

Hepatic neovascularization after partial portal vein ligation: novel mechanism of chronic regulation of blood flow

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Yokoyama, Yukihiro, Rajiv Baveja, Natalie Sonin, Mark G. Clemens, and Jian X. Zhang. Hepatic neovascularization after partial portal vein ligation: novel mechanism of chronic regulation of blood flow. *Am J Physiol Gastrointest Liver Physiol* 280: G21–G31, 2001.—The present study was undertaken to investigate hepatic microcirculatory response following partial portal vein ligation (PPVL) in rats. Portal pressure was markedly increased 2–6 wk after PPVL, but no significant reduction in sinusoidal perfusion and hepatocellular injury were detected. However, marked neovascularization was observed in PPVL rats using intravital microscopy and scanning electron microscopy (SEM). Extremely high red blood cell velocity (2,000–4,900 $\mu\text{m/s}$) was seen in these vessels. Injection of fluorescein sodium via the carotid artery revealed that the neovessels originated from the hepatic arterial vasculature. This was further confirmed by clamping the common hepatic artery and phenylephrine injection from the carotid artery. These vessels maintained sufficient flow after massive sinusoidal shutdown elicited by the portal infusion of endothelin receptor B agonist IRL-1620. SEM also showed extensive neovascularization at the hilum. Additionally, clamping the portal vein decreased sinusoidal perfusion only by 9.5% in PPVL, whereas a 71.2% decrease was observed in sham. These results strongly suggest that the liver maintains its microcirculatory flow by vascular remodeling from the hepatic arterial vasculature following PPVL.

liver microcirculation; intravital microscopy; portal hypertension; hepatic vascular casts

PARTIAL PORTAL VEIN LIGATION (PPVL) has been used as an animal model to induce prehepatic portal hypertension, which is characterized by increased cardiac output, reduced systemic vascular resistance, increased plasma volume, and decreased vascular responsiveness to vasopressors (27). Although much attention has been drawn to the pathophysiological changes in the preportal splanchnic circulation and systemic circulation (2, 3, 7, 22), little information is available on how the liver responds to the sudden and sustained decrease in portal blood flow following PPVL.

The liver receives blood supplies from both the portal vein and the hepatic artery, and these circulations are regulated independently. Portal venous flow is regu-

lated by the resistance vessels in the splanchnic viscera upstream and is determined by the net outflow of the splanchnic organs. The liver does not control portal blood flow. A change in blood perfusion to the liver is brought about by regulation of hepatic arterial blood flow via the hepatic arterial buffer response (HABR) (14, 15). HABR, first reported by Lautt et al. (13) as the primary intrinsic regulator of the hepatic artery, increases hepatic arterial blood flow in response to decreased portal flow. This compensatory mechanism of hepatic blood flow has been proposed to be mediated by adenosine washout. If portal blood flow is severely reduced, less adenosine is washed away into the portal blood and the accumulated adenosine leads to hepatic arterial dilation and increased hepatic arterial flow. Although HABR has been generally accepted as a regulatory mechanism of the total hepatic blood flow with an acute change in portal blood flow, information on how the liver regulates its blood flow under conditions in which portal blood flow is chronically reduced, such as occurs with PPVL, is still lacking. It is conceivable that the liver responds to diminished portal blood flow following PPVL with chronic functional and/or structural changes. Tsoporis et al. (26) reported the structural changes in mesenteric arterioles after chronic treatment with a vasodilator. Another study performed by Sumanovski et al. (24) showed increased angiogenesis in the abdominal cavity in a rat PPVL model. However, little information is available on the effects of PPVL on the microcirculatory hemodynamics and morphological adaptations in the liver. Therefore, the present study was conducted to investigate the chronic response of hepatic microcirculation following PPVL.

Using intravital fluorescence microscopy and scanning electron microscopy (SEM), we discovered that the liver adapted to permanently diminished portal blood flow after PPVL by initiating neovascularization from the hepatic arterial system. The neovessels bear characteristics of arterioles and function similarly to the arterial vasculature. We therefore propose that neovascularization of the hepatic artery may represent a novel mechanism for a long-term regulation of hepatic microcirculatory flow under

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conditions in which portal blood flow is chronically deprived.

MATERIALS AND METHODS

Chemicals

IRL-1620, an endothelin receptor B agonist, was purchased from American Peptide. Batson's No. 17 anatomic corrosion compound was purchased from Polysciences (Warrington, PA). All other chemicals were purchased from Sigma (St. Louis, MO).

