Liver regeneration after portal vein embolization using absorbable and permanent embolization materials in a rabbit model

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Abstract

Background: PVE is used to increase future remnant liver volume preoperatively. Application of temporary, absorbable embolization materials could be advantageous in some situations, provided sufficient hypertrophy is achieved of the non-embolized lobe. The aim of this study was to compare the safety and hypertrophy response following portal vein embolization (PVE) using two absorbable and three permanent embolization materials.

Methods: Six groups of rabbits (n=5) underwent PVE of 80% of the total liver volume using saline (sham), gelatin sponge, fibrin glue, polyvinyl alcohol particles with coils (PVAc), nbutylcyanoacrylate (nBCA), or polidocanol. The rabbits were sacrificed after 7 days. Portography, CT volumetry, Doppler ultrasound, laboratory liver function and damage parameters, (non-embolized) liver–to–body weight ratio, immunohistochemistry, and cytokine and growth factor tissue levels were assessed to examine the differences in the liver regeneration response.

Results: Polidocanol was discontinued because of toxic reactions in 3 rabbits. Gelatin sponge was the only material that was absorbed within 7 days and resulted in less hypertrophy of the non-embolized lobe compared to the other 3 materials. There were no significant differences in hypertrophy response between the other 3 embolization groups. CT volumetry data were supported by liver-to-body weight ratio and the amount of proliferating hepatocytes. The volume gain of the non-embolized lobe was proportional to the volume loss of the embolized liver lobes. The number of Kupffer cells in the embolized liver lobe was significantly higher in the fibrin glue, PVAc, and nBCA groups compared to the sham and gelatin sponge group. However, the levels of IL-6, TNF-α, HGF, and TGF-β1 were significantly lower.

Conclusions: Temporary occlusion using gelatin sponge for PVE resulted in significantly less hypertrophy response compared to the use of permanent embolization materials. Except for polidocanol, none of the embolization materials exhibited evident hepatotoxicity.
Introduction

Portal vein embolization (PVE) is a widely used method to increase the future remnant liver (FRL) before major liver resection.\textsuperscript{1} This is necessary when the amount of FRL is considered too small, thereby increasing the risk of postoperative liver failure.\textsuperscript{2} With PVE the portal vein branches of the to-be-resected liver lobe are occluded, causing atrophy of this liver lobe. This results in a release of regenerative factors that induces a compensatory hypertrophy response in the FRL.\textsuperscript{3-5}

There are 2 main methods to occlude the portal vein: by portal vein ligation (PVL) or by embolization. In a previous study, we compared the effects of PVE and PVL in a rabbit model and concluded that PVE is superior to PVL in terms of the extent of the hypertrophy response.\textsuperscript{6} Many embolization materials are available, the majority of which causes a permanent occlusion of blood vessels. It is believed that permanent occlusion of the portal vein is more effective in inducing a hypertrophy response than transient occlusion.\textsuperscript{7,8} However, there are several clinically relevant drawbacks to the use of permanent embolization agents. First, there is always a risk that the embolization material migrates to contralateral portal vein branches.\textsuperscript{9,10} When the material is absorbable, occlusion of non-targeted vasculature is reversible and therefore safer. Second, in patients in whom the embolized part of the liver is ultimately not resected, occlusion of the portal vein with an absorbable material would be advantageous over a permanent material in order to preserve/regain function of this part of the liver. Third, reversible PVE for the induction of liver regeneration has potential use in living donor liver transplantation, in which the future graft in the donor could be increased without endangering residual liver function of the donor. These points underscore the potential benefit of using absorbable embolization agents for PVE.

Accordingly, there is a need to elucidate whether the hypertrophy response is dependent on the type of embolization material (permanent vs. absorbable) and to determine which material is most suitable for PVE with respect to the extent of liver regeneration and safety. A recent study on the effect of reversible PVE on liver regeneration in monkeys concluded that reversible PVE using gelatin powder efficiently induced a hypertrophy response.\textsuperscript{11} However, it is presently unclear which type of embolization material optimally induces liver regeneration. Consequently, this study investigated the extent of the hypertrophy response following PVE using 2 absorbable and 3 permanent embolization agents in a standardized rabbit model.\textsuperscript{12} In anticipation of potential clinical applicability of the embolization materials in liver surgery, the safety of the embolization agents was evaluated on the basis of post-PVE hepatocellular damage and liver function. Finally, regeneration-specific cytokines and growth factors as well as the cellular constituents responsible for their release were assayed in the atrophic and hypertrophic liver lobes.
Methods

Animals

Experimental protocols were approved by the institute’s animal ethics and welfare committee. In total 38 female New Zealand White rabbits (Harlan, Gannat, France) with a mean weight of 3,108g (range 2,800-3,450g) were acclimatized for 2 weeks under standardized laboratory conditions in a temperature-controlled room with a 12-h light/dark cycle and access to standard chow and water ad libitum.

