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Original Article

Limited therapeutic efficacy of thrombopoietin on the regeneration of steatotic livers

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Abstract: Liver regeneration after partial hepatectomy is impaired in steatotic livers of leptin-deficient ob/ob mice. Previous studies have shown that thrombopoietin (TPO) promotes liver regeneration and improves liver cirrhosis by an increase of platelet counts and the expansion of hepatic progenitor cells. Herein we studied whether TPO exerts pro-proliferative and hepatoprotective effects and thereby improves the regenerative capacity of steatotic livers. For this purpose, we studied hepatic regeneration at day 2, 3, 7 and 10 in a model of 55% hepatectomy in obese (ob/ob) and non-obese (C57BL/6J) mice. Liver function and injury, platelet counts, weight of the regenerated liver, proliferating liver cells as well as the number of hepatic (CK19-positive) oval cells were quantified by biochemical and immunohistochemical analysis. As expected, obese mice had a markedly decreased regenerative capacity of livers compared with lean animals. Pretreatment of mice with recombinant TPO (12.5 μg/kg) had no evident effect on regeneration of fatty livers, but ameliorated acute liver damage in obese mice, as indicated by decreased liver enzyme release early after resection. TPO was unable to enhance hepatocyte proliferation, but increased proliferation of non-parenchymal cells, including CK19-positive oval cells, at later observation time points after resection. Interestingly, TPO completely inhibited the resection-induced increase of plasma triglycerides immediately after resection in non-obese mice. In conclusion, TPO slightly prevents acute liver damage after resection in obese mice, but fails to significantly enhance regeneration of fatty livers.

Keywords: Thrombopoietin, partial hepatectomy, ob/ob, steatosis, oval cells

Introduction

Due to the worldwide problem of shortage in donor organs, the use and transplantation of so called 'marginal' organs such as steatotic livers is increasing. However, hepatic resection or transplantation of a steatotic liver is often associated with immediate graft failure, caused not only by ischemia reperfusion injury, but also by impaired liver regeneration [1, 2]. It has been reported that alteration of signaling mechanisms and cell-cycle–related proliferative disorders [3] are the reasons for a largely impaired regeneration of steatotic livers after hepatectomy [2]. In fatty livers, expansion of hepatic progenitor cell populations, such as oval cells, is suggested to compensate for the increased turnover of senescent hepatocytes [4]. Thus, pharmacological manipulation of compromised liver regeneration via pleiotropic substances might be of high clinical relevance to decrease complications and mortality after resection and transplantation of steatotic livers.

Thrombopoietin (TPO) was first identified as a hematopoietic growth factor involved in megakaryocytopoiesis and platelet production [5]. TPO demonstrates structural homology with erythropoietin (EPO) [6] which has been recognized as antiapoptotic and tissue-protective pleiotropic substance in various nonhematopoietic tissues [7-9]. Few studies have examined the interactions between various hematologic factors and liver regeneration. It has been demonstrated that thrombocytotic conditions mediate anti-fibrotic effects [10] and promote liver regeneration by increasing hepatic concentration of pro-proliferative growth factors [11-14]. In line with this Matsuo et al. [13] could show that the transfusion of platelet-rich plasma promoted liver regeneration. In addition, platelet-derived serotonin has been reported to mediate liver regeneration [11]. The ability of TPO to stimulate cell proliferation is supposedly due to direct effects of platelets on hepatocytes via the release of growth factors, as platelets are a

Table 1. Baseline values of sham-operated mice. Values of 6 animals per group are given as means ± SEM

	lean	ob/ob
platelets (x 10^9/l)	1177 ± 60	997 ± 62
albumin (mg/ml)	23.7 ± 2.4	26.0 ± 1.2
triglycerides (mg/dl)	44.4 ± 4.2	71.6 ± 7.9**
ALT (U/l)	28.9 ± 1.1	275.1 ± 42.8**
GLDH (U/l)	13.0 ± 2.3	201.3 ± 9.3**
BrdU-positive parenchymal cells (n/HPF)	0.23 ± 0.08	0.78 ± 0.24
BrdU-positive non-parenchymal cells (n/HPF)	1.19 ± 0.22	6.02 ± 1.91*
CK19-positive cells (n/HPF)	1.6±0.4	5.2 ± 1.8

*p<0.05 vs. lean; **p<0.01 vs. lean.

major source of HGF during liver regeneration [15, 16]. Hepatocyte proliferation might further be promoted by TPO due to its cooperative effects with sinusoidal endothelial cells or Kupffer cells [15, 16]. Additionally, it is described that TPO acts as a growth factor for hematopoietic stem cells [17, 18] and hepatic progenitor cells [19, 20].

