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PVA = polyvinyl alcohol

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Embolization of Portal Vein Branches Induces Hepatocyte Replication in Swine: A Potential Step in Hepatic Gene Therapy¹

PURPOSE: To determine whether embolization of portal vein branches would stimulate hepatocyte replication in pigs.

MATERIALS AND METHODS: The portal vein branches supplying 50%–70% of the liver were embolized in eight pigs by using a combination of coils and polyvinyl alcohol particles. The extent of embolization was assessed at portography in all animals and at computed tomography in one animal. Hepatocyte replication was determined by calculating the percentage of cells that incorporated bromodeoxyuridine into their nuclei. Animals survived up to 35 days after the procedure.

RESULTS: Embolization of the portal vein branches supplying the left and median lobes caused transient increases of less than 70% in portal vein pressures and of less than 100% in liver enzyme levels. Indocyanine green clearance was measured in two animals and decreased less than 50%. The percentage of replicating hepatocytes in the nonembolized lobe was 0% on day 0, 7% on day 2, 14% on day 7, and 2% on day 12.

CONCLUSION: Substantial hepatocyte replication occurred 2–7 days after embolization of portal vein branches. Further research will help determine if this procedure can facilitate retroviral transduction in large animals. If successful, the low morbidity of this method may allow its use in humans for gene therapy.

Gene therapy involves the transfer and expression of a gene that encodes a particular protein into the appropriate cells of a patient with an inborn error of metabolism or an acquired disease. Hepatic gene therapy might be used to treat serum protein deficiencies, such as hemophilia, or metabolic defects, such as familial hypercholesterolemia (1–3). The liver is a desirable target organ for gene therapy, because it normally supplies most serum proteins and plays an important role in numerous metabolic pathways. The large size of the liver and its abundant blood supply also make it an attractive target.

Genes can be delivered to the liver as plasmid DNA or by means of adenoviral or retroviral vectors (4,5). The viral vectors are made by replacing some or all of the viral genes with the therapeutic gene. The expression with adenoviral vectors or plasmid DNA usually is transient, due to the immunologic destruction of adenoviral-transduced cells and the instability of plasmid vectors (6,7). In contrast, retroviral vectors result in long-term expression because of their ability to integrate genes into the chromosome (8–11). Indeed, investigators have achieved stable expression of a coagulation factor gene (9) and the low-density-lipoprotein receptor (12–14) gene in animals, although the levels were insufficient to have a clinical result. We recently demonstrated that the combination of a strong liver-specific promoter and the introduction of large amounts of the retroviral vector resulted in a much higher level of expression (8,15). This approach generated therapeutic levels of a coagulation protein, factor X (8), and an anticoagulation factor, protein C, in rat gene therapy experiments. The levels have not diminished after 1 year.

Although it is now clear that retroviral vectors can result in stable and therapeutic levels of expression of genes in animals, the risks of the methods that have been used to deliver a retroviral vector to the liver are an impediment to the application of these procedures in humans. Nearly all retroviral vectors require cell division for integration (16,17). Because hepatocytes are normally quiescent (18–20), the protocols for retroviral vector-mediated gene therapy have included steps to induce hepatocyte replication. An *ex vivo* approach (21,22) involved a partial hepatectomy, isolation of hepatocytes, *in vitro* transduction of replicating cells, and introduction of the genetically modified cells into the portal vein. Hepatocyte replication *in vivo* has been induced by means of partial hepatectomy (23,24) or administration of a liver toxin (25–27). These *in vivo* protocols require that the retroviral vector be injected into the portal vein during liver regeneration. The injection must coincide with replication because there is only a short period (<6 hours) during which the gene derived from the vector can become integrated into the genome of dividing cells (16,17).

An exception is the human immunodeficiency virus, which can replicate and integrate its sequences into nondividing cells. Naldini et al (28) recently used a retroviral vector based on the human immunodeficiency virus to stably transduce nondividing cells. Although gene therapy protocols with modified human immunodeficiency virus vectors might eliminate the need to induce hepatocyte replication, there are safety concerns, including the generation of a wild-type virus.

With this in mind, we sought to develop a safe and effective method of inducing hepatocyte replication that could be used for gene therapy in humans. Ideally, the method would include a means of maintaining access to the portal vein for subsequent introduction of the retroviral vector. We have recently demonstrated (29) that surgical ligation of the portal vein branches supplying 70% of the liver induced sufficient hepatocyte replication to allow successful hepatic gene therapy with retroviral vectors in rats. These results suggested that percutaneous occlusion of portal vein branches could be a safe and effective method of inducing hepatocyte replication in larger animals.

Percutaneous embolization of portal vein branches has been performed in humans with hepatic malignancies and

has caused little morbidity (30–38). Animal studies (39–41) of portal vein occlusion have shown that the hepatocytes in the embolized segments undergo apoptosis. Hepatocyte apoptosis likely explains the minimal alterations in liver function tests found in the human and animal studies (32–36,42–44). Apoptosis also would explain why the embolized segments atrophy in humans (31,32,34–37,42). The observed hypertrophy of the nonembolized segments in these same studies almost certainly represents hepatocyte replication, but we know of no human or large animal studies in which the extent and timing of hepatocyte replication after percutaneous embolization of portal vein branches have been investigated.

The purposes of this study were (a) to investigate the technical aspects of the embolization procedure and the maintenance of a portal vein catheter in a large-animal model and (b) to measure the extent and timing of hepatocyte replication after embolization of portal vein branches. The safety of the procedure was also studied. The results in this animal model indicate that this procedure could become one step in human hepatic gene therapy protocols.

MATERIALS AND METHODS

Materials

Female domestic swine (Oak Hill Genetics, Ewing, Ill) and female minipigs (Sinclair Research Center, Columbia, Mo) were obtained. All animals weighed approximately 15 kg. The angiographic catheters, guide wires, and embolization coils were obtained from Cook (Bloomington, Ill) unless otherwise specified. Meglumine iothalamate and ioversol (Conray and Optiray, respectively; Mallinckrodt, St Louis, Mo) were used as contrast agents. Polyvinyl alcohol (PVA) particles (350–500- μ m and 500–750- μ m diameter) were obtained from Neurovena (White Bear Lake, Minn). Cacodylate buffer was obtained from Electron Microscopy Services (Fort Washington, Pa). Indocyanine green was obtained from Becton Dickinson (Cockeysville, Md). Goat anti-bromodeoxyuridine antibody was provided by an independent laboratory at the authors' institution. All other chemicals were obtained from Sigma Chemical (St Louis, Mo).