Experimental design

Six groups of 5 rabbits were planned for PVE, each group corresponding to a different embolization material. Prior to PVE, blood samples were drawn and CT volumetry and digital subtraction portography were performed as described below. The portal blood flow in the caudal and right liver lobe was quantified by Doppler ultrasound (ProSound 3500SX, Aloka, Tokyo, Japan).

PVE was performed as described below using 2 absorbable embolization materials and 3 permanent embolization materials. With respect to the former, fibrin glue (Tissucol, Baxter Healthcare, Deerfield, IL) or gelatin sponge (Spongostan, Ferrosan, Soeborg, Denmark) was used. The gelatin sponge was completely dissolved in sterile physiological saline (Baxter) by repetitively passing the gel foam shred-containing fluid from one 1-mL syringe to another via an interposed stopcock (BD Biosciences, San Jose, CA) while gradually closing the valve in the stopcock in order to produce a viscous fluid. For the permanent materials, a combination of polyvinyl alcohol particles (90-180 μm in diameter followed by 300-500 μm in diameter, Cook, Bloomington, IN) and 3 fibered platinum coils (4.0, 5.0, and 6.0 mm, Boston Scientific, Natick, MA) (PVAc) was used, or the infusion of n-butyl cyanoacrylate (nBCA) (Histoacryl, B. Braun Medical, Melsungen, Germany) or polidocanol (Aethoxysklerol 3%, Kreussler Pharma, Wiesbaden, Germany). It should be noted that PVE with polidocanol was discontinued due to the high level of toxicity; 2 of the first 3 animals died immediately after injection of polidocanol. The control group received sterile physiological saline as placebo embolization material (sham).

Directly after PVE, digital subtraction portography was performed so as to confirm portal vein occlusion. Blood sampling, CT volumetry, and Doppler ultrasound were repeated on days 3 and 7 post-PVE. Digital subtraction portography was performed prior to sacrifice on day 7.

Additionally, 10 rabbits were added to the gelatin sponge and PVAc groups (n=5 per group) and sacrificed 24 hours after PVE in order to obtain liver tissue at the onset of the liver regeneration response.

Portal vein embolization

Animals were anesthetized by intramuscular injection of ketamine (25.0 mg/kg body weight, Nimatek, Eurovet, Bladel, the Netherlands) and medetomidine (0.2 mg/kg body weight, Dexdomitor, Orion, Espoo, Finland). Maintenance anesthesia consisted of 1-2% isoflurane
(Forene, Abbott Laboratories, Kent, UK) mixed with O2: air (0.5:0.5 L/min). Buprenorphine (0.03 mg/kg body weight, Temgesic, Reckitt Benckiser Healthcare, Hull, Great Britain) and Baytril (0.2 mg/kg body weight, Bayer Healthcare, Berlin, Germany) were administered subcutaneously prior to the operation.

The animals were placed in supine position. The eyes were protected from drying out using an eye cream (Oculentum simplex, Pharmachemie, Haarlem, the Netherlands). Heart rate and arterial oxygen saturation were measured by pulse oximetry (Hewlett Packard M1165A, model 56S, Andover, MA) on the hind leg throughout the operative procedure. After a midline laparotomy, a branch of the inferior mesenteric vein was cannulated with an 18-G catheter (Hospira Venisystems, Lake Forest, IL). A Renegade 3-Fr microcatheter (Boston Scientific) with a Transend-ex 0.36 mm × 182 cm guide wire (Boston Scientific) was subsequently introduced into the portal vein. Digital subtraction portography was performed with a mobile C-arm Exposcop 8000 (Ziehm Imaging, Nürnberg, Germany) to identify the individual portal vein branches. A schematic picture of the portal vein branches in the rabbit is shown in Figure 1A. After passing the portal branch to the caudal liver lobe, the microcatheter was positioned in the main portal branch supplying the cranial liver lobes. The portal branches were embolized by transcatheter infusion of the embolization agents. Subsequently, the catheter was flushed with sterile physiological saline or, in case of Histoacryl, with 5% glucose in order to prevent obstruction of the catheter. Following portographic confirmation of PVE, the catheter was removed and the mesenteric vein was closed with a ligature. The abdomen was closed in two layers. Baytril (0.02 mg/kg body weight) was administered subcutaneously once a day up to postoperative day 4.

