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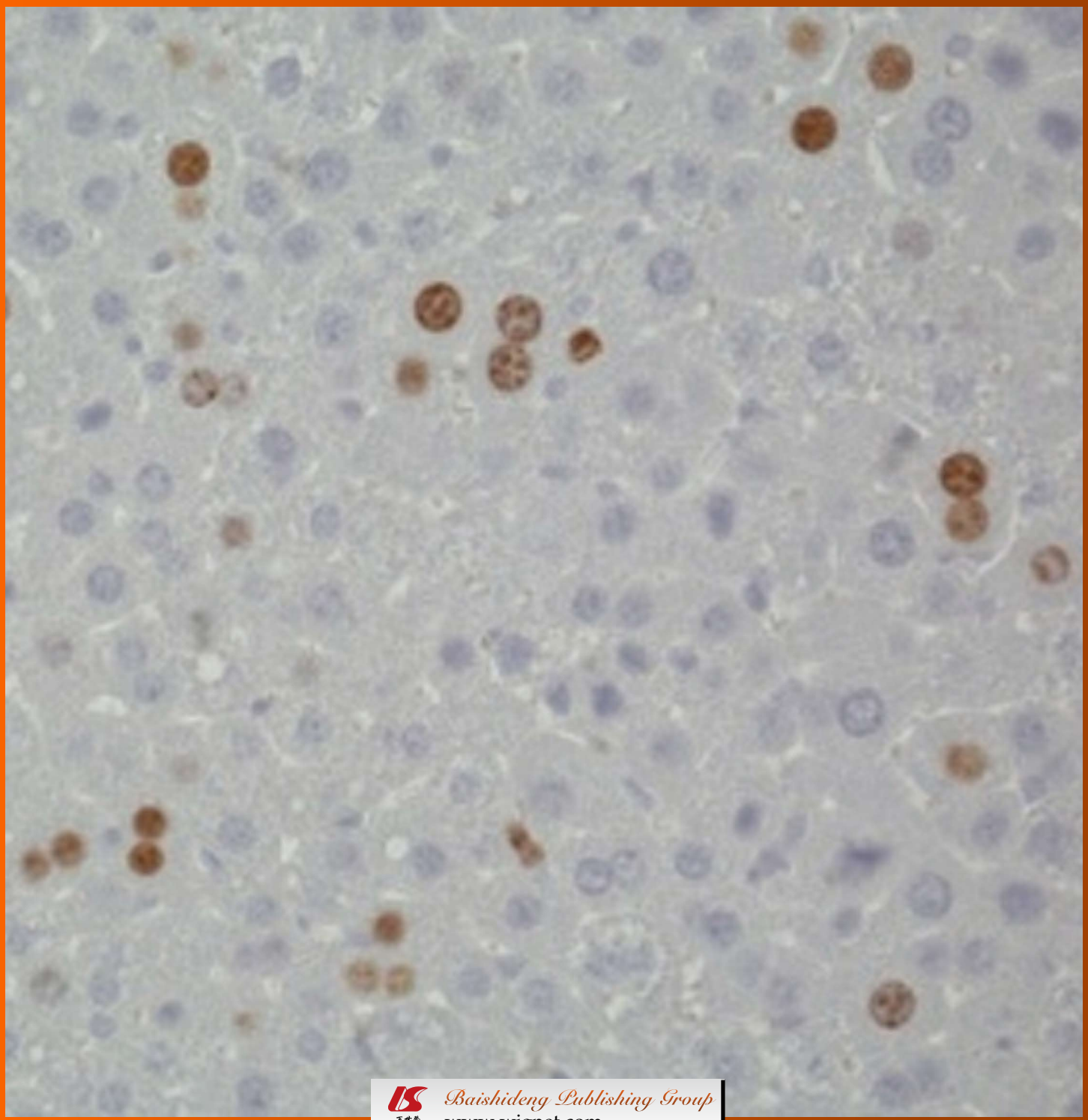

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EDITORIAL

New developments in transplant-acquired allergies

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Abstract

Transplant-acquired allergy (TAA) was firstly described as transplant-acquired food allergy (TAFA) after bone marrow transplantations and mostly observed in a transient form. The picture is complicated by numerous case reports of TAFA after the receipt of liver grafts from donors with no documented history of food allergy. The estimated prevalence of TAFA among young children in the literature has been documented in various studies ranging from 6% to 57%. Although TAA is mostly found to be associated with liver transplantation; it has been recently reported to be related with heart, intestinal, lung and even renal transplantations in adults. Previous reviews of published cases of liver TAA misleadingly emphasized the predominance of children and the absence of TAA in cardiac, pulmonary, and renal transplant recipients. In different studies, the male/female ratio is equal. Literature data suggest that children with TAFA typically present within the first year after surgery and are typically allergic to multiple foods. The pathogenesis of TAA is not still completely understood. Most of the studies support the concept that the functioning liver itself, and not only tacrolimus immunosuppression, is one of the main contributors to TAA in these patients. In the light of recent findings, other possible mechanisms can be summarized as following: (1) the recovery of delayed type hypersensitivity; (2) late manifestation of food allergy; (3) intestinal injury as well as inhibition of cellular energy production by tacrolimus; and (4) transfer of food-specific IgE or lymphocytes. Thus, interplay between hematopoietic cells from the transplanted organ and recipient specific factors (*e.g.*, younger age and atopic background) seem to underlie the development of TAA. Most patients will have symptomatic improvement following reduced immunosuppression and an appropriately restricted diet. Nevertheless, some studies suggest that atopic diseases occur in some of pediatric liver transplant recipients, with manifestations including food allergy, eczema, allergic rhinitis, and asthma. More studies would be needed including greater number of patients to determine whether TAA is transient or not in pediatric/adult solid organ recipients.

Key words: Cyclosporine A; Tacrolimus; Liver; Transplantation; Donor; Recipient; Atopy; Children

Core tip: Transplant-acquired allergy (TAA) was firstly described after bone marrow transplantation and mostly observed in a transient form. Although TAA is mostly found to be associated with liver transplantation; it has been recently reported to be related with heart, intestinal, lung and even renal transplantations in adults. Most studies suggest that the functioning liver itself, and not only tacrolimus immunosuppression, is one of the main contributors to TAA in these patients. Most patients will have symptomatic improvement following reduced immunosuppression and diet. Nevertheless, recent studies suggest that allergic diseases (*e.g.*, eczema, rhinitis and asthma) occur in some of pediatric transplant recipients.

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BACKGROUND

The transfer of allergy from a food allergic solid organ

such as liver donor to a previously non-allergic transplant recipient was firstly reported in 1990's, and has subsequently been reported in one further case[1-3]. The phenomenon is consistent with previous findings of allergy transfer *via* bone marrow transplantation, and the finding that donor-derived stem cells present in a transplanted liver can sustain long-term hematopoiesis in a recipient[4]. The picture is complicated by numerous case reports of transplant-acquired food allergy (TAFA) after the receipt of liver grafts from donors with no documented history of food allergy. An association between tacrolimus therapy after liver transplantation and development of food allergy, TAFA, was first suggested by Lacaille *et al*[1].

What is transplant-acquired allergy?

Transplant-acquired allergy (TAA) was firstly described as TAFA after bone marrow transplantations and mostly seen in a transient form[4]. The estimated prevalence of TAFA among young children in the literature has been documented in various studies ranging from 6% to 57%. TAFA is described mainly after liver, but also after small bowel/intestinal, lung and heart transplantations[5-9]. In different studies, the male/female ratio is equal[1-9]. Literature data suggest that children with TAFA typically present within the first year after surgery and they are typically allergic to multiple foods[4,5,8].

PATHOGENESIS OF TAA

The pathogenesis of TAA is not still completely understood. Most of the studies support the concept that the functioning liver itself, and not only tacrolimus immunosuppression, is one of the main contributors to TAA in this patient population[10-13]. Animal models suggest hepatic mechanisms may be really important for immune tolerance to orally ingested antigens, but there is little direct evidence for this in humans. Watanabe *et al*[14] showed in a mouse model that the liver is found to be one of the sites at which T-helper (Th) 2 lymphocytes specific to a food antigen develop.

A recent study evaluated paired pre- and post-liver transplant sera from children aged 0-36 mo treated at a single centre during 2001-2008. Thirty-five of 50 cases had IgE sensitization to ⩾ 1 food pre-transplant and 18 post-transplant. Food sensitization pre-transplant was associated with severity of liver dysfunction. Young children with severe liver dysfunction appear to have a high prevalence of food sensitization. Hepatic mechanisms may therefore be important for establishing immune tolerance to dietary antigens in humans. However, these findings were not replicated in the renal transplant group[13].

Association with the type of transplantation: liver vs kidney

Liver TAFA has been widely reported now, and is estimated to affect nearly 10% of children who receive a liver transplant. For example, Legendre *et al*[7] described 4 of the 65 children (6%) who underwent liver or combined liver and kidney transplantation acquiring a new-onset food allergy postoperatively. The majority of cases reported have been in young children receiving tacrolimus immunosuppression, and in only a few cases with passive transfer of food allergy from an allergic donor have been documented. Nevertheless, the only reports of liver TAFA in adults have occurred *via* passive transfer from a food allergic liver donor.

Table 1 Predisposing factors for transplant-acquired allergy development in different types of organ transplantation

Predisposing factors	Type of organ transplantation	
	Liver	Renal
Use of MMF/prednisone	-	+
Delayed manifestation of food allergy in recipient	+	+
Recovery of delayed type hypersensitivity	++	+
Transfer of hematopoietic stem and dendritic cells	+	±
Transfer of food-specific IgE	+	+
Passive transfer of food-specific lymphocytes	++	+
Atopic background of recipient	+	+
Younger age of recipient	+	+
Allergy of donor	+	+

MMF: Mycophenolate mofetil; -: No effect; ±: Suspicious effect; +: Positive effect; ++: Strong positive effect.

