Abstract. Background: Steatohepatitis is a type of histopathological liver injury that can be caused by chemotherapy (chemotherapy-associated steatohepatitis (CASH)) and can progress to liver fibrosis or cirrhosis. CASH impairs liver functions, including liver regeneration. Impaired liver regeneration reduces the number of patients who can undergo liver resection and reduces opportunities for curative therapies. Transforming growth factor-beta (TGFβ) is a potent mitotic inhibitor that participates during the last phase of liver regeneration. TGFβ has been studied as a potential solution to the development of liver fibrosis or hepatocellular carcinoma.

Aim: The first aim of our study was to establish a large animal model of toxic liver injury and test the ability of a monoclonal antibody against TGFβ (MAB-TGFβ) to increase liver-regeneration capacity. The second aim was to evaluate the degree to which early preoperative administration of MAB-TGFβ influenced hepatic parenchyma regeneration following healthy liver resection in a swine experimental model.

Materials and Methods: Toxic liver injury was induced by alcohol consumption and regular intraperitoneal administration of carbon tetrachloride (CCl4) to piglets for 10 weeks. After 10 weeks, the piglets underwent liver resection of the left lateral and left medial liver lobes. Twenty-four hours after liver resection, MAB-TGFβ was administered to the experimental group (10 piglets) and a physiological solution to the control group (10 piglets) through an implemented port-a-cath. In the second part of the study, either MAB-TGFβ or a saline solution control were administered at 12 and 4 days prior to resection of the right lobes of healthy liver (six experimental and 10 control group subjects). Observation and follow-up was performed throughout the entire experiment. Ultrasound and biochemical tests (for albumin, cholinesterase, aspartate aminotransferase, alanine aminotransferase, γ-glutamyltransferase, alkaline phosphatase, bilirubin, urea, creatinine and ammonia levels) were performed on postoperative days 1, 3, 7, 10 and 14. A histopathological examination was performed after sacrificing the animals on the 14th postoperative day. Results: No significant differences were observed between groups when using ultrasound volumetry to assess the regenerative volume of the liver in both experiments. The only significant differences found when comparing biochemical parameters between groups were higher serum levels of both creatinine and γ-glutamyl transferase in the experimental group with preoperative administration of MAB-TGFβ. There were no differences in the histological analyses of hepatic lobule cross-sectional area nor in the proliferative index between animals receiving MAB-TGFβ and those treated with physiological saline solution before resection. Hepatocytic cross-sectional areas were larger in animals treated with physiological solution versus those treated with MAB-TGFβ on the operative day; however, these values were comparable between groups by 14 days following resection. Conclusion: We established a large animal model of toxic liver injury that is comparable with CASH. The toxic injury that was induced without pause between administrations was probably more extensive than occurs in CASH, and there was no effect of MAB-TGFβ administration on liver regeneration. MAB-TGFβ administration did not lead to any observable side-effects, indicating that it could be a promising solution for use as an oncologic-targeted treatment.
Transforming growth factor-beta (TGFβ) is a pleiotropic cytokine that regulates a broad spectrum of cellular processes, including cellular differentiation and apoptosis (1). In healthy hepatic tissue, TGFβ also plays an important role during the final phase of liver regeneration, which can be characterized by hepatocytic differentiation and the production of the extracellular matrix. In chronic diffuse liver diseases, the function of TGFβ is related to progression in all stages of the disease, from initial injury through inflammation and fibrosis to cirrhosis and hepatocellular carcinoma (2). In these cases, an increase in the production TGFβ mRNA can be observed in hepatic stellate cells, hepatocytes, Kupffer cells, biliary endothelial cells and macrophages (3). Liver damage induces the production of active TGFβ, which enhances hepatocyte destruction and mediates hepatic stellate cell and fibroblast activation, resulting in a wound-healing response, including myofibroblast generation and extracellular matrix deposition (4). TGFβ also inhibits the production of hepatocyte growth factor, which sustains hepatocytes during proliferation (5).