PPVL

Experiments were performed using male Sprague-Dawley rats (Charles River Labs) weighing 250–350 g at the time of operation. All procedures were performed in accordance with National Institutes of Health guidelines under a protocol approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Charlotte. Prehepatic portal hypertension was induced by PPVL as described by Vorbioff et al. (27). Briefly, rats were anesthetized with metaphane (Schering Plough). After an upper midline abdominal incision, the portal vein proximal to the confluence of the right and left branches was exposed and carefully separated from the hepatic artery. A 20-gauge needle was placed beside the portal vein, and a tight ligature was performed around the needle and portal vein with 4-0 silk. Subsequent removal of the needle resulted in constant calibrated stenosis of the portal vein. After washing the abdominal cavity with physiological saline, the abdomen was closed and the animals were allowed to recover for 2 or 6 wk. Sham animals were operated using the same procedure except for the ligation of the portal vein.

Lactic Dehydrogenase and Alanine Aminotransferase Assays

To determine whether PPVL induces liver injury, blood samples were obtained from the arterial line of PPVL and sham rats for measurement of serum lactic dehydrogenase (LDH) and alanine aminotransferase (ALT) levels. Measurements were made spectrophotometrically using diagnostic kits from Sigma.

Fluorescent Cell Labeling

FITC-labeled red blood cells (RBCs) were prepared as originally described by Zimmerhackl et al. (30) in a modified technique. Briefly, RBCs from 10 ml of heparinized blood of a donor animal were washed three times in Alsever's buffer [in g/l: 20.5 glucose, 8.0 citric acid trisodium salt, 0.55 citric acid, and 3.766 NaCl, pH 6.2] and once in bicine-saline buffer [in g/l: 3.264 bicine, 0.399 NaOH, and 7.288 NaCl, pH 8.3]. For each washing step, 3 min of centrifugation at 2,500 rpm was used. Subsequently, FITC, at a dose of 4 mg/ml RBC suspension, was dissolved in 0.1 ml *N,N*-dimethylformamide and added into 1:1 diluted RBCs in the bicine-saline buffer. After incubation for 3 h at room temperature on a rocking plate, cells were washed once in bicine-saline buffer and 3 times in physiological saline by centrifugation. After resuspension in physiological saline with citrate-phosphate-dextrose (0.14 ml/ml RBC suspension) to a hematocrit of ~50%, RBCs were refrigerated until use within 7 days.

Intravital Microscopy

Surgical preparation. Rats were fasted overnight but were allowed free access to water. Every procedure was performed

on a heating pad to maintain body temperature at 36–37°C. Following induction of anesthesia (pentobarbital sodium, 50 mg/kg body wt ip), a tracheotomy was performed to allow spontaneous breathing. After cannulation of the right carotid artery, a midline laparotomy was performed. The bowel was wrapped with wet gauze and shifted to the left side to expose the portal and splenic veins. After careful dissection of the connective tissue around the portal vein and the splenic vein, a double-lumen catheter (outer catheter PE-90, inner catheter PE-10) was inserted from the splenic vein into the portal vein. The tip of the catheter was positioned at the proximal site of PPVL. This cannulation allowed for the simultaneous pressure measurement and infusion. The bowel was repositioned, and the lower half of the incision was approximated. Each animal was transferred to the stage of an Olympus IX70 inverted microscope and turned on its left side. The left lobe of the liver was gently exteriorized and positioned onto a window, which was covered with a Corning no. 1 micro-cover glass so that the working distance between the objective lens and the specimen could be minimized. To prevent evaporation, the surface of the liver was covered with Saran plastic wrap (Dow Chemical).

In vivo microscopy. The liver surface was epi-illuminated with a 100-W mercury lamp with 460–500 nm excitation and 515–560 nm emission band-pass filters. The images were projected onto a DAGE-MTI SIT 66 camera and processed with an ARGUS-10 image processor (Hamamatsu Photonics). The processed images were viewed on a high-resolution video monitor and recorded with a S-VHS VCR (Panasonic AG 7300). Quantitative analysis of the images was performed offline during video playback using digitized frame-by-frame analysis with Image-Pro (Media Cybernetics, Silver Spring, MD). A total magnification (specimen to monitor) of $\times 760$ was used for the experiment. Mean arterial pressure (MAP), heart rate, and portal pressure (PP) were recorded simultaneously using Digi-Med 200 pressure analyzer system (Micro-Med, Louisville, KY) during the entire procedure. Measurements of sinusoidal diameters and evaluation of sinusoid density (defined as number of perfused sinusoids per 150 μm) were made directly from video playback as previously described (4).

