Liver Regeneration in Rats after Liver Resection and Small-for-size Liver Transplantation from Living Donors

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1 INTRODUCTION

1.1 Living donor liver transplantation using small-for-size grafts

Living donor liver transplantation (LDLT), using a relative or another person closely related to the recipient as a donor, was theoretically proposed by Smith in 1969 to alleviate the critical shortage of cadaveric graft supply for children (Smith, 1969). In 1989, it was introduced into clinical reality with the first successful series of paediatric recipients by Broelsch (Broelsch et al., 1991).

The success in paediatric liver transplantation and a refined surgical technique led to the adaptation of LDLT for adults (Marcos et al., 1999). Since then, LDLT has become a generally accepted procedure with a relevant impact on the treatment of end-stage liver diseases. The major indications expanded from biliary atresia and other paediatric liver diseases to a variety of liver diseases occurring in adults, particularly hepatocellular carcinoma (HCC) and hepatitis B-related liver cirrhosis (Tanaka, 2003). The applicability in terms of timing of surgery has been extended from elective to highly urgent transplants for fulminant, subacute, and acute-on-chronic liver failure (Chen et al., 2003). Up to date, the annual number of LDLT has rapidly increased in Germany since 1997, and it accounts for more than 10% of all liver transplantations (Figure 1). The trend of the greater applicability of LDLT in North America is similar to that seen in Germany (Renz et al., 2003).

However, LDLT requires the use of a partial liver graft, which is small-for-size when transplanted to an adult. Lo considered that the reduced size was a dominant factor contributing to impaired postoperative graft function (Lo et al., 1996), indicating that using a small-for-size graft in adult-to-adult LDLT remains a problem. The minimal graft volume required to meet the metabolic and regenerative demand of the recipient is undefined till now. There is general agreement that the lower limit depends on the recipient's condition, the quality of the graft as well as the technique of transplantation performed (Broering et al., 2003). The graft-to-recipient weight ratio (GRWR) or graft-to-recipient standard liver volume ratio (GSLV) of the recipient has been used to determine the ideal liver volume for recipients. A GRWR of 0.8-1% or a GSLV of 40-50% are considered safe (Nishizaki et al., 2001).

Compared to other organs, the liver has the unique capacity to regenerate and adjust its size according to the requirement of the host (Francavilla et al., 1994a). Therefore, liver regeneration is a very critical factor to the success of LDLT.

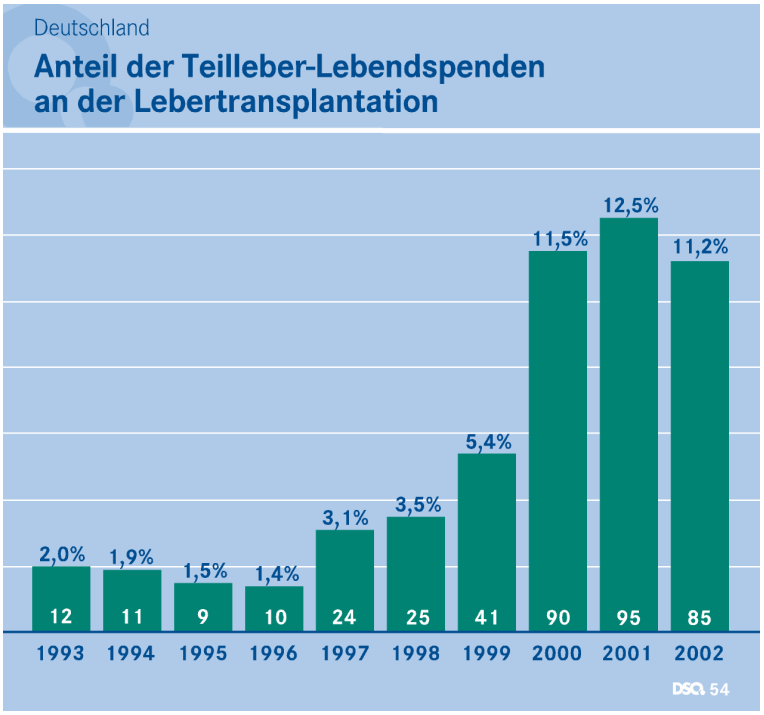


Figure 1. Annual number of LDLT cases in Germany.
(Deutsche Stiftung Organtransplantation. www.dso.de)

1.2 Liver regeneration after hepatectomy and partial liver transplantation

1.2.1 Basic characteristics of liver regeneration

Liver regeneration has long been recognized, as implied by the ancient Greek myth of Prometheus. Having stolen the secret of fire from the gods of Olympus, Prometheus was condemned to having a portion of his liver eaten daily by an eagle. His liver regenerated overnight, thus providing the eagle with eternal food and Prometheus with eternal torture (Figure 2).

In modern times, liver regeneration is described as the remarkable capacity to reestablish its optimal volume in relation to body size after injury and cell loss through DNA synthesis and mitosis (Court FG et al., 2002). The most commonly studied model is the 70% partial hepatectomy (PH) in rodents which was introduced by Higgins and Anderson (Higgins GM et al.,1931). After PH, the

residual liver lobes enlarge to make up for the mass of the removed lobes until the original size has been restored. Once this has been attained, liver regeneration halts abruptly (Steer, 1995). The whole process, which lasts 7 to 10 days in rats, is compensatory because the size of the resultant liver is determined by the demands of the organism.

Figure 2. Liver regeneration and the legend of Prometheus.
(Copied from www.the-pantheon.com)

1.2.2 Factors influencing liver regeneration

In liver resection and in partial liver transplantation, regeneration is essential to restore an adequate liver size. Regeneration of the liver can be affected by a variety of factors. First of all, it depends on the volume of remnant or transplanted liver and the original liver volume of the patient. The extent of regeneration following PH is directly correlated to the extent of liver mass reduction (Masson et al., 1999). In addition, the quality of the remnant or grafted liver is of special concern, e.g. steatosis has been shown to reduce the rate of hepatic regeneration after resection (Selzner and Clavien, 2000).

Ischemia-reperfusion injury is the unavoidable damage specific to the transplantation procedures (Selzner et al., 2002b), which may affect the regeneration process as well. A poor general condition of the recipient as well as

postoperative complications may also influence the regenerative capacity of the graft (Shiffman et al., 2002).

LDLT between adults results in a small-for-size hepatic graft, which in general is subjected to shorter period of cold ischemia than a cadaveric graft. However, the graft needs to regenerate to accommodate the recipient's metabolic requirements. Therefore, it is essential to determine the influence of the transplantation procedure itself as well as the effect of prolonged cold ischemia on the process of liver regeneration.

1.2.3 Immunological regulation of liver regeneration

Recently a feedback mechanism regarding the regulation of liver regeneration was postulated (Dahmen and Dirsch, 2002). It was hypothesized that liver regeneration and immune activation are inversely related.

1. Regeneration is inducing a general immune activation.
2. Immune activation is inhibiting regeneration.

=> Inhibition of immune activation is enhancing the regenerative response.

1.2.3.1 Influence of immune activation

This postulated mechanism might be of special importance when using small-for-size liver grafts in patients transplanted for chronic viral liver disease. Viral hepatitis leads to an ongoing immune activation potentially sufficient to impair regeneration. This question can be best addressed in a surrogate model, such as observing liver regeneration after PH in animals undergoing Hepatitis B vaccination.

1.2.3.2 Effect of inhibiting immune activation

Patients after liver transplantation are always subjected to immunosuppressive therapy to inhibit the immunologic attack of the graft by the recipient immune system. In general a combination therapy is used, seeking to inhibit T cell activation by blocking the interleukin-2 (IL-2) pathway as well as inhibiting T cell proliferation (Tanaka et al., 1993e).

Assessing liver regeneration under the influence of single immunosuppressive drug acting differently on the immune response may help to elucidate how inhibition of immune activation can enhance regeneration.

1.3 Methods for assessing liver regeneration

Liver regeneration can be assessed by a number of tissue-based tests and various serum-based methods. The former includes liver weights, mitotic counts, DNA synthesis rates, immunohistochemical staining of nuclear antigens, gene expressions and certain protein levels. The latter one mainly consists of specific enzyme determinations such as thymidine kinase or documentation of certain proliferation markers.

Measurement of liver weight is the simplest tissue-based test. However, this determination may be influenced by oedematous swelling of the liver subsequent to resection and, as Assy suggested, by the deposition of various materials such as lipid and glycogen (Assy and Minuk, 1997b). The determination of mitotic figures can result in artificially low counts because mitosis may proceed to completion in inadequately fixed tissue samples while new mitotic figures do not develop (Baak, 1990). ^{3}H-thymidine and Bromodeoxyuridine (BrdU) incorporation into DNA are the reference tools for studying DNA synthesis, but their use requires pre-injection with radioactive isotope-labelled or halogenated nucleotides (Baratta et al., 1996). Immunohistochemical staining for nuclear antigens including Ki-67, proliferating cell nuclear antigen (PCNA), DNA polymerase alpha and nucleolar organizer region (NOR) proteins are acceptable and commonly used methods but the antigens in question are expressed in various phases of the cell cycle (Assy and Minuk, 1997a).

Of the serum-based determinations, thymidine kinase (TK), ornithine decarboxylase (ODC), fibronectin, alpha fetoprotein and early pregnancy factor offer practical and non-invasive tools to monitor liver regeneration, but their sensitivity and specificity have yet to be determined (Assy and Minuk, 1997c).

Although there are disadvantages inherent in each method, the gold standard has long been the in vivo labelling of DNA by the modified pyrimidine analogue, a halogenated derivative of thymidine, BrdU (Gratzner, 1982; Muskhelishvili et al., 2003). It is readily incorporated into nuclei during the DNA synthetic phase of the cell cycle and is detected by immunohistochemistry (IHC) with anti-BrdU antibody. It combines the advantages of speed and technical simplicity and it doesn't require exposure to radioactive materials or sophisticated equipment. Thus, determination of BrdU incorporation has been proven to be a powerful parameter in the study of

proliferating cells (Abbasoglu et al., 1995; Miyagawa et al., 1997; Tarao et al., 1991).

As BrdU incorporation is frequently used to assess liver regeneration in experimental models, a variety of different protocols exist regarding the application route and time as well as regarding the immunohistochemical assay. Furthermore, also the evaluation method is subjected to a considerable variability. The results may be given as positive cells irrespectively of cell type/high power field or positive cells of a given type/given number of total cells. Additional variation of the results may be related to inter- and intra- observer variability. Validation of the immunohistochemical assay is therefore considered indispensable prior to a quantitative evaluation.

1.4 Aim of this study

Living liver donation between adults requires an extended resection in the donor to obtain a small-for-size graft for the recipient. This decrease in functional liver mass puts the recipient at risk of transient liver insufficiency. Only regeneration of the liver can restore an adequate functional liver mass.

This animal experiment was designed to investigate liver regeneration after hepatectomy and small-for-size liver transplantation from living donors using rat models.

1. Liver regeneration was assessed using the BrdU incorporation method. Interest was focused on the validation of the quantification method and the immunohistochemical assay by
   a. Comparing conventional and computer-assisted counting
   b. Determining the assay precision of BrdU-IHC staining

2. Liver regeneration after transplantation was studied by investigating
   a. Kinetics of regeneration after small-for-size liver transplantation
   b. Influence of extended cold ischemia on regeneration of a small-for-size graft

3. The feedback mechanism between liver regeneration and immune activation was studied using liver resection as a surrogate model. Special attention was given to study the effect of
   a. Immune activation by vaccination on liver regeneration
   b. Inhibition of immune activation by immunosuppressive drugs
      - Inhibition of IL-2 activation (cyclosporine A (CsA), FK506)
      - Inhibition of T cell proliferation (mycophenolate mofetil (MMF) and Sirolimus (RAD))

2 MATERIALS AND METHODS

2.1 Experimental design

This experiment was designed to investigate liver regeneration after liver resection and small-for-size liver transplantation from living donor using the rat models. The regenerative capacity was evaluated by BrdU incorporation method. Accordingly, the control and experimental groups were set up as follows:

2.1.1 Control groups

a. Naive group (n=10)
b. 70%PH group (n=24, 6 animals from each subgroup were sacrificed at 24, 48, 72 hours and 1 week after 70%PH)
c. 90%PH group (n=8, animals were sacrificed at 48 hours after 90%PH)

2.1.2 Experimental groups

The various experimental groups were designed to answer the following questions:

a. Kinetics of regeneration after small-for-size liver transplantation.

30% nonarterialized partial orthotopic liver transplantation (NAPOLT) was performed in a syngeneic strain combination (Lewis to Lewis). The corresponding course of regeneration was compared with that after 70%PH. 6 animals from each subgroup were sacrificed at 24, 48, 72 hours and 1 week after transplantation (Table 1).

Table 1. Experimental design for investigating the regeneration kinetics after small-for-size liver transplantation

Group	**Observation Periods**			
	24 hours	48 hours	72 hours	1 week
70%PH	*(n=6)*	*(n=6)*	*(n=6)*	*(n=6)*
30%NAPOLT	*(n=6)*	*(n=6)*	*(n=6)*	*(n=6)*

b. Influence of duration of cold ischemia on regeneration of a small-for-size graft.

30%NAPOLT was performed in a syngeneic strain combination (Lewis to Lewis). Cold ischemic time of the graft was set to 1, 3 and 5 hours, respectively. In order

to assess the influence of prolonged ischemia on outcome the 1-week survival rate was assessed. The influence of prolonged ischemia on proliferation rates was assessed at 48h postoperatively and compared with those after 70%PH (Table 2).

Table 2. Experimental design for investigating the influence of prolonged cold ischemia on liver regeneration of a small-for-size graft

Group	Ischemic time	1-week survival rate	Proliferation rate (48h postoperatively)
70%PH	Control	*(n=6)*	*(n=6)*
30%NAPOLT	1 hour	*(n=6)*	*(n=6)*
	3 hours	*(n=10)*	*(n=4)*
	5 hours	*(n=10)*	*(n=3)*

c. Immune activation by vaccination on liver regeneration.

Vaccination with hepatitis B vaccine can induce immune activation in vivo (Keating and Noble, 2003). Thus, the influence of immune activation on liver regeneration was tested by stimulating the animal 7 days prior to 70%PH by vaccination, which was used as a surrogate model of small-for-size partial liver transplantation. The proliferation rate at 24, 48, 72 hours and 1 week postoperative were observed (Table 3).

Table 3. Experimental design for exploring the influence of immune activation by vaccination on liver regeneration

Group	Observation Periods			
	24 hours	48 hours	72 hours	1 week
70%PH-control	*(n=6)*	*(n=6)*	*(n=6)*	*(n=6)*
70%PH-vaccinated	*(n=6)*	*(n=6)*	*(n=6)*	*(n=6)*

d. Inhibition of immune activation by immunosuppressive drugs.

Subtotal resection was performed as a surrogate model to evaluate the regenerative ability of small-for-size graft treated with immunosuppressive drugs. 32 animals were divided into 4 groups. 8 animals in each group were treated with CsA, FK506, MMF and RAD respectively one day prior to 90%PH. Proliferation rate at 48 hours postoperatively was observed (Table 4).

Table 4. Experimental design for investigating the influence of immunosuppressive drugs on liver regeneration

Operation	Observation time point	Treatment
90%PH	48 hours postoperatively	Control *(n=8)*
		CsA *(n=8)*
		FK506 *(n=8)*
		MMF *(n=8)*
		RAD *(n=8)*

2.2 Animals

Experiments were performed in inbred male Lewis (LEW, $RT1^{l}$) rats (Figure 3) weighing 250 to 350g, which were obtained from Charles River Gmbh (Sulzfeld, Germany). Donor and recipient rats of similar weight (+/-10g) were chosen randomly. The animals were housed under standard animal care conditions and fed with standard rat chow (Rat Food 10H10, Firma Nohrlin, Germany) ad libitum before and after operations. Surgical procedures were done mainly under isofluran (CuraMed Pharma GmbH, Karlsruhe, Germany) anesthesia. The rats were maintained behind barriers under controlled environmental conditions. Animal housing and procedures were carried out according to the German Animal Welfare Legislation.

Figure 3. Experiment animal: inbred male Lewis rat

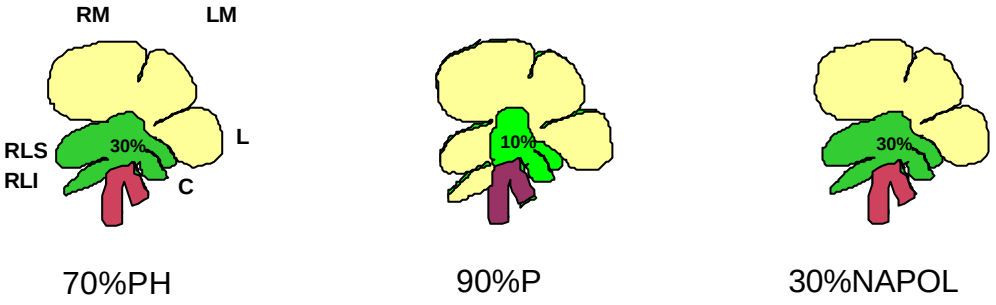


Figure 4. Partial hepatectomy and nonarterialized partial orthotopic liver transplantation (NAPOLT). The rat liver consists of right portion and left portion of median lobe (RM and LM), superior and inferior portion of right lateral lobe (RLS and RLI), left lateral lobe (LL), anterior and posterior caudate lobe (CLA and CLP). The green portion indicates the remnant liver after partial hepatectomy (left and middle) and the liver lobes forming the 30% graft volume (right) in NAPOLT.