Based on this, we hypothesized that TPO improves regeneration of steatotic livers after hepatectomy by activating the hepatic progenitor cell compartment. For this purpose, we used leptin deficient ob/ob mice, which exhibit obesity, severe hepatic steatosis and insulin resistance [21, 22]. They are further characterized by a profoundly impaired liver regeneration [23]. Animals underwent a 55% liver resection and were treated with TPO. Lean littermates and NaCl-treated animals served as controls.

Materials and methods

Experimental groups and protocol

Obese (ob/ob; B6.V-lep^{ob}/J) and non-obese mice (lean C57BL/6J; 10-12 week old, Charles River Laboratories, Sulzfeld, Germany) were treated with recombinant mouse TPO (12.5 µg/kg bw i.p; Sigma-Aldrich, Taufkirchen, Germany) 1 h before and 24 h after resection, and subsequently every 72 h. Control animals received equivalent volumes of physiological saline. Regenerating livers were analyzed 2, 3, 7 and 10 days after partial hepatectomy (PH) (n=6 animals per group and time point). Sham-treated obese and non-obese mice, without resection and receiving only isotonic saline, served as controls (n=6 animals per group) to obtain basal values.

Liver regeneration model

Upon approval by the local government, all experiments were performed in accordance with the German legislation on protection of animals and the National Institutes of Health 'Guide for the Care and Use of Laboratory Animals' (Institute of Laboratory Animal Resources, National Research Council; NIH publication 86-23 revised 1985).

Mice were anesthetized by breathing isoflurane (1.5 vol%) and subjected to a 55% PH. The animals were placed in supine position and a median laparotomy was followed by retraction of the xyphoid cartilage for an appropriate exposure of the liver and transection of hepatic ligaments. The lobus sinister lateralis was resected by placing 4-0 silk suture tie as proximal to the origin of the lobe as possible. A second ligature was placed in the cleavage of the median lobe to remove the lobus dexter medialis. After removing the tied lobes and irrigating the abdomen with warm saline, the peritoneum and the skin were closed with running 5-0 sutures, respectively. Subcutaneous 5 ml-depots of physiological saline served as volume replacement. All surgical procedures were performed under aseptic conditions between 8 AM and 11 AM to avoid circadian variations. The animals were allowed to recover from anesthesia and surgery under a red warming lamp and were held in single cages until the subsequent experiments followed at postoperative days 2, 3, 7 and 10 (n=6 animals per time point).

Hematological measurements and plasma enzyme levels

Animals were exsanguinated by puncture of the inferior vena cava for immediate separation of ethylenediaminetetraacetic acid (EDTA) plasma. Alanine aminotransferase (ALT) and glutamate dehydrogenase (GLDH) activities were measured spectrophotometrically as indicators of hepatocellular disintegration and necrosis. Blood platelet count was assessed with an automated cell counter (Sysmex KX-21, Sysmex).

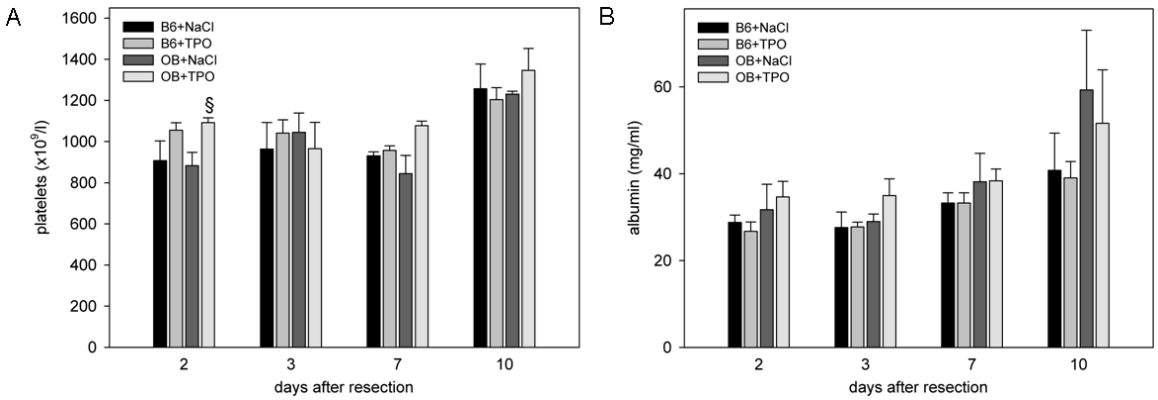

Figure 1. Effect of TPO on platelet counts and liver function. Platelet counts (A) and plasma albumin concentration (B) at multiple time points after hepatic resection of non-obese (B6) and obese animals (OB) receiving either recombinant mouse thrombopoietin (TPO, 12.5 µg/kg) or equivalent volumes of physiological saline (NaCl). Values are given in means ± SEM of six independent experiments per group and time point. ANOVA and post-hoc comparison; $^{\S}p<0.05$ vs. OB+NaCl.