CT volumetry

A multiphasic CT scan was performed with a 64-slice CT scanner (Brilliance 64, Philips, Eindhoven, the Netherlands) on anesthetized animals placed in supine position. After a baseline series, contrast solution (3 mL Visipaque, GE Healthcare, Waukesha, WI) was injected through a 22-G venflon catheter in the ear vein followed by a flush with 4 mL sterile physiological saline. A contrast-enhanced scan was performed at 15 s (arterial phase), 30 s (portal phase), and 45 s (venous phase) after injection of contrast solution. 3-D reconstructions of the liver were composed by superimposing sequential reconstructed 2-mm axial slices. The total liver and the caudal liver lobe were manually delineated and the total liver volume (TLV) and caudal liver volume (CLV) were calculated. Before PVE, CLV was expressed as a percentage of TLV (%CLV) using the formula:

\[
%\text{CLV}_{\text{pre}-\text{PVE}} = \frac{\text{CLV}_{\text{pre}-\text{PVE}}}{\text{TLV}_{\text{pre}-\text{PVE}}} \times 100\%
\]

After PVE, the CLV was calculated by:

\[
%\text{CLV}_{\text{post}-\text{PVE}} = \frac{\text{CLV}_{\text{post}-\text{PVE}}}{\text{TLV}_{\text{pre}-\text{PVE}}} \times 100\%
\]
The increase of the CLV was calculated by:

$$\text{Increase CLV} = \frac{\text{CLV}_{\text{post-PVE}} - \text{CLV}_{\text{pre-PVE}}}{\text{CLV}_{\text{pre-PVE}}} \times 100\%$$

The degree of hypertrophy\(^{13}\) at designated time points was calculated by:

$$\text{Degree of Hypertrophy} = \%\text{CLV}_{\text{post-PVE}} - \%\text{CLV}_{\text{pre-PVE}}$$

The decrease of the atrophic liver volume (ALV), i.e., the cranial liver lobes, was calculated by:

$$\text{Degree of Atrophy} = \%\text{ALV}_{\text{post-PVE}} - \%\text{ALV}_{\text{pre-PVE}}$$

Liver to body weight index
After sacrifice the liver was weighed using a precision scale (Sartorius, Göttingen, Germany). The liver weight was divided by the body weight to correct for influences of body weight.

Wet-to-dry weight ratio
After sacrifice liver biopsies of the caudal and left lateral lobes were weighed (wet weight) and subsequently stored in a stove at 60°C. After 4 weeks, the specimens were weighed again (dry weight). The percentage of water was calculated by the formula: \((\text{wet weight} - \text{dry weight}) \times 100 / \text{wet weight}\).

Liver damage and function
Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined as well-established liver damage parameters. Prothrombin time and albumin were used as indirect parameters of liver synthesis function, whereas plasma bilirubin was used as an indirect measure of hepatic uptake and excretory function. All parameters were determined by routine clinical chemistry.

Histological examination
Liver tissue samples from an embolized (left lateral) and the non-embolized (caudal) liver lobe were fixed in buffered formalin, dehydrated in graded steps of ethanol and xylene, embedded in paraffin, and cut in 5-μm sections. The histological specimens were stained with hematoxylin and eosin (H&E). All H&E slides were scored by an experienced pathologist in a blinded fashion for necrosis, inflammation, atrophy/sinusoidal dilatation, and edema using an ordinal scale: grade 0, none; grade 1, mild; grade 2, moderate; grade 3, severe.

In addition, sections were immunostained with diaminobenzidine (DAB)-conjugated anti-Ki-67 antibodies (monoclonal mouse anti-rat Ki-67 antigen, clone MIB-5, Dako Cytomation, Glostrup, Denmark) and with DAB-conjugated antibodies against macrophages (monoclonal mouse anti-rabbit macrophage, clone RAM11, Dako Cytomation) according to the manufacturer’s instructions. The immunostained sections were counterstained with
hematoxylin. Ki-67- and hematoxylin-positive cells were quantified in 10 fields of view per section (20× magnifications) using ImageJ software (Ki-67 plugin, NIH, Bethesda, MD). The proliferation index was defined as the percentage of Ki-67-positive hepatocytes per total hepatocytes in the field of view. The pixels in a field of view occupied by macrophages (RAM11-positive pixels) was determined by ImageJ software and expressed as a percentage of the total amount of pixels in the field of view.