The accumulating data shows that mostly liver transplantations seem to be associated with new onset TAA suggesting the hematopoietic tissue and dendritic cells play a role in this phenomenon. Pluripotential hematopoietic stem cells and dendritic cells are known to be normally resident in the liver. T-cell activation by antigens migrating through the portal vein occurs in the liver and some liver-resident dendritic cells and liver sinusoidal endothelial cells (LSEC) direct naive $CD4^+$-T cells preferentially to Th2 differentiation. Furthermore, it was recently shown in a mouse model that helper $CD4^+$-T cells in the liver induced an IgE response to a food antigen[14].

At the same time, it could be argued that children with kidney transplants receive more prednisone than children with liver transplants, which may down regulate mast cell degranulation in response to exposure to allergenic foods. Furthermore, unlike children with liver transplants, they receive mycophenolate mofetil, which also suppresses humoral immunity, and, thereby, IgE production. Hällgren *et al*[15] also showed the low IgE concentrations in uremia are suggested to reflect altered T-cell regulation of the IgE production in renal transplant recipients (Table 1).

Relation with the type of immunosuppressant: tacrolimus vs cyclosporine A

Another main contributor to TAA in this patient population is immunosuppressant used in prevention of graft rejection. Tacrolimus is a macrolide agent that is now the primary immunosuppressant utilized in transplant recipients. It has been found to be superior to cyclosporine A (CsA) for rescue therapy as well as for earlier weaning of steroids. Both tacrolimus and CsA share similar toxicity profiles; however, their gastrointestinal side effects have received little attention. An increased prevalence of food

Table 2 Side effects of immunosuppressive agents help developing transplant-acquired allergies in solid organ recipients

Types of side effects	Immunosuppressive agents	
	Tacrolimus	Cyclosporine A
Intestinal injury	+	-
Inhibition of cellular energy production in intestine	+	-
Th1/Th2 imbalance	++	+
IL-2 production	↓↓	↓
IL-5 production	↑↑	↑
IL-10 production	↑↑	↑
IL-13 production	↑↑	↑
IgE production	↑↑	↑
Immunosuppression	++	+

IgE: Immunoglobulin E; IL: Interleukin; Th: T-helper cells; -: No effect; +: Positive effect; ++: Strong positive effect; ↓: Decrease; ↑: Increase; ↓↓: Strong decrease; ↑↑: Strong increase.

allergy noted specifically in children receiving tacrolimus immunosuppression supports the hypothesis that selective suppression of Th1 lymphocytes by the interleukine (IL)-2 inhibitor immunosuppressants such as CsA, and the more potent drug, tacrolimus, promotes Th2 lymphocytes and an allergic immune response. Tacrolimus, however, is more potent than CsA and, in addition, augments the production of IL-5 and IL-13-eosinophil- and IgE-promoting cytokines. It is also known to increase intestinal permeability, which may lead to increased exposure to allergenic proteins and a further shift toward Th2 cytokines and IgE production against these proteins[11,12]. As a result; the immunomodulatory effects of tacrolimus, including its propensity to skew toward a Th2 phenotype by inhibiting production of IL-2, as well as its effects on intestinal permeability, are potentially important (Table 2).

It looks like that under the tacrolimus or immunosuppressive therapy, independent of transplantation type; there is always a chance for TAA development. Insufficient control of allergen-specific responses *via* the Treg-cell compartment under systemic immunosuppression has recently been demonstrated by Eiwegger *et al*[16] as one of the triggering factors.

A present study by Gruber *et al*[17] directly compared the occurrence of allergic sensitization and disease under tacrolimus- *vs* CsA-based immunosuppressive therapy in kidney-transplanted patients. The rate of clinically relevant allergy in patients receiving tacrolimus was twice that in patients receiving CsA (15% *vs* 8%). Their results suggest that post-transplant immunosuppression with tacrolimus is associated with an increased occurrence of IgE-mediated sensitization and probably manifestation of allergic disease.

A recent study by Granot *et al*[18] was performed to demonstrate an association with asymptomatic eosinophilia, elevated total and specific IgE levels under tacrolimus immunosuppression. This study was undertaken to characterize the IgE-mediated immune response, in CsA and tacrolimus-treated, post- orthotopic liver transplanted children. Thirty children and adolescents aged 2-21 years, (6-year post-transplantation), were studied. Immunosuppression-CsA: 10 patients, tacrolimus; 20 patients. Eosinophilia was present in 10/20 of patients treated with tacrolimus and 1/10 treated with CsA. IgE levels were found to be elevated in 8/10 tacrolimus-treated patients and in 2/10 CsA patients. Specific IgE levels to a wide panel of food allergens were positive in 5 tacrolimus-treated patients and to both food and inhaled allergens in 3 patients (2, tacrolimus-, 1, CsA-treated). Four children (tacrolimus-treated) had symptoms of food allergy.

Other mechanisms for TAA

In addition, none of the hypotheses would clearly explain why food allergy develops specifically in tacrolimus- but not CsA-immunosuppressed children if the mechanism was only the Th1/Th2 imbalance and immunosuppression. I think that Th1/Th2 imbalance caused by tacrolimus could not be the only cause for TAA. Although the exact mechanism is still not clear, the reported series confirm their role in triggering allergy in post transplant children. In the light of recent findings, possible mechanisms can be summarized as following: (1) the recovery of delayed type hypersensitivity in patients who could have been in a state of relative immune deficiency, *e.g.*, cirrhosis before transplantation[19]; (2) delayed manifestation of food allergy may be due to limited exposure to dietary allergens prior to transplant, which happens especially in the context of anergy caused by chronic liver disease. Acute and chronic liver disease particularly cirrhosis have long been recognized to be associated with absent delayed cutaneous hypersensitivity responses, which is called as the immune anergy of liver failure. Thus, some food allergic children fail to manifest their food allergy due to the immune anergy caused by their liver failure; (3) intestinal injury as well as inhibition of cellular energy production by tacrolimus in the intestine plays a significant role allowing penetration of protein antigens and skewing the immune response towards Th2 *via* induction of cytokines like IL-10[20-22]; and (4) transfer of food-specific IgE or lymphocytes with specificity for particular food antigens from donors.

In summary, interplay between hematopoietic cells from the transplanted organ and recipient specific factors underlie the development of TAA.

RISK FACTORS?

Transplant recipient-specific factors

Some cases presented in the literature are remarkable for the discordant development of liver TAA in two recipients of the same liver[23]. This highlights the importance of transplant recipient specific factors in this condition.

Younger age: These studies suggest that immature infant immune responses play an important part in their predisposition to allergic disease. Most of the children were less than 1 year of age at the time of transplantation, and the appearance of allergy might be explained by their limited exposure to dietary antigens[13,23]. The reported cases sug-

gest that liver TAA occurs when patients with immature immunoregulatory responses undergo transplantation and fail to suppress the clinical expression of new food allergies.

Atopic background: Those who develop liver TAA may also have greater background risk of allergic disease than those who fail to develop TAA. The majority of patients had a family history of atopy, which might be another risk factor for food allergy after transplantation[13,24].

Transplant donor-specific factors

The occurrence of TAA has also found to be associated with young donor age and donor's atopic diseases[7,11,24].

OTHER ROUTES FOR DEVELOPING TAA: HEMATOPOIETIC STEM CELL, CORD BLOOD STEM CELL, LUNG, HEART TRANSPLANTATIONS

Previous reviews of published cases of liver TAA misleadingly emphasized the predominance of children and the absence of TAA development in cardiac, pulmonary, and renal transplant recipients. Although TAA is mostly found to be associated with liver transplantation; it has been recently reported to be related with heart and even adult renal transplantations[6-9].

Consistently, the absence of new-onset food allergy in the children with isolated kidney transplants is compatible with the earlier literature. Search of the literature till 2006 by Dehlink *et al*[24] yielded only one report of food allergy in a child after kidney transplantation receiving tacrolimus therapy. Furthermore, a recent article by Chehade *et al*[8] reported *de novo* food allergy after intestinal transplantation.

The finding that mostly liver and small bowel transplantations seem to be associated with new onset TAA suggests that the pluripotent hematopoietic stem cells and dendritic cells play a role in this phenomenon. The nature of these transplants also involves transfer of mature donor lymphocytes into recipient tissues. Transfer of donor Th2-B lymphocytes producing specific IgE antibodies in recipient tissue can result in ongoing cellular and humoral activity against the allergen. Transferred cell populations are not deleted by post-transplant immunosuppression[24].

Given the histology of lung tissue, lung transplantation results in limited transfer of pluripotent hematopoietic cells and mature lymphocytes into recipient tissues. As a result, the mechanism of allergy transfer following lung transplantation was postulated to involve passive transfer of IgE-sensitized donor mast cells within the transplanted lung into the recipient. Schuller *et al*[9] reported a case transferring of peanut allergy following lung transplantation. They supposed two mechanisms may explain the observations described for the patient reported in this study: *de novo* development of peanut allergies after transplantation, or passive transfer of peanut allergies from a peanut-sensitized organ donor. Moreover, Bhinder *et al*[25] reported a case developing transient peanut allergy following lung transplantation as well. An alternate mechanism was proposed for passive transfer of immunoglobulin E-sensitized mast cells and/or basophils within the transplanted tissue that subsequently migrate into recipient tissues. The gradual decline in the magnitude of the peanut skin prick test and its return to negative over the course of 1 year suggested the gradual depletion of sensitized cells (B lymphocytes and, possibly, mast cells) in the recipient and supported the initial passive transfer of sensitized cells from donor tissue during transplantation.

We described one of the first patients developing TAA after heart transplantation. This patient was receiving tacrolimus subsequent to heart transplantation and developed angioedema after consumption of dairy products at 12 mo after transplantation. The patient was found to be allergic to multiple foods by both radioallergosorbent test and Immuno Solid-phase Allergy Chip tests[26].