Detection of neovascularization. The neovessels were first identified using epi-illumination with 460- to 500-nm excitation/515- to 560-nm emission band-pass filters. Neovessels exhibit characteristics that easily distinguish them from sinusoids, i.e., larger diameters and clearly defined vascular boundaries (Fig. 1). The blood flow in those vessels was extremely fast. To determine whether those vessels originated from the hepatic artery or the portal vein, 1 ml of fluorescein sodium (0.2 mg in saline) was injected from the carotid artery while the sequential images of appearance of the fluorescent dye in those vessels were recorded. In addition, the origin of the neovessels was confirmed by clamping the common hepatic artery that was isolated during the surgical preparation while the blood flow rate in those vessels was monitored. In separate experiments, 0.2 ml of FITC-labeled RBCs (hematocrit = 50%) was injected intravenously for measurement of the RBC velocity in both the neovessels and sinusoids.

Response of the neovessels to vasoconstrictors. To compare the responses of the neovessels and sinusoids to vasoconstrictors, the α -adrenergic receptor agonist phenylephrine (PE) and endothelin receptor B agonist IRL-1620 were used. A bolus of PE (4×10^{-7} mol) was injected via the carotid artery ($n = 3$) or portal vein while the response of the neovessels was monitored. IRL-1620 was infused through the portal vein at a rate of $5 \text{ pmol} \cdot \text{min}^{-1} \cdot 100 \text{ g}^{-1}$ for 20 min, and responses of the sinusoids and neovessels were continuously monitored for 1 h (sham, $n = 7$; PPVL, $n = 8$).

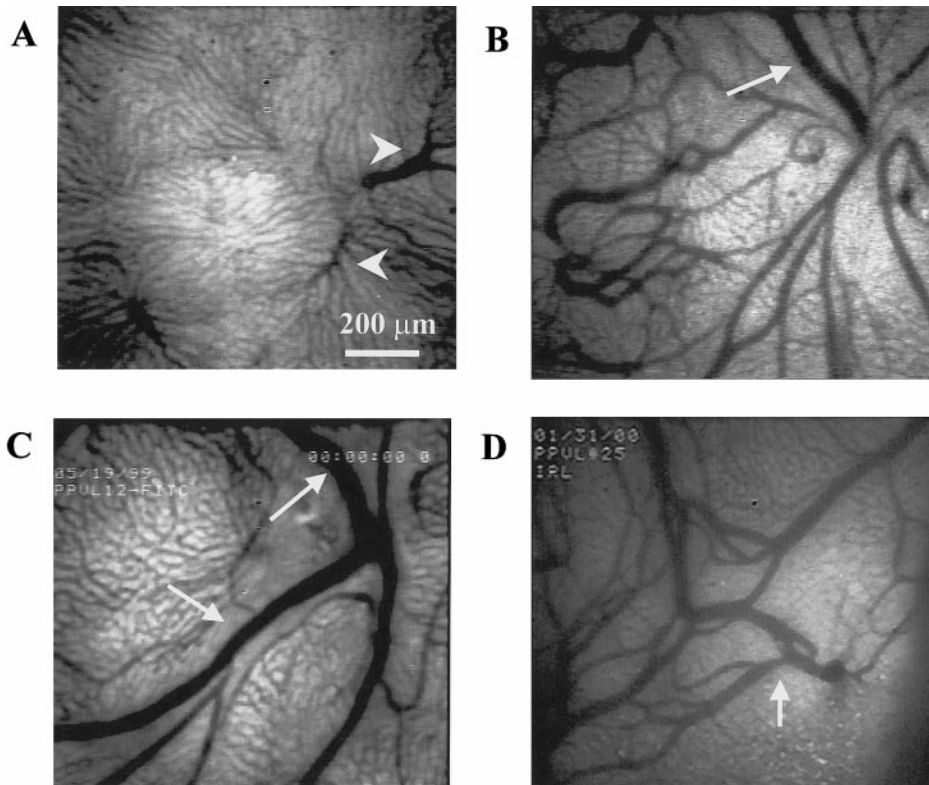


Fig. 1. Intravital microscopic images of the hepatic microcirculation in sham-controlled (A) and partial portal vein ligation (PPVL; B, C, and D) rats (FITC epifluorescence; original magnification $\times 100$). No neovascularization was detected in the sham-controlled liver, whereas extensive neovascularization was observed in the livers of PPVL rats. The neovessels (arrows) display smooth borders of vascular wall and conduct brisk blood flow. Some branches of the neovessels drain into sinusoids, but a few vessels directly connect with the terminal hepatic venules. Arrows indicate the neovessels, and arrowheads represent terminal hepatic venule (THV). Maximum size of these vessels was 20–60 μm in diameter.