Assays

EDTA plasma further served for the analysis of albumin as a parameter of liver function using a commercially available enzyme-linked immunosorbent assay kit in accordance with the manufacturer's instructions (Assaypro, MO, USA). Measurement of plasma triglycerides, serving as an indicator of dyslipidemia, was performed using the triglyceride assay kit method according to the manufacturer's instructions (Cayman Chemical Company, MI, USA).

Regeneration study

Animals were sacrificed and the remnant livers were harvested, weighed and processed for subsequent analysis. Two markers of liver regeneration were evaluated: (i) The weight of regenerated liver was used to calculate the growth of residual liver lobes according to weight of regenerated liver/preoperative liver weight × 100 (%). As determined by sham-treated animals, preoperative liver weight was assumed as 4.3% of body weight for lean mice and as 8% of body weight for obese mice. (ii) Being aware that liver weight is influenced by various extrinsic and intrinsic factors that are often unrelated to hepatic regeneration, we additionally used 5-bromo-2-deoxyuridine (BrdU) incorporation to study DNA synthesis upon liver resection by immunohistochemistry. For this purpose, mice were given BrdU (50 mg/kg bw ip) 1 h prior to harvest of liver tissue.

Histology and immunohistochemistry

Liver tissue of animals was excised, fixed in 4% phosphate buffered formalin for 2-3 days and then embedded in paraffin. From the paraffin-embedded tissue block, 5 µm sections were cut. To identify hepatic oval cells we performed immunostaining using an antibody against CK19 (TROMA3) (1:50, DSHB, Iowa, USA), a marker for hepatic oval cells [19]. Over night incubation (4°C) with the first antibody (monoclonal rat anti-CK19) was followed by alkaline-phosphatase (AP) conjugated goat anti-rat immunoglobulin (1:200, Santa Cruz). The sites of AP-binding were detected by Permanent Red (Dako Cytomation, Hamburg, Germany). The sections were counterstained with hemalaun and the number of oval cells was counted within 50 consecutive high power fields (HPF) (x 40 objective, numerical aperture 0.65) per specimen and were given as cells/HPF.

For the demonstration of DNA-incorporated BrdU in liver cells, sections collected on poly-L-lysine-coated glass slides were incubated with monoclonal mouse anti-BrdU antibody (1:50; Dako) over night at 4°C followed by horseradish-peroxidase (HRP)-conjugated goat anti-mouse immunoglobin (LSAB kit plus; Dako). The sites of peroxidase-binding were detected by 3,3'-diaminobenzidine (Dako). The sections were counterstained with hemalaun. BrdU-positive hepatocellular nuclei were counted within 50 HPF and were given as cells/HPF. In analogy, BrdU-expressing non-parenchymal

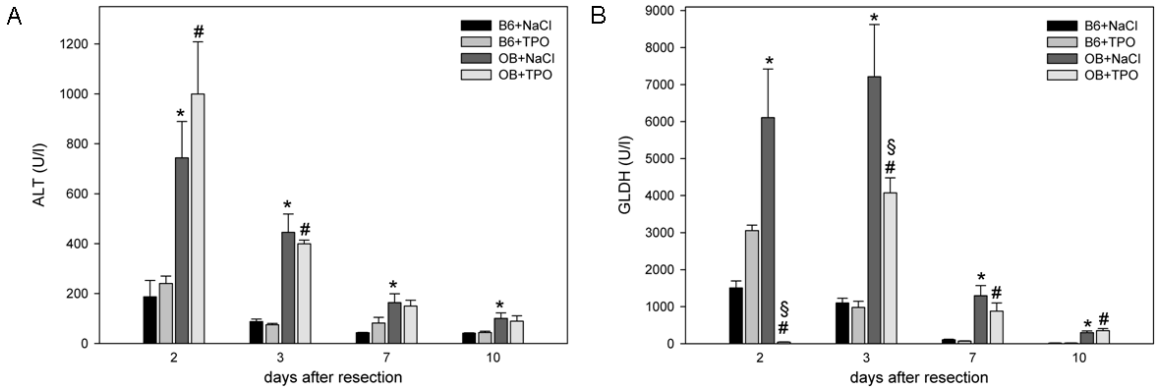

Figure 2. Effect of TPO on hepatic injury. Plasma activities of alanine aminotransferase (ALT) and glutamate dehydrogenase (GLDH) at multiple time points after hepatic resection in non-obese (B6) and obese animals (OB) receiving either recombinant mouse thrombopoietin (TPO, 12.5 µg/kg) or equivalent volumes of physiological saline (NaCl). Values of six animals per group and time point are given in means ± SEM. ANOVA and post hoc comparison; *p<0.05 vs. B6+NaCl; #p<0.05 vs. B6+TPO; §p<0.05 vs. OB+NaCl.

cells were assessed and also given as cells/HPF.