2.3 Operations

2.3.1 Partial hepatectomy

Partial hepatectomy was performed according to the technique of Higgins' (Higgins GM et al.,1931). Briefly, the median lobe and left lateral lobe were resected for a 70%PH while the median lobe, left lateral lobe and right lateral lobe were resected for a 90%PH (Figure 4).

2.3.2 Nonarterialized small-for-size orthotopic liver transplantation

Orthotopic liver transplantation was performed according to the cuff technique of Kamada and Calne (Kamada and Calne, 1983). Graft reduction was performed in situ prior to perfusion of the graft with physiological saline. The 30% graft that included the superior and inferior portion of the right lateral lobe (RLS and RLI) and the caudate lobe (CL) were stored at 4°C for either 1 hour, 3 hours or 5 hours before implantation (Figure 4).

2.4 Treatment

2.4.1 Standard perioperative treatment

All animals were treated immediately with 100 mg/kg/d Mezlocillin (Baypen, Bayer AG, Leverkusen, Germany) by intramuscular injection after the operation.

2.4.2 Glucose supplementation

A dose of 5ml 10% glucose solution (Fresenius, Germany) was injected subcutaneously immediately after operation. Besides normal tap water, 20% glucose solution (Fresenius, Germany) was supplied for drinking for 3 days after 90%PH and after 30%NAPOLT.

2.4.3 Vaccination

Recombinant hepatitis B Vaccine ENGERIX-B (20mcg/1ml, GlaxoSmithKlein Biologicals, Rixensart, Belgium) was administered intramuscularly in a dose of 0.2ml 7 days prior to 70%PH.

2.4.4 Immunosuppressive treatment

Animals received one of the following 4 immunosuppressive drugs one day prior to 90%PH: CsA (5mg/kg/d, subcutaneously, Sandimmune™, Novartis, Switzerland),

FK506 (1mg/kg/d, subcutaneously, Prograf^{TM}, Fujisawa, Japan), MMF (40mg/kg/d, subcutaneously, CellCept^{TM}, Roche, Switzerland) or RAD (2.5 mg/kg/d, by gavage, obtained directly from Novartis, Switzerland).

2.5 Sampling of animals

Animals were injected with BrdU (Sigma Inc, USA. 50mg/kg body weight, dissolved in 1.0 ml of 0.9% sodium chloride solution) intravenously one hour before sacrifice, followed by laparotomy under anesthesia. After complete inspection of the abdominal cavity to detect surgical complications, liver tissue was removed and fixed in 10% buffered formalin for immunohistochemical staining.

2.6 Evaluation of liver regeneration

2.6.1 Immunohistochemical staining of BrdU

Incorporated BrdU was detected by immunohistochemical staining to allow calculation of the BrdU labelling index (LI). The staining procedure was based on a modified protocol of Sigma Inc (Table 5). After deparaffinization and rehydration, tissue sections were treated with prewarmed 0.1% trypsin solution at 37°C for 40 minutes, followed by denaturation of the DNA with 2N HCl at 37°C for 30 minutes. In the next step sections were incubated with 1:50 monoclonal anti-BrdU antibody at 37°C for 1 hour, then blocked with avidin solution and subsequently with biotin solution for 5 minutes, respectively, followed by anti-mouse linked antibody for 1 hour at room temperature, prior to the application of Neufuchsin solution for 20 minutes. The sections were counterstained with hematoxylin, and coverslipped using ImmuMount (Shandon Inc).

Table 5. Protocol for BrdU immunohistochemical staining

Reagents	**Company**	**Order No.**
Trypsin tablets	Sigma, Germany	T-7168
2N HCl	Merck, Germany	1.09063
TBS as wash-buffer	DAKO, USA	S1968
Avidin-biotin blocking System	DAKO, USA	X0590
Monoclonal anti-BrdU-Ab	DAKO, USA	M0744
PowerVision	Immuno Logic, Netherland	DPVM-110 AP
DAKO Antibody Diluent	DAKO, USA	S0809
0.2M Aminomethylpropandiol Buffer	Merck, Germany	801464
0.05M Tris buffer	Merck, Germany	8382
5% Neufuchsin solution	Chroma, Germany	1B467
4% Natriumnitrit solution	Sigma, Germany	S-2252
Dimethylformamid solution	Merck, Germany	822275
NaCl	Merck, Germany	6404
Levamisole	Sigma, Germany	L-9756
Naphtol-As-Bis-Phosphat	Sigma, Germany	N-2250

Equipments and Materials:

wet chambers, staining jar, tissue drying oven, scale, 691 PH meter, Pipette (100ul, 1000ul), test tubes, ImmuMount

Procedures:

1. Deparaffinization and rehydration
   - Xylene 30 minutes
   - 100% methanol 3 minutes
   - 90% methanol 3 minutes
   - 70% methanol 3 minutes
   - Distilled water 3 minutes
   - Rinse in TBS for 5 minutes
2. Enzymatic pre-treatment
   - Prepare trypsin-solution: dissolve 1 tablet in 1ml distilled water, prewarm it to 37°C
   - Apply 100µl of trypsin-solution on each slide, incubate it in the wet chamber at 37°C for 40 minutes
   - Rinse in TBS
3. DNA denaturation
   - Put slides in pre-warmed (37°C) 2N HCl solution, incubate for 30 minutes at 37°C
   - Rinse thoroughly in TBS
4. First antibody
   - Put the slides into the wet chamber,
   - Dilute BrdU-antibody with Antibody-Diluent 1:50

- Add 100µl/slide anti-BrdU antibody, incubate for 1 hour at 37°C
- Rinse in TBS buffer for 3 minutes

5. Blocking
   - Add 2 drops/slide of Avidin solution, incubate for 5 minutes
   - Rinse in TBS
   - Add 2 drops/slide of Biotin solution, incubate for 5 minutes
   - Rinse in TBS
6. Link and label
   - Add 2 drops/slide of PowerVision (anti-mouse), incubate for 60 minutes at room temperature in the wet chamber
   - Rinse in TBS for 15 minutes
7. Colour-reaction
   - Prepare the neufuchsin solution

   Solution 1:
   - 18ml Aminomethylpropandiol buffer
   - 50ml 0.05M Tris buffer, pH 9.7
   - 0.6g NaCl
   - 28mg Levamisole

     Mix thoroughly

   Solution 2:
   - 35mg Naphtol-As-Bis-Phosphat
   - 0.5ml Dimethylformamid

     Mix thoroughly

   Solution 3:
   - 8 drops 5% Neufuchsin-solution
   - 20 drops 4% Natriumnitrit-solution
   - Mix solution 1 with solution 3, then pour the mixture into solution 2 and mix them. Use 2N HCl to adjust the pH-value to 8.7
   - Filter the solution before using
   - Pour the solution into a staining jar, then put the slides into it
   - Incubate for 20 minutes at room temperature
   - Rinse with tap water
8. Counterstaining
   - Put the slides in Haematoxylin, incubate for 30 seconds
   - Rinse in tap warm water to enhance the blue staining of the nuclei.
   - Apply coverslips with ImmuMount

2.6.2 Analysis of BrdU LI

BrdU LI was determined by analysing 10 digital pictures which were taken at 200x magnification. A computer-assisted image analysis system was used (Table 6). After transferring the digital pictures to the computer, BrdU-labeled hepatocyte nuclei were counted using ImageTool 3.0, followed by the quantification of unlabeled hepatocyte nuclei using SigmaScan Pro 5.0 with an adapted macro. In this way, more than 3000 hepatocytes were counted per slide. The LI was calculated as the percentage of BrdU-labeled nuclei of hepatocytes out of counted hepatocytes (labeled + unlabeled hepatocytes).

Labeling index (LI) = (number of BrdU-labeled nuclei in hepatocytes / number of counted hepatocytes) X 100%

2.7 Validation of quantification method and immunohistochemical assay

2.7.1 Comparison of methods to quantify unlabeled hepatocyte nuclei

As the staining intensity varied within the same section and between different sections, the colour threshold must be adapted for each image to include 95% of the true hepatocyte nuclei.

Three steps were accomplished to validate the quantification of unlabeled hepatocyte nuclei, including the comparison between conventional and computer-assisted counting, followed by assessing the intra- and inter-observer reproducibility with computer-assisted counting.

2.7.1.1 Correlation between conventional and computer-assisted counting

1. Select 1 set of BrdU pictures randomly (Animal No. REG-032).
2. Count the total numbers of unlabeled hepatocyte nuclei using ImageTool 3.0 and Sigmascan Pro 5.0 respectively.
3. Compare and analyse the differences statistically.

2.7.1.2 Intra-observer reproducibility in computer-assisted counting

1. Select 1 set of BrdU pictures randomly (REG-032).
2. Count the total numbers of unlabeled hepatocyte nuclei using Sigmascan Pro 5.0 by 1 skilled observer on 2 different days (once per day).
3. Compare and analyse the differences statistically.

Table 6. Protocol for analysis of BrdU LI

Materials:

- Digital camera (Nikon Coolpix 995, Tokyo 100-8331, Japan)
- Compact Flash card (128MB, 206510, Japan)
- Microscope (Olympus BX50)
- Computer

Hardware:

- CPU: AMD Athlon ™ 1.67GHz
- RAM: 640MB
- Hard disk: 110GB

Software:

- ImageTool 3.0 (developed in 2002 by the Department of Dental Diagnostic Science, The University of Texas Health Science Center, San Antonio, Texas, USA)
- SigmaScan Pro 5.0 (developed in 1999 by SPSS Inc., Chicago, USA) with adapted macro

Procedures:

1. Digital pictures
   - Select 5 representative areas of portal tract and of central vein respectively, preferably away from major blood vessels.
   - Take 10 digital pictures totally with above areas at 200x magnification.
   - Transfer the pictures to the computer.
   - Organize the pictures of different animals into folders named with animal numbers.
2. Quantification of BrdU-labeled hepatocyte nuclei
   - Import the digital pictures into ImageTool 3.0 one by one.
   - Identify and subsequently mark the BrdU-labeled nuclei of hepatocytes which are stained red.
   - Note the number given by ImageTool 3.0 automatically
3. Quantification of unlabeled hepatocyte nuclei
   - Parametrize the unlabeled nuclei of hepatocytes based on
     - Colour***** (blue)
     - shape (>0.6)
     - size (400-2500 pixels)
   - Use SigmaScan Pro 5.0 to count the number of the unlabeled nuclei of hepatocytes which are stained blue.
   - Record the number.
4. Calculation of LI according to the above formula

***** Value for saturation was fixed while the hue value was adapted for each picture to include 95% of true hepatocyte nuclei.

2.7.1.3 Inter-observer reproducibility in computer-assisted counting

1. Select 1 set of BrdU pictures randomly (REG-032).
2. Count the total numbers of unlabeled hepatocyte nuclei using Sigmascan Pro 5.0 by 2 skilled independent observers.
3. Compare and analyse the differences statistically.

2.7.2 Assay precision of BrdU-IHC staining

2.7.2.1 Quality criteria of BrdU-IHC staining

The quality criteria for BrdU staining were standardized according to a scoring system, in which the intensity of positive signals in small bowel and liver tissue are the key points (Table 7). The sections which did not fulfil the criteria were excluded from further analysis and restained.

Table 7. Quality criteria of BrdU-IHC staining

Intensity of positive signals		**Consequence**	**Explanation**
small bowel	liver		
+~++ ++	++ +~++ +	Accept it	Acceptable staining quality
+~++ ++	(-) (including hepatocytes, bile duct cells and other non-parenchymal cells)	Repeat it. If the intensity of positive signals are same, then accept it	The possibility that liver tissue did not receive staining solution is unlikely to happen twice to the same slide
(+) +	(-) (including hepatocytes, bile duct cells and other non-parenchymal cells)	Repeat it. If the intensity of positive signals are same, then exclude it	Potential staining or BrdU incorporation problems
(+)	Single hepatocytes clearly positive	Repeat it. If the intensity of positive signals are same, then accept it	Incorporation problem happens in small bowel but not in liver tissue

++: strong positivity; +~++: moderate positivity; +: poor positivity;
(+): very poor positivity; (-): negativity.

2.7.2.2 Intra-assay variation of BrdU-IHC staining

To assess the intra-assay variation, a frequently used control sample, BLH-021, was stained in the same assay. The procedures were as follows:

1. Cut 5 sections from the same paraffin block (BLH-021) as experimental slides.
2. Perform BrdU-IHC staining in the same assay.
3. Analyse the BrdU LI of each slide using computer-assisted counting respectively.
4. Evaluate the intra-assay precision by calculating the coefficient of variance (CV).

2.7.2.3 Inter-assay variation of BrdU-IHC staining

To assess the inter-assay variation, BLH-021 was stained in different assays. The procedures were as follows:

1. Cut 5 sections from the same paraffin block (BLH-021) as control slides.
2. Perform BrdU-IHC staining in different assays.
3. Analyse the BrdU LI of each slide using computer-assisted counting respectively.
4. Evaluate the inter-assay precision by calculating the CV.

2.8 Statistical analysis

The data were analyzed with SPSS 11.0. Differences between 2 paired groups and between 2 independent groups were analyzed using paired- and independent-samples Student's *t* test, respectively. Multiple groups were compared using ANOVA followed by S-N-K analysis. Correlation between 2 data sets was evaluated using bivariate correlations analysis. $P < 0.05$ was considered significant.

3 RESULTS

3.1 Validation of quantification method and immunohistochemical assay

3.1.1 Comparison of methods to quantify unlabeled hepatocyte nuclei

The total numbers of unlabeled hepatocyte nuclei generated automatically by computer-assisted counting were compared with those derived manually by ImageTool 3.0. The time required to quantify the number of unlabeled hepatocyte nuclei in one picture automatically was considerably less than that required to perform the counting manually (1-2 minutes vs. 8-10 minutes). Furthermore, no statistically significant difference was found between the two data sets using paired-samples t test (p=0.269) and a strong correlation between them was observed (r=0.868, p=0.001). (Table 8 and Figure 5)

Table 8. Comparison between numbers obtained conventionally and automatically.

Animal No.	Picture No.	Number of unlabeled hepatocyte nuclei		Differences
		ImageTool 3.0	Sigmascan Pro 5.0	
REG-032	CV032-1257	349	343 (135,190)*	6
	CV032-1258	327	310 (115,190)	17
	CV032-1259	355	367 (110,190)	-12
	CV032-1260	341	334 (105,190)	7
	CV032-1261	369	366 (115,190)	3
	PT032-1247	357	339 (115,190)	18
	PT032-1253	354	350 (135,190)	4
	PT032-1254	322	329 (135,190)	-7
	PT032-1255	335	343 (125,190)	-8
	PT032-1256	315	305 (135,190)	10
	Sum	**3424**	**3386**	**38**
	Statistical analysis			**P=0.269**

* In computer-assisted counting, the numbers together with their corresponding hue values of color threshold set by the observer were given.

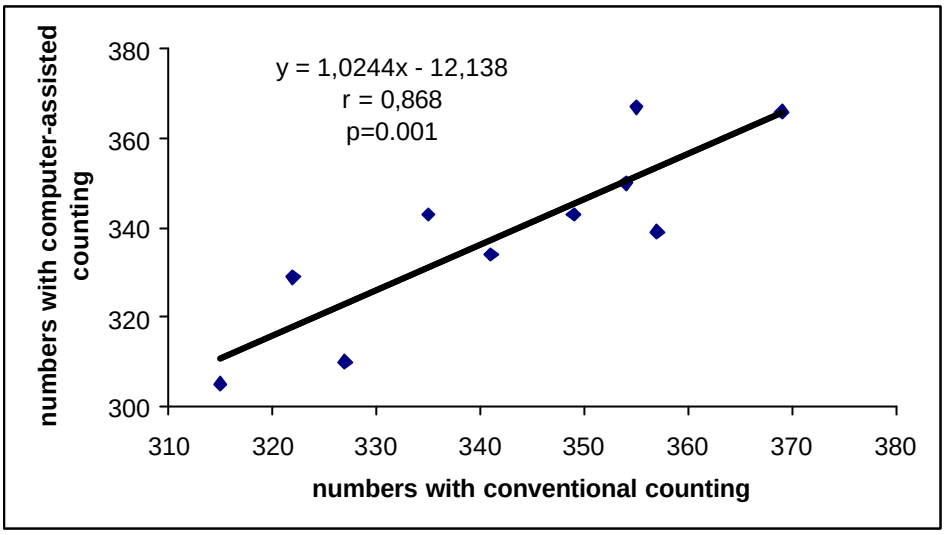


Figure 5. Correlation between the numbers obtained conventionally and automatically.