Effect of portal vein clamping. To determine the relative significance of the blood supply to the hepatic microcirculation from the hepatic arterial system following PPVL or sham operation, acute portal vein clamping was performed to occlude the portal flow while the blood perfusion in sinusoids was monitored using intravital microscopy. Before portal clamping, 0.2 ml of FITC-labeled RBCs was injected from the carotid artery for measurement of the RBC velocity (V_{RBC}). Sinusoidal diameters (D_s) and V_{RBC} were measured during offline analysis. Perfusion index (PI), which reflects the blood perfusion in the hepatic microcirculation, was calculated using the following equation

$$\text{PI} = P_s \times [V_{\text{RBC}} \times \pi \times (D_s/2)^2]$$

where P_s is number of perfused sinusoids across a 150- μm microscopic field. PI was measured at the baseline and 2 min after clamping.

Hepatic Vascular Casting

Hepatic vascular casting was carried out using a modified method of Lim et al. (16). Under pentobarbital anesthesia, the portal vein and thoracic aorta were cannulated with a PE-160 catheter. A cannula for the thoracic aorta was inserted caudally to position the catheter tip at the radix of the celiac artery. Tight ligation was performed on abdominal aorta just proximal to the branching point of the renal artery. The inferior vena cava was also ligated at the level proximal to the right renal vein. After dissecting the right atrium as a drainage route of perfusate, the liver was perfused with sterilized saline containing 10,000 U/l heparin for 10 min to wash out the blood. Pressure of the arterial line was monitored by Digi-Med 200 pressure analyzer system (Micro-Med, Louisville, KY). During the perfusion, Batson's No. 17 anatomic corrosion compound (Polysciences) was formulated for use as the casting medium. The ingredients of the mixture

consisted of 10.0 ml of monomer base, 2.0 ml of catalyst, and 2 drops of promoter. The casting medium of different colors was injected from portal vein (blue) and thoracic aorta (red) manually by using a 10-ml disposable polyethylene syringe. The pressure of the arterial line was kept under 180 mmHg to prevent the formation of arterial aneurysm. Infusion was terminated when the casting medium appeared on the surface of the liver. The liver was covered with Saran plastic wrap and left in the abdominal cavity overnight to allow complete polymerization. After polymerization, the liver was excised carefully and soaked in potassium hydroxide (6 M) to macerate the tissue. The solution was changed at least once a day. More than 2 days were required to dissolve the tissue surrounding the cast entirely. Additional maceration was carried out in a 60°C incubator when the room temperature digestion of the tissue was incomplete. The casts were then dehydrated in ethanol and dried.

SEM. Dried casts of the liver were mounted on aluminum stubs and sputter-coated with gold (96%)-palladium (4%). Samples were viewed and photographed by Cambridge scanning electron microscope (Cambridge, MA) at an accelerating voltage of 15 kV.

Statistical Analysis

Statistical significance was tested using one-way ANOVA, with individual means detected by Student-Newman-Keuls test. A P value < 0.05 was considered significant. All results are presented as means \pm SE.

RESULTS

Hemodynamic and Enzymatic Data

The hemodynamic data of PPVL and sham-operated rats are shown in Table 1. PP was markedly increased

Table 1. Hemodynamic data of sham-operated and PPVL rats

	Sham	PPVL
Portal pressure, mmHg	5.4 ± 0.6	13.2 ± 0.9*
Mean arterial pressure, mmHg	117.4 ± 9.9	105.6 ± 5.8
Sinusoidal diameter, μm	8.4 ± 0.2	8.2 ± 0.3
Sinusoidal density	6.05 ± 0.2	6.82 ± 0.4
n	4	8

Values are means ± SE. PPVL, partial portal vein ligation. Sinusoidal density is the number of perfused sinusoids/150 μm . * $P < 0.05$ vs. sham.