Despite this anti-proliferative effect, it was proven that a high level of TGFβ in the serum represents a negative prognostic factor in patients with hepatocellular carcinoma. Increased serum levels of TGFβ were found to correlate with an infiltrating form of hepatocellular carcinoma versus its nodular form (6). During hepatocellular carcinoma progression, TGFβ may switch from acting as a tumor suppressor at the beginning of the malignant process, into a tumor promoter that exacerbates invasive and metastatic behaviour (2, 7). Accordingly, TGFβ represents an attractive option for the targeted biological treatment of hepatocellular carcinoma (8, 9). There exist promising pre-clinical studies in which TGFβ signaling pathways were blocked in oncological treatment (10, 11). Thus far, there are no clinical trials examining the use of monoclonal antibody against TGFβ (MAB-TGFβ) therapy to treat hepatocellular carcinoma have been completed. It remains unknown how therapy targeted against TGFβ in a neoadjuvant model influences hepatic tissue regeneration following liver resection and what effects such therapy may have on the timing of surgical intervention. In our previous experimental research in a porcine model, we demonstrated that the use of MAB-TGFβ accelerated the growth of non-occluded liver parenchyma after partial portal vein ligation, and this effect occurred between the third and seventh postoperative days (12). A simillar effect was observed in biliary obstructed rats (13).

Half of the patients afflicted by colorectal cancer have or will develop liver metastases (14, 15). In cases of synchronous liver metastasis, only 15 to 20% of patients can undergo resection at the time of diagnosis (16). In this situation, neoadjuvant chemotherapy is used for the down-sizing of tumor mass to convert the disease to a resectable stage. Oxaliplatin, irinotecan and 5-fluorouracil are included in this neoadjuvant regime. However, the toxic effect of neoadjuvant regimes on liver is well known; nevertheless, surgical treatments and chemotherapy combinations have achieved the best results. Preoperative chemotherapy use can also be uncertain because of hepatotoxicity. Patients who are treated with leucovorin and 5-fluorouracil are more likely to develop steatosis. Oxaliplatin can be responsible for sinusoidal obstruction syndrome, and irinotecan can lead to steatohepatitis (17-19).

Steatohepatitis is the histopathological impairment of the liver parenchyma that is marked not only by steatosis but also by inflammatory cells in lobules and ballooning hepatocytes (18). Steatohepatitis genesis is described by a two-hit theory. The first step is the involvement of hepatocytes usually as the result of metabolic syndrome or toxic liver disability (i.e. alcohol consumption). The second hit is usually produced by free oxygen radicals, which are created following mitochondrial dysfunction during oxidative stress. Free radicals injure hepatocytes and mediate cytokine and chemoattractant releasing, which attracts inflammatory cells. When steatohepatitis develops as the result of alcohol consumption, it is called alcoholic steatohepatitis; if the patient’s history is free of alcohol consumption, it is called non-alcoholic steatohepatitis. Steatosis can be also caused by chemotherapy (chemotherapy-associated steatohepatitis; CASH). A typical inducer of CASH is irinotecan. Steatohepatitis and CASH are advanced stages of chronic diffuse liver disease. Hepatocyte mitochondrial function is impaired during CASH. It has been suggested that oxidative stress compromises the function of the mitochondrial respiratory chain (20). Moreover, oxygen radicals also cause cell membrane lipid peroxidation, and hepatocytes succumb to necrosis. Subsequently, there is the development of necrosis and inflammation, stellate cell activation with connective tissue replacing dead hepatocytes, and progression to fibrosis and cirrhosis (20, 21), which are the end-stages of toxic liver injury.

Patients with normal liver function are able to undergo resection, with a reduction of parenchymal liver of up to 20% of the total liver volume (22, 23). Nevertheless, this future liver remnant volume (FLRV) is inadequate in chemotherapy-treated patients. In such patients with induced liver injury, a minimum of 30% of the liver parenchyma function must be retained, and in patients with fibrosis or cirrhosis, it is necessary to retain more than 40% of the total liver volume (22, 24). A small FLRV leads to a reduction or damage in the liver-regenerative capacity, which is why leaving an adequate FLRV, in situ prevents acute liver failure or postoperative liver insufficiency and reduces postoperative complications (19).