With computer-assisted counting, the total numbers of unlabeled hepatocyte nuclei counted by one skilled observer on two different days did not show any statistically significant difference using paired-samples t test (p=0.954) (Table 9). Similarly, to the same set of BrdU pictures, there was no significant difference between the numbers determined by two independent skilled observers using paired-samples t test (p=0.711) (Table 10).

Table 9. Intra-observer reproducibility with computer-assisted counting

Animal No.	Picture No.	Number of unlabeled hepatocyte nuclei		Differences
		Sigmascan-01	Sigmascan-02	
REG-032	CV032-1257	343 (135,190)	343 (135,190)*	0
	CV032-1258	310 (115,190)	319 (110,190)	-9
	CV032-1259	367 (110,190)	367 (110,190)	0
	CV032-1260	334 (105,190)	334 (105,190)	0
	CV032-1261	366 (115,190)	353 (120,190)	13
	PT032-1247	339 (115,190)	339 (115,190)	0
	PT032-1253	350 (135,190)	350 (135,190)	0
	PT032-1254	329 (135,190)	329 (135,190)	0
	PT032-1255	343 (125,190)	346 (120,190)	-3
	PT032-1256	305 (135,190)	305 (135,190)	0
	Sum	**3386**	**3385**	**1**
	Statistical analysis			**P=0.954**

* In computer-assisted counting, the numbers together with their corresponding hue values of color threshold set by the observer were given.

Table 10. Inter-observer reproducibility with computer-assisted counting

Animal No.	Picture No.	Number of unlabeled hepatocyte nuclei		Differences
		Observer-01	Observer-02	
REG-032	CV032-1257	343 (135,190)	342 (125,225)*	1
	CV032-1258	310 (115,190)	312 (120,235)	-2
	CV032-1259	367 (110,190)	360 (120,225)	7
	CV032-1260	334 (105,190)	306 (130,240)	28
	CV032-1261	366 (115,190)	372 (120,235)	-6
	PT032-1247	339 (115,190)	359 (120,225)	-20
	PT032-1253	350 (135,190)	371 (130,215)	-21
	PT032-1254	329 (135,190)	335 (130,223)	-6
	PT032-1255	343 (125,190)	327 (130,220)	16
	PT032-1256	305 (135,190)	321 (130,225)	-16
	Sum	**3386**	**3405**	**-19**
	Statistical analysis			**P=0.711**

* In computer-assisted counting, the numbers together with their corresponding hue values of color threshold set by the observer were given.

3.1.2 Assay precision of BrdU-IHC staining

The intra- and inter-assay LI were in the range of 3.2-3.7% and 2.7-3.5% respectively, from which the corresponding CV were obtained. Compared to the inter-assay CV, the intra-assay CV is lower (7.6% vs. 9.7%). There were no significant differences among the intra-assay LI as well as inter-assay LI using one way ANOVA test (p=0.962 and p=0.889 respectively) (Table 11 and 12).

Table 11. Intra-assay variation of BrdU-IHC staining

Animal No.	Assay No.	Slide No.	Intensity of small bowel	LI (%)	Mean	SD	CV (%)	Statistical analysis
BLI-021	BrdU-075	W03-1485-1	+-++	3.7	3.4	0.3	7.6	ANOVA: p=0.962
		W03-1485-2	+-++	3.7				
		W03-1485-3	+-++	3.4				
		W03-1485-4	+-++	3.2				
		W03-1485-5	+-++	3.2				

Table 12. Inter-assay variation of BrdU-IHC staining

Animal No.	Slide No.	Assay No.	Intensity of small bowel	LI (%)	Mean	SD	CV (%)	Statistical analysis
BLI-021	W03-1485	BrdU-063	+-++	2.9	3.1	0.3	9.7	ANOVA: p=0.889
		BrdU-065	+-++	3.1				
		BrdU-067	++	3.1				
		BrdU-068	++	3.5				
		BrdU-070	++	2.7				

3.2 Kinetics of regeneration after small-for-size liver transplantation

The proliferation rate of normal rat liver was 0.2%. After 70%PH, a peak LI of 13% was reached at 24 hours, then the proliferation rate decreased gradually, returning to the preoperative value at 1 week (Figure 6). Compared to 70%PH, the onset of regeneration following 30%NAPOLT was delayed until 24 hours (13.1±8.4% vs. 0.5±0.8%, $p < 0.05$) and peaked at 48 hours (5.6±4.3% vs. 15.8±9.3%, $p < 0.05$). There were no significant differences between 70%PH and 30%NAPOLT at 72 hours and 1 week. In addition, the difference between the peak LI of 70%PH and 30%NAPOLT group had no statistical significance (Table 13). The corresponding BrdU pictures are shown in Figure 7 and 8.

Table 13. Kinetics of liver regeneration following 70%PH and 30%NAPOLT (data are expressed as mean ± SD %).

Group	**Observation Periods**				
	0h	24h	48h	72h	1w
Naive	0.2±0.1% (n=10)				
70%PH		13.0±8.4% (n=6)	5.6±4.3% (n=6)	4.4±1.6% (n=6)	0.3±0.2% (n=4)
30%NAPOLT		0.5±0.8%***** (n=5)	15.8±9.3%***** (n=4)	4.2±1.7% (n=5)	2.1±2.3% (n=4)

Compared to 70%PH group, * $p<0.05$.

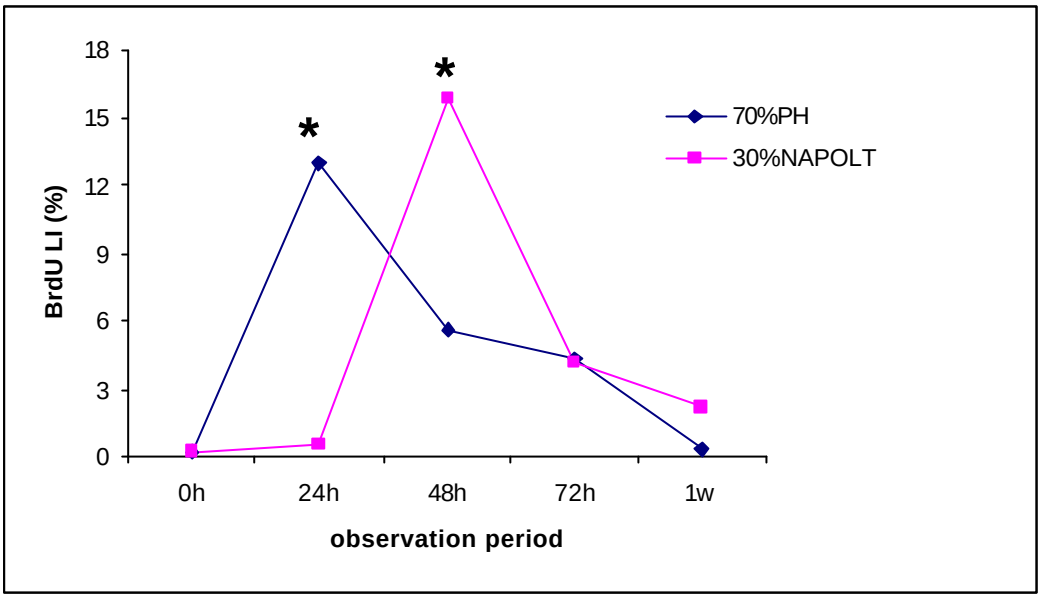
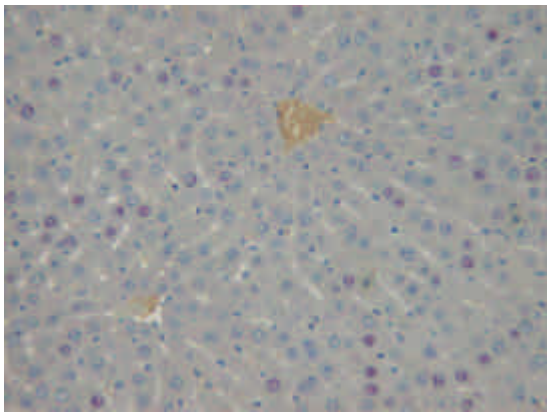
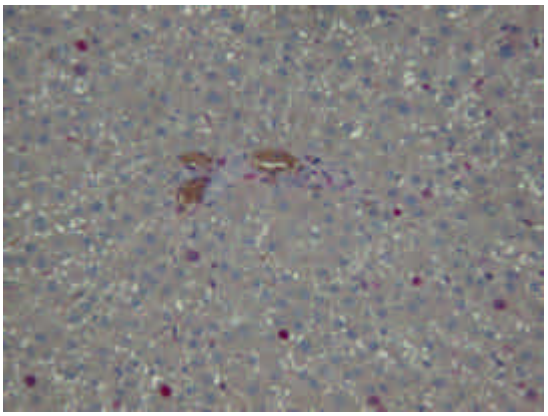


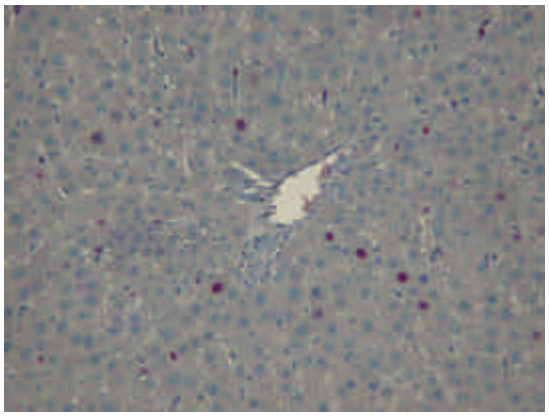
Figure 6. Kinetics of regeneration following 70%PH and 30%NAPOLT. The BrdU LI showed a peak value of 13% at 24 hours after 70%PH, which declined gradually thereafter. The onset of liver regeneration was delayed after 30%NAPOLT, starting at 24 hours and reaching a peak value at 48 hours. Its magnitude was similar to that at 24 hours after 70%PH. The regeneration rates in both groups decreased to the same values at 72 hours postoperatively. (Compared to 70%PH group, * $p<0.05$)



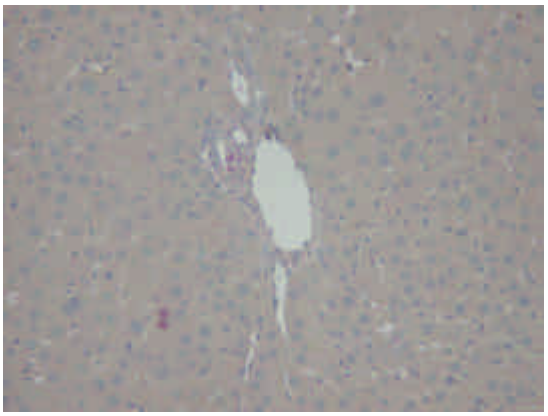
70%PH-24h (REG-038-DSCN6688)



70%PH-48h (REG-144-DSCN0351)



70%PH-72h (REG-155-DSCN0648)



70%PH-1w (REG-199-DSCN0139)

Figure 7. BrdU LI at different time points after 70%PH (control group): 24h (upper left), 48h (upper right), 72h (lower left) and 1 week (lower right).
BrdU LI peaked at 24 hours and decreased gradually thereafter. Proliferating hepatocytes were uniformly distributed in the lobules at 24h. At 48h and 72h, not only hepatocytes but also biliary epithelial cells, Kupffer cells as well as sinusoidal endothelial cells underwent proliferation. (BrdU, 200x).

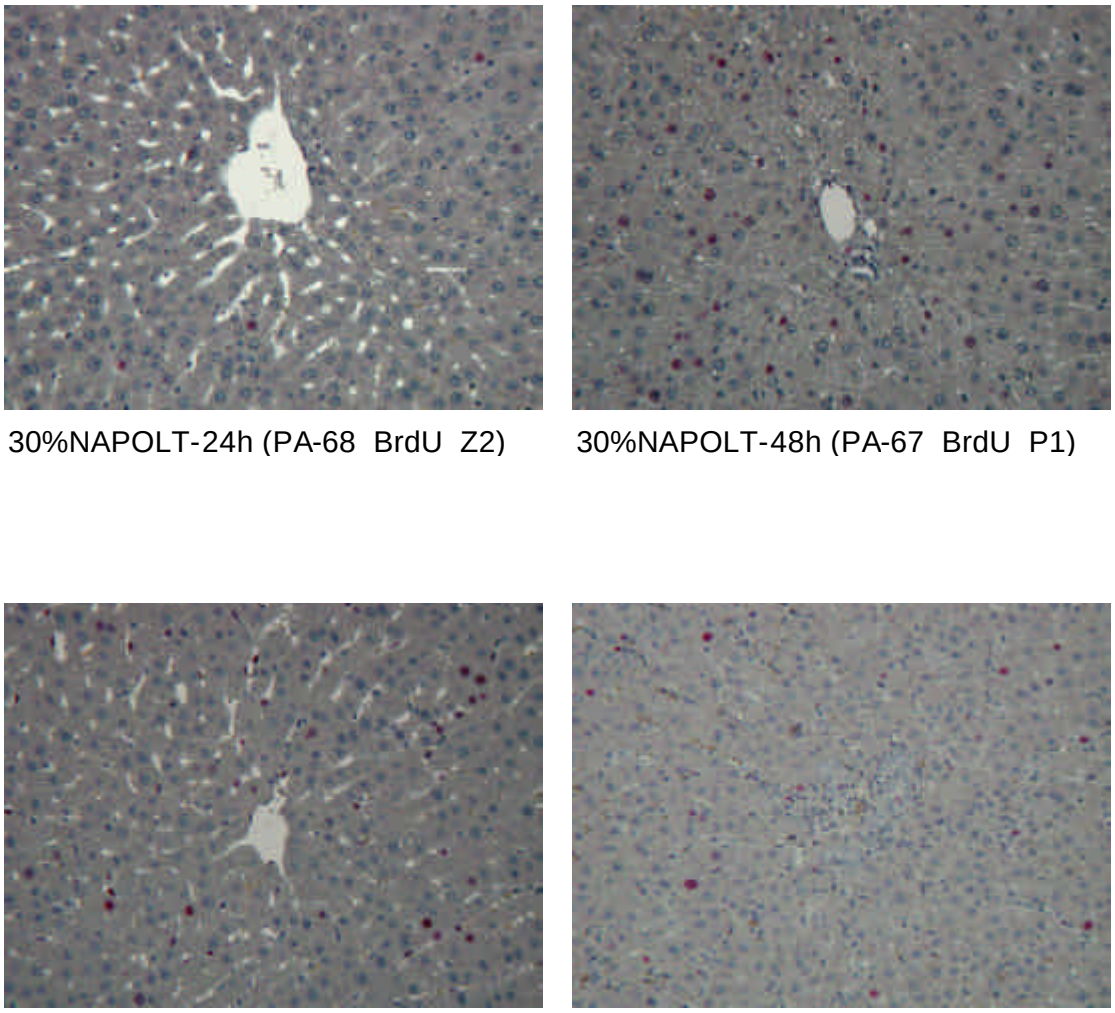


Figure 8. BrdU LI at different time points after 30%NAPOLT: 24h (upper left), 48h (upper right), 72h (lower left) and 1 week (lower right).
The onset of proliferation following 30%NAPOLT started at 24h and peaked at 48h postoperatively, decreasing gradually thereafter. Proliferating cells were uniformly distributed in the lobules at 48h, majority of them being hepatocytes. At 72h proliferating Kupffer cells were detected. (BrdU, 200x).

3.3 Influence of extended cold ischemia on regeneration of a small-for-size graft

Transplantation of a 30% partial liver graft subjected to1 hour of cold ischemia was tolerated by all animals. Extending cold ischemia to both 3 and 5 hours reduced the 1-week survival rate to 50% and 30%, respectively (Table 14). The BrdU LI tested at 48 hours after 70%PH showed 5.6%. The proliferative rate of a 30% liver graft subjected to 1-hour cold ischemia was significantly higher at the same time point ($p<0.05$), as onset and peak of proliferation were delayed. The influence of prolonged ischemia on proliferation rates was assessed at 48h postoperatively (n=3-4/group) and was associated with a lower mean LI, although the difference did not reach statistical significance (Table 14 and Figure 9). The corresponding BrdU pictures are shown in Figure 10.

Table 14. Effects of cold ischemia on the survival rate and regenerative ability of the liver (data are expressed as percentage and mean ± SD %, respectively).

Group	Ischemic time	1 week SVR	BrdU LI at 48h
70%PH	control	100% (6/ 6)	5.6±4.3%
30%NAPOLT	Ischemia 1h	100% (6/ 6)	15.8±9.3%*
	Ischemia 3h	50% (5/10)	9.1±1.3%
	Ischemia 5h	30% (3/10)	9.3±7.8%

*: Compared to the control group, $p<0.05$.