Embolization of Portal Vein Branches

The study protocol was approved by the institutional animal study commit-

tee. Animals were sedated with a mixture of telazol, ketamine, and xylazine before they were intubated and ventilated with a mixture of isoflurane, oxygen, and nitrous dioxide. Placement of a peripheral intravenous catheter was established, and a preprocedural dose of cefazolin (25 mg/kg) was administered. The skin in the right upper abdomen and flank was sterilely prepared, and, with fluoroscopic guidance, a 21-gauge needle was advanced into the liver. The needle was slowly pulled back until blood return was obtained, and contrast material was injected to determine whether the needle tip was within a portal vein or a hepatic vein branch. When the needle tip was within a portal vein branch, a 0.018-inch guide wire was advanced into the main portal vein, and the track was dilated to 5 F by using a tapered dilator set. The 0.018-inch guide wire was exchanged for a 0.038-inch Bentson guide wire, which was used to secure access while the 5-F dilator was exchanged for a 6-F vascular sheath.

Before embolization, a 5-F angiographic catheter was advanced into the main portal vein, and digital subtraction portography was performed. Portograms were routinely obtained during suspended respiration by injecting 5–10 mL of contrast material. The portal vein catheter was also used to obtain baseline blood samples.

Embolization of portal vein branches was performed by injecting a 1:1 mixture of 350–500- μ m and 500–750- μ m particles suspended in diluted contrast medium into a 5-F angled catheter whose tip was within the portal vein branches supplying the left and median lobes. Typically, 200 mg of particles was needed. Once stasis or near stasis was achieved, a series of 3–10-mm-diameter embolization coils were deployed in the portal vein branches. After embolization of 60%–70% of the liver, a catheter was positioned in the main portal vein, and a follow-up portogram was obtained.

The catheter was exchanged over a 0.038-inch guide wire for a 7- or 8-F Dawson-Mueller catheter (Cook), which was then secured in place with a polypropylene skin suture (0-Prolene; Ethicon, Somerville, NJ), flushed with 5 mL of 100 U/mL of heparin, and covered with a sterile dressing. Animals routinely received 10 mg/kg aspirin on the morning after the procedure, and the catheter was flushed daily with 5 mL of 100 U/mL heparin. Both multi-side-hole and non-side-hole Dawson-Mueller catheters were used. Blood samples were collected every 1–3 days for determination of the levels

of creatinine, serum transaminases, alkaline phosphatase, γ -glutamyl transpeptidase, and total bilirubin.

On the day of sacrifice, animals received 50 mg/kg bromodeoxyuridine (10 mg/mL solution prepared in phosphate buffered saline and filter sterilized). The animals were anesthetized approximately 3 hours later, and portography was performed by using the indwelling portal vein catheter. The animal was then euthanized, and tissue specimens were immediately collected from each lobe of the liver. The specimens were fixed or frozen (as described later) and the remainder of the liver was removed and fixed in 10% neutral buffered formalin.

Computed Tomography

Computed tomography (CT) was performed 6 hours after the embolization procedure in one animal. The animal was sedated by using telazol, ketamine, and xylazine before receiving a propofol infusion (15 mg/kg/hr) to maintain anesthesia. The animal was placed supine in the CT scanner (Siemens, Munich, Germany), and oblique axial images through the liver were obtained during injection of meglumine iohalamate into the portal vein by using the portal vein catheter. The fraction of the liver embolized was calculated by measuring the area of the enhancing and nonenhancing portions of the liver on a series of images that included the entire liver. The embolized fraction was calculated by dividing the summed areas that did not enhance by the summed area of the entire liver.

Immunohistochemistry and Cytochemistry

Hepatocyte replication was assessed after bromodeoxyuridine administration as described by Bowling et al (29). A single animal was sacrificed at each of the time points used to assess hepatocyte replication (0, 2, 3, 4, 7, and 12 days), a small wedge-biopsy specimen of the liver was immediately frozen in a cutting compound (OCT; Miles, Elkhart, Ind), and 8- μ m-thick sections were prepared by using a cryostat. The sections were mounted on slides and fixed in formalin. The slides were treated with trypsin (0.05 mg/mL in tromethamine [TRIS]-buffered saline solution [100 mmol/L sodium chloride, 50 mmol/L TRIS at pH 7.5]) for 10 minutes before being washed with TRIS-buffered saline solution. Endogenous peroxidase was blocked by incubating for 10 minutes

in 0.3% hydrogen peroxide in TRIS-buffered saline solution. The slides were treated with 2-normal hydrochloric acid with 0.3% Triton-X-100 (Sigma) for 30 minutes and were washed with water before application of a goat anti-bromodeoxyuridine antibody at a 1:500 dilution in TRIS-buffered saline solution with 0.3% Triton-X-100 and 5% dry milk. The slides were incubated at room temperature overnight with the primary antibody and then were washed with TRIS-buffered saline solution and incubated with horseradish peroxidase-coupled antigoat sheep immunoglobulin G at a 1:200 dilution in a solution of TRIS-buffered saline, 0.3% Triton-X-100, and 5% milk. After a 60 minute incubation, the slides were washed in TRIS-buffered saline solution and were developed in 3,3'-diaminobenzidine tetrahydrochloride. The slides were examined, and 20 random fields containing more than 100 hepatocytes each were analyzed for the number of brown-stained hepatocyte nuclei. The same slides were then stained with hematoxylin to identify all hepatocyte nuclei in the corresponding fields. The fraction of hepatocytes undergoing replication was calculated by dividing the number of brown-stained hepatocyte nuclei by the total number of hepatocyte nuclei.

Representative images were recorded on film (Tri-X; Eastman Kodak, Rochester, NY) and digitized by using a scanner (Sprint Scan-35; Polaroid, Cambridge, Mass). The images were composited by using a Power Macintosh 6100 computer (Apple, Cupertino, Calif) running ADOBE PHOTOSHOP (version 3.0; Adobe Systems, San Jose, Calif) and printed by using a ColorEase printer (Eastman Kodak).

Light and Electron Microscopy

Tissue wedges from each lobe of the liver and the catheter track were fixed in 10% neutral buffered formalin and were submitted to the histology laboratory at our institution for paraffin embedding and sectioning. A series of 4- μ m-thick sections were prepared, mounted on slides, and stained with either hematoxylin-eosin or Masson trichrome. Representative images were recorded on film (Royal Gold 25; Eastman Kodak) by using a microscope (model OM-10; Olympus, Tokyo, Japan) equipped with a photoport and exposure control module. These images were digitized, composited, and printed as described for the immunohistochemistry and cytochemistry specimens.

Tissue specimens from each lobe were

fixed in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate at pH 7.4 and were submitted for sectioning and staining. Representative images were recorded, digitized, composited, and printed as described for the immunohistochemistry and cytochemistry specimens.