There exist many experimental models that induce steatohepatitis or liver fibrosis. The classical animal model of
liver fibrosis that is based on toxic insult is the carbon tetrachloride (CCl₄) model (25). Hepatocyte cytochrome enzymes transform CCl₄ into CCl₃ radicals. These radicals invade hepatocytes and induce necrosis, and this liver damage leads to the subsequent progression of fibrosis or cirrhosis. The effects of CCl₄ can be accelerated by ethanol or phenobarbital (25). TGFβ can be coupled with this experimental model.

Parenchyma regeneration following liver resection in a swine partial hepatectomy in animals afflicted by steatohepatitis. We evaluated the effect of MAB-TGFβ on liver regeneration after partial hepatectomy in animals subjected to toxic steatohepatitis. The aim of our current study was to establish a porcine model of toxic steatohepatitis that corresponds with CASH. Subsequently, we wanted to evaluate the effect of MAB-TGFβ on liver regeneration after partial hepatectomy in animals afflicted by steatohepatitis.

The second aim was to evaluate how early preoperational application of MAB-TGFβ influences healthy hepatic parenchyma regeneration following liver resection in a swine experimental model.

Materials and Methods

The experimental study was conducted under the control of the Ministry for Education of the Czech Republic, and all procedures were approved by the Commission for Work with Experimental Animals at the Pilsen Medical Faculty, Charles University in Prague (approval number 17100/2008-30). Additionally, this study was conducted in accordance with the law of the Czech Republic, which is compatible with European Union legislation.

First experiment. Regeneration after liver surgery with regard to toxic steatohepatitis. Before liver resection: Three-month-old piglets were included in the experimental study (10 animals in the experimental and 10 animals in control group). For 10 weeks, toxic injury was induced by administration of CCl₄ and alcohol to all of the piglets. Every Monday and Thursday, CCl₄ was administered by intraperitoneal application in doses according to piglet weight (0.25 ml of 40% CCl₄ per kilogram). The piglets drank 5% alcohol during the induction period. Access to granular feed was not restricted.

Premedication and general anesthesia: For CCl₄ application, analgesic sedation with 10 mg/kg ketamine (Narkamon - Spofa, AS, Prague, Czech Republic), 5 mg/kg azaperon (Stresnil - Jannssen Pharmaceutica NV, Beerse, Belgium) and 1 mg atropine (Atropin Biotika- Hoechst Biotika, spol. s.r.o., Martin, Slovak Republic) was used. The same method was used for intramuscular pre-medication before surgery. General anesthesia was induced and maintained by propofol (1% mixture 5-10 mg/kg/h Propofol, Fresenius Kabi Norges AS Halden, Norway). Fentanyl (1-2 μg/kg/h Fentanyl Torrex, Chiesi cz s.r.o, Prague, Czech Republic) was used for continuous analgesia. Myorelaxation was induced with a bolus of pancuronium (0.1-0.2 mg/kg Pavulon, N.V.ORGANON, Oss, the Netherlands). The piglets were intubated and mechanically ventilated during the surgical procedure and received infusion and volume substitution when needed with Plasmalyte (Baxter Healthcare Ltd., Compton, UK) and Gelofusine (B. Braun Melsungen AG, Melsungen, Germany). Aminopenicillin and clavulanic acid (Augmentin 1.2 g per pig, GlaxoSmithKline Slovakia s.r.o., Bratislava, Slovak Republic) were used as an antibiotic prophylaxis throughout the procedure. Electrocardiogram, oxygen saturation and central venous pressure monitoring were also conducted.