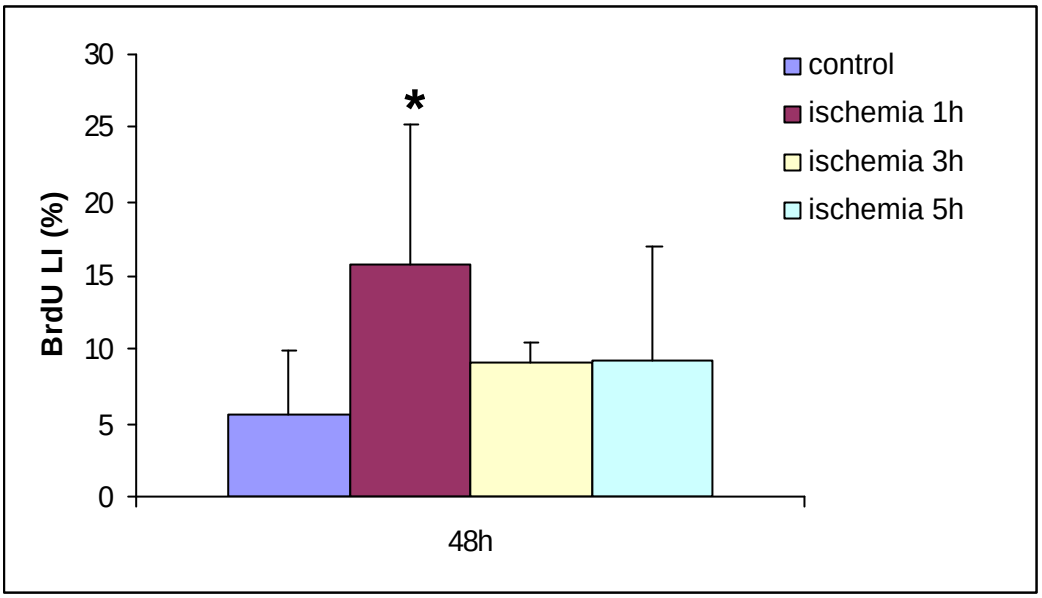
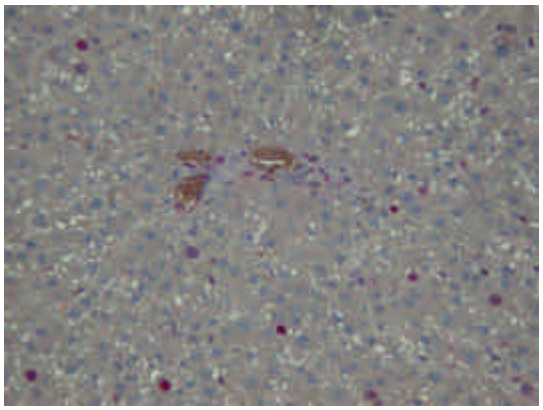
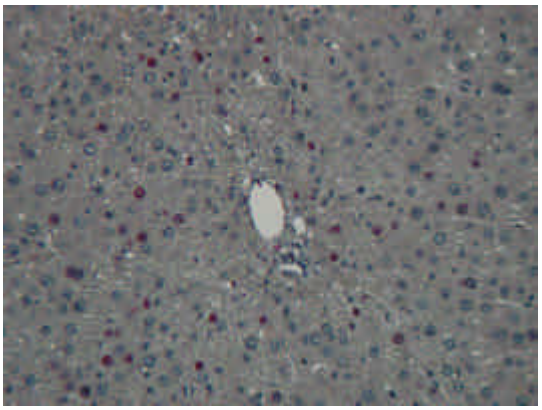


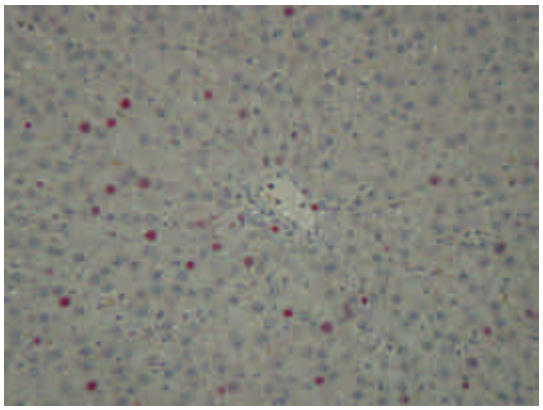
Figure 9. Effect of cold ischemia on the regenerative capacity of a 30% partial liver graft (compared to the control group, *p<0.05).



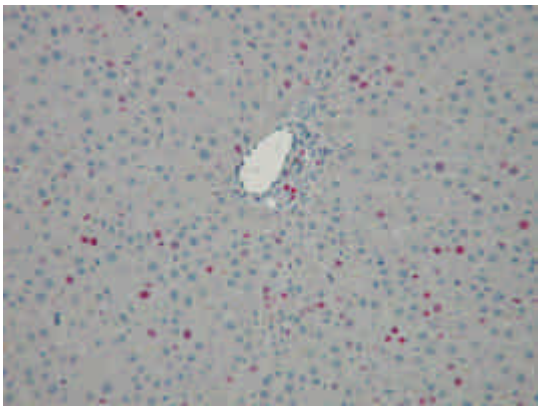
70%PH-Control (REG-144-DSCN0351)



30%NAPOLT-Ischemia 1h (PA-067_BrdU_P1)



30%NAPOLT-Ischemia 3h (PI-067_BrdU_P1)



30%NAPOLT-Ischemia 5h (PI-071_BrdU_P1)

Figure 10. Influence of cold ischemia on BrdU LI (48 hours postoperatively). Control group: 70% PH (upper left), experimental groups: 30%NAPOLT subjected to 1h cold ischemia (upper right), to 3h (lower left) and to 5h (lower right). Labeling indices were reduced in animals subjected to Prolonged ischemia. (BrdU 200x)

3.4 Immunological regulation of liver regeneration

3.4.1 Immune activation by vaccination on liver regeneration

After 70%PH, both the control and the vaccinated group reached their regeneration peaks at 24h. However, compared to the control group, the peak value of the vaccinated group was significantly lower (13.0±8.4% vs. 4.4±4.1%, $p<0.05$). Moreover, the LI at 48h and 72h postoperatively of the vaccinated group was significantly lower than those of the control group (5.6±4.3% vs. 1.6±1.0%, $p<0.05$, and 4.4±1.6% vs. 1.2±0.7%, $p<0.01$, respectively). Thereafter the proliferation rates of both groups decreased gradually, returning to baseline at 1 week postoperatively (Table 15 and Figure 11). The corresponding BrdU pictures are shown in Figure 12.

Table 15. Regeneration kinetics of control and vaccinated group following 70%PH (data are expressed as mean ± SD %).

Group	**Observation Periods**				
	0h	24h	48h	72h	1w
Naive	0.2±0.1% (n=10)				
Control		13.0±8.4%* (n=6)	5.6±4.3%* (n=6)	4.4±1.6%** (n=6)	0.3±0.2% (n=4)
Vaccinated		4.4±4.1% (n=6)	1.6±1.0% (n=6)	1.2±0.7% (n=6)	0.4±0.2% (n=6)

Compared to vaccinated group: * $p<0.05$. ** $p<0.01$

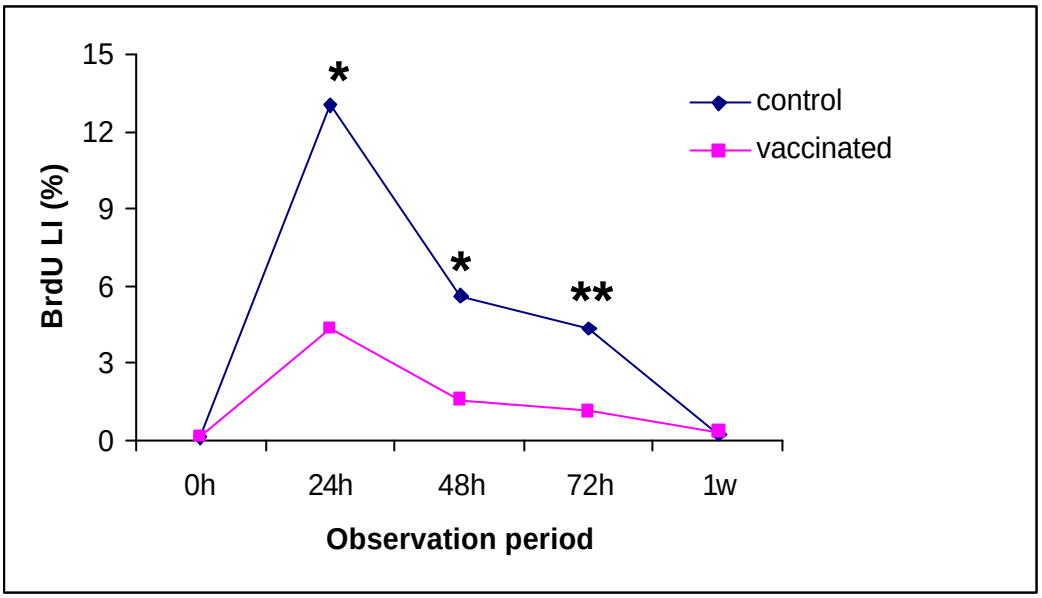
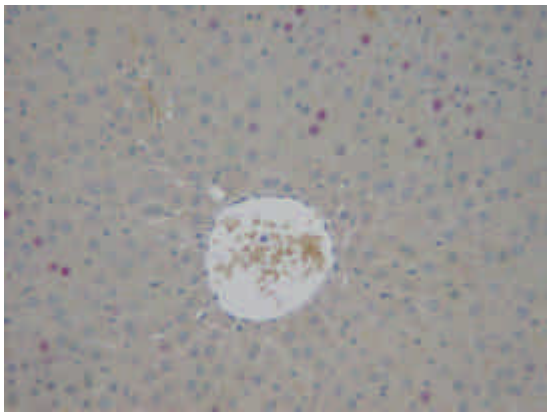
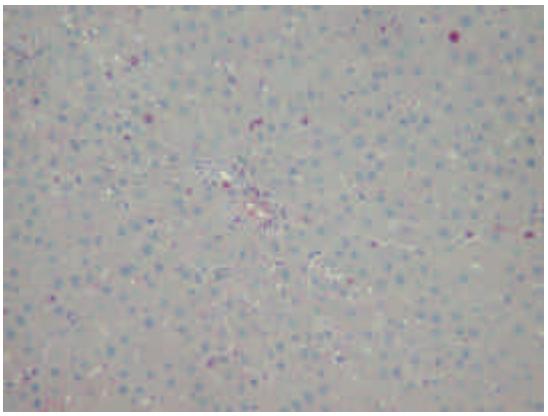


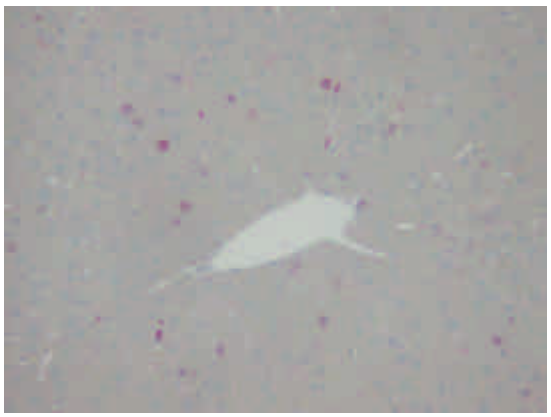
Figure 11. Kinetics of regeneration in control and vaccinated group following 70%PH. Although the kinetics of regeneration in the experimental group was similar to the control group, the LI at 24h, 48h as well as 72h were significantly lower. (Compared to vaccinated group: ***** $p<0.05$; ****** $p<0.01$)



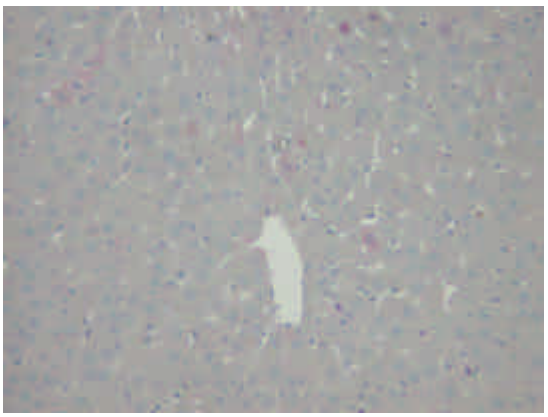
70%PH-24h (REG-029-DSCN8509)



70%PH-48h (REG-192-DSCN0049)



70%PH-72h (REG-204-DSCN0330)



70%PH-1w (REG-210-DSCN0287)

Figure 12. BrdU LI at different time points after 70%PH in animals subjected to hepatitis B vaccination 1 week before. 24h (upper left), 48h (upper right), 72h (lower left) and 1 week (lower right).
Compared to the control group, proliferation reached a lower peak at 24 hours and decreased gradually thereafter. At 48h and 72h, majority of the proliferating cells were biliary epithelial cells, Kupffer cells and sinusoidal endothelial cells. (BrdU, 200x).

3.4.2 ffect of inhibiting immune activation by immunosuppressive drugs on liver regeneration

Subtotal reduction of the liver mass was followed by a survival rate of 50% in the control group. Treatment with calcineurin inhibitors increased the short-term survival in both groups. 75% of the animals treated with CsA and 63% of those treated with FK506 survived up to 48 hours postoperatively. Treatment with RAD did not show any effects on the survival rate when compared with the control group, whereas MMF reduced the survival rate dramatically to 21%, which was the reason to increase the group size (Table 16).

Treatment with CsA augmented liver regeneration as indicated by the increased BrdU LI compared to the control group ($p<0.05$). Animals receiving FK506 presented with a regenerative rate of 15.5%, which was similar to that of the control group. However, animals treated with either MMF or RAD showed a much lower regenerative rate ($p<0.05$ and $p<0.01$, respectively) (Figure 13). The corresponding BrdU pictures are shown in Figure 14.

Table 16. Effects of different immunosuppressive drugs on the survival and regeneration rate of the liver after 90%PH (data are expressed as percentage and mean ± SD %, respectively).

PH	Treatment	48 hours SVR	BrdU LI
90%PH	Control	50% (4/8)	12.2±2.3%
	CsA	75% (6/8)	18.5±7.5%*****
	FK506	63% (5/8)	15.5±7.6%
	MMF	21% (3/14)	4.3±4.3%*****
	RAD	50% (4/8)	1.1±1.2%******

Compared to the control group, *****$p<0.05$, ******$p<0.01$.

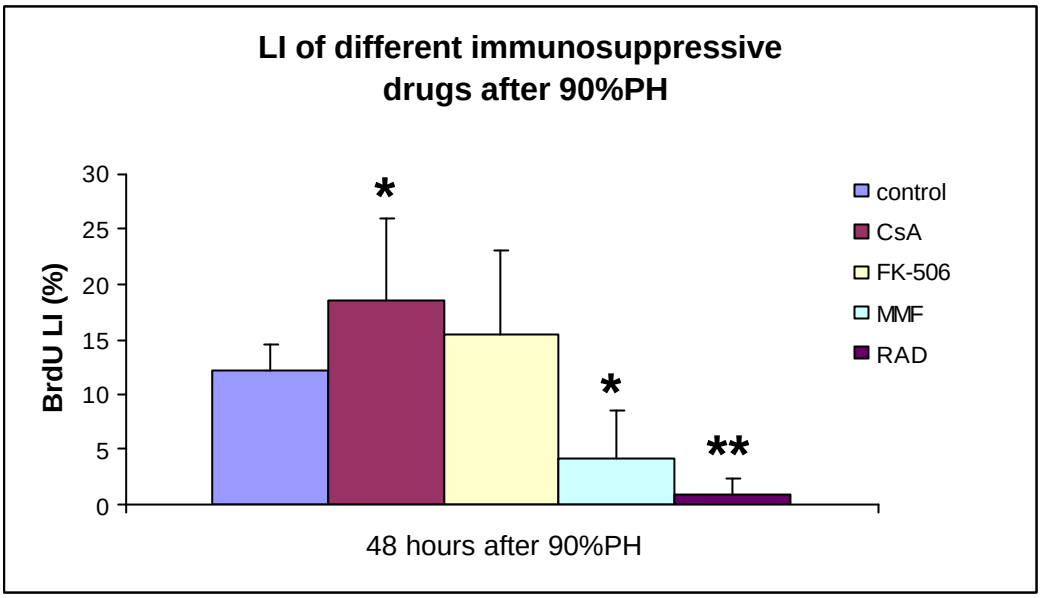
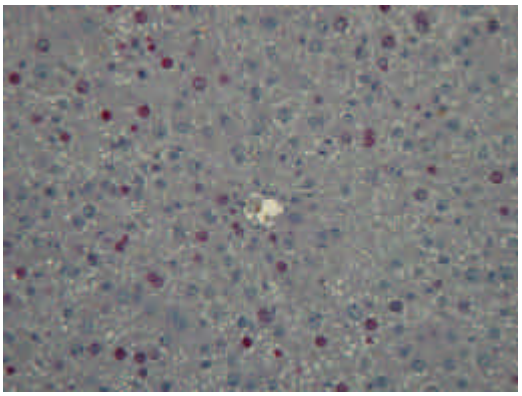
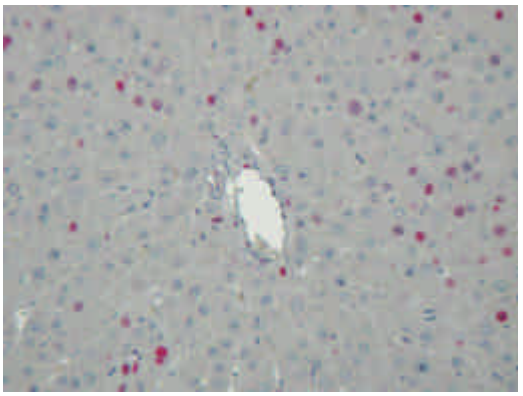


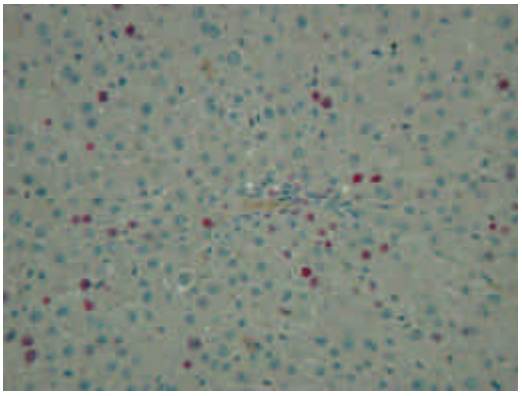
Figure 13. Effects of different immunosuppressive drugs on the regeneration rate of the liver at 48 hours after 90%PH (Compared to the control group, ***p<0.05, ****p<0.01).