Cytokines and growth factors

Several liver regeneration-specific cytokines and growth factors were quantified from liver tissue obtained from the caudal and left lateral liver lobe. The levels of interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), hepatocyte growth factor (HGF), and transforming growth factor beta 1 (TGF-β1) were measured in homogenized liver tissue using an ELISA kit for the respective antigen (USCN Life, Wuhan, China) according to the manufacturer’s instructions. Antibodies were diluted 4× in phosphate buffered saline (PBS). All samples were measured in duplicate and the concentrations were calculated from a standard curve. Protein concentrations were determined with a BCA Protein Assay kit (Pierce, Rockford, IL). Hepatic cytokine and growth factor content was normalized to protein content.

Confocal microscopy

Biopsies of the caudal and left lateral liver lobe were snap frozen in liquid nitrogen and stored at -80°C until histological processing. The sections were cryocut and equilibrated at room temperature for 30 min and fixed in ice cold acetone (-20°C) for 5 min. After drying in air, the sections were washed twice in PBS for 2 min. Subsequently, the sections were immunostained with anti-macrophage antibodies (1:500 dilution in PBS-1% bovine serum albumin (BSA), clone RAM11, Dako Cytomation) and either polyclonal goat anti-rabbit IL-6 or TNF-α antibodies (1:125 dilution in PBS-1%BSA, USCN Life) for 1 h at room temperature. The sections were washed 3× for 2 min in PBS after which the anti-macrophage and anti-cytokine primary antibodies were secondarily labeled with Cy3-conjugated donkey anti-mouse IgG (500 μg/mL, 1:50 dilution in PBS-1%BSA, Millipore, Billerica, MA) and Alexa488-conjugated chicken anti-goat IgG (H+L chains, 2 mg/mL, undiluted, Invitrogen, Carlsbad, CA), respectively, for 15 min in the dark. Control sections were incubated with the fluorophore-conjugated secondary antibody only to rule out unspecific binding and to set the background fluorescence intensity. The sections were washed 3× in PBS for 2 min, mounted (Vectashield, Vector Laboratories, Burlingame, CA), and stored in the dark at 4°C until used for confocal microscopy.

Confocal microscopy was performed with a Leica SP2 system equipped with an argon laser and OATB transmission filters (Wetzlar, Germany). Alexa488-labeled constituents were imaged at λex = 476 nm, λem = 498-552 nm and Cy3-labeled constituents were imaged at λex = 561 nm, λem = 568-627 nm. A Normanski filter set was used to generate differential interference contrast images with the 561-nm laser line.
Statistical analysis

Statistical analysis was performed with the Statistical Package for Social Sciences (SPSS, Chicago, IL). Tests were performed for equal variances (Levene’s test) and normality (Shapiro-Wilks test), in consequence to which statistical differences (p<0.05) were tested nonparametrically. Overall differences between groups were assessed by the Kruskal Wallis test. If the Kruskall Wallis indicated a significant difference between groups, separate Mann-Whitney U tests were used to compare the groups individually. In the latter we used a Bonferroni-Holm adjustment to correct for multiple testing with an adjusted alpha of 0.05 denoting the level of significance. Repeated measurements were analyzed using linear mixed model analysis based on rank-transformed data. A Mann-Whitney U test was used to analyze differences between the atrophic and caudal liver lobe. Correlation between variables was tested using the Spearman’s ρ coefficient. Repeated measurement and correlation tests were two-tailed and differences were considered significant at a p-value of <0.05. Data are expressed as mean ± SD unless otherwise stated.