Liver resection: Surgery was performed under aseptic and antisepsis conditions. Firstly, we performed a port-a-cath into the superior vena cava by open method. Initially a mid-line laparotomy incision was performed. After Pringle’s maneuver, the left medial and left lateral liver lobes were resected, which included approximately 40% of the total liver volume. Biopsic samples from liver lobes were taken and stored in 10% formaldehyde (methanal) and also frozen below −70°C. Electrocauter and bipolar scissors were used for these procedures. Bile ducts and vessels on the resection surface were ligated. The laparotomy was closed in anatomical layers. The animals were then extubated and monitored each day for the next 14 days, with a focus on clinical examination (particularly through attention to wound healing and gastrointestinal system functionality) to diagnose any possible surgical complications.

Follow up: Twenty-four hours after the operation, MAB-TGFβ (MAB1032, Chemicon International, Inc., Billerica, MA, USA) at 40 μg/kg of body weight (experimental group) or a physiological solution (control group) was injected through the port-a-cath. This was performed in accordance with reports by Demene and Liska (12,13). The animals were examined every week following induction of toxic injury. Observations and follow-up after surgery were performed on postoperative days −1, 3, 7, 10 and 14. Ultrasound examinations were performed for verification of toxic insult. After surgery, we used ultrasound examinations for liver growth evaluation and for liver volume calculations. We measured the livers in B-mode in the axial, sagittal and coronal planes. Liver volume was calculated using an ultrasonographic formula that is used in human medicine (axial × sagittal × coronal/2) and then recalculated for relative comparisons.

Blood samples were collected four times during the toxic injury and continued immediately before operation, after resection and 2 hours after resection. Blood samples were also collected on postoperative days-1, 3, 7, 10 and 14. Serum albumin, cholinesterase, aspartate aminotransferase, alanine aminotransferase, γ-glutamyltransferase, alkaline phosphatase, bilirubin, urea and creatinine levels were obtained by biochemical analysis. Immediately after each blood draw, we assessed the serum ammonia levels.

On the fourteenth day after surgery, the piglets were sacrificed under general anesthesia by intravenous administration of a potassium chloride solution. Liver tissue samples were obtained and were histologically examined by haematoxylin-eosin and periodic acid Schiff staining. We assessed hepatocyte size and lobuli length from 4 μm-thick histological sections, and a pathologist counted the number of binucleated hepatocytes in 20 microscopic fields with an eyepiece micrometer.

Second experiment. Early application of MAB-TGFβ before liver resection.
Sixteen piglets were included in this study (ten in the control group and six in the experimental group). It was not necessary to exclude any piglet due to untimely death or surgical complications.

**Before liver resection**: At the beginning of each experiment we introduced a port-a-cath into the superior caval vein under general anaesthesia as described in the first experiment. On the second and eighth days of the experiment, MAB-TGFβ (MAB1032; Chemicon International, Inc.) at 40 μg/kg of body weight was injected into the central venous catheter (experimental group) (13). For the control group, saline solution was similarly administered via a central venous catheter.

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**Figure 1. a, b**: Histological quantification of tissue samples. Assessment of the area of the profile of classical lobules. Hematoxylin-eosin staining. Scale bars: 1 mm (a), 25 μm (b). c: Assessment of the proliferative index using Ki-67-positive hepatocytes. Most of the positive Ki-67 nuclei were those of mesenchymal cells, but only Ki-67-positive nuclei of hepatocytes (arrow) were counted. Scale bar=50 μm.

**Figure 2.** Irregular and uneven liver lobe sizes, the mass of inflammatory cells in the hepatic lobulus and steatosis are typical features of toxic steatohepatitis (arrows) (a). Some tissue samples exhibited toxic hepatitis with fibrotic stripes (arrows) that were separate from a small cirrhotic nodule in the central part of the image (b). In detail, the central part of the liver lobulus with ballooning hepatocytes (arrow) and Mallory (hyalin) bodies (c) can be clearly seen.
Liver resection: Liver resection was performed two weeks following the initiation of the experiment under the same conditions as described for the first experiment.