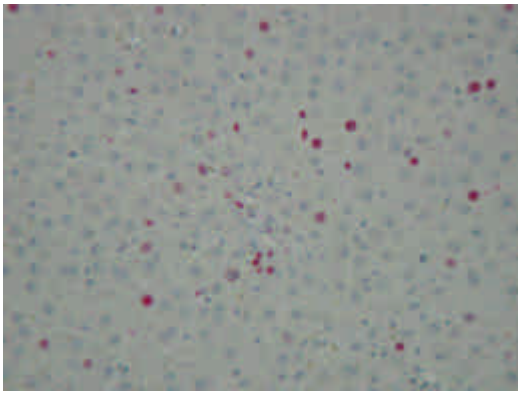
90%PH-CsA (RIL-018 BrdU Z2)



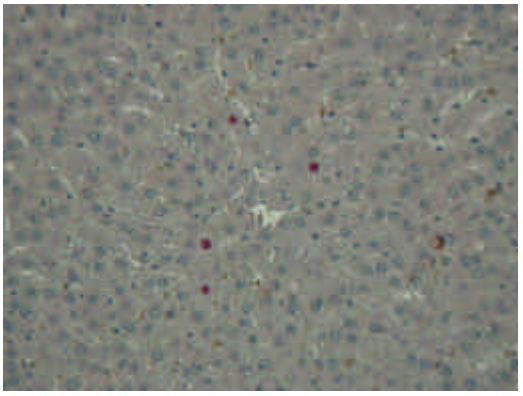
90%PH-FK (RIL-043 BrdU P2)



90%PH-Control (RIL-026 BrdU P1)



90%PH-MMF (RIL-053 BrdU P2)



90%PH-RAD (RIL-010 BrdU Z1)

Figure 14. BrdU LI in rats subjected to 90%PH and treated with different immunosuppressive drugs (48h postoperatively). Treatment with CsA (upper left), with FK506 (upper right), control animal receiving no treatment (middle), with MMF (lower left), and with RAD (lower right).
Labeling index was increased in animals subjected to CsA treatment and almost abrogated in animals receiving MMF and RAD (BrdU, 200x)

4 DISCUSSION

4.1 Validation of quantification method and immunohistochemical assay

4.1.1 Correlation between conventional and computer-assisted counting

The interest in cell proliferation in response to partial hepatectomy or toxic injury has led to a rapidly growing demand for assessment of cell turnover. A widely used technique in animal models is to administer a halogenated pyrimidine, BrdU, and to visualize its incorporation into the DNA during the S phase by immunohistochemistry (Magavi and Macklis, 2002). Labelled and unlabeled hepatocyte nuclei are then counted by an observer and the percentage of cells that incorporated the nucleotide during S phase are calculated.

Some progress has been made in automating this process. Soames indicated that automated image analysis can reduce inter-observer variation and should minimize intra-observer error, as well as reducing the tedium of measuring the labelling indices in the liver (Soames et al., 1994b). A macro with object-targeted adaptation of SigmaScan Pro 5.0 was developed previously in our group which enabled us to automate the counting of unlabeled hepatocyte nuclei and generate LI subsequently. The time required to perform the computer-assisted counting is much less than that required performing the counting conventionally using ImageTool 3.0.

Since the pioneering report by Soames et al. (Soames et al., 1994a) on quantifying hepatocyte nuclei by image analysis, no other reports characterized a similar work. Using a monochrome camera, which provides a gray scale image only, they developed an algorithm based on nuclear size and shape to distinguish hepatocyte nuclei from other cell types leading to an inclusion of 90% true hepatocytes (Table 17). We used a consumer camera to acquire colour images for data documentation and analysis. Therefore our parametrization included colour threshold besides nuclear size and shape. In order to compensate for slight differences in staining intensity between different slides, the hue values for the colour threshold were adapted for each image prior to quantification until 95% of the hepatocyte nuclei were included.

In both studies, a strong correlation was shown between conventional and computer-assisted counting. Furthermore, in the current study, no statistically significant differences were found in either intra- or inter-observer data sets, indicating that a reproducible and reliable result was achieved using computer-assisted counting.

Table 17. Computer-assisted quantification of hepatocyte nuclei in rodents

Author	Object	Camera/ Program	Parameter	Quality control	Statistical results
Soames 1994	Total hepatocytes nuclei in rat and mouse liver (>90%)	Sony XC-7CE monochome CCD	Nuclear size (>25 um^2) Shape (>0.7)	Correlation between conventional and computer-assisted counting	Strong correlation
		Kantron Vidas 2.1			
Current study	Unlabeled hepatocytes nuclei in rat liver (>95%)	Nikon Coolpix 995	Nuclear size (400-2500 pixels) Shape (>0.6) Colour (blue)	Correlation between conventional and computer-assisted counting	Strong correlation
		SigmaScan Pro 5.0 with adapted macro		Intra- and inter-observer reproducibility	No significant difference

4.1.2 Assay precision of BrdU-IHC staining

In recent years, IHC has become an indispensable technique in modern scientific research. However, the technique is very sensitive to a variety of factors such as tissue fixation and processing, antigen retrieval, choice of antibodies and detection systems (Ruiter et al., 1998). In order to achieve comparable results between different laboratories, but also within the same laboratory, these factors have to be standardized. Furthermore, differences in results can also be related to the evaluation method. To achieve consistent results, a standard staining protocol was used and only slides fulfilling the quality criteria were included in the analysis (Table 7).

The interpretation of immunohistochemical staining is based on the use of appropriate positive and negative internal and/or external controls. O'Leary indicated that the control specimen should not exhibit intense immunoreactivity for

the antigen in question, but should ideally demonstrate immunoreactivity that is weak in some places and strong in others. This is most readily accomplished through the use of multi-tissue blocks (O'Leary, 2001). Accordingly all experimental samples were embedded in multi-tissue blocks containing liver, small bowel as well as kidney tissue as internal control. The intensity of positive signals in small bowel and liver tissue were the key points of the quality criteria (Table 7). In addition, an animal with a moderate regeneration rate following hepatectomy (BLI-021) was used as control sample.

To assess the assay precision of BrdU-IHC staining, the control sample was stained and evaluated repeatedly to test the intra- and inter-assay variations. The intra- and inter-assay CV were 7.6% and 9.7%, respectively. Being unaware of any published data characterizing the assay precision of BrdU-IHC staining, we evaluated these results in two aspects. On one hand, intra- and inter-assay CV below 10% in enzyme-linked immunosorbent assay (ELISA) is usually considered acceptable and reproducible (Austin et al., 2001). Since IHC shares the basic principle with ELISA using enzymatic colour reaction to detect the binding of antigen and antibody, this criteria might be adapted to the evaluation of assay precision of BrdU-IHC staining. In this aspect, the results suggested an acceptable intra- and inter-assay variability. On the other hand, statistical methods can be used to judge the significance of the assay variability. Using one way ANOVA test, there were no significant differences among the intra-assay LI as well as inter-assay LI ($p=0,962$ and $p=0,889$ respectively). Therefore, we concluded that our BrdU-IHC staining was reproducible, reliable and of high precision.

4.2 Kinetics of regeneration after small-for-size liver regeneration

4.2.1 Delayed onset of regeneration after transplantation

Loss of liver mass is being compensated by liver regeneration which starts in any case after a reduction of functional liver mass by any means, irrespectively of the etiology (Andiran et al., 2000). The final size of the liver is determined by the needs of the host, as the liver is growing to adjust its size according to the requirement of the host (Francavilla et al., 1994b). As transplantation of a small-for-size graft is unavoidable in living liver donation between adults, recovery of

functional liver mass by hepatocyte proliferation is one of the crucial factors for the success of the procedure.

According to the experience obtained from the standard resection model and confirmed in this experiment, liver regeneration starts within hours after resection, and the maximal proliferation index is observed between 18 and 30 hours (Assy et al., 1998). In this study, the BrdU LI at 24 hours after 70%PH showed a maximal value of 13%, which declined gradually thereafter. The maximal LI, which was observed after transplanting a graft of similar size (30%) as the remnant liver after 70% resection, reached the same magnitude. However, initiation of regeneration was delayed. The peak was reached 24 hours later on postoperative day (POD) 2 than in the resection model. This observation suggested that the small-for-size liver graft is capable of regeneration similarly to a remnant liver of the same size, but follows a different kinetics.

Few other reports addressed this question (Table 18). In addition, comparison of results is difficult as the experimental design varies considerably in respect to species, graft size, number and time point of observation during the course of regeneration. Furthermore, although determination of regeneration rate is based on the BrdU LI, the assay and its evaluation varies in respect to the primary antibody as well the detection system and the quantification method. Results were expressed as hepatocytes/HPF and in percentage of proliferating hepatocytes.

The group of Kikuchi and Yamaguchi (Kikuchi et al., 1994; Yamaguchi et al., 1996) performed a detailed kinetic study with observation points every 12 h post operation. They used the same graft volume, a similar transplantation technique (perfusion of liver graft with heparinized saline solution, non-arterialized transplantation) but a different syngeneic model. In contrast to our reported results, initiation of regeneration was not delayed. However, the peak proliferation rate was also postponed. They observed 15% proliferating hepatocytes in the periportal areas at 24h followed by the maximal proliferation rate of 25% at 36h, which was 12 hours later than after 70%PH.

The other authors reported a peak proliferation rate at 48 hours or in case as late as 72 hours, but did not obtain the time course of liver regeneration, as they only measured the proliferation rate on day 2 and day 4 or on day 1 and 3 after transplantation (Conzelmann et al., 2003; Selzner et al., 2002a; Uchiyama et al., 1999).

Table 18. Kinetics of liver regeneration following syngeneic POLT with various graft volumes in two rodent models (using BrdU LI as proliferation parameter)

Author and publish year	Graft volume	Rat strain	Regeneration rate			
			d[1]1	d2	d3	d4
Tanaka et al., 2003	20%	DA-DA	30%	28%	27%	Not done
Kikuchi et al., 1994 Yamaguchi et al., 1996	30%	Wistar-Wistar	Periportal areas			
			15%	5%	5%	Not done
			Pericentral areas			
			5%	8%	5%	Not done
Selzner et al., 2002	30%	Lewis-Lewis	Not done	43 (pos[2] HC[3] / HPF[4])	Not done	38% (pos HC/ HPF)
Conzelmann et al., 2003	50%	Male C57B1/6 mice	Not done	32%	Not done	Not done
Uchiyama et al., 1999	60%	Lewis-Lewis	0.7%	Not done	1.6%	Not done
Own study	30%	Lewis-Lewis	0.5%	15.8%	4.2%	Not done

d[1] : day; d pos[2] : positive; HC[3] : hepatocytes; HPF[4]: high power fields (200x).

4.2.2 Possible explanations for the delayed onset of regeneration

There are several possible explanations for the delay in initiation of regeneration in animals undergoing partial liver transplantation. First of all, the small functional liver volume subsequent to extended resection and/or impaired by the additional ischemia-reperfusion injury during the transplantation procedure may limit liver regeneration, as the liver must be capable of maintaining its normal metabolic functions at the same time as undergoing cell division (; Broering et al. 2003).

Furthermore, initiation of liver regeneration requires the presence of a variety of growth factors, which are released locally during liver resection. As in this experiment the liver was flushed during the transplantation procedure with 10ml of saline solution, these growth factors might have been washed out leading to the delayed onset of regeneration as suggested by Bolitho (Bolitho et al., 1995). However, the importance of flushing the liver in respect to the onset of regeneration remains questionable, as Kikuchi and Yamaguchi did not observe delayed initiation in their experiments.

In conclusion, the small-for-size liver graft is capable of regeneration similar to the situation following PH, with a slightly postponed time course.

4.3 Influence of extended cold ischemia on regeneration of a small-for-size graft

Although LDLT is associated with shorter periods of cold ischemia compared to cadaveric transplantation, ischemia-reperfusion injury cannot be completely avoided. As the recipient only receives a partial graft, which has to regenerate in order to compensate for the size reduction, it is of importance to assess the impact of the duration of cold ischemia on regeneration.

4.3.1 Extended ischemia reducing liver regeneration

This question was addressed using the 30% partial liver transplantation model and subjecting the graft to different periods of cold ischemia. In order to compare the results to the previous kinetic study, where grafts were subjected to 1-hour cold ischemia without the use of a preservation solution, a prolonged cold ischemia time of 3- and 5-hour was selected. The 1-week survival rate was reduced to 50% and 30% respectively after 30%NAPOLT, when grafts were subjected to 3- and 5-hour cold ischemia. Analysis of LI at POD 2 showed a reduced rate of proliferating hepatocytes after extended cold ischemia although not reaching statistical significance. This result is supported by the observations of Selzner (Selzner et al., 2002c), who is up to now the only other author investigating this problem. He used the same model of rat partial liver transplantation (30%NAPOLT in Lew-Lew), but he subjected the graft to considerably longer cold ischemic time. In contrast to the present study, where grafts were flushed with saline, he used University of Wisconsin (UW) as organ preservation solution to reduce ischemia-reperfusion injury (Table 19).

Although Selzner's experimental setup was different from ours, the key observation, that prolonged ischemia reduced the hepatocyte proliferation rate on POD 2, was similar. In his experiment, proliferation was reduced to one fourth compared to the control group, whereas our results showed a reduction albeit not reach statistical significance. This may be due to several reasons. First of all, he subjected the graft to 10-16 hours of cold ischemia, whereas we used only 3-5

hours. This prolonged ischemia time might have had a more pronounced effect, although he applied UW solution for organ preservation. Furthermore, as we mentioned before, the route and dosage of BrdU administration as well as the evaluation method between the two studies were different (Table 19). He used a dose twice as high, as well as an in-vivo incubation time in the animal, which was twice as long. Instead of calculating an index based on a reasonable total number of hepatocytes (we counted at least 3000 HC/rat), he only quantified the number of positive hepatocytes per high power field (200x). Depending on the selection of the high power field (size of portal field or central vein or confluent necrosis) the result may be inaccurate.

Table 19. Influence of duration of cold ischemia on regeneration

Author	Model	Ischemic time	Preservation solution	BrdU LI (d2)	BrdU dose	Route of injection	Evaluation method
Selzner 2002	Lew-Lew 30%NAPOLT	30 min[1] 10h[2] 16h	UW	43 10 5	100mg/kg	Ip[4]. 2h before sacrifice.	No. of labelled HC / HPF
own study	Lew-Lew 30%NAPOLT	1h 3h 5h	saline	15% 9% 9%	50mg/kg	Iv[3]. 1h before sacrifice.	(No. of labelled HC[5]) / (total HC in 10 HPF[6]) x 100%

min[1] : minutes; h[2] : hour(s); iv[3] : intravenously; ip[4] : intraperitoneally;
HC[5] : hepatocytes; HPF[6] : high power fields (200x).

4.3.2 Speculation regarding the mechanism

Tumor necrosis factor alpha (TNF-alpha) and interleukin-6 (IL-6) are major and initial growth factors involved in regeneration in vivo (Cressman et al., 1996; Fausto et al., 1995; Yamada et al., 1997). Selzner postulated that short period of cold ischemia may stimulate nonparenchymal cells, possibly sinusoidal endothelial and Kupffer cells, through yet unknown mechanisms, to produce growth factors such as TNF-alpha and IL-6 (Selzner et al., 2002d). This could be one of the mechanisms explaining the slightly elevated proliferation rate after full-size liver transplantation, where the recipient did not experience a major loss of functional liver mass (Takata et al., 1993).