Results

Portal vein occlusion, liver damage, and liver function

Digital subtraction portography performed before PVE did not reveal any notable anatomical variations in hepatic vasculature. Portography performed directly after PVE confirmed complete occlusion of the portal vein branches to the cranial liver lobes in all treatment groups (i.e., the gelatin sponge, fibrin glue, PVAc, and nBCA groups). On day 1 after PVE (animals included a posteriori), 3 out of 5 rabbits in the gelatin sponge group exhibited reperfusion of the portal vein to the cranial liver lobes, whereas this branch of the portal vein remained occluded in all PVAc animals. On day 7, the cranial segment of the portal vein had remained completely occluded in the fibrin glue, PVAc, and nBCA groups (from here onward collectively termed “long-term occluding embolization materials”). However, in the gelatin sponge group, recanalization of the portal vein was observed in all animals on day 7, leading to extensive parenchymal perfusion of the cranial liver lobes (Figure 1).

Doppler ultrasonography showed an increase in portal blood flow to the caudal liver lobe directly after PVE in all groups that had received an embolization agent, albeit the flow did not differ statistically from the control group. No portal blood flow was detected in the cranial liver lobes directly after PVE. Three and 7 days after PVE, portal blood flow in the cranial liver lobes was detected in all rabbits of the gelatin sponge group. In concordance with the portography findings, the cranial liver lobes of the fibrin glue, PVAc, and nBCA groups had remained deprived from portal blood flow (data not shown).

Serum liver transaminases and LDH showed a transient increase after PVE in the 4 treatment groups with a concentration peak on day 1. AST levels on day 1 were significantly higher in all treatment groups compared to the sham group (p≤0.016) (Figure 2). The
synthesis, uptake, and/or excretory functions of the liver, assessed by prothrombin time, albumin, and bilirubin were not significantly affected by the procedures in any of the groups (data not shown). Histopathological examination of H&E-stained liver biopsies of the left lateral and caudal liver lobes did not reveal necrotic regions in any of the groups, and no significant differences in scores were found for atrophy/sinusoidal dilatation and edema (data not shown).

Figure 1. Representative portographs acquired 7 days after PVE. A schematic picture of the rabbit liver anatomy is shown in panel A (CL=caudal liver lobe, LL=left lateral liver lobe, LM=left medial liver lobe, and RL=right liver lobe). In (B), a radiographic image is shown of the total portal tree corresponding to the liver shown in A. Portal blood flow to the embolized cranial liver lobes was almost completely restored following PVE with gelatin sponge (C). In the fibrin glue (D), PVAc (E), and nBCA (F) groups, the portal vein to the cranial liver lobes did not fill with contrast fluid, indicating that the embolized branches were still occluded. The level of embolization is indicated by white arrows (D-F).

Figure 2. Liver damage following PVE. Plasma AST, ALT, and LDH exhibited a transient increase that peaked 1 day (d1) after PVE. Only the AST levels on day 1 were significantly higher in all treatment groups compared to control (*, p≤0.016).
Liver regeneration response

CT volumetry data are presented in Table 1 for the caudal, hypertrophic liver lobe. The CLV increased significantly in the first 3 days after PVE in all 4 treatment groups and further increased from day 3 till 7 in the fibrin glue, PVAc, and nBCA groups.

The degree of hypertrophy was significantly higher in all treatment groups compared to the sham group on day 3 (p ≤ 0.016), whereas on day 7, the degree of hypertrophy was significantly higher for the long-term occluding embolization materials compared to the gelatin sponge group 7 days after PVE (*, p ≤ 0.016).

Figure 3. The degree of hypertrophy as determined by CT volumetry plotted as a function of time after PVE. The degree of hypertrophy of the caudal liver lobe is significantly higher in all treatment groups compared to the sham group on days 3 and 7 after PVE (# and *, respectively, p ≤ 0.016). The degree of hypertrophy of the fibrin glue, PVAc, and nBCA groups was significantly higher compared to the gelatin sponge group 7 days after PVE (*, p ≤ 0.016).

