DNA became undetectable after 16 mo of antiviral treatment with TDF. Virologic and biochemical breakthroughs occurred at 43 mo after treatment initiation.

pressure[4,5]. Mutation and resistance are determined by three factors: viral fitness, nucleos(t)ide analogue potency, and the genetic barrier to resistance[15]. Viral fitness refers to the ability for viral replication in a defined environment. The potency of a nucleos(t)ide analogue describes its ability to inhibit HBV replication by acting as a substrate. The genetic barrier to resistance refers to the number of substitutions in the HBV polymerase reverse transcriptase (RT) domain required for the development of resistance[14]. Of the above three factors, nucleos(t)ide analogue potency and the genetic barrier to resistance are properties of antiviral agents. A higher nucleos(t)ide analogue potency and genetic barrier corresponds to a lower mutation rate[16].

The results of longitudinal study of TDF therapy demonstrated no resistance development throughout 8 years of treatment[17]. This result is possible because TDF provides the combination of a high genetic barrier, potent viral suppression, and reduced fitness of resistant viruses. Thus, TDF has been one of the drugs recommended as a first-line therapy for CHB patients[18,19]. TDF is also recommended for patients who have developed resistance to LAM, ETC, or LdT[20]. Several case reports and retrospective cohort studies also demonstrated the clinical efficacy of TDF in ETC-resistant or ETC-refractory patients[12,21]. In one study, the HBV DNA in some patients who experienced a virologic breakthrough while on TDF therapy contained an RT mutation site such as rtL101L/F, rtA307A/T, rtV173L + rtL180M + rtM204V, or rtA181T. However, these substitutions did not result in reduced susceptibility to TDF, and most of the patients did not adhere to treatment[11]. The rtA194T HBV polymerase mutation that was recently identified in HIV/HBV-coinfected patients treated with TDF did not confer resistance to TDF as the sole mutation *in vitro*[22]. In another study, phenotypic analyses revealed that the presence of the rtA194T mutation combined with the rtL180M and rtM204V mutations resulted in a greater than 10-fold increase in the IC_{50} for TDF compared to the wild type[23]. However, those sites have not been confirmed as mutation sites affecting TDF resistance because TDF resistance was not reported. Further studies are needed to assess the extent to which these mutations are associated with TDF resistance in HBV infection.

Reports on TDF resistance are difficult to find because TDF resistance is rare. A few years ago, a case of TDF resistance was reported in a chronic hepatitis B (CHB) patient who received sequential nucleos(t)ide therapy[24]. TDF resistance with virologic and biochemical breakthroughs had occurred during TDF rescue therapy after consecutive LAM, ETC, and LAM+ADV treatment failures. The identified HBV DNA mutation sites were rtL80M, rtL180M, rtM204V/I, rtA200V, rtF221Y, rtS223A, rtT184A/L, rtR153Q, and rtV191I, which were previously known as mutation sites related to LAM, ETC and ADV resistance. Recently, Lee *et al*[13] reported two TDF-resistant patients during "The Liver Week 2017" symposium in the United States. The patients described in this report had also previously taken other antiviral drugs and demonstrated multidrug resistance. However, the authors detected seven mutations in the HBV DNA, including three new substitutions, namely, rtS106C, rtH126Y, and rtD134E, which were collectively termed CYE. The TDF IC_{50} values for wild-type HBV and the CYE mutant were 3.8 ± 0.6 μmol/L and 14.1 ± 1.8 μmol/L, respectively. However, the CYE mutation site was not definitively identified as the site related to TDF resistance, although the TDF IC_{50} was higher in the CYE mutant than in the wild type. The TDF resistance described in the previous two reports developed after the failure of treatment with other nucleos(t)ide analogues. The patient in the present study had no history of nucleos(t)ide analogue treatment and showed complete viral suppression before the development of TDF resistance. It is not clear whether all 9 HBV RT mutation sites identified in the

current patient were associated with TDF resistance. However, the accumulation of this mutational data is helpful for confirming the sites associated with TDF resistance. Further in vitro study is needed to reveal whether the 9 mutation sites are associated with an increased TDF IC_{50}.

ARTICLE HIGHLIGHTS

Case characteristics

The patient had not complained of any specific symptoms.

Clinical diagnosis

The hepatitis B virus DNA titer rebounded to 7.5 log_{10} copies/mL at 43 mo after TDF treatment in a treatment-naive patient.

Differential diagnosis

We performed full genome sequencing to find other mutation sites to know it is associated with tenofovir disoproxil fumarate (TDF) resistance.

Laboratory diagnosis

We performed full genome sequencing to find TDF mutation sites and the results showed mutations at 9 sites, namely, rtY9H, rtL91I, rtS106C, rtS106G, rtT118C, rtT118G, rtQ267L, rtI269L, rtA317S, rtK333Q, and rtN337H.

Treatment

We changed the therapy from tenofovir to entecavir with a regimen of 0.5 mg once daily.

Experiences and lessons

We have to consider possibility of TDF resistance although its rarity.

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