An interesting patient, 2-mo-old Japanese male, developed hemophagocytic lymphohistiocytosis. At 7 mo of age, cord blood stem cell transplantation was performed. He developed veno-occlusive disease (VOD) on day 6 after transplantation. Liver damages due to VOD might contribute to the development of TAFA in this case. It has been shown that Kupffer cells, LSEC and liver dendritic cells uptake and present gut-derived antigens, including food allergens, to naïve T cells, thus resulting in immune tolerance both in $CD8^+$-T cells and $CD4^+$-T cells. Therefore, it is possible that VOD -associated damages to the liver, especially to these cells that can induce immune tolerance, might have suppressed oral tolerance to food allergens and promoted the development of TAFA in these patients[27].

VARIOUS CLINICAL PRESENTATIONS OF TAA

Is this just happening as a food allergy or allergy to other substances such as airborne allergens?

Current literature data suggest that children developing TAA typically present to be allergic to multiple foods and aeroallergens[4,5,8]. For instance: Dehlink *et al*[24] showed food allergy in 2, both food and inhalant allergy in 2; inhalant allergy in 7 cases after different solid organ transplantations.

Eosinophilic gastroenterocolitis

New-onset TAA, whether immediate hypersensitivity type or eosinophilic gastroenteropathy, is an infrequent but potentially serious complication of organ transplantation. Eosinophilic gastroenteropathy is common after transplantation and should be considered in all children with gastrointestinal symptoms undergoing transplantation. The colitis in a study appeared to be mediated by food allergies. Most of the patients had symptomatic improvement following reduced immunosuppression and an appropriately restricted diet[23,28].

Urticaria/angioedema

Our group described one of the first patients developing TAA after heart transplantation. This patient presented

to us with angioedema after consumption of dairy products at 12 mo after transplantation[26].

Atopic disease (atopic dermatitis, allergic rhinitis and asthma)

Shroff *et al*[29] demonstrated presentation of atopic disease in a large cohort of pediatric liver transplant recipients. Food allergy and atopic skin disease symptoms were present in 40% and 56% of cases, respectively. Asthma, allergic rhinitis, or both were found in 66% of cases. The onset of symptoms of food allergy and eczema (median, 12 mo post-transplantation) preceded symptoms of allergic rhinitis and asthma (median of 27 and 30 mo post-transplantation, respectively).

LONG-TERM OUTCOME OF TAA?

The long-term prognosis of TAA after solid organ transplantations is currently obscure. As you imagine, TAA may be transient or persist long period of time and turn into manifestation of an atopic disease.

Transient TAA

Several modes of TAA may be envisaged. Some reports in adults and children with liver transplants attributed the development of food allergy to passive transfer of food-specific IgE antibodies from the allergic donors to the recipients. Passive transfer of food allergy has been described in association with bone marrow transplants and solid organ (liver, combined liver and kidney) transplants, all in adult patients. Passive transfer of donor IgE is unlikely, because the half-life of IgE is only a few days, whereas the allergic reaction occurred 3-12 mo after transplantation. However, it cannot be ruled out the possibility that donor IgE bound to the recipient's mast cells and basophils could have persisted for more than a few days.

The findings were explained by the presence of specific IgE-producing B cells in the donor bone marrow and by the presence of IgE producing B cells and specific IgE antibodies or sensitized mast cells with allergen-specific IgE in the donor organ. For transient cases of anaphylaxis occurring only shortly after transplantation, it has been postulated that passive transfer of donor mast cells or basophils sensitized by donor allergen-specific IgE occurred from donor to recipient *via* transplanted tissues[1-3,7]. The transfer of allergen-specific donor lymphocytes is a more likely possibility[9,19,20,25]. In mice, a secondary hapten-specific IgE response can be elicited by the adoptive transfer of primed B lymphocytes, T lymphocytes, or both[30]. The occurrence of immune hemolytic anemia and autoimmune thrombocytopenia after liver transplantation from donors with such diseases indicates that the transfer of functionally active donor-type B or T lymphocytes can occur in humans.

Persistent TAA

Some studies describe the long-term outcome of food allergy in this population, demonstrating that although a substantial number of food sensitivities are lost, most children remain sensitized to at least a subset of foods for an extended period. For instance: Mavroudi *et al*[31] reported long term outcome of acquired food allergy in 3 pediatric liver recipients as a single center experience. The symptoms of food allergy persisted for 8 years in one of the cases and for 2 years in the other two cases. The long-term prognosis in their cases was excellent and food allergy resolved in all the patients. In Granot *et al*'s[18] study, eosinophilia was present in up to 50% of children and adolescents receiving tacrolimus immunosuppression. The majority of these patients also had elevated levels of total and specific (mainly to food allergens) IgE antibodies. However, most patients were asymptomatic and did not manifest food allergy or asthma[18,32,33].

Nevertheless, Shroff *et al*[29] utilized for 176 orthotopic liver transplanted pediatric recipients at a single institution for manifestations of allergic disease. They demonstrated that atopy occurs in approximately 14% of pediatric liver transplant recipients, with manifestations including food allergy, eczema, allergic rhinitis, and asthma.

CONCLUSION

At the end, most patients will have symptomatic improvement following reduced immunosuppression and an appropriately restricted diet. Nevertheless, some studies show that atopic diseases may occur in some of pediatric liver transplant recipients, with manifestations including food allergy, eczema, allergic rhinitis, and asthma. I think that more studies would be needed including greater number of patients to determine whether TAA is transient or not in pediatric/adult solid organ recipients.

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ORIGINAL ARTICLE

mTOR signaling in liver regeneration: Rapamycin combined with growth factor treatment

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Abstract

AIM: To investigate the effects of mammalian target of rapamycin (mTOR) inhibition on liver regeneration and autophagy in a surgical resection model.

METHODS: C57BL/6 mice were subjected to a 70% partial hepatectomy (PH) and treated intraperitoneally every 24 h with a combination of the mTOR inhibitor rapamycin (2.5 mg/kg per day) and the steroid dexamethasone (2.0 mg/kg per day) in phosphate buffered saline (PBS) or with PBS alone as vehicle control. In the immunosuppressant group, part of the group was treated subcutaneously 4 h prior to and 24 h after PH with a combination of human recombinant interleukin 6 (IL-6; 500 μg/kg per day) and hepatocyte growth factor (HGF; 100 μg/kg per day) in PBS. Animals were sacrificed 2, 3 or 5 d after PH and liver tissue and blood were collected for further analysis. Immunohistochemical staining for 5-Bromo-2'-deoxyuridine (BrdU) was used to quantify hepatocyte proliferation. Western blotting was used to detect hepatic microtubule-associated protein 1 light chain 3 (LC3)-Ⅱ protein expression as a marker for autophagy. Hepatic gene expression levels of proliferation-, inflammation- and angiogenesis-related genes were examined by real-time reverse transcription-polymerase chain reaction and serum bilirubin and transaminase levels were analyzed at the clinical chemical core facility of the Erasmus MC-University Medical Center.

RESULTS: mTOR inhibition significantly suppressed regeneration, shown by decreased hepatocyte proliferation (2% *vs* 12% BrdU positive hepatocyte nuclei at day 2, $P < 0.01$; 0.8% *vs* 1.4% at day 5, $P = 0.02$) and liver weight reconstitution (63% *vs* 76% of initial total liver weight at day 3, $P = 0.04$), and furthermore increased serum transaminase levels (aspartate aminotransferase 641 U/L *vs* 185 U/L at day 2, $P = 0.02$). Expression of the autophagy marker LC3-Ⅱ, which was reduced during normal liver regeneration, increased after mTOR inhibition (46% increase at day 2, $P = 0.04$). Hepatic gene expression showed an increased inflammation-related response [tumor necrosis factor (TNF)-α 3.2-fold upregulation at day 2, $P = 0.03$; IL-1Ra 6.0-fold upregulation at day 2 and 42.3-fold upregulation at day 5, $P < 0.01$] and a reduced expression of cell cycle progression and angiogenesis-related factors (HGF 40% reduction at day 2; vascular endothelial growth factor receptor 2 50% reduction at days 2 and 5; angiopoietin 1 60% reduction at day 2, all $P \leq 0.01$). Treatment

with the regeneration stimulating cytokine IL-6 and growth factor HGF could overcome the inhibitory effect on liver weight (75% of initial total liver weight at day 3, $P = 0.02$ *vs* immunosuppression alone and $P = 0.90$ *vs* controls) and partially reversed gene expression changes caused by rapamycin (TNF-α and IL-1Ra levels at day 2 were restored to control levels). However, no significant changes in hepatocyte proliferation, serum injury markers or autophagy were found.

CONCLUSION: mTOR inhibition severely impairs liver regeneration and increases autophagy after PH. These effects are partly reversed by stimulation of the IL-6 and HGF pathways.

Key words: Hepatocyte proliferation; Autophagy; Microtubule-associated protein 1 light chain 3; Partial hepatectomy; Rapamycin

Core tip: Interference of immunosuppressive medication with liver regeneration is a highly relevant issue for transplantation of small-for-size liver grafts. Inhibition of mammalian target of rapamycin (mTOR) represents an important immunosuppressive strategy after transplantation, yet as mTOR regulates cell proliferation and autophagy, concerns remain regarding a negative impact on regeneration. The exact role of mTOR signaling after living-donor liver transplantation is largely unknown. Here we report that mTOR inhibition by rapamycin severely impairs liver regeneration and increases autophagy after liver resection in mice. The most novel finding of this study is that this impaired regeneration can be partly reversed by treatment with exogenous growth factors.

Fouraschen SMG, de Ruiter PE, Kwekkeboom J, de Bruin RWF, Kazemier G, Metselaar HJ, Tilanus HW, van der Laan LJW, de Jonge J. mTOR signaling in liver regeneration: Rapamycin combined with growth factor treatment. *World J Transplant* 2013; 3(3): 36-47 Available from: URL: http://www.wjgnet.com/2220-3230/full/v3/i3/36.htm DOI: http://dx.doi.org/10.5500/wjt.v3.i3.36

INTRODUCTION

The liver has the remarkable ability to regenerate in order to compensate for lost or damaged liver tissue after injury and thereby restore liver function and maintain homeostasis. This process is ultimately required after living-donor liver transplantation, in which a small-for-size graft is subjected to ischemia and reperfusion injury and transplanted into a recipient with urgent metabolic needs. In this situation, both loss of a substantial part of the initial liver mass as well as oxidative stress after reperfusion are central mechanisms of hepatic injury[1,2].