Table 1. CT volumetry data of the caudal liver lobe

<table>
<thead>
<tr>
<th>Group</th>
<th>Measurement (mean±SD)</th>
<th>Measurement time point</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre-PVE</td>
</tr>
<tr>
<td>Sham</td>
<td>Absolute CLV [cm³]</td>
<td>17.8 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>%CLV</td>
<td>26.3 ± 1.4</td>
</tr>
<tr>
<td>Gelatin sponge</td>
<td>Absolute CLV [cm³]</td>
<td>15.8 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>%CLV</td>
<td>25.7 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>CLV increase [%]</td>
<td>-</td>
</tr>
<tr>
<td>Fibrin glue</td>
<td>Absolute CLV [cm³]</td>
<td>17.9 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>%CLV</td>
<td>22.6 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>CLV increase [%]</td>
<td>-</td>
</tr>
<tr>
<td>PVAc</td>
<td>Absolute CLV [cm³]</td>
<td>17.3 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>%CLV</td>
<td>22.4 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>CLV increase [%]</td>
<td>-</td>
</tr>
<tr>
<td>nBCA</td>
<td>Absolute CLV [cm³]</td>
<td>17.1 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>%CLV</td>
<td>22.6 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>CLV increase [%]</td>
<td>-</td>
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</tbody>
</table>
The CT volumetry data were supported by the liver-to-body weight index of the caudal liver lobes, which was also significantly higher for the long-term occluding embolization materials compared to the gelatin sponge and sham groups on day 7 (p≤0.016). The wet-to-dry weight ratio was not different between the groups (data not shown), precluding the possibility that edema caused the volume/weight gain.

In concordance with these findings, PVE performed with fibrin glue, PVAc, and nBCA induced significantly more hepatocyte proliferation in the caudal liver lobe compared to the absorbable gelatin sponge group as assessed by Ki-67 staining on day 7 (p≤0.016) (Figure 4). Moreover, the number of proliferating hepatocytes was significantly higher on day 7 in the caudal, non-embolized liver lobe compared to the cranial, embolized liver lobe for the permanent occluding embolization materials (p<0.05).

In summary, long-term occlusion of the portal vein leads to a more profound hepatocyte proliferation in the non-embolized liver lobe and thus to a higher hypertrophy response compared to short-term occlusion.

Figure 4. Extent of liver regeneration in the caudal and left lateral lobe expressed as the percentage of Ki-67-positive hepatocytes (A). In the fibrin glue, PVAc, and nBCA groups, significantly more proliferating hepatocytes were found in the hypertrophic, caudal liver lobe compared to the atrophic, left lateral liver lobe (#, p<0.05). Significantly more proliferating hepatocytes were present in the caudal liver lobes of the fibrin glue, PVAc, and nBCA groups, compared to the gelatin sponge and sham group (*, p<0.016). Ki-67-stained sections are depicted of the caudal (B) and cranial (C) liver lobe embolized with nBCA.

Mechanistic features of the differential hypertrophy response

The hypertrophy response is believed to be triggered by the lobular atrophy induced by PVE in a proportional manner. Accordingly, correlation analysis was performed between the degree of atrophy and the degree of hypertrophy. A positive correlation was found on day 7 after PVE (Spearman’s ρ=0.65, p=0.001).
Furthermore, liver regeneration is known to be mediated by several cytokines released by activated Kupffer cells. Therefore, the amount of macrophages/Kupffer cells stained with a macrophage-specific antibody (RAM11) in liver tissue obtained on day 7 after PVE was visualized by light microscopy (Figure 5) and quantitated on the basis of the positively stained pixel fraction in the field of view (Figure 6).

**Figure 5.** H&E (top row) and stained sections with a macrophage-specific antibody (RAM11, bottom row) of serially sectioned embolized portal vein segments of the left lateral liver lobe 7 days after PVE. Perilobular and periportal inflammation was predominantly observed in the fibrin glue group, whereas extensive inflammatory infiltration into the embolization material was observed in the gelatin sponge group (p=0.032 Mann-Whitney U test). RAM11 staining in the long-term occluding embolization material groups (PVAc and nBCA) was primarily confined to the vascular lumen.

**Figure 6.** Quantification of Kupffer cells in the caudal and left lateral lobes following PVE. Histological sections were stained with a Kupffer cell-specific antibody (RAM11) and analyzed with ImageJ software for the number of ‘positive pixels.’ The area filled with macrophages was significantly greater in the atrophic, left lateral liver lobe compared to the hypertrophic caudal lobe of the fibrin glue, PVAc, and nBCA groups (#, p<0.05). The differences between the atrophic liver lobes of the fibrin glue, PVAc, and nBCA groups was significantly greater compared to the gelatin sponge and sham groups (*, p<0.025).
Part of the macrophages in the portal fields were characterized as multinucleated giant cells, positioned in direct contact with the embolization materials. The RAM11-positive area in the left lateral, atrophic liver lobe was significantly greater than in the caudal, hypertrophic liver lobe for the fibrin glue, PVAc, and nBCA groups (p=0.014, p=0.009, and p=0.004, respectively), whereas no differences in macrophage/Kupffer cell density were found in the sham and gelatin sponge groups. Similarly, the RAM11-positive area in the left lateral liver lobe was significantly greater in the fibrin glue, PVAc, and nBCA groups compared to the atrophic lobe of the sham group (p<0.025).