Follow up: Blood samples were collected from the central vein catheter at the beginning of the experiment, on days 7 and 12, immediately before resection, immediately after resection, 2 hours after resection, and on experimental days 15, 17, 21, 24 and 28. The evaluation of serum biochemical parameters focused on analysis of liver function in order to detect any influence of the applied MAB on each experimental animal and to distinguish any differences between experimental and control groups. Serum levels of albumin, cholinesterase, aspartate aminotransferase, alanine aminotransferase, γ-glutamyltransferase, alkaline phosphatase, bilirubin, urea and creatinine were assessed. Ultrasonographic imaging of liver was performed immediately following surgery and on experimental days 17, 21, 24 and 28. The piglets were sacrificed on the 28th day of the experiment (14th postoperative day) under the same conditions as described for the first experiment.

Histological examination. Liver tissue blocks fixed in 4% formalin were embedded in paraffin. The cutting plane was randomised prior to the embedding. From each block, 4 µm-thick histological sections were taken and stained with haematoxylin and eosin and periodic acid Schiff. To visualise hepatocytic proliferation, we used immunohistochemical detection of the Ki-67 antigen (monoclonal mouse anti-human, clone MIB-1; Dako, Glostrup, Denmark). Ki-67-negative cells are considered to be in G0-phase.

To assess the size of classical morphological lobules, four micrographs with a ×20 objective were taken in a systematic uniformly random manner per sample. They represented the whole area of the tissue section. Profiles of lobules sampled for size estimation were measured using the Nucleator plugin in Ellipse software (ViDiTo, Kosice, Slovak Republic). The nucleator method has been devised as a local stereological probe to estimate the volume of objects provided that each object has a unique arbitrary point and the sections are either isotropically or vertically uniform (29-31). A 2-D nucleator probe with four isotropically oriented rays was utilised and the intersections of these rays were marked to delineate the borders of at least 20 classical morphological lobules (Figure 1). When assessing the mean area of hepatocyte profiles, four micrographs per tissue sample were taken with a ×40 objective in a systematic uniform random manner. In each micrograph, the profiles of at least 20 nucleated hepatocytes were sampled using a pattern of unbiased counting frames (32) covering the whole micrograph. Using the nucleator principle, sampled nucleated cell profiles were measured by marking the intersections of four isotropically oriented rays with the cell borders (Figure 1). For this area estimate, a sample of two-dimensional isotropic uniform section and content gives an estimated mean area of the hepatocytic profile. Neither the area of the liver lobules nor the area of the nucleated hepatocyte profiles should be considered as values characterising the in vivo condition. No correction factor was applied to compensate for tissue shrinkage caused by histological processing.

To assess hepatic proliferation, 10 high-power image fields per tissue sample were viewed with a ×40 objective in a systematic uniformly random manner. They represented the whole area of the tissue section. At least 1000 nucleated cell profiles were counted. Epithelial vs. connective tissue cells were discriminated and only Ki-67-positive hepatocytes were considered (Figure 1). We calculated the proliferative index (PI) as a ratio between the Ki-67-positive nuclear profiles and the total number of nuclear profiles of hepatocytes.

Statistical analysis. The assessed biochemical and ultrasonographic parameters were analysed with CRAN 2.4.0 and STATISTICA 98 Edition (StatSoft, Inc., Tulsa, OK, USA). As normality was rejected for some of the variables in the analysed groups, non-parametric methods were applied. To compare the distribution of study parameters over the groups and for paired tissue samples taken from the same animal a distribution-free test was used (Wilcoxon). The Mann–Whitney U-test was used for testing the equality of population medians between the groups when assessing histological data. As a measure of the statistical relationships between biochemical, ultrasonographic and histological variables, Spearman’s rank-order correlation was used. The development of the studied parameters over time was compared between the groups using ANOVA. Values were considered to be statistically significant for p<0.05.