Quantification of the Number of Cells per Lobule

The average volume of the lobule was calculated as follows: The diameter of 20 lobules was determined on a formalin-fixed, paraffin-embedded section by using a micrometer. The radius (r) was used to calculate the lobule volume by using the formula $\text{Volume} = 4/3\pi r^3$. The average lobule volume and the SD were then determined. These values were normalized to the lobule volume of a normal pig liver to give the relative lobule volume. The relative lobule volume alone could not be used to determine the number of cells per lobule, because the number of hepatocytes per area was greater in the left (embolized) lobes than in a normal lobule, and the number of cells in the right (nonembolized) lobes was less than that in a normal lobule. The number of cells per high-power field was, therefore, determined for five fields, and the average and the SD were determined. The number of cells per high-power field was then normalized to the number of cells in a normal lobule to give the relative cell density. To determine the relative number of cells per lobule, the relative lobule volume was multiplied by the relative cell density.

Indocyanine Green Clearance

Indocyanine green was prepared according to the manufacturer's instructions and was injected (1.5 or 12.5 mg/kg) into a peripheral vein in two animals. Serial blood samples (2 mL each) were collected from the main portal vein at the indicated times and were allowed to clot. Serum was prepared by means of centrifugation, and the absorbance at 800 nm (optical density 800, or OD₈₀₀) was determined. Results in a series of control samples indicated a linear relationship between indocyanine green concentration and absorbance as long as the OD₈₀₀ remained below 2.5. Serum samples from the animal that received the higher dose of indocyanine green were diluted with water to bring the OD₈₀₀ into this range. Rate constants were calculated with 0.693 divided by the half-life, and only the 2-10-minute data were used because the

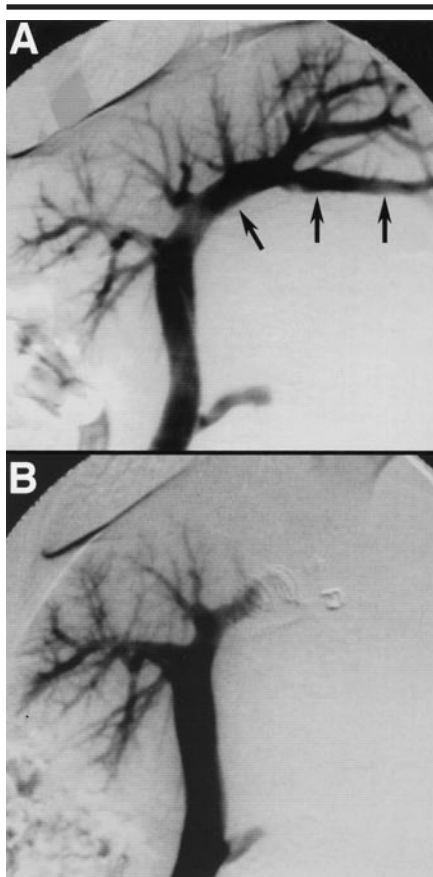


Figure 1. Animal 4. Embolization of portal vein branches. The portal vein in this minipig was accessed by means of transhepatic puncture, and a catheter was advanced into the main portal vein. *A*, Digital subtraction portogram obtained before embolization was performed shows the portal vein branches (arrows) supplying the left lobe and the majority of the median lobe. These branches were then embolized with PVA particles and appropriately sized embolization coils. *B*, Portogram obtained after embolization shows occlusion of the portal vein branches.

rapid decrease in serum indocyanine green concentration between 1 and 2 minutes likely reflected equilibration within the intravascular space.

RESULTS

Occlusion of Portal Vein Branches: Feasibility and Extent

Both domestic swine and minipigs were used in this study. Three domestic swine were used initially to assess the feasibility of the procedures. Subsequent studies were conducted with minipigs (five animals), because their smaller size better suited our plans for future long-term survival studies. Details about the animals

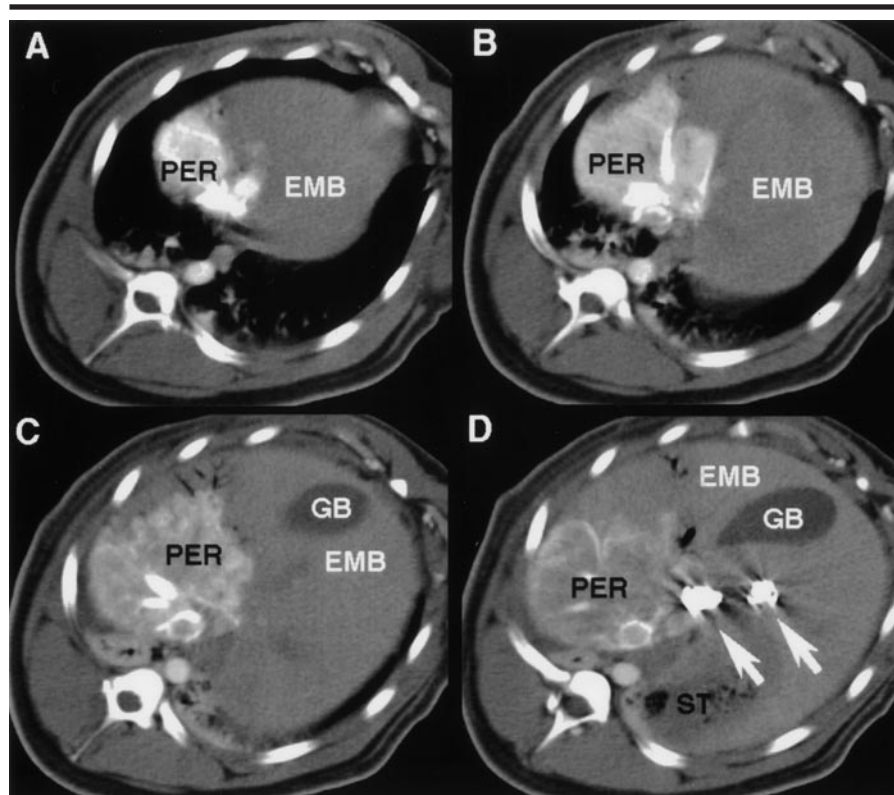


Figure 2. Animal 8. *A-D*, Contrast-enhanced CT scans of the liver in this minipig were obtained approximately 6 hours after embolization with coils (arrows in *D*) of the portal vein branches (*EMB*) supplying the median and left lobes. Contrast material was injected through the portal vein catheter. The images were obtained in an oblique axial plane to best demonstrate the enhancement difference between the right and left lobes. *GB* = gallbladder, *PER* = hepatic segments still perfused by the portal vein, *ST* = stomach.