Statistical analysis

All data are expressed as means ± SEM. After testing for normality and equal variance across groups, differences between the groups were assessed by two-way ANOVA, followed by the appropriate post-hoc comparison test, including Bonferroni probabilities to compensate for multiple comparisons. For reasons of clarity and comprehensiveness, only statistically significant differences between groups at each time point are indicated in the figures. Basal values of sham-treated lean and obese mice were compared using either t-test or Mann-Whitney U test (**Table 1**). Statistical significance was set at $P<0.05$. Statistics were performed using the software package Sigma-Stat (Jandel Corporation, San Rafael, CA, USA).

Results

Effect of TPO on platelet counts

When compared to basal values of sham-operated mice (**Table 1**), the peripheral platelet count decreased early after resection in both NaCl-treated mice strains (**Figure 1A**). In turn, TPO administration increased platelet counts 2 days after resection by 23% in obese and 16% in lean mice. Subsequently, platelet count did not markedly differ between the groups and showed a general trend to higher numbers at the end of the observation time period (**Figure 1A**).

Effect of TPO on liver function

In both obese and lean mice, hepatic resection did not impair liver function, as given by plasma albumin levels (**Figure 1B**) which were in the range of values found in sham-animals (**Table 1**). Administration of TPO was without any noteworthy effect (**Figure 1B**).

Effect of TPO on liver injury

Biochemical analysis of plasma ALT (**Figure 2A**) and GLDH activities (**Figure 2B**) reflected the immediate, but transient resection-induced tissue damage. Notably, obese mice showed in general significantly higher levels of both ALT and GLDH compared to lean mice. In line with this, ob/ob mice exhibited markedly higher plasma ALT and GLDH activities already at baseline (**Table 1**). Pretreatment with TPO significantly reduced GLDH, but not ALT concentrations of obese mice at day 2 and 3 after resection (**Figure 2**).

Effect of TPO on hepatic steatosis

At baseline, levels of plasma triglycerides were significantly higher in ob/ob mice than in lean controls (**Table 1**). While in lean NaCl-treated mice triglycerides increased dramatically at day 2, indicating the resection-induced release of triglycerides from adipose tissue deposits, obese mice failed to respond in this way (**Figure**

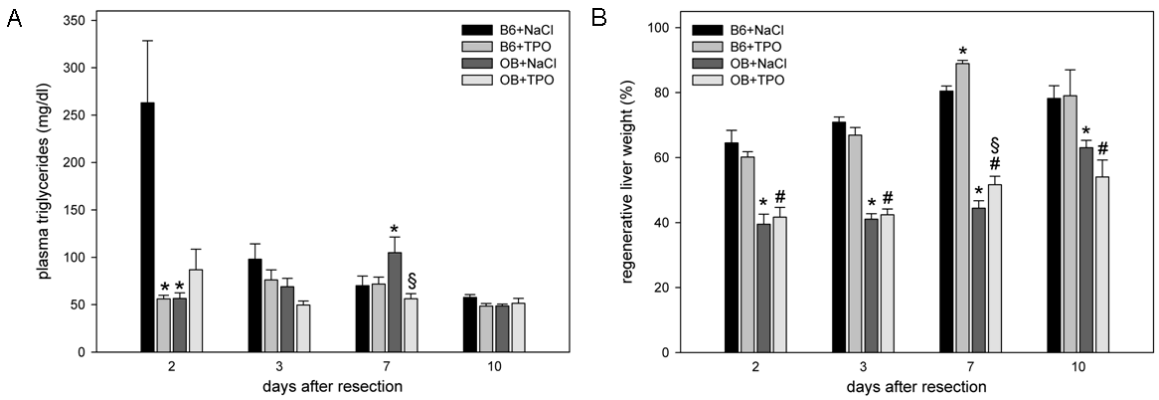

Figure 3. Effect of TPO on hepatic steatosis and regenerative liver weight. Plasma triglycerides (A) and ratio of regenerated to preoperative liver weight (B) at multiple time points after hepatic resection in non-obese (B6) and obese animals (OB) receiving recombinant mouse thrombopoietin (TPO, 12.5 μg/kg) or equivalent volumes of physiological saline (NaCl). The weight of the regenerating livers was used to calculate the growth of residual liver lobes as ratio of regenerated liver/preoperative liver weight x 100 (%). Pre-operative liver weight was assumed to be 4.3% of the body weight in non-obese and 8.0% of the body weight in obese mice. Values of six animals per group and time point are given in means ± SEM. ANOVA and post hoc comparison; *p<0.05 vs. B6+NaCl; #p<0.05 vs. B6+TPO; §p<0.05 vs. OB+NaCl.