It was reported repeatedly that prolonged cold ischemia followed by reperfusion of the liver graft impaired Kupffer cell function and increased apoptotic cell death of

sinusoidal endothelial cells (Caldwell-Kenkel et al., 1989; Clavien, 1998; Gao et al., 1998; Thurman et al., 1988). Injury of nonparenchymal cells during reperfusion may result in decreased production of the acute reactant cytokines TNF-alpha and IL-6, thereby preventing adequate hepatocyte proliferation. Kupffer cell blockade also caused impaired regeneration, possibly through decreased availability of TNF-alpha and IL-6 (Meijer et al., 2000; Selzner et al., 2003).

As cold ischemia is impairing liver regeneration, special efforts should be undertaken to keep the already short ischemic time in living donation even shorter, especially when the graft-to-liver weight ratio is low.

4.4 Immunological regulation of liver regeneration

Recently we proposed a feedback mechanism regarding the immunological regulation of liver regeneration (; Dahmen and Dirsch 2002). It was hypothesized that liver regeneration and immune activation are inversely related, and inhibition of immune activation by administration of immunosuppressive drugs could enhance the regenerative response (Figure 15).

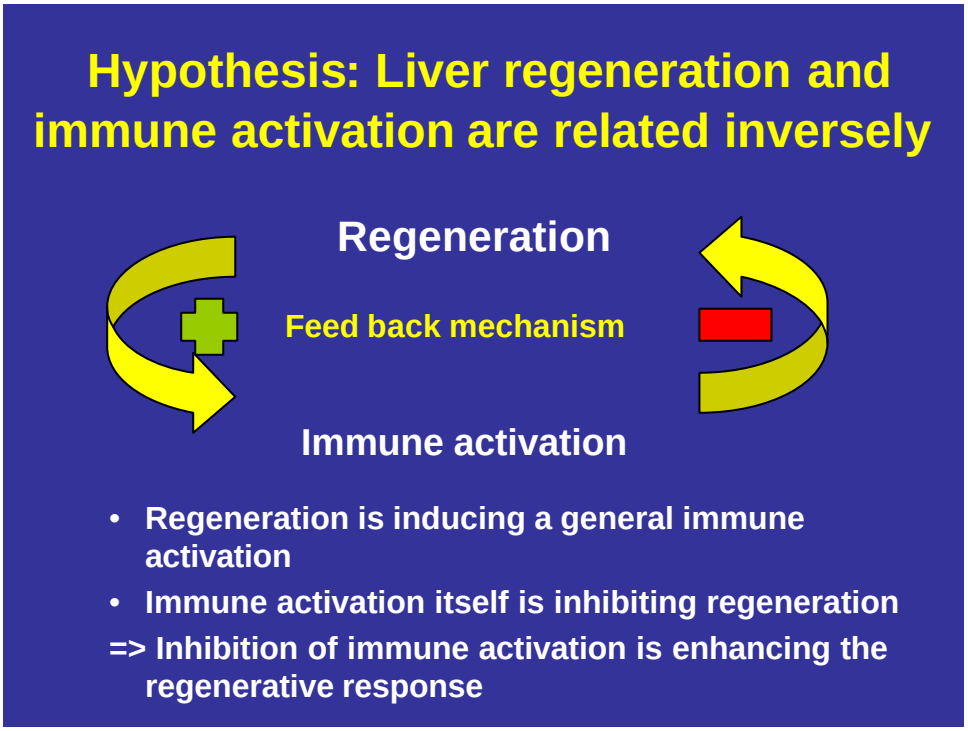


Figure 15. The feedback mechanism between liver regeneration and immune activation

4.4.1 Feedback mechanism between liver regeneration and immune activation

4.4.1.1 Experimental evidence supporting the hypothesis

It has been demonstrated that the liver has the function of a hematolymphoid organ closely associated with the systemic hematolymphoid system (Sakamoto et al., 1992). Several phenomena such as alteration of extrathymic T cells in the liver (Sato et al., 1993a), activation of natural killer cells (NK cells) and autoreactive T cells (Ono et al., 1984) as well as suppressor T cells against the allogeneic response (Pinto et al., 1987) were observed in animal experiments after PH. Therefore, it was hypothesized, that liver regeneration following hepatectomy is inducing systemic immunological activation.

On the other hand, there is considerable evidence that immune activation associated factors on the cellular as well as the humoral level may inhibit liver regeneration. Three observations are described here. Ohnishi suggested that NK cells activated by IFN-gamma may be involved in killing the regenerating liver cells (Ohnishi et al., 1993). Tanaka reported that infusion of lymphokine activated killer (LAK) cells was followed by inhibition of liver regeneration after partial hepatectomy in mice (Tanaka et al., 1993a). Vujanovic studied the phenotype and function of NK cells residing in the liver after 70%PH, and suggested that these NK cells may be involved in the regulation of the extent of liver regeneration (Vujanovic et al., 1995). All the above experimental results suggested that immune activation inhibited liver regeneration inversely.

4.4.1.2 Animal model and experimental design

We wanted to confirm this hypothesis using Hepatitis B vaccination as stimulus for systemic immune activation prior to liver resection. As hepatitis B-related liver diseases represent a major indication for liver transplantation, it is of interest to assess the influence of a viral-related immune activation on the course of regeneration. In order to exclude other effects such as flushing of the remnant liver and/or additional ischemia-reperfusion injury during the transplantation procedures, the standard 70%PH model was chosen for this experiment.

Hepatitis B vaccination leads to a similar albeit weaker stimulation of the immune system as in the host during the infection with the hepatitis B virus, when the

immune system tries to eliminate the virus (; Keating and Noble 2003) although the T-cell compartment is not involved to the same extent .

ENGERIX-B (GlaxoSmithKlein Biologicals, Belgium) was selected for vaccinating the rats prior to liver resection, as it is a noninfectious recombinant DNA hepatitis B vaccine containing purified surface antigen (S protein). Following recognition by B cells, this antigen induces production of specific anti-HBs antibodies not only in humans, but also in the rat as demonstrated previously (Dahmen et al., 2002; Dahmen et al., 2003; Gu et al., 2002; Li et al., 2002).

4.4.1.3 Possible mechanism of the inhibitory effort of immune activation on liver regeneration

Indeed, we observed that liver regeneration was impaired in the vaccinated animals. The course of regeneration remained unaffected, but the peak of proliferation was significantly lower, suggesting an inhibitory effect of immune activation on the proliferating kinetics following 70%PH.

Processing and presentation of the HBsAg via the major histocompatibility complex (MHC) class II pathway results in the priming of the CD4+ T cell response and provides efficient T cell ‘’help’’ for B lymphocytes to synthesize specific anti-HBs antibodies (Schirmbeck et al., 1994; Wild et al., 1999). Th1 cells induce an immune response by secreting cytokines such as IFN-gamma and IL-2 (Jafarzadeh and Shokri, 2003). Two recent studies revealed that vaccination with recombinant HBsAg induced the increased production of these two cytokines (Akbar et al., 1999; Vingerhoets et al., 1994).

IFN-gamma is a glycoprotein produced mainly by T cells in response to specific sensitizing antigens. It was proven to be a substance which activates NK cells (Trinchieri, 1989). Sato reported that IFN-gamma inhibits liver regeneration by stimulating MHC II antigen expression in the regenerating liver (Sato et al., 1993b). They subsequently revealed IFN-gamma mRNA expressions decreased in the liver on day 1 after 70%PH but increased clearly thereafter (Sato et al., 1999). In addition, Lai observed that the elevation of serum IFN-gamma on day 5 after 70%PH was three times higher than the preoperative data (Lai et al., 1996). These studies suggested that IFN-gamma may negatively regulate liver regeneration.

IL-2 belongs to lymphohematopoietic cytokines and plays a critical role in the promotion and enhancement of cellular response. It is responsible for the

regulation of T lymphocytes proliferation and the activation of cytotoxic T lymphocytes (CTL), NK cells, macrophages as well as granulocytes (Lyszkiewicz and Pajtasz-Piasecka, 2002).

Okamura (Okamura et al., 1992a) reported already in 1992, that liver regeneration was inhibited by treatment with IL-2. His observation was confirmed by Tanaka (Tanaka et al., 1993d) and later by others, who also reported that regeneration was suppressed following treatment with IL-2 (Liu et al., 2002; Wadamori et al., 1996) (Table 20).

Table 20. IL-2 treatment inhibits liver regeneration

Author/ Publish year	Model	Treatment	Effect on liver regeneration	Mechanism
Okamura et al., 1992	70%PH	IL-2, intramuscularly injection	Liver regeneration was blocked by IL-2	IL-2 participates in converting NK cells into activated killer cells
Tanaka et al., 1993	70%PH	IL-2, intraperitoneal injection	BrdU LI at 36 hours was suppressed in a dose-dependent manner	Not mentioned
Wadamori et al., 1996	70%PH	IL-2, portal infusion	Liver regeneration was suppressed	IL-2 not impaired cell proliferation, but reappearance of gap junctions, which could be a kind of maturation of hepatocytes, was affected by IL-2
Liu et al., 2002	66%PH	IL-2	Liver regeneration was blocked by IL-2	IL-2 enhanced NK activity

In summary, vaccination induced immune activation impaired liver regeneration, possibly through the increased production of IFN-gamma and IL-2.

4.4.2 Enhancement of the regenerative response by inhibition of immune activation

4.4.2.1 Experimental evidence supporting the hypothesis

Complementary to the studies describing the inhibitory effect of IL-2 administration on liver regeneration are the reports describing the hepatotrophic effects of the IL-2–blocking calcineurin inhibitors.

The first observation regarding the effect of CsA on liver regeneration was obtained in 1988 and attributed the hepatotrophic effect to an increased activity of

ODC and TK induced by CsA (Kahn et al., 1988). In the same year Kim postulated a direct trophic effect on hepatocytes (Kim et al., 1988a; Kim et al., 1988b), but also suggested that the inhibition of the T cell response was involved in the enhancement of regeneration. Since 1989 some evidence was given that the effect was related to the inhibition of IL-2 production (Francavilla et al., 1989b; Morii et al., 1999; Tanaka et al., 1993c). However, there is also evidence that the hepatotrophic effect is related to other mechanism such as mediated by increasing the cellular Ca^{++} pools (Provencher and Gascon-Barre, 2002) (Table 21).

Table 21. Effect of CsA on liver regeneration

Author/ Publish year	Model	Effect on liver regeneration	Mechanism	
			cytokine related	Cell related
Kahn et al., 1988	70%PH	Hepatotrophic	Not mentioned	Not mentioned
Kim et al., 1988	70%PH	Hepatotrophic	Not mentioned	-Direct trophic effect on hepatocytes -Inhibition of T cell responses
Francavilla et al., 1989	40%PH 70%PH	Hepatotrophic	Inhibition of IL-2 production and binding	Inhibition of T cell response
Kim et al., 1990	70%PH	Hepatotrophic	Not mentioned	-Activation of hepatic metabolism -Inhibition of T cell responses
Tanaka et al., 1993	70%PH	Hepatotrophic	Inhibition of IL-2 production and binding	Inhibition of NK cell responses
Morii et al., 1999	70%PH	Hepatotrophic	-Inhibition of IL-2 production -Decrease of TGF-ß production in liver tissue -Inhibition of TNF-a expression	-Inhibition of T cell, with minimal influence against B cell functions
Provencher and Gascon-Barre, 2002	70%PH	Accelerate hepatic compensatory growth	Not mentioned	In part by increasing the cellular Ca^{++} pools

As FK506 influences the IL-2 production negatively similarly to CsA, comparable results were obtained when evaluating the effect on liver regeneration. Enhancement of liver regeneration was demonstrated repeatedly and was also attributed to the inhibition of IL-2 production (Bendahan et al., 1994; Francavilla et al., 1989a; Francavilla et al., 1990; Okamura et al., 1992b; Sato et al., 1992; Tanaka et al., 1993b). Other mechanism was also suggested such a regeneration

promoting effect mediated by up-regulating of the insulin receptors in the regenerating rat liver (Escribano et al., 2002) (Table 22).

Table 22. Effect of FK506 on liver regeneration

Author/ Publish year	Model	Effect on liver regeneration	Mechanism	
			cytokine related	Cell related
Francavilla et al., 1989	40%PH 70%PH	Hepatotrophic	Inhibition of IL-2 production and binding	Inhibition of T cell response
Francavilla et al., 1990	70%PH	Hepatotrophic	Inhibition of IL-2 production	Inhibition of the T cell component
Sato et al., 1992	70%PH	Hepatotrophic	Inhibition of IL-2 production	Not mentioned
Okamura et al., 1992	70%PH	Hepatotrophic	Inhibition of IL-2 production	Not mentioned
Tanaka et al., 1993	70%PH	Hepatotrophic	Inhibition of IL-2 production and binding	Inhibition of NK cell responses
Bendahan et al., 1994	70%PH	Return of the regenerative response to normal time scale	Inhibition of IL-2 production	Not mentioned
Escribano et al., 2002	70%PH	Promotion of liver regeneration	Not mentioned	Up-regulates insulin receptors in hepatocytes

In contrast, little information is available regarding the effect of MMF and RAD on liver regeneration. Both are new drugs, introduced recently in clinical immunosuppressive regimen. The first report regarding the use of MMF in clinical liver transplantation dated from 1996 (McDiarmid, 1996), whereas RAD was only introduced in 1999 by Watson (Watson et al., 1999). Both drugs exert their immunosuppressive activity by blocking T lymphocyte proliferation (Brazelton and Morris, 1996).

4.4.2.2 Animal model and experimental design

The reason to select the four drugs with different mechanism was to evaluate how immune activation must be blocked in order to influence liver regeneration, either by inhibition of IL-2 production or by influencing the T cell response.

As enhancement of liver regeneration using the standard liver resection model by calcineurin inhibitors has been observed in the past, the current study was designed using a model of subtotal hepatectomy, where the course of liver regeneration is altered.

4.4.2.3 Inhibition of immune activation and liver regeneration

In the present study, both CsA and Fk506 had a slightly promoting effect on the survival rate. However, only the former one augmented liver regeneration significantly at 48 hours after subtotal PH. Proliferation was almost completely abrogated when using drugs blocking T-cell proliferation.

4.4.2.3.1 Inhibition of immune activation by calcineurin inhibitors

Calcineurin inhibitors act at an early stage after T cell receptor signalling by inhibiting calcineurin, a serine threonine phosphatase involved in the activation of transcription factors nuclear-factor-of-activated-T-cells (NFAT), nuclear factor kappa B (NF-kB) and activator protein-1 (AP-1), which induce the expression of several cytokines including IL-2 (Goddard and Adams, 2002).

As mentioned before, it has been reported by different authors that liver regeneration is impaired in the presence of IL-2 and enhanced when the IL-2 pathway is pharmacologically blocked. The underlying mechanism has not yet been revealed (Table 20). Our own observations: firstly, the inhibition of regeneration in an immune activated animal, - with supposingly elevated IL-2 levels-, and secondly, the increased proliferation rate in the CsA-treated rats, where IL-2 production is blocked, is supporting this concept.

Although these results do not contribute to elucidating the mechanism, they are nevertheless of clinical importance. If immune activation by Hepatitis B vaccination is impairing regeneration as observed and if active hepatitis would exert a similar effect on regeneration, the use of small-for-size grafts should be critically discussed in patients with viral liver disease. One recent report describes that Lamivudine, an antiviral drug reducing viral replication and thus activity of the disease as indicated by a reduction in the necroinflammatory activity (Suzuki 1999), leads to rapid regeneration of the atrophic liver in decompensated cirrhosis due to hepatitis B (Saito et al., 2002), an observation which further supports the concept.

4.4.2.3.2 Inhibition of immune activation by antiproliferative drugs

Although MMF has already replaced Azathioprine in standard triple immunosuppressive protocols used in partial liver graft recipients nowadays (Gummert et al., 1999), up to now its influence on liver regeneration was not

addressed. Besides the inhibition effect on both T and B lymphocytes proliferation, recent evidence suggested that MMF is also capable of inhibiting the proliferation of non-immune cells, such as smooth muscle cells, renal tubular cells and mesangial cells (Morath and Zeier, 2003). The antiproliferative effect is mediated via the selective, non-competitive and reversible inhibition of inosine monophosphate dehydrogenase (IMPDH) and of the type II isoform in particular. IMPDH is the rate-limiting enzyme in the de novo biosynthesis of guanosine nucleotides.

Up to now, only one group studied the effect of MMF on liver regeneration. In contrast to the results of this study, they observed an enhanced mitotic rate and a delayed, but increased peak in the TK activity in MMF-treated animals undergoing standard 70%PH. However, comparison between the two studies is hardly possible. They did not give any details regarding the kinetics of regeneration. They used a different surgical model and assessed regeneration using other parameters than in the present study. Before coming to a definite conclusion regarding the effect of MMF on hepatocyte proliferation, the time course of liver regeneration must be investigated in direct comparison to the other drugs.