Liver resection triggers release of the cytokines tumor necrosis factor (TNF) and interleukin 6 (IL-6), crucial priming factors for the initiation of hepatocyte proliferation by activation of the janus activated kinases/signal transducer and activator of transcription (JAK/STAT) pathway[3-5]. This priming phase stimulates resting hepatocytes to enter the G_1 phase of the cell cycle. Simultaneously, growth factors including hepatocyte growth factor (HGF), contribute to the passage of hepatocytes from the G_1 into the S phase by activating the phosphoinositide-3 kinase (PI3K)/Akt signal transduction pathway[6-8]. PI3K/Akt interacts with the mammalian target of rapamycin (mTOR), involved in the control of protein synthesis, cell size and proliferation[9,10]. Both cascades lead to activation of a variety of signaling pathways, including upregulation of several downstream cyclins like cyclin D1, which is associated with the G_1-S phase transition of hepatocytes[3,4,6,11,12].

Besides being a key regulator of cell growth and proliferation, mTOR was recently identified to play an important role in the control of autophagy[13-15]. Autophagy is an evolutionarily conserved lysosomal degradation pathway that plays an important protective role in case of cellular injury by mediating the elimination of damaged cellular components[13]. In non-hepatic cells, autophagy has not only been implicated as a survival response, but also as a mediator of cell death during stress conditions[16,17]. Autophagy might therefore play a role in liver regeneration, though this has not been thoroughly studied. This is of special interest to the field of liver transplantation as mTOR inhibition, in combination with a short course of steroids, is an attractive alternative for current calcineurin inhibitor based immunosuppressive strategies. Calcineurin inhibitors are neurotoxic, associated with a 20% incidence of chronic kidney dysfunction and carry a cumulative risk for *de novo* malignancy of up to 55% at 15 years after liver transplantation[18-22]. mTOR inhibitors like rapamycin therefore represent an important immunosuppressive option, especially in patients with calcineurin inhibitor-induced neurotoxicity, poor renal function and possibly also in patients with hepatocellular carcinoma. However, in the initial phase after liver transplantation, the mTOR inhibitor rapamycin is rarely used, since it is reported to delay liver regeneration[23-25].

Rapamycin inhibits mTOR complex 1 (mTORC1) by complex formation with FK506 binding protein 12, thereby acting on its downstream messengers and abrogating translation initiation and protein synthesis, which results in cell cycle arrest at the G_1 to S phase[23-25]. Cyclin D1 as well as p21 are shown to be important downstream messengers of the rapamycin-mediated cell cycle arrest[26-28]. The exact underlying cellular and molecular mechanisms by which mTOR inhibition attenuates liver regeneration and the interplay between mTOR inhibition and autophagy in liver regeneration needs to be further characterized.

Both after kidney as well as deceased liver transplantation, mTOR inhibition in combination with steroids has proven an efficient immunosuppressive strategy. Addition of an mTOR inhibitor to steroid treatment might therefore also show beneficial effects after living-donor

liver transplantation, especially in patients with compromised renal function. Aim of this study is to investigate the effects of mTOR inhibition, in combination with the steroid dexamethasone, on liver regeneration and autophagy in a surgical resection model and in particular its involvement in IL-6 and HGF stimulated pathways. Besides mimicking the post-transplant treatment strategy, this combination of immunosuppressants also allowed more specific investigation of the effects of exogenous IL-6 and HGF, since steroids are multi-potent inhibitors of endogenous production of pro-inflammatory cytokines like TNF and IL-6[29]. Effects on body and liver weight, hepatocyte proliferation, autophagy and hepatic function and injury were analyzed at specific time points after surgery in a 70% partial hepatectomy (PH) model in mice.

MATERIALS AND METHODS

Animals

Male C57Bl/6 mice (age 12-15 wk) were obtained from Charles River (Maastricht, Netherlands) and maintained in the animal facility on a 12/12 h light/dark schedule. The animals had free access to food and drinking water and received care according to the Guide for the Care and Use of Laboratory Animals. All animal experiments were performed with approval of the institutional animal welfare committee.

PH and treatments

Liver regeneration was induced in C57BL/6 mice by performing a 70% PH as first described by Higgins and Anderson in 1931. Animals were anaesthetized with isoflurane and, after a midline laparotomy, the left lateral and median lobes of the liver were ligated and resected. The peritoneum and skin were sutured separately. All procedures were performed under clean conditions. Animals were treated intraperitoneally every 24 h, starting at time of PH, with a combination of the immunosuppressants rapamycin (2.5 mg/kg per day; sirolimus oral solution, Wyeth Pharmaceuticals, Louvain-la-Neuve, Belgium) and dexamethasone (2.0 mg/kg per day, Organon, Oss, The Netherlands) in phosphate buffered saline (PBS) (Lonza, Verviers, Belgium; total volume 0.5 mL) or with PBS alone as vehicle control. In the immunosuppressant (Rapa-Dex) group, part of the group was treated subcutaneously 4 h prior to and 24 h after PH with a combination of human recombinant IL-6 (500 μg/kg per day; Peprotech, London, United Kingdom) and HGF (100 μg/kg per day; Peprotech) in PBS. Animals (n = 5-9 per group) were sacrificed 2, 3 or 5 d after PH and liver tissue and blood were collected for further analysis. To investigate the effects of dexamethasone alone, an additional group was treated with dexamethasone alone (Dex) as described above and sacrificed at day 2 after PH.

Weight calculations

Animals were weighed daily prior to treatment and the resected liver mass was weighed after PH. The initial total liver weight was calculated as follows: resected liver weight/70 × 100 (g).

At time of sacrifice, animals and their regenerated liver mass were weighed and the percentage of reconstitution of the liver was calculated by: regenerated liver weight/initial total liver weight × 100 (%).

Immunohistochemistry

One hour prior to sacrifice, animals were injected intraperitoneally with 50 mg/kg 5-Bromo-2'-deoxyuridine (BrdU; B5002, Sigma-Aldrich, Zwijndrecht, Netherlands). Livers were harvested and processed to 4 μm thick formalin fixed, paraffin embedded sections. Immunohistochemical staining for BrdU was achieved using monoclonal mouse anti-BrdU (Bu20a; DakoCytomation, Glostrup, Denmark; 1:80 in blocking buffer) as primary antibody and polyclonal anti-mouse IgG/HRP (P0161; DakoCytomation; 1:1000 in blocking buffer) as secondary antibody (see Supplemental Information for a full description of the protocol). Per animal 4 high power fields (HPF; × 400) were analyzed for BrdU positive hepatocytes.

Real-time quantitative reverse transcription-polymerase chain reaction

At time of sacrifice, liver tissue was stored overnight at 4 ℃ and thereafter at -80 ℃ in Allprotect Tissue Reagent (Qiagen, Hilden, Germany) for RNA preservation. After RNA extraction and reverse transcription (see Supplementary Information for the protocol), real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed with a SensiMix SYBR and Fluorescein Kit (Bioline, London, United Kingdom) and MyIQ real time PCR detection system (Bio-Rad Laboratories) according to the manufacturer's instruction. PCR primers (Table 1) were synthesized by Isogen Life Science (Maarssen, Netherlands) and Biolegio (Nijmegen, Netherlands). Gene expression levels were normalized using the ΔΔCT method and TATA binding protein as reference gene, because it is shown to be stable during different phases of liver regeneration[30].

Western blotting

Liver tissue, preserved in Allprotect as described, was assessed for autophagy by investigating hepatic protein levels of microtubule-associated protein 1 light chain 3 (LC3)-Ⅱ using rabbit polyclonal LC3A/B (1:1000, Cell Signaling Technology, Danvers, United States) and mouse purified IgG C4/actin (1:2500, BD Biosciences, Franklin Lakes, United States) as primary antibodies and goat-anti-mouse IgG IRDye 680 and goat-anti-rabbit IgG IRDye 800CW (both 1:5000; LI-COR Biosciences, Lincoln, United States) as secondary antibodies (See Supplemental Information for a full description of the protocol). Blots were scanned using an Odyssey Infrared Imager (LI-COR Biosciences) and the results were analyzed using Odyssey software.

Serum analysis of enzyme levels

Blood samples were collected at time of sacrifice in heparin coated microtubes. After collection, samples were

Table 1 Reverse transcription-polymerase chain reaction primer sequences

Gene	Name	Accession number	Primer (forward/reverse)
CCND1	Cyclin D1	NM_007631	GCGTACCCTGACACCAATCTC
			CTCCTCTTCGCACTTCTGCTC
PCNA	Proliferating cell nuclear antigen	NM_011045	CTTGGTACAGCTTACTCTGCG
			AGTTGCTCCACATCTAAGTCCAT
TNFA	Tumor necrosis factor alpha	NM_013693	CCCTCACACTCAGATCATCTTCT
			GCTACGACGTGGGCTACAG
IL1RN	Interleukin 1 receptor antagonist	NM_031167	GCTCATTGCTGGGTACTTACAA
			CCAGACTTGGCACAAGACAGG
IL6	Interleukin 6	NM_031168	TAGTCCTTCCTACCCCAATTTCC
			TTGGTCCTTAGCCACTCCTTC
HGF	Hepatocyte growth factor	NM_010427	ATGTGGGGGACCAAACTTCTG
			GGATGGCGACATGAAGCAG
TGFB	Transforming growth factor β	NM_011577	CTCCCGTGGCTTCTAGTGC
			GCCTTAGTTTGGACAGGATCTG
KDR	Vascular endothelial growth factor receptor 2	NM_010612	TTTGGCAAATACAACCCTTCAGA
			GCAGAAGATACTGTCACCACC
ANGPT1	Angiopoietin 1	NM_009640	CACATAGGGTGCAGCAACCA
			CGTCGTGTTCTGGAAGAATGA
VEGFA	Vascular endothelial growth factor A	NM_009505	GCACATAGAGAGAATGAGCTTCC
			CTCCGCTCTGAACAAGGCT
FLT1	Vascular endothelial growth factor receptor 1	NM_010228	TGGCTCTACGACCTTAGACTG
			CAGGTTTGACTTGTCTGAGGTT
TBP	TATA binding protein	NM_013684	AGAACAATCCAGACTAGCAGCA
			GGGAACTTCACATCACAGCTC

centrifuged (19 min, 1800 r/min) to separate the serum, which was further analyzed at the clinical chemical core facility of the Erasmus MC-University Medical Center to determine bilirubin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels.