Results

First experiment. Regeneration after liver surgery with regard to toxic steatohepatitis. Toxic liver injury negatively influenced liver regeneration. Carbon tetrachloride is one of the many toxic substances known to damage liver parenchyma. In our porcine experimental model, the effect of CCl4 was potentiated by ethanol consumption. After 10 weeks, toxic injury of the liver was proven by histopathological examinations (Figure 2). In this impaired context, we studied the effect of MAB-TGFβ on liver regeneration.

During the postoperative follow-up, there were no statistically significant differences in the liver lobe volumes between the experimental and control groups, although the median values were generally higher in the experimental group.
Figure 4. Continued
Figure 4. Comparisons of the studied biochemical parameters in serum over time (model of toxic steatohepatitis): a: albumin, b: alkaline phosphatase, c: alanine aminotransferase, d: aspartate aminotransferase, e: γ-glutamyltransferase, f: bilirubin, g: cholinesterase, h: creatinine, i: ammonia, j: urea. The following time points are included: (1) before the experiment, (2) on the 14th day following toxic injury induction, (3) on the 28th day of toxic injury, (4) on the 42nd day of toxic injury, (5) preoperative, (6) at liver resection, (7) 2 hours after resection, (8) on the 1st postoperative day (PD), (9) on the 3rd PD (10) on the 7th PD, (11) on the 10th PD and (12) on the 14th PD. PD: Postoperative day. Vertical bars denote 0.95 confidence intervals, means shown in graph.

Figure 5. Comparison of hepatocyte sizes in the hypertrophic lobes. There were no significant differences between the observed groups (model of toxic steatohepatitis).

Figure 6. Growth of liver volume following resection. The volume is expressed as a percentage. Vertical bars denote 0.95 confidence intervals, means shown in graph.
Figure 7. Continued
(Figure 3). We compared all the serum parameters between the experimental and control groups, and there were no significantly different values for any of the measurement points (Figure 4), except one, which was on the first postoperative day for serum ammonia level (Figure 4i). It should be noted that blood was drawn before MAB-TGFβ administration.

Tissue samples from the resected livers and from the dissected piglet livers were histopathologically examined. There were no statistically significant differences between the studied groups in the estimated histological factors, including hepatic lobule length, hepatocyte size and binucleated hepatocyte number (Figure 5).

**Second experiment. Early application of MAB-TGFβ before liver resection.** The volumes of regenerated liver tissue, as measured by ultrasonography and physical examination on day 28 demonstrated no significant differences between experimental and control groups (Figure 6). The only significant differences observed in the measured biochemical parameters (Figure 7a-h) were in serum levels of creatinine and γ-glutamyltransferase. We observed elevated concentrations of creatinine ($p=0.0139$, Figure 7a) and γ-glutamyltransferase ($p=0.0125$, Figure 7b) in the experimental group. Wilcoxon testing demonstrated significant differences at separate time points for serum levels of creatinine (higher in the experimental group on the day 1, $p=0.0220$), γ-glutamyltransferase (higher in the experimental group on day 1, $p=0.0304$, immediately before resection, $p=0.0221$; immediately after resection, $p=0.0046$, and 2 h after resection, $p=0.0305$), bilirubin (higher in the experimental group on day 9 ($p=0.0418$, Figure 7g) and aspartate aminotransferase (higher in the experimental group 2 hours post resection ($p=0.0197$, Figure 7f).

**Histology.** Treated vs. untreated animals: When pooling data obtained from all of the animals from both end time points of the experiment (days 14 and 28), the cross-sectional area of hepatic lobules (Figure 8a) and the PI (Figure 8b) were comparable in animals that received either the MAB-TGFβ or the control physiological saline solution. Hepatocytic cross-sectional areas were greater in animals treated with the physiological solution compared to animals treated with the MAB-TGFβ (Figure 8c).