RAD, also known as Sirolimus, is causing G1-phase cell cycle arrest by inhibition of the mammalian target of rapamycin, which is a downstream effector of the phosphatidylinositol 3-kinase / protein kinase B signalling pathway mediating cell survival and proliferation (Panwalkar et al., 2004). The antiproliferative effect of RAD is nowadays not only used to suppress immune activation but is also evaluated for inhibiting smooth muscle cell proliferation (McKeage et al., 2003) to prevent in-stent restenosis and, much more important, to treat malignant growth.

Some information is available on the effect of RAD on liver proliferation in vivo (Table 23). Francavilla observed the inhibitory effect of RAD not only on liver, but also on kidney as well as intestine regeneration (Francavilla et al., 1992). This observation was confirmed by Chavez in 1999, who stated that the hepatotrophic effect of cyclosporine and FK506 was not mimicked by RAD (Chavez et al., 1999). Jiang identified which signal transduction pathway was disrupted by RAD-treatment explaining the inhibition of liver regeneration in a rat model of 70%PH (Jiang et al., 2001).

Table 23. Effect of RAD on liver regeneration

Author/ Publish year	Model	Effect on liver regeneration	Mechanism
Francavilla et al., 1992	70%PH	Antihepatotrophic	Not mentioned
Chavez et al., 1999	70%PH	Reduced regeneration	Not mentioned
Jiang et al., 2001	70%PH	Attenuation of regeneration	Growth-suppressive effect due to dephosphorylation of p70 S6 kinase and/or 4E-BP1

RAD acts directly on the proliferative capacity of the cells by inhibiting the cell cycle. The observed inhibition of liver regeneration might well be a direct effect of this drug via the above mentioned pathway. It seems that this effect is of much more biological relevance than the hypothesized liver growth promoting effect via the inhibition of T cell proliferation, even more as the key step in promoting or impairing regeneration by inhibition of immune activation seems to be related to the absence or presence of liver growth promoting cytokines.

The clinical recommendation resulting from these studies is to restrict the use of both antiproliferative drugs when using small-for-size grafts, but to promote its use, especially of RAD, in cancer patients undergoing full size liver transplantation. In this situation a growth inhibitory effect would be rather beneficial for the patient.

In summary, inhibition of immune activation by calcineurin inhibitors enhanced liver regeneration, possibly through the suppressed production of IL-2. The antiproliferative drugs inhibited T cell activation effectively, but seemingly also exerted a direct effect on proliferation of parenchymal cells, which explains the observed inhibitory effect on liver regeneration.

5 SUMMARY

Introduction: Using a partial liver graft obtained from a living donor takes advantages of the unique capability of the liver to regenerate. This study aims to validate the immunohistochemical (IHC) assay to quantify liver regeneration, to investigate the kinetics of regeneration after transplantation and to study the negative feedback mechanism between immune activation and liver regeneration.

Materials and Methods: Lewis rats were used as donors for the 30% partial liver grafts (POLT) and as recipients, as well as for 70% and 90% liver resection (PH). Kinetics of regeneration was compared after 70%PH and 30%POLT. The influence of prolonged ischemia was assessed by comparing the hepatocyte proliferation rate in liver grafts subjected to either 1h, 3h or 5h of cold ischemia. The influence of immune activation on liver regeneration was studied by subjecting the animals to hepatitis B vaccination prior 70%PH in comparison to the untreated hepatectomized rats. The effect of inhibition of immune activation was investigated by using either drugs suppressing IL-2 production (Calcineurin inhibitors CsA and FK506) or inhibiting T-cell proliferation (MMF and RAD). BrdU 50mg/kg was injected intravenously 1h prior to sacrifice. Paraffin embedded liver samples were used for IHC detection of the incorporated halogenated nucleotide.

Results: Comparison between the conventional and computer-assisted quantification of the hepatocyte nuclei revealed a strong correlation between the two methods (r=0.868). Intra- and Inter-assay variation were low (CV<10%), demonstrating the high precision of the assay. Regeneration following 70%PH peaked at day 1 postoperatively. Transplantation delayed the onset of liver regeneration by 24 hours. Prolonged ischemia reduced the maximal LI. Immune activation prior to 70%PH did not alter the kinetics of regeneration, but reduced the maximal proliferation rate, possibly related to the increased IL-2 secretion in response to vaccination. Inhibition of IL-2 production by calcineurin inhibitor tended to increase the proliferation rate, whereas the use of antiproliferative drugs blocked liver regeneration almost completely.

Conclusions: Transplantation as well as prolonged ischemia postponed and impaired regeneration. The hypothesis regarding the immunological regulation of liver regeneration was supported, as immune activation inhibited regeneration and inhibition of IL-2 production by CsA enhanced the regeneration rate.

6 REFERENCE LIST

1. Abbasoglu, O., Berker, M., Ayhan, A., Palaoglu, S., Sayek, I. (1995): The effect of the pineal gland on liver regeneration in rats. J. Hepatol. 23, 578-581.

2. Akbar, S.M., Abe, M., Masumoto, T., Horiike, N., Onji, M. (1999): Mechanism of action of vaccine therapy in murine hepatitis B virus carriers: vaccine-induced activation of antigen presenting dendritic cells. J. Hepatol. 30, 755-764.

3. Andiran, F., Ayhan, A., Tanyel, F.C., Abbasoglu, O., Sayek, I. (2000): Regenerative capacities of normal and cirrhotic livers following 70% hepatectomy in rats and the effect of alpha-tocopherol on cirrhotic regeneration. J. Surg. Res. 89, 184-188.

4. Assy, N., Gong, Y., Zhang, M., Pettigrew, N.M., Pashniak, D., Minuk, G.Y. (1998): Use of proliferating cell nuclear antigen as a marker of liver regeneration after partial hepatectomy in rats. J. Lab Clin. Med. 131, 251-256.

5. Assy, N., Minuk, G.Y. (1997): Liver regeneration: methods for monitoring and their applications. J. Hepatol. 26, 945-952.

6. Austin, E.B., Smith, L.C., Walker, R.Y. (2001): An anti-idiotopic antibody-based enzyme-linked immunosorbent assay for the quantification of the monoclonal anti-D BRAD-5. Vox Sang. 80, 179-183.

7. Baak, J.P. (1990): Mitosis counting in tumors. Hum. Pathol. 21, 683-685.

8. Baratta, B., Rizzoli, R., Galliani, I., Vitale, M., Rizzi, E., Matteucci, A., Galanzi, A., Zamai, L., Mazzotti, G. (1996): Early events of liver regeneration in rats: a multiparametric analysis. Histochem. Cell Biol. 105, 61-69.

9. Bendahan, J., Tyler, M., Lotz, Z., McLeod, H., Engelbrecht, G.H., Kahn, D., Hickman, R. (1994): The effect of administration of FK506 on delayed

regeneration in flushed partially hepatectomized livers. Transplantation 57, 655-658.

10. Bolitho, D.G., Engelbrecht, G.H., Lotz, Z., Tyler, M., McLeod, H., Hickman, R. (1995): Regeneration after in situ flushing of partially hepatectomised rat livers. S. Afr. J. Surg. 33, 78-81.

11. Brazelton, T.R., Morris, R.E. (1996): Molecular mechanisms of action of new xenobiotic immunosuppressive drugs: tacrolimus (FK506), sirolimus (rapamycin), mycophenolate mofetil and leflunomide. Curr. Opin. Immunol. 8, 710-720.

12. Broelsch, C.E., Whitington, P.F., Emond, J.C., Heffron, T.G., Thistlethwaite, J.R., Stevens, L., Piper, J., Whitington, S.H., Lichtor, J.L. (1991): Liver transplantation in children from living related donors. Surgical techniques and results. Ann. Surg. 214, 428-437.

13. Broering, D.C., Sterneck, M., Rogiers, X. (2003): Living donor liver transplantation. J. Hepatol. 38 Suppl 1, S119-S135.

14. Caldwell-Kenkel, J.C., Currin, R.T., Tanaka, Y., Thurman, R.G., Lemasters, J.J. (1989): Reperfusion injury to endothelial cells following cold ischemic storage of rat livers. Hepatology 10, 292-299.

15. Chavez, R., Jamieson, N., Takamori, S., Nivatvongs, S., Pino, G., Metcalfe, A., Watson, C., Romero, D., Metcalfe, S. (1999): Hepatotrophic effect of cyclosporine and FK 506 is not mimicked by rapamycin. Transplant. Proc. 31, 2429-

16. Chen, C.L., Fan, S.T., Lee, S.G., Makuuchi, M., Tanaka, K. (2003): Living-donor liver transplantation: 12 years of experience in Asia. Transplantation 75, S6-11.

17. Clavien, P.A. (1998): Sinusoidal endothelial cell injury during hepatic preservation and reperfusion. Hepatology 28, 281-285.

18. Conzelmann, L.O., Zhong, Z., Bunzendahl, H., Wheeler, M.D., Lemasters, J.J. (2003): Reduced-size liver transplantation in the mouse. Transplantation 76, 496-501.

19. Court FG, Wemyss-Holden, S.A., Dennison, A.R., Maddern, G.J. (2002): The mystery of liver regeneration. Br. J. Surg. 89, 1089-1095.

20. Cressman, D.E., Greenbaum, L.E., DeAngelis, R.A., Ciliberto, G., Furth, E.E., Poli, V., Taub, R. (1996): Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. Science 274, 1379-1383.

21. Dahmen, U., Dirsch, O. (2002): Mechanism and modulation of the regeneration-induced augmentation of the allo-immune response after living liver donation. Investigation of the interaction between regeneration and allo-immune response. DFG-grant application Da 251/3-1,

22. Dahmen, U., Gu, Y., Dirsch, O., Li, J., Polywka, S., Doebel, L., Shen, K., Broelsch, C.E. (2002): Adoptive transfer of HBV immunity by kidney transplantation and the effect of postoperative vaccination. Antiviral Res. 56, 29-37.

23. Dahmen, U., Li, J., Dirsch, O., Gu, Y.L., Polywka, S., Doebel, L., Shen, K., Broelsch, C.E. (2003): Adoptive transfer of donor-derived immunity by liver transplantation: a potential avenue to prevent hepatitis B virus reinfection. J. Viral Hepat. 10, 31-36.

24. Escribano, O., Fernandez-Moreno, M.D., Pina, M.J., Fueyo, J., Menor, C., Roman, I.D., Guijarro, L.G. (2002): Pretreatment with FK506 up-regulates insulin receptors in regenerating rat liver. Hepatology 36, 555-561.

25. Fausto, N., Laird, A.D., Webber, E.M. (1995): Liver regeneration. 2. Role of growth factors and cytokines in hepatic regeneration. FASEB J. 9, 1527-1536.

26. Francavilla, A., Barone, M., Starzl, T.E., Zeevi, A., Scotti, C., Carrieri, G., Mazzaferro, V., Prelich, J., Todo, S., Eiras, G., . (1990): FK 506 as a growth control factor. Transplant. Proc. 22, 90-92.

27. Francavilla, A., Barone, M., Todo, S., Zeng, Q., Porter, K.A., Starzl, T.E. (1989): Augmentation of rat liver regeneration by FK 506 compared with cyclosporin. Lancet 2, 1248-1249.

28. Francavilla, A., Starzl, T.E., Scotti, C., Carrieri, G., Azzarone, A., Zeng, Q.H., Porter, K.A., Schreiber, S.L. (1992): Inhibition of liver, kidney, and intestine regeneration by rapamycin. Transplantation 53, 496-498.

29. Francavilla, A., Zeng, Q., Polimeno, L., Carr, B.I., Sun, D., Porter, K.A., Van Thiel, D.H., Starzl, T.E. (1994): Small-for-size liver transplanted into larger recipient: a model of hepatic regeneration. Hepatology 19, 210-216.

30. Gao, W., Bentley, R.C., Madden, J.F., Clavien, P.A. (1998): Apoptosis of sinusoidal endothelial cells is a critical mechanism of preservation injury in rat liver transplantation. Hepatology 27, 1652-1660.

31. Goddard, S., Adams, D.H. (2002): New approaches to immunosuppression in liver transplantation. J. Gastroenterol. Hepatol. 17, 116-126.

32. Gratzner, H.G. (1982): Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. Science 218, 474-475.

33. Gu, Y.L., Dahmen, U., Li, J., Dirsch, O., Polywka, S., Broelsch, C.E. (2002): Prolonged suppression of humoral immune response after organ transplantation. Eur. Surg. Res. 34, 260-265.

34. Gummert, J.F., Ikonen, T., Morris, R.E. (1999): Newer immunosuppressive drugs: a review. J. Am. Soc. Nephrol. 10, 1366-1380.

35. Jafarzadeh, A., Shokri, F. (2003): The antibody response to HBs antigen is regulated by coordinated Th1 and Th2 cytokine production in healthy neonates. Clin. Exp. Immunol. 131, 451-456.

36. Jiang, Y.P., Ballou, L.M., Lin, R.Z. (2001): Rapamycin-insensitive regulation of 4e-BP1 in regenerating rat liver. J. Biol. Chem. 276, 10943-10951.

37. Kahn, D., Lai, H.S., Romovacek, H., Makowka, L., Van Thiel, D., Starzl, T.E. (1988): Cyclosporine A augments the regenerative response after partial hepatectomy in the rat. Transplant. Proc. 20, 850-852.

38. Kamada, N., Calne, R.Y. (1983): A surgical experience with five hundred thirty liver transplants in the rat. Surgery 93, 64-69.

39. Keating, G.M., Noble, S. (2003): Recombinant hepatitis B vaccine (Engerix-B): a review of its immunogenicity and protective efficacy against hepatitis B. Drugs 63, 1021-1051.

40. Kikuchi, N., Yamaguchi, Y., Mori, K., Takata, N., Goto, M., Makino, Y., Hamaguchi, H., Hisama, N., Ogawa, M. (1994): Liver regeneration after orthotopic reduced-size hepatic transplantation in the rat. Hepatogastroenterology 41, 170-173.

41. Kim, Y.I., Calne, R.Y., Nagasue, N. (1988a): Cyclosporin A stimulates proliferation of the liver cells after partial hepatectomy in rats. Surg. Gynecol. Obstet. 166, 317-322.

42. Kim, Y.I., Salvini, P., Auxilia, F., Calne, R.Y. (1988b): Effect of cyclosporin A on hepatocyte proliferation after partial hepatectomy in rats: comparison with standard immunosuppressive agents. Am. J. Surg. 155, 245-249.

43. Lai, H.S., Chen, W.J., Chen, K.M. (1996): Changes in T-lymphocyte subpopulations and serum lymphokine concentrations after partial hepatectomy in rats. Nutrition 12, 700-705.

44. Li, J., Dahmen, U., Dirsch, O.R., Gu, Y., Polywka, S., Fiedler, M., Doebel, L., Roggendorf, M., Broelsch, C.E. (2002): The potential role of bone marrow transplantation in augmenting donor-derived immunity to hepatitis B after rat liver transplantation. Liver Transpl. 8, 397-404.

45. Liu, L., Sakaguchi, T., Cui, X., Shirai, Y., Nishimaki, T., Hatakeyama, K. (2002): Liver regeneration enhanced by orally administered ursodesoxycholic acid is mediated by immunosuppression in partially hepatectomized rats. Am. J. Chin Med. 30, 119-126.

46. Lo, C.M., Fan, S.T., Chan, J.K., Wei, W., Lo, R.J., Lai, C.L. (1996): Minimum graft volume for successful adult-to-adult living donor liver transplantation for fulminant hepatic failure. Transplantation 62, 696-698.

47. Lyszkiewicz, M., Pajtasz-Piasecka, E. (2002): [Contribution of interleukin 2 and interleukin 12 receptors in signal transduction during cell activation of the immune system]. Postepy Hig. Med. Dosw. 56, 707-731.

48. Magavi, S.S., Macklis, J.D. (2002): Identification of newborn cells by BrdU labeling and immunocytochemistry in vivo. Methods Mol. Biol. 198:283-90., 283-290.

49. Marcos, A., Fisher, R.A., Ham, J.M., Shiffman, M.L., Sanyal, A.J., Luketic, V.A., Sterling, R.K., Posner, M.P. (1999): Right lobe living donor liver transplantation. Transplantation 68, 798-803.

50. Masson, S., Daveau, M., Hiron, M., Lyoumi, S., Lebreton, J.P., Teniere, P., Scotte, M. (1999): Differential regenerative response and expression of growth factors following hepatectomy of variable extent in rats. Liver 19, 312-317.

51. McDiarmid, S.V. (1996): Mycophenolate mofetil in liver transplantation. Clin. Transplant. 10, 140-145.

52. McKeage, K., Murdoch, D., Goa, K.L. (2003): The sirolimus-eluting stent: a review of its use in the treatment of coronary artery disease. Am. J. Cardiovasc. Drugs 3, 211-230.