Statistical analysis

Data are presented as mean ± SE. Statistical analysis was performed using the Mann-Whitney test or student *t*-test after checking for normal distribution. A *P*-value ≤ 0.05 was considered statistically significant.

RESULTS

Inhibition of mTOR causes progressive body weight loss after liver resection

As shown in Figure 1A, significant and progressive body weight loss was seen after PH in animals treated with Rapa-Dex compared to control treated animals (15% *vs* 6% loss, $P < 0.01$ at day 2; 11% *vs* 2%, $P = 0.04$ at day 3 and 25% *vs* 7%, $P < 0.01$ at day 5). No significant body weight loss was seen in animals treated with Dex alone (9% loss, $P = 0.11$ at day 2; data not shown). Combined treatment with Rapa-Dex and IL-6/HGF could not overcome the progressive weight loss and showed a similar effect on body weight (14% loss, $P < 0.01$ at day 2; 14%, $P = 0.06$ at day 3 and 24%, $P < 0.01$ at day 5).

Reduced liver mass reconstitution by mTOR inhibition can be overcome with exogenous IL-6 and HGF

After 70% PH in the control group, liver mass recovered to 54% of the initial total liver weight by day 2 and to 76% by day 3 (Figure 1B). Treatment with Rapa-Dex caused a significant inhibition in the reconstitution of liver mass at day 3 *vs* control treatment (63% of initial total liver weight, $P = 0.04$). A similar trend was seen at day 5, but differences did not reach statistical significance. Treatment

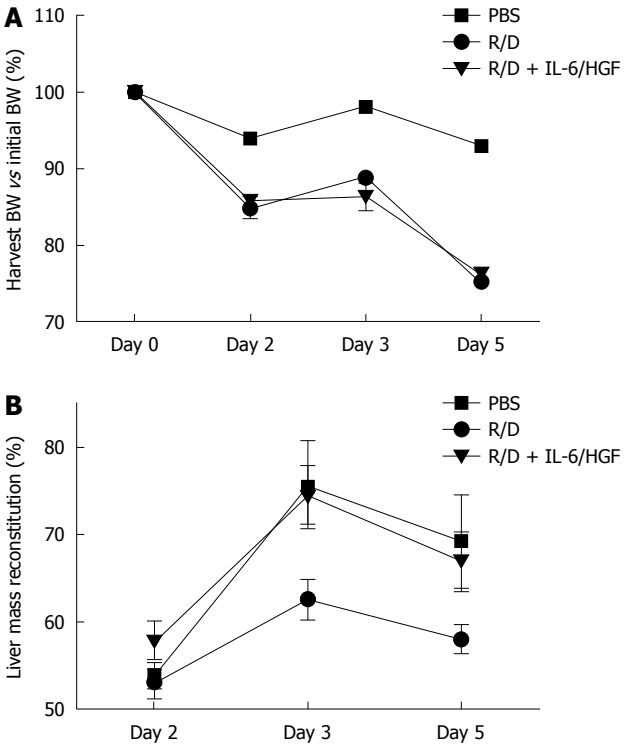

Figure 1 Effects of mammalian target of rapamycin inhibition on body and liver weight. A: Harvest body weight at days 2, 3 and 5 after partial hepatectomy (PH) *vs* initial body weight; B: Harvest liver weight at days 2, 3 and 5 after PH *vs* total liver weight prior to PH. Data are shown as mean ± SE. BW: Body weight; R/D: Rapa-Dex; IL-6: Interleukin 6; HGF: Hepatocyte growth factor; PBS: Phosphate buffered saline.

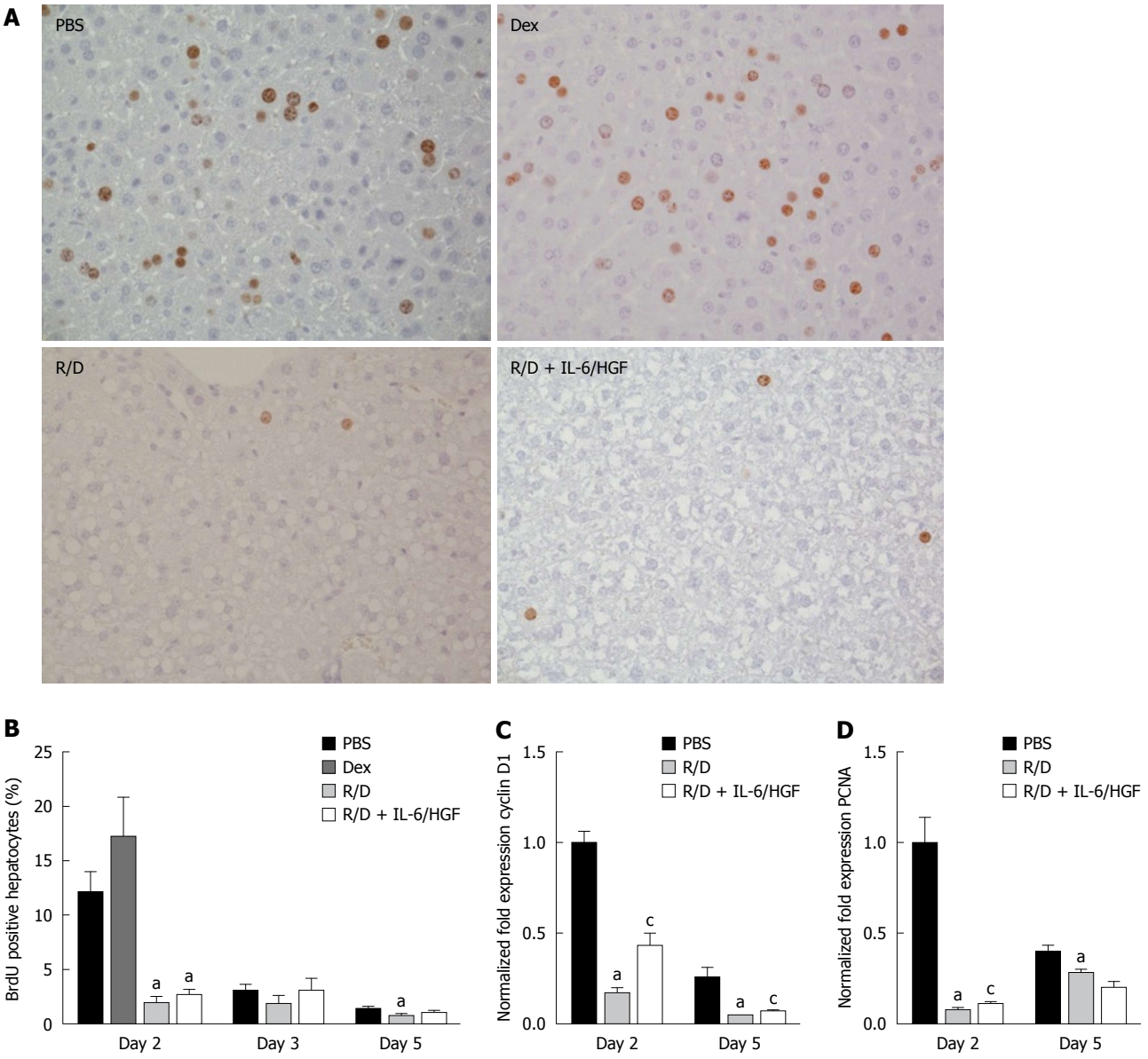

Figure 2 Effects of mammalian target of rapamycin inhibition on hepatocyte proliferation. A, B: Livers were processed for immunohistochemistry on 5-Bromo-2'-deoxyuridine (BrdU) to quantify hepatocyte proliferation. A: Representative pictures (× 400) of hepatocyte proliferation at day 2 after partial hepatectomy (PH); B: Quantification of hepatocyte proliferation at day 2, 3 and 5 after PH; C, D: Hepatic gene expression levels of cyclin D1 and proliferating cell nuclear antigen (PCNA) were determined by quantitative reverse transcription-polymerase chain reaction and normalized against TATA binding protein. C: Expression levels of cyclin D1 at day 2 and 5 after PH; D: Expression levels of PCNA at day 2 and 5 after PH. Data are shown as mean ± SE. $^{a}P \leq 0.05$ *vs* phosphate buffered saline (PBS); $^{c}P \leq 0.05$ *vs* Rapa-Dex (R/D). HGF: Hepatocyte growth factor; IL-6: Interleukin 6.

with Dex alone did not show significant differences compared to controls (57% of initial total liver weight at day 2, $P = 0.30$; data not shown). Combination of IL-6/HGF with Rapa-Dex completely restored liver reconstitution to control levels (75% of initial total liver weight at day 3, $P = 0.02$ *vs* Rapa-Dex and $P = 0.90$ *vs* controls).

IL-6 and HGF treatment upregulates cell cycle progression-related gene expression of cyclin D1 and proliferating cell nuclear antigen, but does not restore mTOR-induced inhibition of hepatocyte proliferation

Hepatocyte proliferation, quantified by the percentage of BrdU positive hepatocyte nuclei, was significantly reduced at day 2 after PH in animals treated with Rapa-Dex compared to control treated animals (2% *vs* 12%, $P < 0.01$; Figure 2A and B). mTOR inhibition delayed hepatocyte proliferation at least until day 5 (0.8% *vs* 1.4%, $P = 0.02$). In contrast, treatment with Dex alone had no significant effect on proliferation at day 2. Addition of exogenous IL-6/HGF to Rapa-Dex treatment did not significantly stimulate hepatocyte proliferation at any time point after PH, although no significant difference compared to control treatment was seen at days 3 and 5. Combined treatment of Rapa-Dex with IL-6/HGF did, however, cause a decrease in the number of hepatocytes per HPF compared to treatment with Rapa-Dex alone (170 cells/HPF *vs* 206 cells/HPF, $P = 0.05$; data not shown), suggesting an increase in cell size.