Animals, Materials, and Selected Procedural Details

Animal No.	Type of Pig	Embolization Material [†]	Portal Venous Pressure (mm Hg)*		Embolization-Sacrifice Interval (d)
			Before Embolization	After Embolization	
1	Domestic	Coils	NR	NR	0
2	Domestic	Coils	4	4	28
3	Domestic	PVA particles and coils	NR	NR	3
4	Minipig	PVA particles and coils	6	10	7
5	Minipig	PVA particles and coils	8	12	2
6	Minipig	PVA particles and coils	9	15	4
7	Minipig	PVA particles and coils	NR	NR	12
8 [‡]	Minipig	PVA particles and coils	8	11	35

* NR = not recorded.

[†] PVA particles were a 1:1 mixture of 350–500- μ m-diameter and 500–750- μ m-diameter PVA particles.

[‡] Liver specimens were obtained in this animal by means of needle biopsy at day 0 and day 8.

and some of the procedures used in this study are summarized in the Table.

The portal vein was accessed by using a transhepatic route, and a baseline portogram was obtained. The porcine liver can be divided into right, median, and left lobes (45). The porcine portal vein courses

superomedially and contributes branches to the right and median lobes before it terminates in a series of branches to the left lobe (Fig 1, *A*). The results in the first two animals suggested that some recanalization of portal vein branches occurred if the portal vein branches were occluded

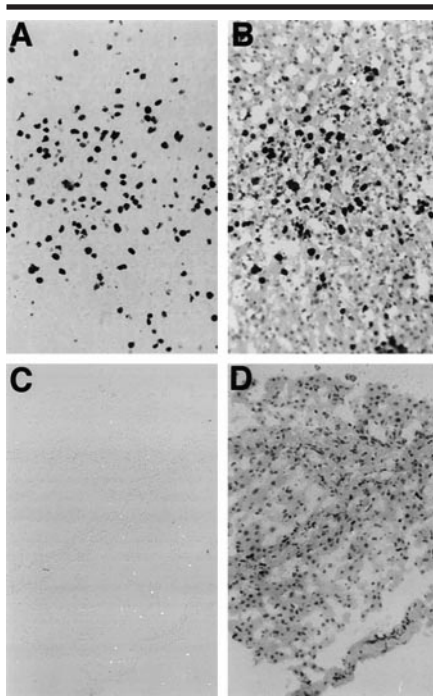


Figure 3. Photomicrographs show bromodeoxyuridine incorporation into hepatocytes after embolization. Bromodeoxyuridine was injected either 7 days after (A, B) or immediately after (C, D) embolization of the portal vein branches supplying the left and median lobes. Liver tissue from the right (nonembolized) lobe was collected 4 hours later, and bromodeoxyuridine incorporation into hepatocyte nuclei was assessed as described in Materials and Methods. A, C, No counterstain was applied, and the dark reaction product indicating DNA synthesis is seen only in the tissue obtained 7 days after the embolization procedure (A). B, D, The sections have been stained with hematoxylin to better visualize the nuclei of all the cells in the section. (Original magnification, $\times 125$.)

with embolization coils alone. The portal vein branches supplying the median and left lobes were, therefore, embolized with a combination of 350–700- μm PVA particles and embolization coils in the remaining animals. The postembolization portogram showed essentially no portal blood flow to the embolized segments when PVA particles and embolization coils were used (Fig 1, B). Portograms obtained just before sacrifice showed no recanalization of portal vein branches when PVA particles and embolization coils were used.

The extent of embolization was further assessed with CT scans in one animal. As shown in Figure 2, injection of a contrast agent into the main portal vein 6 hours after the embolization procedure resulted in substantial enhancement of the right lobe but essentially no enhancement of the left and median lobes. The CT images

were used to estimate what fraction of the liver had been embolized. In this animal, the occluded left and median lobes accounted for approximately 67% of the liver.

Portal vein pressures were measured before and after embolization in five animals (Table). Pressures were increased after embolization in four animals; however, the increase was small (less than twofold).

The animals tolerated the procedure well, with minimal untoward effects. There were no instances of portal vein catheter thrombosis. All animals resumed their normal level of activity within 4 hours after the procedure. A single animal (animal 2) developed fever and lethargy 5 days after the procedure. The white blood cell count in this animal was within normal limits, but empiric administration of antibiotics was initiated. The fever and lethargy resolved within 2 days, and the postmortem examination 23 days later showed no evidence of infection.

Portal Branch Embolization Stimulates Hepatocyte Replication

Hepatocyte replication was assessed by measuring the fraction of cells that incorporated bromodeoxyuridine. Bromodeoxyuridine is a thymidine analogue that is incorporated into newly synthesized

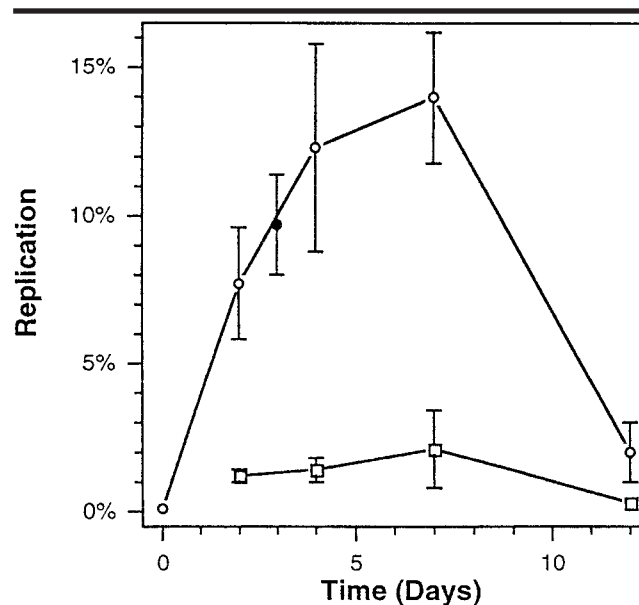


Figure 4. Graph shows the time course of hepatocyte replication. The time course of hepatocyte replication in a series of pigs was assessed as described in Materials and Methods. ○ = replication in right (nonembolized) lobe of minipigs, ● = replication in right (nonembolized) lobe of a domestic pig (animal 3), □ = replication in left (embolized) lobe, error bars = SEM for different regions of each lobe from an individual animal.

DNA (46). It was injected into the portal vein, and, approximately 4 hours later, liver tissue was collected from both the right (nonembolized) and left (embolized) lobes. Frozen sections were prepared, and bromodeoxyuridine incorporation was detected by using immunohistochemical techniques. Multiple hepatocyte nuclei contained the dark brown reaction product, which indicated DNA synthesis in the nonembolized portions of the liver at 7 days after embolization of portal vein branches (Fig 3, A). Although the dividing hepatocytes were present throughout the nonembolized lobe, they were more prevalent in the periportal regions of individual lobules, and some lobules contained a substantially greater number of dividing cells than others. As a control, bromodeoxyuridine was injected immediately before the embolization procedure in animal 8, and a needle biopsy specimen was obtained 2 hours later. This specimen showed essentially no bromodeoxyuridine incorporation (Fig 3, C). The lack of bromodeoxyuridine incorporation agrees well with results of other studies (18–20), which have shown that, under normal conditions, fewer than 0.1% of hepatocytes are replicating.