When comparing the samples taken at days 14 (Figure 8d) and 28 (Figure 8e) separately, no differences were found in the cross-sectional area of hepatic lobules and in PI (Figure 8f and 8g). The larger cross-sectional area of hepatocytes in animals treated with physiological solution was found only at day 14 (Figure 8h). The area of hepatocytes at day 28 showed a higher variability in animals treated with MAB-TGFβ but there was no statistical difference between the groups (Figure 8i).
Figure 8. Continued
Figure 8. Model of early application of MAB-TGFβ before liver resection.

a: Area of hepatic lobules in experimental and control groups on the 14th and the 28th day (p=0.55‡).

b: Proliferative index of hepatocytes in experimental and control groups on the 14th and the 28th day (p=0.56‡).

c: Area of hepatocytes in experimental and control groups on the 14th day and the 28th day (p=0.01‡). Area of hepatic lobules on the 14th day (p=0.129‡) (d) and on the 28th day (p=0.914‡) (e). Proliferative index on the 14th day (p=0.123‡) (f) and on the 28th day (p=0.437‡) (g).

h: Area of hepatocytes on the 14th day (p=0.030‡).

i: Area of lobules on the 28th day (p=0.193‡). Area of lobules on the 14th and 28th day in animals treated with MAB-TGFβ (p=0.028§) (j) and in animals treated with physiological solution (k) (p=0.203§). Area of hepatocytes on the 14th and 28th day in animals treated with MAB-TGFβ (p=0.753§) (l) and in animals treated with physiological solution (m) (p=0.445§). Proliferative index of hepatocytes on the 14th and 28th day in animals treated with MAB-TGFβ (p=0.208§) (n) and in animals treated with physiological solution (p=0.441§) (o).‡Mann-Whitney U-test; §Wilcoxon matched-pairs.
Comparing paired samples collected from the same animals on days 14 and 28: In animals treated with MAB-TGFβ, hepatic lobule cross-sectional areas were greater on day 28 than day 14 (Figure 8j). In animals treated with the control physiological solution, hepatic lobule cross-sectional areas were comparable on days 14 and 28 (Figure 8k). In animals treated with either MAB-TGFβ (Figure 8l) or with physiological solution (Figure 8m), hepatocytic cross-sectional areas were comparable on days 14 and 28. In animals treated with either MAB-TGFβ (Figure 8n) or with physiological solution (Figure 8o), the hepatocytic PIs were comparable on days 14 and 28.

**Discussion**

Herein we showed that administration of CCl₄ together with consumption of 5% ethanol induced toxic liver injury that was comparable with steatohepatitis. The liver parenchymal changes achieved were similar to those found in CASH. The toxic injury effect was achieved in piglets, in which physiological and pathophysiological processes are analogous to processes in the human body. This is very important because the obtained results may relate to human medicine.

In the experiment with CCl₄, liver lobe growth after surgery was faster in the experimental group; however, there were no statistically significant differences found between the studied groups. This result did not validate our hypothesis regarding the acceleration of growth of liver parenchyma after administration of MAB-TGFβ prior to liver resection, although we previously showed this effect on healthy livers (12). We hypothesise that long-term toxic liver injury induction without compensation of the insult led to irreversible liver damage. Patients treated with chemotherapy usually have a break between each chemotherapy cycle, which allows for restoration of liver function.

The studied serum parameters in experiment with CCl₄ were without statistically significant differences at all of the measurement points, except for the serum ammonia levels on the first postoperative day in the experimental group. Blood sample collections and ammonia measurements were performed the same way for all of the samples; therefore, we do not believe that a mistake was made in the methods or measurements. However, we are not able to explain the mechanism and cause of this different result. The blood samples were collected before administration of MAB-TGFβ, which is why we can conclude that administration of MAB-TGFβ was without side-effects.

Hepatic lobule length, hepatocyte size and binucleated hepatocyte number reflect liver regeneration status, and we previously recorded a greater number of binucleated hepatocytes in healthy piglet livers (12). In our experimental study here, there were no significant differences in these parameters. Because the liver growth volume had a trend for increasing, we hypothesize that liver regeneration was limited by the toxic injury and was completed before the piglets were sacrificed.