53. Meijer, C., Wiezer, M.J., Diehl, A.M., Schouten, H.J., Schouten, H.J., Meijer, S., Van Rooijen, N., van Lambalgen, A.A., Dijkstra, C.D., van Leeuwen, P.A. (2000): Kupffer cell depletion by CI2MDP-liposomes alters hepatic cytokine expression and delays liver regeneration after partial hepatectomy. Liver 20, 66-77.

54. Miyagawa, M., Katsuta, O., Tsuchitani, M., Yoshikawa, K. (1997): Measurement of replicative DNA synthesis (RDS) by a 5-bromo-2'-deoxyuridine (BrdU) labeling technique for detection of hepatocyte proliferation. J. Vet. Med. Sci. 59, 45-49.

55. Morath, C., Zeier, M. (2003): Review of the antiproliferative properties of mycophenolate mofetil in non-immune cells. Int. J. Clin. Pharmacol. Ther. 41, 465-469.

56. Morii, Y., Kawano, K., Kim, Y.I., Aramaki, M., Yoshida, T., Kitano, S. (1999): Augmentative effect of cyclosporin A on rat liver regeneration: influence on hepatocyte growth factor and transforming growth factor-beta(1). Eur. Surg. Res. 31, 399-405.

57. Muskhelishvili, L., Latendresse, J.R., Kodell, R.L., Henderson, E.B. (2003): Evaluation of cell proliferation in rat tissues with BrdU, PCNA, Ki-67(MIB-5) immunohistochemistry and in situ hybridization for histone mRNA. J. Histochem. Cytochem. 51, 1681-1688.

58. Nishizaki, T., Ikegami, T., Hiroshige, S., Hashimoto, K., Uchiyama, H., Yoshizumi, T., Kishikawa, K., Shimada, M., Sugimachi, K. (2001): Small graft for living donor liver transplantation. Ann. Surg. 233, 575-580.

59. O'Leary, T.J. (2001): Standardization in immunohistochemistry. Appl. Immunohistochem. Mol. Morphol. 9, 3-8.

60. Ohnishi, H., Muto, Y., Maeda, T., Hayashi, T., Nagaki, M., Yamada, T., Shimazaki, M., Yamada, Y., Sugihara, J., Moriwaki, H. (1993): Natural killer cell may impair liver regeneration in fulminant hepatic failure. Gastroenterol. Jpn. 28 Suppl 4, 40-44.

61. Okamura, N., Tsukada, K., Sakaguchi, T., Ohtake, M., Yoshida, K., Muto, T. (1992): Enhanced liver regeneration by FK 506 can be blocked by interleukin-1 alpha and interleukin-2. Transplant. Proc. 24, 413-415.

62. Ono, M., Tanaka, N., Orita, K. (1984): Activation of NK activity and auto-reactive cytotoxicity after hepatectomy. Acta Med. Okayama 38, 207-213.

63. Panwalkar, A., Verstovsek, S., Giles, F.J. (2004): Mammalian target of rapamycin inhibition as therapy for hematologic malignancies. Cancer 100, 657-666.

64. Pinto, M., Herzberg, H., Barnea, A., Shenberg, E. (1987): Effects of partial hepatectomy on the immune responses in mice. Clin. Immunol. Immunopathol. 42, 123-132.

65. Provencher, S.J., Gascon-Barre, M. (2002): Effect of cyclosporine a on hepatic compensatory growth: role of calcium status. J. Pharmacol. Exp. Ther. 303, 58-65.

66. Renz, J.F., Yersiz, H., Farmer, D.G., Hisatake, G.M., Ghobrial, R.M., Busuttil, R.W. (2003): Changing faces of liver transplantation: partial-liver grafts for adults. J. Hepatobiliary. Pancreat. Surg. 10, 31-44.

67. Ruiter, D.J., Ferrier, C.M., van Muijen, G.N., Henzen-Logmans, S.C., Kennedy, S., Kramer, M.D., Nielsen, B.S., Schmitt, M. (1998): Quality control of immunohistochemical evaluation of tumour-associated plasminogen activators and related components. European BIOMED-1 Concerted Action on Clinical Relevance of Proteases in Tumour Invasion and Metastasis. Eur. J. Cancer 34, 1334-1340.

68. Saito, T., Shinzawa, H., Watanabe, H., Sugahara, K., Okumoto, K., Togashi, H., Kawata, S. (2002): Lamivudine and rapid regeneration of the atrophic liver in decompensated cirrhosis due to hepatitis B. Am. J. Gastroenterol. 97, 493-495.

69. Sakamoto, T., Saizawa, T., Mabuchi, A., Norose, Y., Shoji, T., Yokomuro, K. (1992): The liver as a potential hematolymphoid organ examined from modifications occurring in the systemic and intrahepatic hematolymphoid system during liver regeneration after partial hepatectomy. Reg Immunol. 4, 1-11.

70. Sato, Y., Farges, O., Buffello, D., Bismuth, H. (1999): Intra- and extrahepatic leukocytes and cytokine mRNA expression during liver regeneration after partial hepatectomy in rats. Dig. Dis. Sci. 44, 806-816.

71. Sato, Y., Tsukada, K., Iiai, T., Ohmori, K., Yoshida, K., Muto, T., Watanabe, H., Matsumoto, Y., Abo, T. (1993a): Activation of extrathymic T cells in the liver during liver regeneration following partial hepatectomy. Immunology 78, 86-91.

72. Sato, Y., Tsukada, K., Matsumoto, Y., Abo, T. (1993b): Interferon-gamma inhibits liver regeneration by stimulating major histocompatibility complex class II antigen expression by regenerating liver. Hepatology 18, 340-346.

73. Sato, Y., Tsukada, K., Yoshida, K., Muto, T., Matumoto, Y. (1992): FK 506 suppresses class II antigen expression in regenerating livers following partial hepatectomy in the rat. Transplant. Proc. 24, 1628-1630.

74. Schirmbeck, R., Melber, K., Mertens, T., Reimann, J. (1994): Antibody and cytotoxic T-cell responses to soluble hepatitis B virus (HBV) S antigen in mice: implication for the pathogenesis of HBV-induced hepatitis. J. Virol. 68, 1418-1425.

75. Selzner, M., Clavien, P.A. (2000): Failure of regeneration of the steatotic rat liver: disruption at two different levels in the regeneration pathway. Hepatology 31, 35-42.

76. Selzner, N., Selzner, M., Odermatt, B., Tian, Y., Van Rooijen, N., Clavien, P.A. (2003): ICAM-1 triggers liver regeneration through leukocyte recruitment and Kupffer cell-dependent release of TNF-alpha/IL-6 in mice. Gastroenterology 124, 692-700.

77. Selzner, N., Selzner, M., Tian, Y., Kadry, Z., Clavien, P.A. (2002): Cold ischemia decreases liver regeneration after partial liver transplantation in the rat: A TNF-alpha/IL-6-dependent mechanism. Hepatology 36, 812-818.

78. Shiffman, M.L., Brown, R.S., Jr., Olthoff, K.M., Everson, G., Miller, C., Siegler, M., Hoofnagle, J.H. (2002): Living donor liver transplantation: summary of a conference at The National Institutes of Health. Liver Transpl. 8, 174-188.

79. Smith, B. (1969): Segmental liver transplantation from a living donor. J. Pediatr. Surg. 4, 126-132.

80. Soames, A.R., Lavender, D., Foster, J.R., Williams, S.M., Wheeldon, E.B. (1994): Image analysis of bromodeoxyuridine (BrdU) staining for measurement of S-phase in rat and mouse liver. J. Histochem. Cytochem. 42, 939-944.

81. Steer, C.J. (1995): Liver regeneration. FASEB J. 9, 1396-1400.

82. Takata, N., Yamaguchi, Y., Goto, M., Makino, Y., Kikuchi, N., Hamaguchi, H., Hisama, N., Otsuka, Y., Mori, K., Ogawa, M. (1993): Hepatocyte regeneration during liver allograft rejection in the rat. Transplant. Proc. 25, 1975-1977.

83. Tanaka, K. (2003): Progress and future in living donor liver transplantation. Keio J. Med. 52, 73-79.

84. Tanaka, N., Tatemoto, A., Urabe, T., Ono, M., Hizuta, A., Naomoto, Y., Gotoh, K., Moreira, L.F., Orita, K. (1993a): Inhibition of liver regeneration in mice following extended hepatectomy by transfusion of lymphokine activated killer cells. Acta Med. Okayama 47, 21-28.

85. Tanaka, N., Yamamoto, H., Tatemoto, A., Urabe, T., Orita, K. (1993b): Regulation of liver regeneration by interleukin-2 and its inhibitors: cyclosporine A and FK 506. Int. J. Immunopharmacol. 15, 211-218.

86. Tarao, K., Shimizu, A., Ohkawa, S., Harada, M., Ito, Y., Tamai, S., Kuni, Y., Nagaoka, T., Hoshino, H. (1991): Increased uptake of bromodeoxyuridine by hepatocytes from early stage of primary biliary cirrhosis. Gastroenterology 100, 725-730.

87. Thurman, R.G., Marzi, I., Seitz, G., Thies, J., Lemasters, J.J., Zimmerman, F. (1988): Hepatic reperfusion injury following orthotopic liver transplantation in the rat. Transplantation 46, 502-506.

88. Trinchieri, G. (1989): Biology of natural killer cells. Adv. Immunol. 47, 187-376.

89. Uchiyama, H., Yanaga, K., Nishizaki, T., Soejima, Y., Yoshizumi, T., Sugimachi, K. (1999): Effects of deletion variant of hepatocyte growth factor on reduced-size liver transplantation in rats. Transplantation 68, 39-44.

90. Vingerhoets, J., Vanham, G., Kestens, L., Penne, G., Leroux-Roels, G., Gigase, P. (1994): Deficient T-cell responses in non-responders to hepatitis B vaccination: absence of TH1 cytokine production. Immunol. Lett. 39, 163-168.

91. Vujanovic, N.L., Polimeno, L., Azzarone, A., Francavilla, A., Chambers, W.H., Starzl, T.E., Herberman, R.B., Whiteside, T.L. (1995): Changes of liver-resident NK cells during liver regeneration in rats. J. Immunol. 154, 6324-6338.

92. Wadamori, K., Oka, M., Tokuda, N., Fujikura, Y., Hazama, S., Fukumoto, T., Suzuki, T. (1996): Influence of continuous interleukin-2 administration via the

portal vein on liver regeneration following partial hepatectomy in rats. Hepatology 23, 1578-1583.

93. Watson, C.J., Friend, P.J., Jamieson, N.V., Frick, T.W., Alexander, G., Gimson, A.E., Calne, R. (1999): Sirolimus: a potent new immunosuppressant for liver transplantation. Transplantation 67, 505-509.

94. Wild, J., Grusby, M.J., Schirmbeck, R., Reimann, J. (1999): Priming MHC-I-restricted cytotoxic T lymphocyte responses to exogenous hepatitis B surface antigen is CD4+ T cell dependent. J. Immunol. 163, 1880-1887.

95. Yamada, Y., Kirillova, I., Peschon, J.J., Fausto, N. (1997): Initiation of liver growth by tumor necrosis factor: deficient liver regeneration in mice lacking type I tumor necrosis factor receptor. Proc. Natl. Acad. Sci. U. S. A 94, 1441-1446.

96. Yamaguchi, Y., Kikuchi, N., Miyanari, N., Ichiguchi, O., Goto, M., Mori, K., Ogawa, M. (1996): Technique for orthotopic reduced-size hepatic transplantation combined with ex vivo liver cut down in the rat. Dig. Dis. Sci. 41, 1713-1721.

7 ABBREVIATIONS

AP-1 – activator protein-1

BrdU – bromodeoxyuridine

CL – caudate lobe

CsA – cyclosporin A

CTL – cytotoxic T lymphocytes

CV – coefficient of variance

ELISA – enzyme-linked immunosorbent assay

GRWR – graft-to-recipient weight ratio

GSLV – graft-to-recipient standard liver volume ratio

HCC – hepatocellular carcinoma

IFN-gamma – interferon-gamma

IHC – immunohistochemistry

IMPDH – inosine monophosphate dehydrogenase

IL-2 – interleukin-2

IL-6 – interleukin-6

LAK – lymphokine-activated killer cells

LI – labeling index

LEW – Lewis

LDLT – living donor liver transplantation

MHC – major histocompatibility complex

MMF – Mycophenolate mofetil

NAPOLT – non-arterialized partial orthotopic liver transplantation

NFAT– nuclear-factor-of-activated-T-cells

NK cells – natural killer cells

NF-kB – nuclear factor kappa B

NKT cells – natural killer T cells

NOR – nucleolar organizer region

ODC – ornithine decarboxylase

PCNA – proliferating cell nuclear antigen

PH – partial hepatectomy

POD – postoperative day

RAD – Sirolimus

RLI – inferior portion of the right lateral lobe

RLS – superior portion of the right lateral lobe

TK – thymidine kinase

TNF-alpha –-tumor necrosis factor alpha

UW – University of Wisconsin

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9 PUBLICATIONS

1. He Q, Zeng QY, Mai WY. Clinical effects of treating unstable angina petoris with low molecular weight heparin and aspirin. New Chinese Medicine, 1998, 29 (suppl 2): 18
2. Li YJ, He Q, Zeng QY, et al. Antihypertensive effect and the changes of relevant humoral factors in hypertension treated with Benazepril. Chinese Journal of Hypertension, 1998, 6(3): 196
3. Mai WY, He Q, Zeng QY, et al. Clinical effects of treating unstable angina petoris with batroxobin and it's effect on platelet aggregation. Journal of Clinical Cardiology, 1999, 15(1): 9
4. Zeng QY, Mai WY, He Q, et al. Treatment of acute myocardial infarction accompanied by arrhythmia with dosage amiodarone. New Chinese Medicine, 1999, 30(2): 75
5. Zeng QY, Mai WY, He Q, et al. Losartan in the treatment of mild to moderate essential hypertension. New Chinese Medicine, 1999, 30(6): 317
6. Mai WY, He Q, Zeng QY, et al. Effects of Cilazapril on plasma postacycline and endothelin in patients with essential hypertension. International Journal of Cardiovascular Medicine,1999, 1(2): 35
7. Zeng QY, Mai WY, He Q, et al. Study on the efficacy and safety of two doses of simvastatin in treating patients with dyslipidemia. New Chinese Medicine, 1999, 30(11): 631
8. Li YJ, He Q, Zeng QY, et al. Clinical observation of benazepril for antihypertensive effect trough to peak ratio. U.S. Chinese Journal of Angiocardiopathy (UCJA), 2000, 2(4): 232
9. He Q, Zeng QY, Mai WY, et al. Clinical observation of valsartan in the treatment of mild to moderate essential hypertension. Foreign Medical Sciences (Fascicle of cardiovascular disease), 2000, 27(6): 353
10. Li YJ, He Q, Zeng QY, et al. Study of platelet aggregations in patients with coronary heart disease and the effect of ticlopidine treatment. Chinese Journal of Pathophysiology, 2000, 16(8): 702
11. Mai WY, He Q, Zeng QY, et al. Clinical study on effects of trimetazidine on improving heart function in patients with ischemic cardiomyopathy. Journal of Clinical Cardiology, 2000, 16(8): 382

12. Mai WY, He Q, Zeng QY, et al. Effects of two calcium channel blockers on heart rate variability in essential hypertension patients with left ventricular hypertrophy. New Chinese Medicine, 2000, 31(9): 530
13. Zeng QY, Mai WY, He Q, et al. Study on the efficacy and safety of two doses of Fluvastatin in treating patients with coronary diseases accompanied by dyslipidemia. Chinese Journal of Medicine, 2000, 35(9): 42
14. Zeng QY, Mai WY, He Q, et al. Clinical study on treating mild to moderate essential hypertension with two AT_1 antagonists. Chinese Journal of Medicine Guide,2000,2(5): 11
15. Zeng QY, He Q, Mai WY, et al. Clinical study on treating mild to moderate essential hypertension with valsartan. New Chinese Medicine, 2000, 31(12): 716
16. Zeng QY, Mai WY, He Q, et al. Clinical study on treatment of mild to moderate essential hypertension with two AT_1 antagonists. The Chinese Journal of Clinical Pharmacology, 2001, 17(3): 182
17. Zeng QY, Mai WY, He Q, et al. Effect of treatment and follow-up in patients with dyslipidemia. Chinese Journal of Medicine Guide, 2001, 3(4): 239
18. He Q, Zeng QY, Mai WY, et al. Effects of calcitonin gene related peptide on the Monitoring of essential hypertension. Academic Journal of Sun Yat-sen University of Medical Sciences, 2001, 22(4): 280
19. He Q, Dirsch O, Madrahimov N, Kleinert R, Ji Y, Broelsch CE, Dahmen U. Restoration of glycogen after 70% and 90% hepatectomy. Tag der Forschung, Universitäts-Klinikum Essen, 18. July 2003.

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