The time course of hepatocyte replication after embolization of portal vein branches was analyzed by using a series of animals. As shown in Figure 4, replication

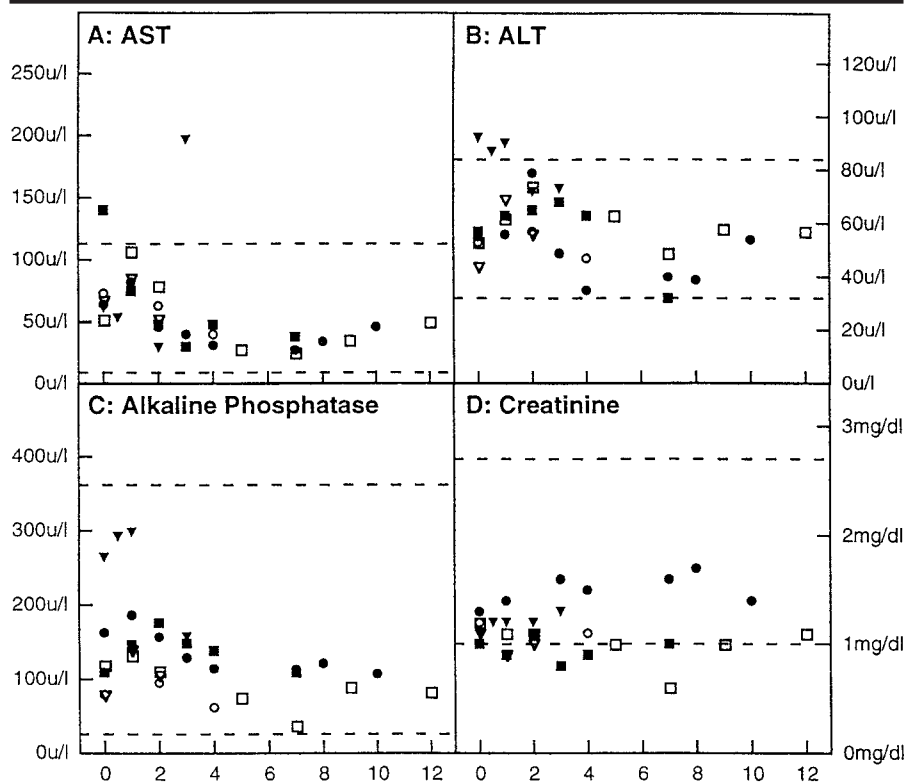


Figure 5. Graphs show laboratory values obtained before and after embolization. A series of blood samples were analyzed for evidence of hepatic or renal damage after embolization in two domestic swine (animals 2 and 3) and in four minipigs (animals 4-7). The upper and lower limits of normal levels are indicated by the dotted lines. The different animals are indicated by different symbols. *ALT* = alanine aminotransferase, *AST* = aspartate aminotransferase, *u/l* = IU/L.

in the right (nonembolized) lobe peaked at 14% approximately 1 week after embolization and decreased to low levels by 12 days. Replication also occurred in the left (embolized) lobe, albeit in lesser amounts.

Liver and Renal Function after Embolization of Portal Vein Branches

A series of blood samples were analyzed for evidence of hepatic or renal damage. As shown in Figure 5, little evidence of hepatocellular damage or cholestasis was seen after embolization. Slight elevations of liver transaminases alanine aminotransferase (Fig 5, A) and aspartate aminotransferase (Fig 5, B) or a bile canalicular marker alkaline phosphatase (Fig 5, C) were seen in some animals, but, in every case, the elevations were less than two-fold over the baseline levels. No alteration in serum creatinine level (Fig 5, D) was seen, which indicates that the procedure did not cause renal damage by means of contrast material-induced nephropathy or other mechanisms.

The plasma clearance of indocyanine

green is mediated by hepatocytes and is a validated measure of hepatic blood flow and hepatocyte function (47-49). Figure 6 demonstrates that plasma clearance decreased 16%-50% after embolization of portal branches supplying 60%-70% of the liver in two animals (animals 4 and 7).

Liver Histologic Findings after Embolization of Portal Vein Branches

The histologic features of embolized and nonembolized hepatic segments were analyzed at various times after embolization of portal vein branches. As detailed subsequently, few changes were seen in the right (nonembolized) lobe. Changes in the left (embolized) lobe included a decrease in lobule size, PVA particles occluding portal vein branches, occasional regenerative nodules, and rare apoptotic hepatocytes. Light and electron microscopy failed to demonstrate evidence of widespread coagulative (lytic) necrosis in either lobe. These results agree well with laboratory data that showed only minor increases in serum levels of hepatocellular enzymes (Fig 5).

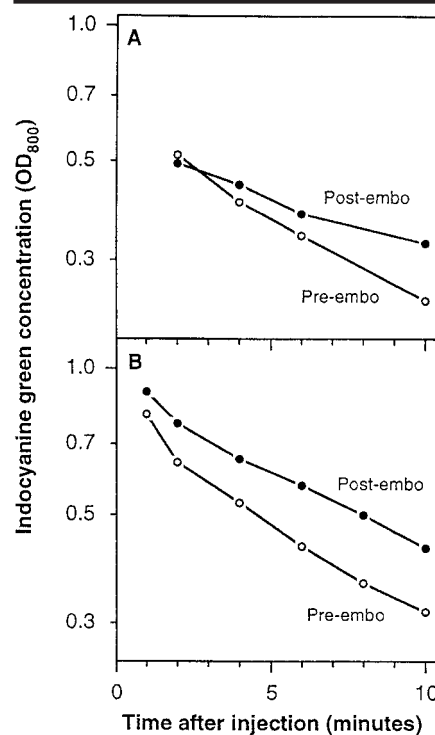


Figure 6. Graphs show indocyanine green clearance before and after embolization. The serum clearance rate of indocyanine green was measured as described in Materials and Methods. The clearance rates immediately before (*Pre-embo*) and after (*Post-embo*) embolization are shown for animal 4 (A) and animal 7 (B). ● = clearance before embolization, ○ = clearance less than 1 hour after occlusion of the portal vein branches supplying 60%-70% of the liver.