3A). Of note, lean mice treated with TPO exhibited significantly decreased plasma triglycerides at day 2 compared to NaCl-treated mice (**Figure 3A**).

Effect of TPO on liver regeneration

There was a constant increase of liver weight upon 55% PH with return to almost preoperative values at day 10 after resection in lean mice (**Figure 3B**). Obese animals showed a significantly impaired and delayed regeneration until day 10 after resection with a limited restoration up to 60% of the initial weight. Pretreatment with TPO led to an only temporary but significant increase of the liver weight at day 7 in both mice strains. In ob/ob mice undergoing 55% PH, mortality was 7.7% (4 mice out of 52 died within 48 h after PH), while there was no mortality in lean littermates (0 mice out of 48).

Effect of TPO on cell proliferation in liver regeneration

The cumulative hepatocyte DNA-synthesis, as histochemically determined in liver sections by BrdU incorporation, was significantly decreased in obese mice compared to lean mice early after resection (**Figure 4A**), although obese mice show increased rates of cell proliferation at baseline (**Table 1**). At 2 days post-PH there were rare and significantly less BrdU positive hepatocytes in obese mice than in lean mice. We observed a shift of the proliferation maximum of parenchymal cells from day 2 in lean mice to day 3 in obese mice. TPO administration failed to augment the proliferation of hepatocytes both in steatotic and in normal livers (**Figure 4A**). Similarly, proliferation of non-parenchymal cells was also markedly reduced in obese mice, as demonstrated by the rare occurrence of BrdU positive cells, particularly 3 days after PH (**Figure 4B**). While TPO showed no stimulative effect on the early proliferation of non-parenchymal cells, TPO induced a doubling of proliferating non-parenchymal cells at day 10 upon resection (**Figure 4B**). Unexpectedly, in lean mice TPO treatment resulted in reduced proliferation of hepatocytes and non-parenchymal cells 3 days after resection.

Effect of TPO on hepatic oval cells

Immunohistochemistry for CK19, a well established marker for oval cells and biliary epithelial cells [19], demonstrated small cells with an oval nucleus and little cytoplasm which were found as cells located singular or organized in ductular structures in the vicinity of the portal area of the liver (**Figure 5B**). Under basal conditions more oval cells could be found in the liver of ob/ob mice than in livers of lean animals (**Table 1**). Quantitative analysis of CK19-