The inhibitory effect of mTOR inhibition on cell proliferation was also reflected in the hepatic gene expression levels of cyclin D1 and proliferating cell nuclear antigen (PCNA), known to be relevant for cell cycle progression and DNA synthesis. Compared to control treatment, Rapa-Dex treatment significantly downregulated expression of cyclin D1 (80% reduction, $P < 0.01$; Figure 2C) and PCNA (90% reduction, $P < 0.01$; Figure 2D) at day 2 after PH. Downregulation of cyclin D1 and PCNA gene expression after Rapa-Dex treatment continued at least until day 5 (80% and 30% reduction respectively, $P < 0.01$). Addition of IL-6/HGF to Rapa-Dex treatment significantly upregulated both cyclin D1 (2.6-fold, $P = 0.04$ at day 2 and 1.4-fold, $P = 0.03$ at day 5) and PCNA (1.3-fold, $P = 0.03$ at day 2) gene expression after PH compared to treatment with Rapa-Dex alone, but did not restore expression to control levels.

Inhibition of mTOR increases autophagy and hepatocyte injury during liver regeneration

During autophagy, the cytosolic form of LC3 (LC3-Ⅰ) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-Ⅱ), which is recruited to autophagosomal membranes and therefore a quantitative marker for autophagy. As shown in Figure 3A, LC3-Ⅱ protein levels in control animals were significantly reduced at day 2 after PH compared to levels before resection (48% reduction, $P = 0.05$). This finding suggests that baseline autophagy levels are reduced during liver regeneration. Compared to control treated animals, animals treated with Rapa-Dex showed a significantly higher LC3-Ⅱ protein expression at day 2 (46% increase, $P = 0.04$; Figure 3B and C). At day 5, LC3-Ⅱ levels were back at pre-resection levels in control treated animals, but appeared further increased in Rapa-Dex treated animals. Treatment with Dex alone did not cause significant differences in hepatic LC3-Ⅱ levels at day 2 (data not shown). Addition of exogenous IL-6/HGF to Rapa-Dex treatment had no significant effect on autophagy compared to Rapa-Dex alone, as LC3-Ⅱ protein levels remained significantly elevated.

As shown in Figure 4A-C, treatment with Rapa-Dex furthermore significantly increased serum AST levels at day 2 (641 U/L *vs* 185 U/L, $P = 0.02$) and caused a non-significant increase in ALT and bilirubin levels, compared to control treatment. Treatment with Dex alone did not cause changes in serum levels of these liver injury markers. Combined treatment with Rapa-Dex and IL-6/HGF significantly elevated levels of AST (1387 U/L, $P < 0.01$), ALT (823 U/L *vs* 67 U/L, $P < 0.01$) as well as bilirubin (39 μmol/L *vs* 18 μmol/L, $P = 0.04$). In accordance with serum levels of these injury markers, treatment with Rapa-Dex, either with or without IL-6/HGF, caused progressive changes in liver histology with formation of necrotic areas (Figure 4D).

mTOR inhibition alters expression of genes relevant for cell proliferation and inflammation

At day 2 after PH, treatment with Rapa-Dex significantly upregulated hepatic gene expression of the pro-inflammatory cytokine TNF-α (3.2-fold, $P = 0.03$; Figure 5A) and the anti-inflammatory cytokine IL-1 receptor antagonist (IL-1Ra; 6.0-fold, $P < 0.01$; Figure 5B) compared to control treatment. No significant effects were seen for IL-6 gene expression (Figure 5C). In contrast, gene expression of HGF was significantly downregulated (40% reduction, $P < 0.01$; Figure 5D), whereas the observed reduced expression of transforming growth factor β (TGF-β) was not statistically significant (Figure 5E). Addition of IL-6/HGF to Rapa-Dex treatment restored the upregulated expression of TNF-α and IL-1Ra to control levels. Combined treatment did however not reverse the downregulated expression of HGF or TGF-β. At day 5, treatment with Rapa-Dex led to progressive upregulation

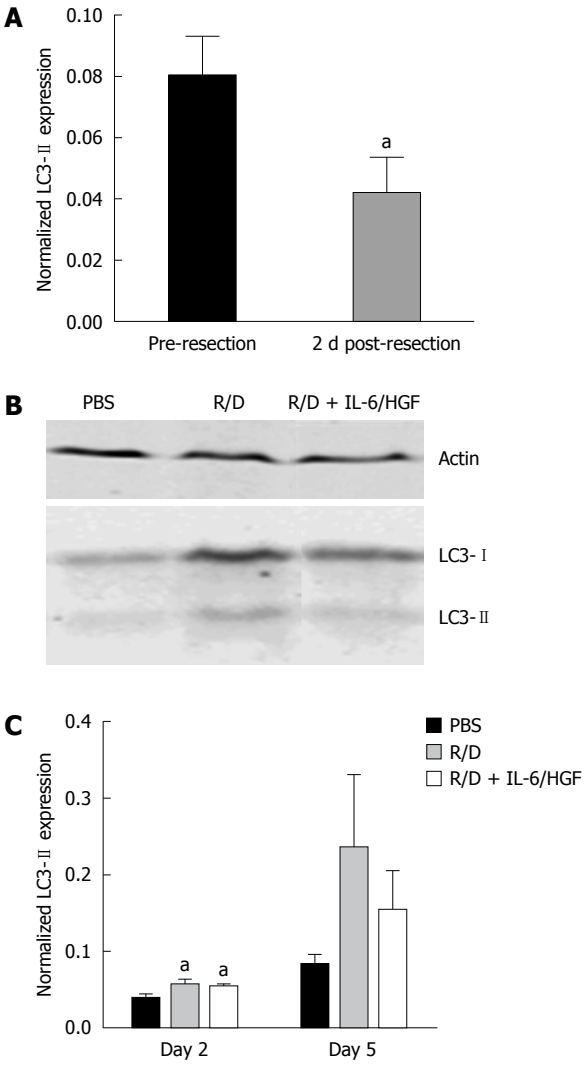

Figure 3 Effects of partial hepatectomy and mammalian target of rapamycin inhibition on hepatic autophagy. Hepatic protein levels of the autophagy marker microtubule-associated protein 1 light chain 3 (LC3)-Ⅱ were determined by Western blotting analysis and normalized against actin. A: Effects of liver resection on autophagy at day 2 after partial hepatectomy (PH); B: Western blotting showing effects of mammalian target of rapamycin inhibition on autophagy at day 2 after PH; C: Quantification of autophagy at day 2 and 5 after PH. Data are shown as mean ± SE. $^aP \leq 0.05$ *vs* phosphate buffered saline (PBS). R/D: Rapa-Dex; HGF: Hepatocyte growth factor; IL-6: Interleukin 6.

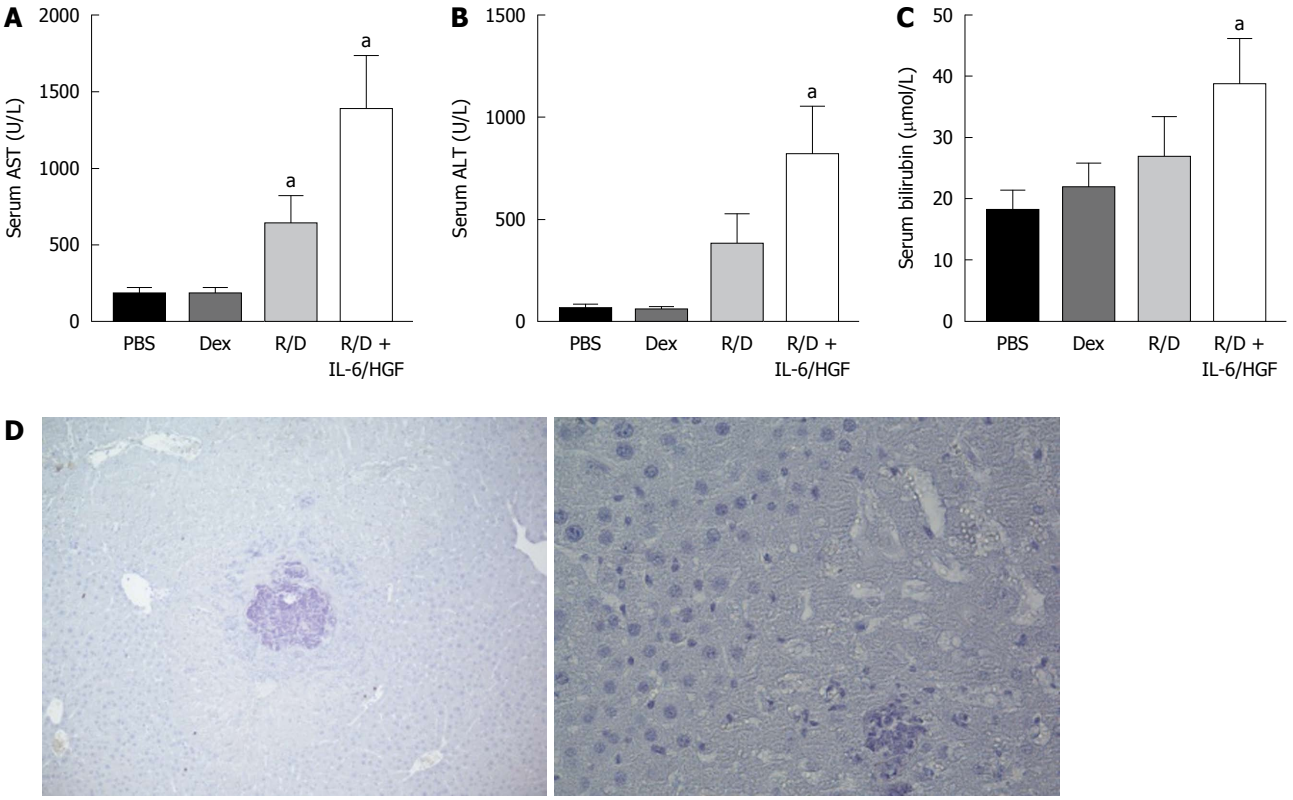

Figure 4 Effects of mammalian target of rapamycin inhibition on hepatocyte injury. Serum levels at day 2 after partial hepatectomy (PH) for aspartate aminotransferase (AST) (A), alanine aminotransferase (ALT) (B) and bilirubin (C); D: Histologic changes (× 400) at day 5 after PH in liver tissue from Rapa-Dex (R/D) treated animals. Data are shown as mean ± SE. $^{a}P \leq 0.05$ *vs* phosphate buffered saline (PBS). HGF: Hepatocyte growth factor; IL-6: Interleukin 6.