In the right lobe, the majority of hepatic lobules maintained their normal architecture (Fig 7, A, C). Although the bromodeoxyuridine incorporation data indicated that hepatocytes in these lobules were replicating (Figs 3, 4), little change was seen in lobule size (Fig 8). Together with the overall increase in the size of the right lobe, these data suggest that new lobules were added to the right lobe. A few sections from the right lobe contained PVA particles. This likely represents reflux of the particles from the left-sided portal vein branches into the right-sided branches during the latter stages of embolization. Small areas of coagulative necrosis were seen in two animals. These could result from occlusion of the portal vein branches by means of the catheter and/or embolic material coupled with catheter-related trauma to hepatic artery branches.

In the left lobe, the most striking change was hepatocyte atrophy. Lobule size decreased after embolization of por-

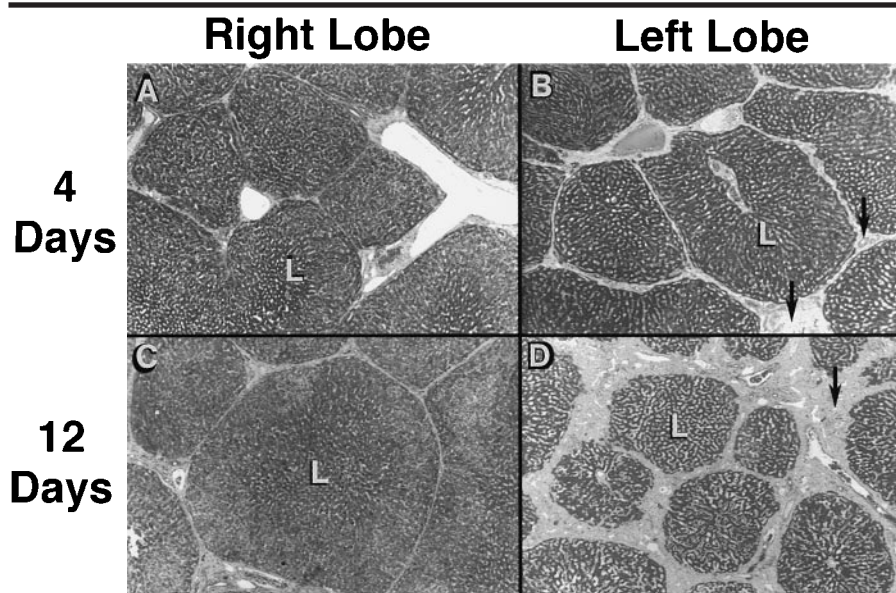


Figure 7. Photomicrographs of tissue collected at autopsy and stained with the Masson trichrome technique provide an overview of the histologic results of embolization. *A, B*, Tissue from the right lobe (*A*, nonembolized) and left lobe (*B*, embolized) obtained 4 days after embolization of the portal vein branches supplying the left and median lobes in animal 6. *C, D*, Tissue from the right lobe (*C*, nonembolized) and left lobe (*D*, embolized) obtained 12 days after embolization of the portal vein branches supplying the left and median lobes in animal 4. The hepatic lobules (*L*) are normal in the right lobe (*A* and *C*). After embolization, changes seen in the left lobe (*B* and *D*) include fibrosis (arrows) separating atrophic lobules. (Original magnification, $\times 50$.)

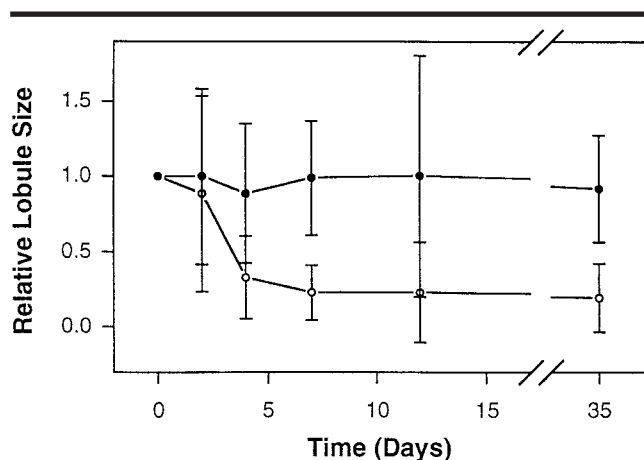


Figure 8. Graph shows relative lobule size after embolization. The relative hepatic lobule volume in lobules from the nonembolized right (\circ) and embolized left (\bullet) lobes was calculated as described in Materials and Methods.

tal vein branches (Fig 7). Quantitative analysis demonstrated that by 12 days, the relative size of the lobules in the left lobe had decreased to 23% of normal (Fig 8). Hepatocyte atrophy at the lobular level led to a similar decrease in the size of the left lobe. Light microscopy at later time points also demonstrated abundant fibrous tissue separating the lobules (Fig 7). This appearance could have resulted

from selective hepatocyte atrophy or a proliferative fibrotic response to embolization of portal vein branches.

Other notable findings in the left lobe included the presence of PVA particles and nodular regenerative hyperplasia and evidence of apoptosis. PVA particles were frequently seen within portal vein branches (Fig 9). Mild inflammatory changes surrounded these particles. The lobules adja-

cent to these particles contained viable hepatocytes. This indicates that occlusion of portal vein branches does not cause hepatocellular necrosis. Nodular regenerative hyperplasia (Fig 10) was occasionally found in the left lobe, likely reflecting hypertrophy of the hepatic cords in response to the adjacent atrophic cords. Finally, in the one animal that was sacrificed 3 days after portal branch occlusion, acidophilic bodies, consistent with apoptosis, were found. Further evidence of hepatocyte apoptosis was found at electron microscopy (Fig 11). Direct evidence of hepatocyte apoptosis was not seen in animals sacrificed at other time points.

DISCUSSION

Hepatic gene therapy with the use of retroviral vectors holds great promise for the treatment of genetic disorders, because it affords long-term expression of the missing protein. Indeed, we have previously demonstrated (8) that stable and therapeutic levels of expression of a coagulation factor can be achieved in rats after transfer of a retroviral vector into the liver (8). However, because retroviral vectors only transduce dividing cells, and hepatocytes are normally quiescent, efficient *in vivo* transfer of retroviral vectors into hepatocytes has required a partial hepatectomy to induce hepatocyte replication. This is an invasive procedure that poses some risks for patients. Our results demonstrate that percutaneous embolization of portal vein branches stimulates hepatocyte division in the nonoccluded segments, and it does so with few untoward effects.