Next, the intrahepatic levels of regeneration-triggering cytokines (IL-6 and TNF-α) were quantitated by ELISA in liver tissue obtained 7 days after PVE. Tissue levels of IL-6 and TNF-α in the caudal liver lobe of the fibrin glue, PVAc, and nBCA groups were not significantly different from the sham and gelatin sponge groups (Figure 7A,B). Liver tissue acquired 1 day after PVE also revealed no significant differences in cytokine levels between the groups (data not shown).

Additionally, histological sections of the left lateral liver lobes were fluorescently immunostained with the antibodies used in the ELISA assays in order to assess protein expression patterns and to determine the localization of the antigens. The left lateral lobes were chosen because these contained the cells that produced the cytokines (Figure 6). The left lateral liver lobes of day 1 samples contained fewer RAM11-positive cells than liver lobes excised 7 days post-PVE. Moreover, the RAM11-positive cells exhibited very little-to-no expression of either cytokine (Figure 7C-F), whereas the RAM11-positive cells in the day 7 liver samples abundantly expressed IL-6 and TNF-α (Figure 7G-J). Incubation of liver tissue with the fluorophore-conjugated secondary antibodies confirmed that no unspecific antibody binding had occurred (Figure 7K-N).

Lastly, the intrahepatic growth factor levels were quantified by ELISA in liver tissue samples obtained at day 7. HGF, which activates DNA synthesis3, showed significantly lower levels in the caudal liver lobe of the fibrin glue, PVAc, and nBCA groups compared to the sham and gelatin sponge groups (p≤0.016, data not shown). Similarly, the levels of TGF-β1, which is important in terminating liver regeneration, were significantly lower in the caudal liver lobes embolized with the long-term occluding materials compared to the gelatin sponge group (p≤0.016, data not shown). No significant differences in growth factor levels were found between the embolization groups on day 1 after PVE.
Figure 7. Levels of IL-6 (A) and TNF-α (B) measured by ELISA in homogenized caudal liver tissue obtained 7 days after PVE, normalized to protein content. Confocal microscopy was performed on immunostained sections derived from the left lateral liver lobes on day 1 (C-F) and day 7 (G-J) after PVE. Representative images of TNF-α are shown from the gelatin sponge group. Kupffer cells were labeled with anti-macrophage antibodies secondarily labeled with Cy3-conjugated IgG, appearing red. TNF-α was labeled by antibodies raised against the respective epitope and secondarily labeled with Alexa488-conjugated IgG, appearing green. Kupffer cells (white arrowheads) expressed little TNF-α 1 day after PVE, whereas on day 7 TNF-α abundantly colocalized with Kupffer cells. Incubation with secondary antibodies only revealed no unspecific binding (K-N). Differential interference contrast (DIC) images were acquired to provide anatomical detail. CV=central vein, S=sinusoids.
Discussion

In this study the use of three permanent (PVAc, nBCA, and polidocanol) and two biodegradable (fibrin glue and gelatin sponge) embolization materials for portal vein embolization was investigated in the context of the degree of hypertrophy and material safety. For these purposes, a validated rabbit model was used in which the hypertrophy response could be studied under controlled circumstances for a period of 7 days, corresponding to the end of post-PVE liver regeneration in this species. Polidocanol produced a lethally toxic reaction in 3 rabbits. The hepatotoxic effect of polidocanol has been described previously and therefore this material seems unsuitable for PVE.

Fibrin glue, PVAc, and nBCA induced total occlusion of the portal vein branches up to 7 days after PVE, which was associated with a significantly greater hypertrophy response compared to the gradually degraded gelatin sponge. With the exception of polidocanol, the embolization agents inflicted minimal hepatocellular damage and were not found to impair liver function, confirming that the PVE materials tested in this study are appropriate for clinical application. Furthermore, the degree of hypertrophy was positively correlated with the degree of atrophy of the embolized liver lobe and was associated with increased inflammatory cell influx into the atrophic liver lobe. Neither the molecular triggers for liver regeneration, IL-6 and TNF-α, nor the proteins responsible for propagation (HGF) and termination (TGF-β1) of liver regeneration were found to be elevated in atrophic liver tissue. However, an elevated expression of cytokines was found in activated macrophages/Kupffer cells in the atrophic liver lobe.