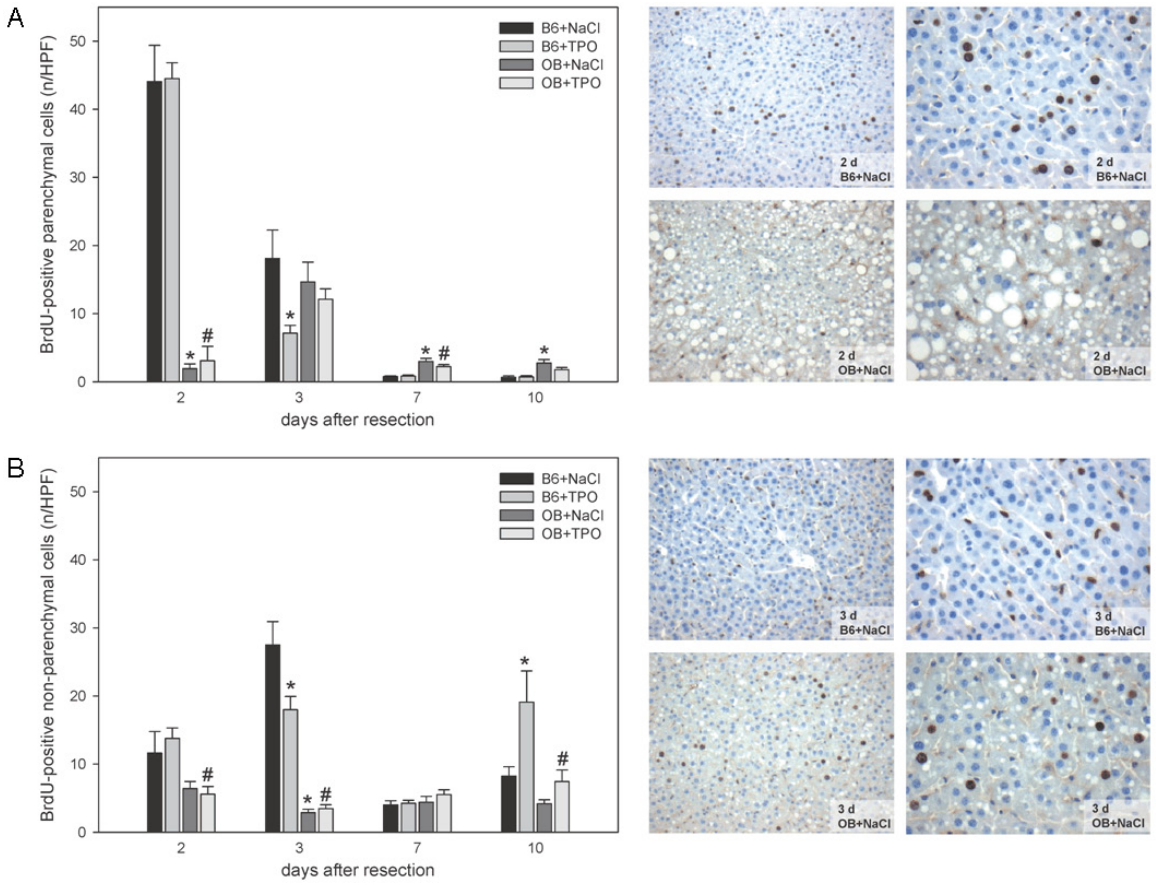

Figure 4. Effect of TPO on proliferation of liver cells. Quantitative analysis of BrdU positive parenchymal (A) and non-parenchymal cells (B) and representative images of BrdU immunohistochemistry (original magnification x 200 (left panel) and x 400 (right panel)) of liver tissue at multiple time points after hepatic resection in non-obese (B6) and obese animals (OB) receiving either recombinant mouse thrombopoietin (TPO, 12.5 μg/kg) or equivalent volumes of physiological saline (NaCl). Values of six animals per group and time point are given in means ± SEM. ANOVA and post hoc comparison; *p<0.05 vs. B6+NaCl; #p<0.05 vs. B6+TPO.

positive cells (**Figure 5A**) upon liver resection revealed much less oval cells in steatotic livers than in normal livers, whereas the number of oval cells just slightly increased with time (**Figure 5**). Treatment with TPO significantly enlarged the number of oval cells at day 10 after resection in both mice strains. At this time point it is distinguishable that oval cells penetrated deep into the liver acinus (**Figure 5B**, right panel).

Discussion

Currently, steatosis is the most common chronic liver disease in the world [24, 25]. Because of the epidemic increase of obesity, hepatic steatosis will increasingly hamper the outcome in liver surgery. It has been noted that resection of steatotic livers is associated with high mortality and impaired liver regeneration [26, 27]. In our experiments, restitution of the liver mass and the proliferative response were profoundly impaired and delayed in ob/ob mice compared to lean mice. The mechanisms underlying impaired regeneration are incompletely known, but investigations in different experimental models of steatosis have implicated abnormalities in the cell cycle progression associated with ATP dysfunction [28] and interruption in various signaling pathways [2, 29]. Therefore, improvement of liver regeneration and postoperative outcome of patients with steatosis by pharmacological strategies is of high interest and clinical relevance.

In the face of structural and biological similarities between TPO and EPO [30], the latter of which is known as a molecule with multiple