Extent and Time Course of Hepatocyte Replication

Percutaneous embolization of portal vein branches was effective at inducing hepatocyte replication. The time course and extent of replication were determined, because the results of previous experiments (29) in rats demonstrated that the efficiency of retroviral vector transduction is directly related to the percentage of cells that are replicating at the time of infusion. The regenerative response after occlusion of portal vein branches occurred considerably later in pigs than it did in rats. In this study, porcine hepatocyte replication peaked at 14% of the cells at 7 days after 60%–70% portal vein branch occlusion, whereas in a previous study (29), rat hepatocyte rep-

lication peaked at 25%–30% at 1.5–2.0 days after 70% portal branch occlusion. This demonstrates that the regenerative response varies among species and may need to be determined empirically for different animal models.

The regenerative response after portal vein branch occlusion in pigs was later than the regenerative response after partial hepatectomy in large animals. Sigel et al (20) studied hepatocyte regeneration in dogs after a 70% partial hepatectomy by analyzing tritiated thymidine incorporation. They found that the percentage of nuclei containing the radiolabel increased to its maximum of 1.5% on day 4 and decreased to 0.6% on day 7 and 0.5% on day 12. Kahn et al (18) used hepatic levels of thymidine kinase and mitotic indexes to assess hepatocyte replication in pigs after a 50% partial hepatectomy. Both parameters began increasing 2 days after the procedure and peaked at 3–4 days. In a subsequent study, Kahn et al (50) found that the maximum regenerative response occurred on the 3rd postoperative day, regardless of the size of the partial hepatectomy.

In contrast, we observed that hepatocyte replication peaked 7 days after partial portal branch occlusion in pigs. In similar fashion, hepatocyte replication in rats peaked 1.5–2.0 days after portal vein branch occlusion, which is later than the peak of replication at 1 day after partial hepatectomy. This delay in hepatocyte replication after partial portal vein branch occlusion as compared with that after partial hepatectomy may be due to the fact that hepatocytes are not removed at the time of the portal branch occlusion procedure, resulting in a delay in the stimulus to replicate.

Our data indicate that high levels of hepatocyte replication occur 2–7 days after occlusion of portal vein branches in a large-animal model, the pig. The achievement of therapeutic levels of a protein by means of *in vivo* hepatic gene therapy will, therefore, likely require multiple infusions of the retroviral vector at 2–7 days after occlusion. This can best be accomplished by using an indwelling portal vein catheter, and we are currently testing this approach. If these experiments are successful in a large animal such as the pig, similar gene therapy experiments in patients would require knowledge about the timing of hepatocyte replication in humans. Francavilla et al (51) used plasma levels of thymidine kinase to assess hepatocyte replication in humans after major liver resections. They found that levels of this enzyme in-

creased 4–5 days after the procedure and concluded that it was a useful index of hepatic regeneration. It is likely, therefore, that humans will have a regenerative response after portal vein branch occlusion that is similar to or slightly later than the response observed in pigs.

Safety of Embolization of Portal Vein Branches

Our data suggest that percutaneous embolization of portal vein branches is safe. The portal vein was easily accessed in all eight animals, and the branches supplying the majority of the liver were completely occluded by using a combination of PVA particles and embolization coils. No complications related to the procedure were observed.

Embolization caused portal vein pressures to increase in some but not all animals. However, the extent of the increase was variable and mild. Other investigators (43,44,52,53) embolized all branches of the portal vein to create animal models of portal hypertension and found that portal pressure increased two- to fivefold after embolization. The difference in portal pressure elevations between these studies and ours most likely reflects differences in the degree of portal vein embolization. The long-term effects are likely to be similar to those of partial hepatic resections or portal vein ligations, with respect to the development of chronic portal hypertension. Experience with animals and humans (33,54) suggests that this would not occur.

Results of laboratory evaluation, as well as results of examination of the liver tissue, indicated that the procedure did not cause widespread hepatocellular necrosis or hepatic insufficiency. Percutaneous embolization of portal vein branches did lead to atrophy of the embolized segments. The histologic and serum enzyme analysis data suggest that this atrophy resulted from hepatocyte apoptosis rather than from lytic necrosis. Results of previous studies (40,41,55) have shown that hepatocyte apoptosis is the major histologic change after surgical ligation of the portal vein, although varying degrees of lytic necrosis in the pericentral regions can occur. However, the results of one study (39) suggested that lytic necrosis could be avoided by minimizing trauma to the hepatic artery.

Although percutaneous occlusion of portal vein branches should minimize hepatic artery injury, we did find rare areas of lytic necrosis in the right lobe. The most likely explanation is that place-

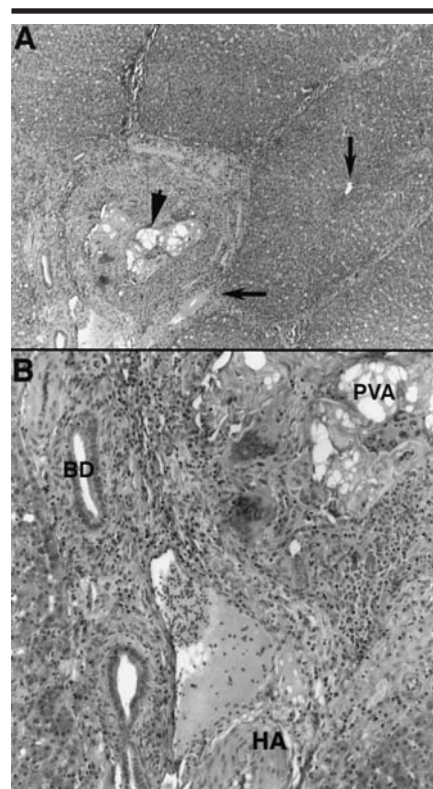


Figure 9. Animal 3. Photomicrographs show an embolized segment from the left lobe obtained 3 days after embolization. *A*, Low-magnification image shows a PVA particle (arrowhead) within a portal vein branch. Horizontal arrow = a portal triad, vertical arrow = a central vein. (Hematoxylin-eosin stain; original magnification, $\times 50$.) *B*, Higher magnification image shows the portal triad in more detail. The PVA particle, a bile duct (*BD*), and a hepatic arterial branch (*HA*) are shown. An inflammatory cell infiltrate surrounds the PVA particle. (Hematoxylin-eosin stain; original magnification, $\times 125$.)

ment of the catheter led to occlusion of peripheral portal vein and hepatic artery branches. The slight elevation in hepatic enzyme levels after the procedure further indicates that lytic necrosis occurred but was not widespread. Although it is possible that more frequent serum sampling might have revealed evidence of substantial hepatic injury, our results and those of studies of portal branch occlusion in other animals (32–36,42–44) suggest that little lytic necrosis occurs.