MAB-TGFβ is a new potential substance for targeted-therapy. Because we observed no side-effects after MAB-TGFβ applications, we predict that usage of MAB-TGFβ in clinical practice will not result in liver regeneration side-effects. It would be of value to develop a neoadjuvant treatment that could be administered immediately before surgical resection. This could be another step to prevent malignant disease, including hepatocellular carcinoma.

In the experiment with preoperative administration of MAB-TGFβ, volumetric analyses of regenerating liver tissue did not reveal any significant influence of MAB-TGFβ treatment on liver volume expansion following liver resection. According to previous studies performed by our group, as well as recent studies regarding the physiological role of TGFβ (12), the inhibition of this cytokine via monoclonal antibody should theoretically accelerate liver tissue regeneration following the resective procedure. A possible explanation for why the current study did not reveal increased liver volumes within the experimental group could be that any effects of TGFβ inhibition were lost by the time of surgery. The latent TGFβ complex has a relatively long half-life (33) and it is possible that neutralised TGFβ could be replaced by the production of new TGFβ during the 5-day period between the final administration of MAB-TGFβ and liver resection. Our results also revealed higher serum levels of parameters used to measure liver damage in the hours following surgery (γ-glutamyltransferase and aspartate aminotransferase) in the experimental group. Nevertheless, these values do not represent a condition of acute liver failure. Another observation that indicated changes in liver function had occurred following the administration of monoclonal antibody was the measurement of elevated bilirubin concentrations within experimental group subjects at one week following the first dose of MAB-TGFβ. However, the overall physiological effects produced by MAB-TGFβ administration are questionable, as there were no significant differences observed between experimental and control groups one week after the second application of antibody. The higher serum concentrations of creatinine measured following liver resection could reflect lower glomerular filtration in the group. While we cannot exclude a causal relationship between MAB-TGFβ application and elevated serum levels of creatinine, confirming the clinical impact of long-term application would be necessary for moving forward. Currently, only a single clinical trial utilising systemic anti-TGFβ1 has been completed, and no mention of altered renal function has been discussed (34).

Histological analyses of hepatic lobule cross-sectional areas and PIs did not differ between animals receiving MAB-
TGFβ versus those receiving control physiological solution in experiments with preoperative administration of MAB-TGFβ. Hepatocytic cross-sectional areas were greater in animals treated with physiological solution versus those treated with the MAB-TGFβ on day 14, but both groups were comparable on day 28. This outcome suggests that treating the animals with MAB-TGFβ caused a reduction in hepatocyte size during the first two weeks following treatment but that this effect was compensated for by no later than four weeks into the experiment. When comparing paired samples collected from the same animals on days 14 and 28, the only difference found was that hepatic lobules had a larger cross-sectional area on day 28 in animals treated with MAB-TGFβ as compared to measurements taken on day 14. However, measurements of both hepatocyte area and PI were comparable at the two time points regardless of the treatment received by the animals. This outcome would suggest a lower growth rate in animals treated with MAB-TGFβ during the first two weeks; however, the cross-sectional area of the lobules did not differ between animals that received the MAB-TGFβ or physiological solution.

Conclusion

In our study, we did not find any effect of MAB-TGFβ on liver regeneration following toxic injury, although we had hypothesized that there would be an effect on the basis of our previous study. The cause of this discrepancy could be extensive toxic injury induced without pauses between the toxic insults, similarly to that which would occur in patients undergoing chemotherapy treatments. However, it remains unclear whether CASH and liver fibrosis are reversible, or if it is possible to alleviate these conditions. Our usage of the MAB-TGFβ treatment did not show that this is possible.

We demonstrated that systemic early preoperative administration of MAB-TGFβ does not significantly influence liver regeneration in a porcine experimental model of liver resection. The data presented herein support the organisation of future studies to examine the effects of neoadjuvant biological therapy on liver malignancy in human medicine.

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