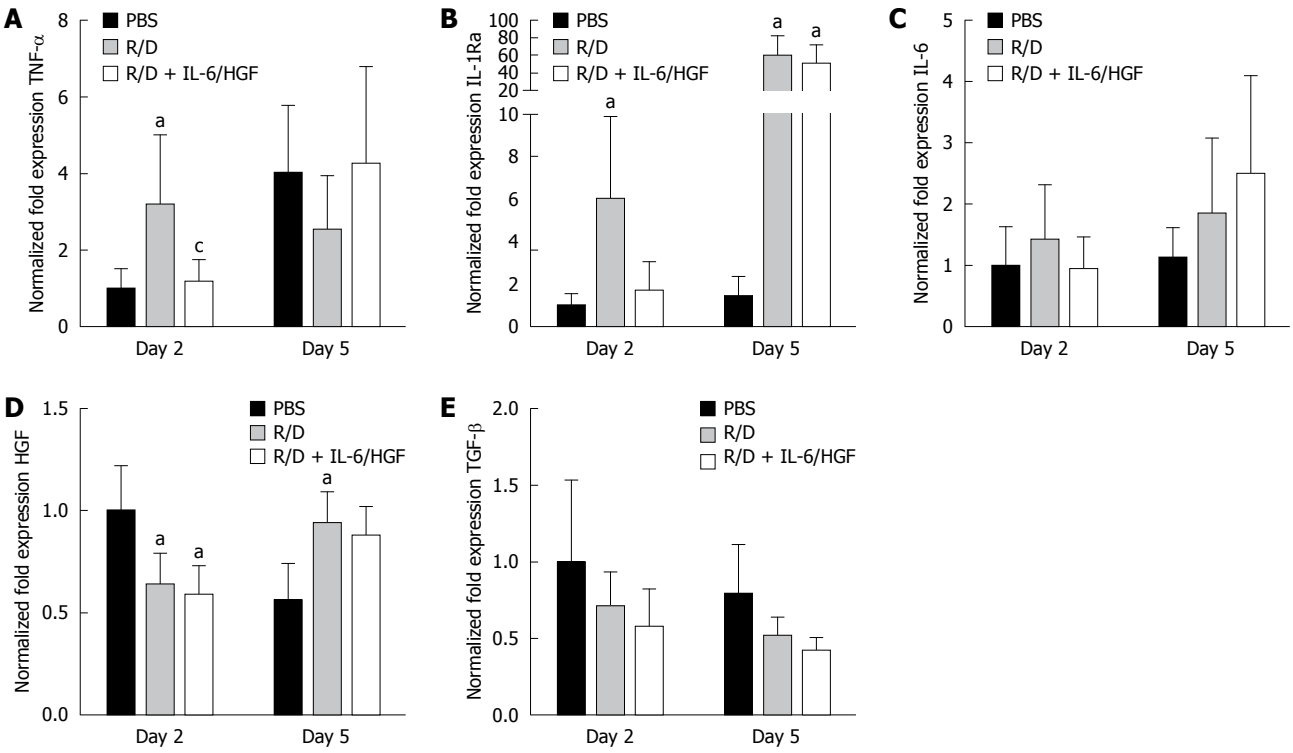

Figure 5 Effects of mammalian target of rapamycin inhibition on inflammation and cell cycle related gene expression. Hepatic gene expression levels were determined by quantitative reverse transcription-polymerase chain reaction and normalized against TATA binding protein. A: Expression levels of tumor necrosis factor α (TNF-α) at day 2 and 5 after partial hepatectomy (PH); B: Expression levels of interleukin 1 receptor antagonist (IL-1Ra) at day 2 and 5 after PH; C: Expression levels of interleukin 6 (IL-6) at day 2 and 5 after PH; D: Expression levels of hepatocyte growth factor (HGF) at day 2 and 5 after PH; E: Expression levels of transforming growth factor β (TGF-β) at day 2 and 5 after PH. Data are shown as mean ± SE. $^{a}P \leq 0.05$ *vs* phosphate buffered saline (PBS); $^{c}P \leq 0.05$ *vs* Rapa-Dex (R/D).

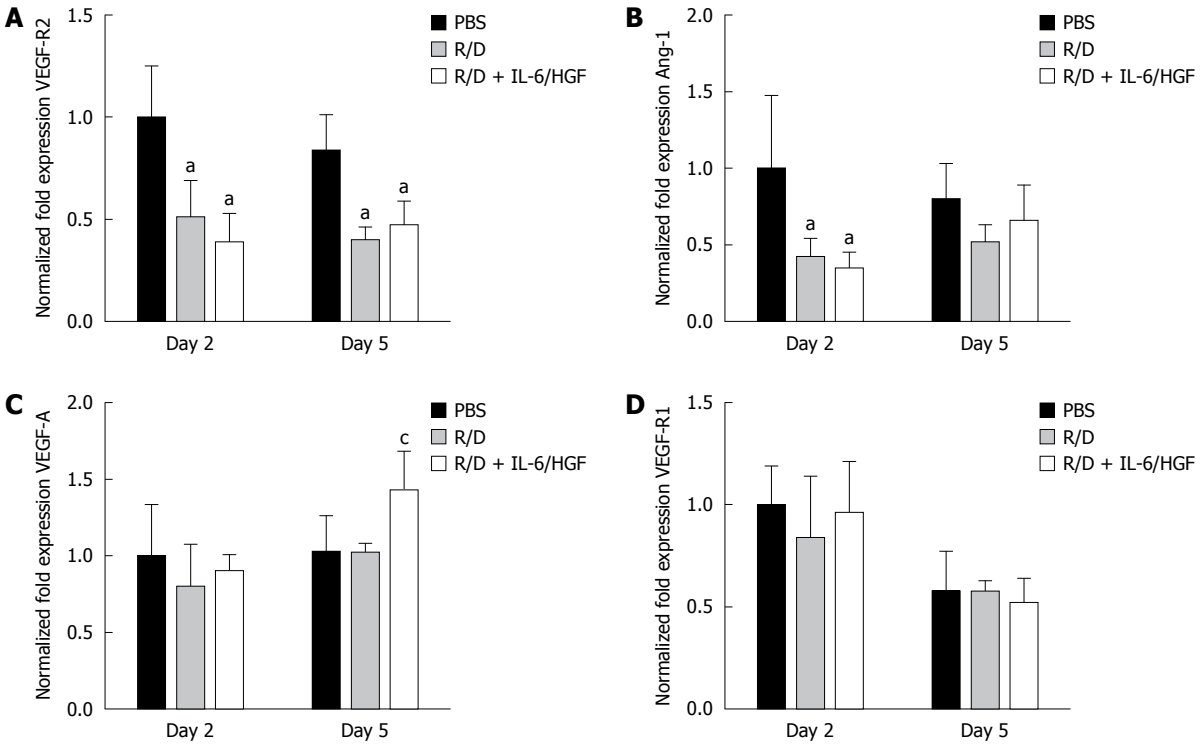

Figure 6 Effects of mammalian target of rapamycin inhibition on angiogenic gene expression. Hepatic gene expression levels were determined by quantitative reverse transcription-polymerase chain reaction and normalized against TATA binding protein. A: Expression levels of vascular endothelial growth factor receptor 2 (VEGF-R2) at day 2 and 5 after partial hepatectomy (PH); B: Expression levels of angiopoietin 1 (Ang-1) at day 2 and 5 after PH; C: Expression levels of VEGF-A at day 2 and 5 after PH; D: Expression levels of VEGF-R1 at day 2 and 5 after PH. Data are shown as mean ± SE. $^{a}P \leq 0.05$ *vs* phosphate buffered saline (PBS); $^{c}P \leq 0.05$ *vs* Rapa-Dex (R/D). HGF: Hepatocyte growth factor; IL-6: Interleukin 6.

of IL-1Ra gene expression (42.3-fold, $P < 0.01$) as well as upregulation of HGF gene expression (1.7-fold, $P = 0.03$) compared to control treatment. Addition of IL-6/HGF to Rapa-Dex could not restore IL-1Ra and HGF gene expression at this time point.

Treatment with Rapa-Dex impairs pro-angiogenic gene expression

As shown in Figure 6, treatment with Rapa-Dex significantly downregulated hepatic gene expression levels of vascular endothelial growth factor receptor 2 (VEGF-R2; 50% reduction, $P = 0.01$) and angiopoietin 1 (Ang-1; 60% reduction, $P < 0.01$) at day 2 after PH compared to control treatment. Downregulation of VEGF-R2 expression continued at least until day 5 (50% reduction, $P < 0.01$). Addition of IL-6/HGF to Rapa-Dex treatment did not affect the downregulated expression levels of VEGF-R2 or Ang-1. Gene expression levels of VEGF-A and VEGF-R1 were not significantly reduced after Rapa-Dex treatment.

DISCUSSION

Current immunosuppressive strategies in the first period after liver transplantation mostly involve treatment with steroids in combination with mycophenolic acid, IL-2 receptor antagonists or calcineurin inhibitors[31]. These regimes are however associated with chronic renal failure, with an incidence of up to 20% kidney dysfunction over time[18]. The mTOR inhibitor and immunosuppressant rapamycin, in contrast to the calcineurin inhibitors tacrolimus and cyclosporin, does not cause nephrotoxicity and is suggested to be a good alternative in transplant patients with deteriorating renal function[32-34].