It remained possible that embolization caused an acute decrease in liver function that would not be reflected in serum assays. This possibility was assessed by measuring the plasma clearance of indocyanine green before and immediately after embolization. Despite the embolization of the portal branches supplying approximately 60% of the liver, only a

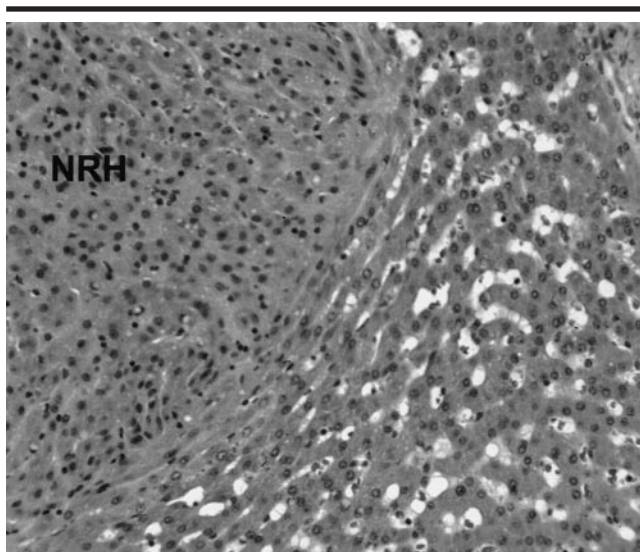


Figure 10. Animal 6. Photomicrograph shows nodular regenerative hyperplasia (NRH) in the left (embolized) lobe 4 days after embolization. The interface between nodular regenerative hyperplasia and normal liver can be seen. (Hematoxylin-eosin stain; original magnification, $\times 200$.)

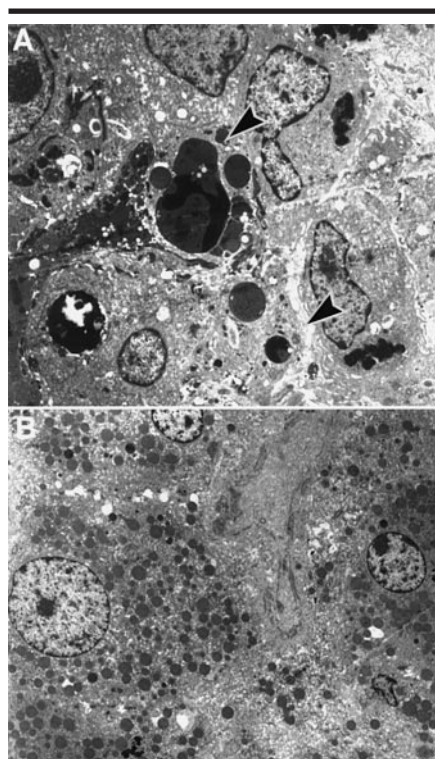


Figure 11. Animal 3. Electron microscopic images of hepatocyte apoptosis 3 days after embolization. *A*, Tissue from the left lobe (embolized) shows cells with condensed nuclear material (arrowheads). These cells likely represent apoptotic hepatocytes adjacent to normal hepatocytes. (Original magnification, $\times 2,200$.) *B*, A representative image of tissue from the right lobe (nonembolized) shows normal hepatic ultrastructure. (Original magnification, $\times 1,650$.)

16%–50% decrease in plasma clearance was seen, which was indicative of minimal alteration in overall hepatic function. A combination of an increase in hepatic arterial flow to the embolized segments and an increase in portal blood flow to the nonembolized segments could explain this discrepancy.

Although we did not directly compare the safety of percutaneous embolization of portal vein branches with that of other possible methods of stimulating hepatocyte division, we expect it to compare favorably with partial hepatectomy, surgical ligation of portal vein branches, or administration of hepatotoxins. Hepatectomy is a major surgical procedure with substantial morbidity and mortality. Savage et al (56) reported a 14.7% mortality rate for major hepatic resections and a 36.9% complication rate. Tsao et al (57) reported a 1.2% mortality rate for all hepatic resections and a 15% complication rate. Whereas ligation of portal vein branches could conceivably be performed laparoscopically, dissection of the portal vein could cause hepatic arterial injury and thus lead to lytic necrosis of the embolized segments. Administration of hepatotoxins, such as carbon tetrachloride (25) or an adenoviral vector that encodes a protein that is toxic to the liver (26, 27), would lead to hepatocellular necrosis, and it would be difficult to control the extent of hepatic injury.

The long-term consequences of the induction of hepatocyte replication by

means of portal branch occlusion were not addressed in this study. Because the nonembolized liver regenerates to restore the original liver mass, it is doubtful that portal hypertension or hepatic insufficiency would develop, unless the clot extended to occlude the entire portal venous system. We believe it unlikely that this will occur, since none of eight pigs in this study developed complete occlusion of the portal venous system. A second concern is the development of malignancy, since occasional nodular regenerative hyperplasia was observed in the embolized lobes of the liver. Nodular regenerative hyperplasia is a histopathologic finding that can be observed in humans with various conditions, including portal vein thrombosis (58,59). Although regeneration occurred in the embolized liver, it is unlikely that malignancy would develop, because the stimulus for replication was short-lived, as evidenced by the fact that the percentage of replicating hepatocytes had decreased to very low levels by 12 days. Additional studies will be needed to assess the long-term consequences of this procedure.

Practical application: We have previously shown (29) that retroviral transduction is proportional to hepatocyte replication in rats after portal branch occlusion. We have now defined the hepatocyte replication timing after portal vein branch embolization in a porcine model. Currently, we are testing percutaneous embolization of portal vein branches in a gene therapy experiment with large animals. If these experiments are successful, these methods may well be used to perform gene therapy in humans. We expect that it should be possible to perform percutaneous embolization of portal vein branches at nearly any point in life. Transhepatic approaches could be used to access the portal veins in adults and children. Our experience with animal models suggests that it should be possible to access the portal vein in a 15-kg child by using a transhepatic puncture. In infants, the umbilical vein could be used to gain access to the portal vein. This route would be particularly useful when the diagnosis of an inborn error of metabolism is made before or shortly after birth.

Whereas the current study used embolization of portal vein branches supplying 60%–70% of the liver to induce hepatocyte replication, it may be possible to induce a similar degree of replication by using a combination of hepatocyte growth factors and occlusion of portal vein branches. Hepatocyte growth factors are most effective when administered after a

“priming” stimulus (60,61), and it is hoped that less extensive embolization would fulfill the priming role. Ueno et al (62) recently tested this approach in dogs and found that hepatocyte growth factor infusion combined with ligation of the left portal vein induced 2.7% of hepatocytes in the nonligated lobe to incorporate bromodeoxyuridine 72 hours after the ligation procedure. As a control, some animals underwent ligation but did not receive the growth factor. In these animals, only 1.5% of the hepatocytes in the nonligated lobe incorporated bromodeoxyuridine. Although these results are encouraging, the growth factor dosing schedule, the time course of replication, and the safety of such protocols must be better defined.

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