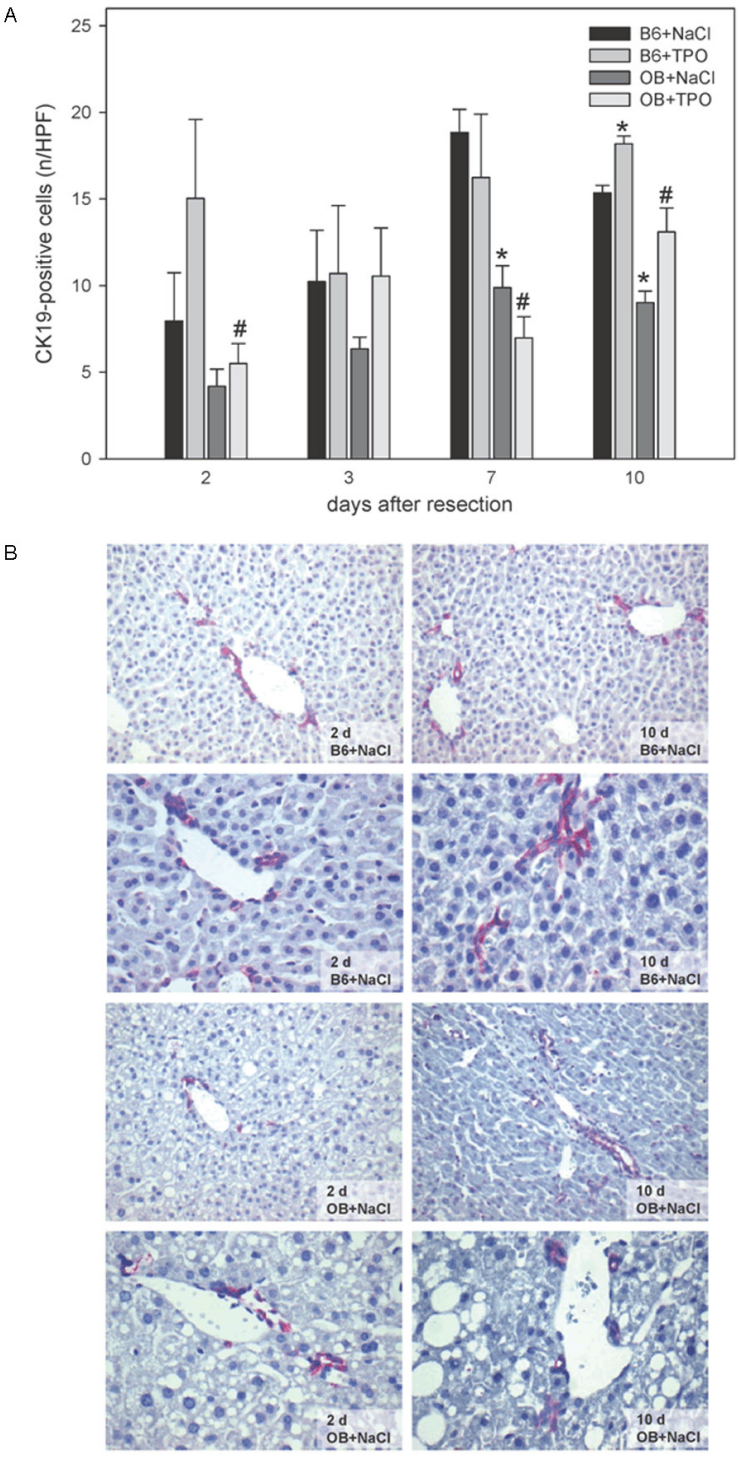

Figure 5. Effect of TPO on the number of hepatic oval cells. Quantitative analysis of CK19-positive cells (A) and representative images of CK19 immunohistochemistry (B) (original magnification x 200 (1. and 3. panel) and x 400 (2. and 4. panel)) of liver tissue at multiple time points after hepatic resection in non-obese (B6) and obese animals (OB) receiving either recombinant mouse thrombopoietin (TPO, 12.5 µg/kg) or equivalent volumes of physiological saline (NaCl). Values of six animals per group and time point are given in means ± SEM. ANOVA and post hoc comparison; *p<0.05 vs. B6+NaCl; #p<0.05 vs. B6+TPO.

actions, some relevance of the hepatic hormone TPO in extrahematopoietic processes has also been recognized. Thus, positive effects on liver regeneration by the pretreatment with TPO have been described in several experimental studies [11, 13, 15, 31]. However, the beneficial effect described for TPO is in part controversial to what we demonstrated in the present study. TPO was unable to increase hepatocyte proliferation and finally failed to enhance liver regeneration of steatotic livers.

To determine whether regeneration-promoting effects were due to TPO administration itself or the increase in platelets, some groups analyzed the effect of a combined injection of anti-platelet serum and TPO [11, 13]. By doing so, they could demonstrate that the acceleration of liver regeneration is due to the increase of platelets and not caused by TPO itself. Lesurtel and coworkers indicated that platelet-derived serotonin mediated liver regeneration in mice [11]. In contrast to others, which have shown an up to 3-fold increase of the systemic platelet counts by TPO [13, 31], the administration of TPO in the present study caused only an increase in platelet counts of 23% 2 days after hepatic resection in obese mice. It can be assumed that the thrombocytotic effect of TPO, although using an equivalent dosage (12.5 µg/kg) as in other studies [13, 32], was to less to induce such positive platelet-derived effects on liver regeneration.