Our study was set up according to the suggestions of Lesurtel et al. published in the Journal of Hepatology, who posited that the use of temporary embolization agents should be evaluated against permanent embolization materials and a sham group. Accordingly, the most important conclusion of this study was that permanent embolization materials induce the most prolific hypertrophy response. Although we showed that reversible PVE with gelatin also induced a hypertrophy response of the non-embolized liver lobe, as was recently demonstrated by Lainas et al. in monkeys, the hypertrophy response was significantly less compared to the permanent embolization materials.

Interestingly, fibrin glue, which is marketed as an absorbable embolization agent, was not absorbed after 7 days of PVE and yielded a hypertrophy response that was comparable to that after PVAc and nBCA. This effect is ascribable to differences in the rate of liver regeneration in rabbits versus humans. Aside from possible inter-species differences in fibrin degradation kinetics, the regeneration response is faster in rabbits compared to humans and evidently reached a plateau before the fibrin glue was degraded. In human livers the fibrin glue is typically absorbed before liver regeneration plateaus at approximately 21 days post-PVE, as a result of which the extent of hypertrophy is reduced compared to that of permanent embolization agents. Consequently, fibrin glue should be classified as a permanent embolization material in this rabbit model and the implications of results should be interpreted accordingly.
In the clinical setting, the plateau phase signifies the end of the waiting time between PVE and liver resection and therefore constitutes the most important time point. In light of the possibility of tumor growth during the time between PVE and resection,\textsuperscript{17} it is imperative to use an embolization material that induces the most profound hypertrophy response in the shortest time frame without inflicting excessive hepatocellular or systemic damage. We have shown that none of the permanent embolization materials, including fibrin glue, caused considerable hepatocellular/histological damage. Additionally, liver synthetic function, and liver uptake and excretory function were preserved. In human livers, fibrin glue is absorbed before 3 weeks, i.e., before the time the plateau phase has been reached,\textsuperscript{16} and hence comprises an inferior embolization material compared to PVAc and nBCA for clinical use. Consequently, for the purposes of post-PVE resection procedures, PVAc and nBCA should be employed to induce the most extensive hypertrophy response in a minimum amount of time.

Additionally, we assessed several growth-promoting mediators of liver regeneration in liver tissue to explain the difference in hypertrophy response. Kupffer cells and recruited, activated macrophages are known to release cytokines and growth factors that trigger and propagate liver regeneration.\textsuperscript{3,18} The amount of macrophages was significantly higher in the embolized liver lobes of the fibrin glue, PVAc, and nBCA groups. However, the groups with the highest hypertrophy response exhibited lower intrahepatic IL-6, TNF-\(\alpha\), and HGF levels compared to the sham and gelatin sponge group, despite the fact that the former groups exhibited the greatest degree of hepatocyte proliferation. Although we did not investigate these contradictory findings any further, we hypothesize that these factors were not yet released on day 1 after PVE in these groups and that these factors had been extensively depleted after 7 days. On the other hand, it might also be that these factors do not play a prominent role in mediating liver regeneration after PVE. To our knowledge, no studies have been performed shedding light on this issue.

Embolization with long-lasting or permanent occlusion materials leads to a higher hypertrophy response than a temporary occlusion material. However, the use of an absorbable embolization material still can be advocated in cases where the portal vein ultimately needs to be patent, such as in living donor liver transplantations. After PVE with gelatin sponge, the hypertrophy response will lead to more hepatic function in the part of the liver that is going to be transplanted, albeit to a lesser extent than would be the case with permanent embolization materials. The remnant donor liver will gradually regain portal blood flow before the hypertrophy plateau phase has been reached and sustains optimal functionality following the explantation procedure. However, an embolization material that is absorbed after 3 weeks at the earliest would be more ideal. PVE with such an embolization agent would result in a greater hypertrophy response with the added benefit of recovery of the portal blood flow to the embolized liver lobes before transplantation.

In conclusion, we found that the use of permanent or at least long-lasting embolization materials leads to a greater hypertrophy response of the FRL compared to an absorbable material. The clinical implication is that absorbable (gelatin-based) embolization materials
should only be used for PVE when only little liver regeneration is needed or when the portal blood flow to the embolized liver lobes should preferably be restored.
References