Recently, mTOR inhibition has gained wide interest in the treatment of cancer[35,36]. Therefore, also in patients transplanted for hepatocellular carcinoma, mTOR inhibitors are an attractive alternative with reported inhibitory effects on tumor growth and recurrence[37-40]. However, mTOR is a key regulator of cell growth and proliferation and its inhibition is reported to have detrimental effects on liver regeneration[23-25]. There may however be a more intricate relation as mTOR also regulates metabolism and inhibition of mTOR may preserve energy supplies for the remaining hepatocytes after liver resection to keep up metabolic function. This is supported by a recent publication showing excellent results in patients treated *de novo* with rapamycin after living-donor liver transplantation as well as data from animal experiments showing no increase in mortality with rapamycin treatment, even after a 90% liver resection and despite inhibited hepatocyte proliferation[41,42].

Additionally, mTOR has been implicated to be of paramount importance in the control of autophagy, a general term for pathways in which cytoplasmic material, including soluble macromolecules and organelles, are delivered to lysosomes for degradation[13,43-45]. Autophagy is thought to have evolved as a stress response mechanism

that allows organisms to survive during harsh conditions, probably by regulating energy homeostasis[16]. Early histomorphologic studies showed a decrease in autophagic bodies of up to 98% at day 1 after PH[46-48]. This can support the hypothesis that the inhibition of intracellular autophagic degradation in regenerating liver has its biochemical equivalent, *i.e.*, inhibited protein catabolism, and is interpreted as an important and adequate mechanism to shift from the physiological steady state to compensatory growth of the liver after PH. Degli Esposti *et al*[49] showed the presence of autophagy in 21% of good functioning human liver grafts 2 h after reperfusion, without differences between normal and steatotic livers. Ischemic preconditioning in this study increased autophagy only in steatotic livers, which appeared to have a protective effect on post-operative function. Wang *et al*[50] showed that autophagy is essential for hepatocyte resistance to oxidant stress and that loss of macroautophagy led to overactivation of the c-Jun N-terminal kinase signaling pathway that induced cell death. Therefore we studied the interplay between liver regeneration, mTOR inhibition and autophagy in a transplant-related 70% PH model. In accordance with the findings of others, we found a significant decrease in proliferating hepatocytes from 12% to 2% after mTOR inhibition, with concomitant decreases in hepatic gene expression of the cell cycle genes cyclin D1 and PCNA[25,42,51]. This was furthermore accompanied by increased serum transaminases, suggesting increased liver injury.

Rupertus *et al*[40] recently described that rapamycin had no detrimental effects on liver regeneration, yet in their study hepatocyte proliferation was not actually measured, but only estimated from wet liver weight at 12 d after hepatectomy. In our experiment, wet liver weight after mTOR inhibition was still lower at day 5 after liver resection. In the study of Dahmen *et al*[42] BrdU incorporation decreased from 17% to less than 1% at 2 d after 90% hepatectomy, without effects on survival. In the study of Palmes *et al*[25] the same effects were found, with decreased gene expression levels of TNF-α, HGF and TGF-β at day 2 after a 70% liver resection. Interestingly, in our series, we found a significant upregulation of TNF-α, downregulation of HGF, but no significant changes in IL-6 and TGF-β gene expression.

Similar to the Palmes study, gene expression of the angiogenic factors VEGF-R2 and Ang-1 was downregulated in our experiments. Inhibition of angiogenesis is suggested to be one of the most relevant mechanisms by which tumor growth and recurrence is inhibited[39,40].

In our study, mTOR inhibition furthermore resulted in a profound upregulation of IL-1Ra gene expression, which was not reported before. IL-1Ra is an anti-inflammatory cytokine, reported to be released in response to both surgical as well as toxic liver injury and to have a protective effect after CCl4-induced toxic liver injury[52-54].

We investigated whether the inhibition in hepatocyte proliferation could be overcome by kick-starting the priming phase of liver regeneration by pre-resection administration of IL-6 and HGF, both described to stimulate liver regeneration, especially in combined treatment[55-57]. It appeared that treatment with exogenous IL-6 and HGF partly reversed the negative effects of rapamycin by restoring TNF-α and IL-1Ra gene expression to control levels, significantly increasing gene expression of Cyclin D1 and PCNA and normalizing liver weight reconstitution. However, no significant increase in hepatocyte proliferation was found and serum transaminases were even further elevated, suggesting increased hepatocyte damage. This is in line with the findings of Haga *et al*[9], who found in their model of LPdk1KO mice that the PI3K/PDK1/Akt/mTOR pathway was regulated independent of the IL-6/JAK/STAT3 pathway. An alternative explanation for the increase in liver weight could be cellular hypertrophy cq. edema, which is supported by the decreased number of hepatocytes per HPF in this treatment group.

For the first time, we describe that mTOR inhibition also significantly increased hepatic autophagy during liver regeneration after PH. Earlier, Kondomerkos *et al*[58] showed that mTOR inhibition by rapamycin increased autophagy in the liver and heart of newborn animals. This effect may compensate for the decreased hepatocyte proliferation, as increased autophagy ameliorates oxidative stress and saves cellular energy.

Finally, the ongoing loss of body weight in mice treated with rapamycin is noteworthy. Similar effects of rapamycin on body weight have previously been reported by DiJoseph *et al*[59] and Zafar *et al*[60]. The role of mTOR in metabolism is complicated; it has been described that chemical inhibitors of glycolysis and mitochondrial function suppress mTORC1 activity, indicating that mTORC1 senses cellular energy[35]. This is crucial, because mTORC1-driven growth processes consume a large fraction of cellular energy and thus could be deleterious to starving cells. The mTORC1 pathway indirectly senses low ATP by a mechanism that is centred on the AMP-activated protein kinase[61]. During starvation, mTOR must be downregulated to avoid energy expenditure in absence of nutrients. Therefore pharmacological inhibition of mTORC1 could lead to a defective energy sensing system, mimicking starvation. On the other hand, rapamycin, as mTORC1 inhibitor, may protect the regenerating liver through this mechanism by slowing down the anabolic processes and saving energy and this may account for the fact that animals survive, despite seriously hampered liver regeneration.

In summary, this study investigated the role of mTOR in liver regeneration *in vivo* and more specific in IL-6 and HGF stimulated signaling pathways. mTOR inhibition resulted in inhibited liver regeneration and increased hepatic autophagy. Although exogenously administered IL-6 and HGF could overcome the rapamycin-induced inhibited reconstitution of liver mass and furthermore upregulated gene expression of factors known to be downstream of mTOR, no significant beneficial effects on body weight, hepatocyte proliferation, autophagy or

markers of liver injury were seen. To interpret these data on mTOR inhibition in relation to the clinical setting of living-donor liver transplantation, it is important to realize that the model used is limiting in that it is purely a liver regeneration model without ischemia and reperfusion injury or alloreactivity. However, from these results, the use of mTOR inhibitors in the early post-transplant setting can currently not be recommended, despite their recently reported beneficial effects on cancer development and kidney function.

ACKNOWLEDGMENTS

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COMMENTS

Background

The liver has a remarkable regenerative capacity to compensate for lost or damaged liver tissue after injury. This process enables living-donor liver transplantation, a setting in which 40%-60% of the liver of a healthy donor is transplanted into a recipient with end-stage liver disease. Treatment of the recipient with immunosuppressive medication is necessary to prevent rejection of the liver graft. Inhibition of the protein mammalian target of rapamycin (mTOR) represents an important immunosuppressive strategy. In the initial phase after living-donor liver transplantation, the mTOR inhibitor rapamycin is rarely used, as mTOR is a key regulator of cell growth and proliferation and concerns have been raised regarding adverse effects on liver regeneration. However, the exact mechanisms by which mTOR inhibition attenuates liver regeneration are largely unknown.

Research frontiers

The mTOR inhibitor rapamycin, in contrast to most immunosuppressive agents, does not cause nephrotoxicity and has recently gained wide interest in the treatment of cancer. mTOR inhibitors are therefore an attractive alternative in patients with deteriorating kidney function and also in patients transplanted for hepatocellular carcinoma. Furthermore, besides being a key regulator of cell growth and proliferation, mTOR was recently identified to play an important role in the control of autophagy. Autophagy is a degradation pathway that plays a protective role in case of cellular injury. It has been implicated as a survival response as well as a mediator of cell death during stress conditions, and might therefore play a role in liver regeneration.

Innovations and breakthroughs

Previous studies have reported detrimental effects of mTOR inhibition on liver regeneration. In contrast, a recent publication shows excellent results in patients treated *de novo* with rapamycin after living-donor liver transplantation. Here we report that mTOR inhibition severely impairs liver regeneration and increases autophagy after liver resection in mice. The most novel finding of this study is that this impaired regeneration can be partly reversed by treatment with the cytokine interleukin 6 (IL-6) and growth factor hepatocyte growth factor (HGF), both described to stimulate liver regeneration, especially if combined.

Applications

From the authors' results, the use of mTOR inhibitors in the early post-transplant setting can currently not be recommended, despite their recently reported beneficial effects on cancer development and kidney function. However, this study contributes to a better understanding of the role of mTOR and autophagy in liver regeneration and more specific in IL-6 and HGF stimulated signaling pathways.

Terminology

Regeneration is the process of restoration, growth and renewal that makes cells, tissues or organisms resilient to natural fluctuations or events that cause injury or loss. mTOR is a protein kinase that regulates cell growth, proliferation and survival, as well as protein synthesis and transcription. Autophagy is the basic catabolic mechanism that involves cell degradation of unnecessary or dysfunctional cellular components through the lysosomal machinery, thereby enabling recycling of cellular components and ensuring cellular survival during starvation.

Peer review

The summary is complete and serves to provide the relevant information of the paper. The introduction is adequate. The methodology is descriptive and logical. The results are very well described. The discussion fully satisfies the requirements to compare the results with existing data from the literature. The current literature is related to the topic. The figures are well prepared and properly described.

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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