It is reported that in thrombocytotic livers platelets accumulate in the hepatic sinusoids and translocate into the space of Disse immediately after hepa-

tectomy [12, 14, 31] providing signals for hepatocyte proliferation [33]. Beside cellular changes, the steatotic liver is characterized by deranged intrahepatic microvasculature [34], in which injury to sinusoidal lining cells may lead to impaired sinusoidal blood flow through adhesion of circulating cells [35]. In addition, sinusoidal lumen becomes narrowed by swollen hepatocytes [36]. Thus, Ohhara et al. [35] showed that the sinusoidal space is decreased by 53% in steatotic livers, causing significant microcirculatory disturbances. Accordingly, it is conceivable that the altered microcirculation hinders translocation of platelets from the sinusoidal lumen into the space of Disse. Consequently, the restricted contact of platelets to hepatocytes limits the exchange of platelet-derived signals and may finally contribute to the low pro-proliferative effects of TPO in steatotic livers.

Remaining cells within the liver acquire sufficient energy substrate to support the metabolic demands of rapid proliferation. Several reports revealed that fatty acids released from the peripheral adipose tissue contribute to the accumulation of intracellular triglycerides within the regenerating liver. Thus, liver regeneration is physiologically associated with transient steatosis, which is mandatory for energy supply and normal liver regeneration [37]. However, resection-induced increase in plasma triglycerides in lean NaCl-treated mice is significantly inhibited by TPO treatment. These findings strongly implicate a causal relationship of the decreased hepatocyte proliferation at day 3 in TPO-treated lean mice with decreased plasma triglyceride levels in these mice at day 2. The data presented here suggest that TPO and/or platelets may modulate the expression of genes involved in transient hepatic adipogenesis.

Mitochondrial dysfunction and lipid peroxidation leading to ATP depletion are well identified pathogenic features of injury in steatosis [38, 39], leading to apoptosis or necrosis in dependence to the energy status of the cell. Thus, cells are unable to meet the increased energy demands required for the proliferative process. Increased ALT and GLDH activities after PH in obese mice represent this impaired energy homeostasis which is considered to further sensitize steatotic livers to surgical stress. Of interest, TPO administration led to improved hepatocellular integrity in obese mice after PH. In accordance to observations of Hisakura and co-workers, showing significantly decreased liver enzyme release in TPO-treated hepatectomized pigs [40], we observed an effect of TPO in protecting steatotic livers from acute damage. TPO might induce mechanisms, which could be involved in the prevention of mitochondrial dysfunction, such as moderate effects on mitochondrial structure and function as well as mitochondrial beta-oxidation enzymes [38, 39].

Beside disruption of the cell cycle and of signaling mechanisms, metabolic adaption to the chronic oxidative stress, to which fatty livers are exposed, renders mature hepatocytes replicatively senescent and might impede the regeneration process [2, 12]. It has been shown in several models of acute or chronic liver diseases that alternatively activation and accumulation of progenitor (oval) cells compensate for increased hepatocyte loss [4, 19, 26, 41, 42]. In accordance to observations of others [41] the number of CK19-positive cells was higher in livers of obese than in lean mice prior to PH (**Table 1**) and was found to be decreased or nearly unchanged immediately after resection [26]. In contrast to findings of others, we rather observed an increased number of oval cells in lean than in obese mice. The role of TPO in the expansion and survival of progenitor cells has been the focus of several recent investigations. Indeed, hematopoietic stem cells [17, 18] and hepatic progenitor cells [19, 20] can be expanded by TPO. At later time points of the regeneration process oval cells tend to increase in both lean and obese mice, which could be boosted by the administration of TPO. This is in accordance to observations of Ichiba et al. in the Solt-Farber model [19], showing an increase of oval cell numbers per se as well as stimulation of oval cells by an adenovirus-mediated TPO gene transfer not until the late phase of regeneration. In this late stage oval cells invade into liver lobule, as also observed in the present study, and subsequently transdifferentiate into hepatocytes and biliary epithelial cells [19]. Nevertheless, we conclude that there is no major active recruitment and accumulation of this progenitor compartment to serve as an adaptive mechanism to compensate for impaired hepatocyte proliferation in obese mice after 55% PH. The well-documented expression of the TPO receptor, c-mpl, on liver

cells [8, 20] and hepatic progenitor cells [19, 20], does therefore not necessarily reflect their capacity to accelerate proliferation.

In conclusion, although sufficient to significantly improve the metabolic phenotype of obese mice, including liver injury, TPO administration was inefficient in correcting the hepato-proliferative defect in response to 55% liver resection. Our data suggest that treatment of impaired liver regeneration in steatotic mice will not be as simple as supplementing a pleiotropic substance, such as TPO, to normalize liver regeneration. The metabolic abnormalities of obese mice may explain the discrepancy between the reports on TPO and liver regeneration. Further studies are required to better understand how the different effects of TPO interact with each other. However, the present study may provide some clues regarding the low capability of TPO to promote regeneration of steatotic livers.

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Disclosure of conflict of interest

The authors declare that there are no conflicts of interest.

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