in PPVL rats compared with sham-operated rats. MAP of the PPVL group shows a trend of decrease but did not reach statistical difference from the sham group. The sinusoidal diameters and number of perfused sinusoids per 150- μm length in each microscopic field (sinusoidal density) showed no significant differences between PPVL rats and sham controls. No deficits in microcirculatory perfusion and necrosis were observed in PPVL rats when compared with sham rats. To determine the level of hepatocellular injury, plasma LDH and ALT levels were measured 2 wk after PPVL or sham operation (Fig. 2). No significant differences were found between PPVL and sham (LDH: sham, 54.0 ± 7.0 ; PPVL, 57.4 ± 10.0 IU/l; ALT: sham, 20.0 ± 6.0 ; PPVL, 31.4 ± 10.0 IU/l), indicating that the procedure of PPVL did not produce significant hepatocellular injury.

Detection of Neovascularization

Using in vivo intravital fluorescence microscopy, we were able to observe extensive neovascularization in livers that were subjected to PPVL for 2 or 6 wk, but no neovessels were found in the liver of sham-operated animals (Fig. 1). The neovessels were usually observed near the edge of the left liver lobe. Because of the difficulty of maneuvering the liver for microscopic viewing, no attempt was made to search for the neovessels in other lobes. The neovascularization was observed in 13 out of 37 PPVL rats (35.1%), with no difference in the frequency of observation between 2 and 6 wk of PPVL.

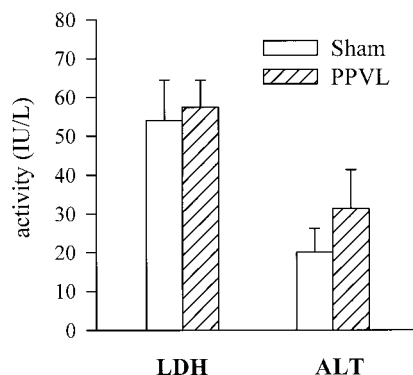


Fig. 2. Plasma lactate dehydrogenase (LDH) and alanine aminotransferase (ALT) levels after 2 wk of PPVL or sham operation. There was no significant difference between the two groups.

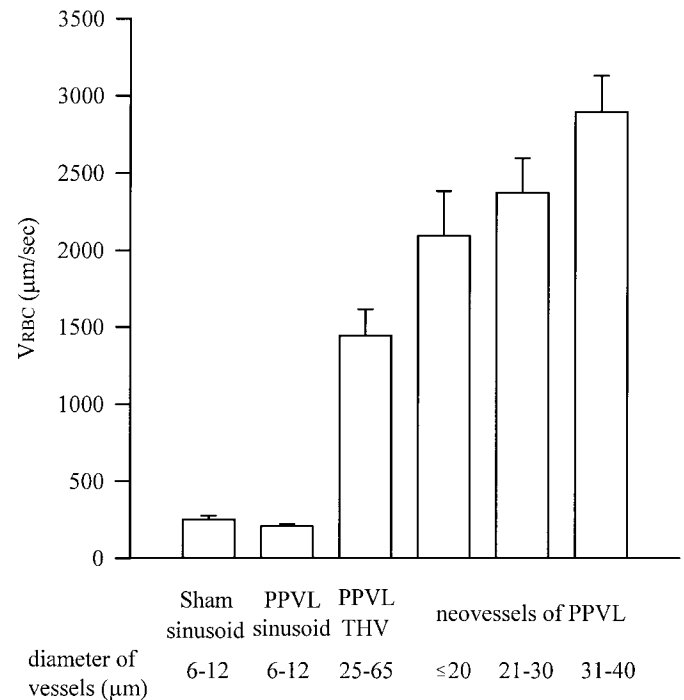


Fig. 3. Comparison of red blood cell velocity (V_{RBC}) in sinusoids, THVs, and neovessels. V_{RBC} was much higher in the neovessels, even with smaller diameter ($\leq 20 \mu\text{m}$) compared with that in sinusoid or THV.

These neovessels showed features that clearly distinguished them from sinusoids. The size was larger than sinusoids, ranging from 8 to 60 μm in diameter, and they conducted flow at extremely high rates (RBC velocity 2,000–4,900 $\mu\text{m/s}$ vs. the normal sinusoid rates of 200–500 $\mu\text{m/s}$). The average RBC velocity was $>2,000 \mu\text{m/s}$ even in the smaller neovessels ($<20 \mu\text{m}$ in diameter) and was higher than that of terminal hepatic venules with similar size (Fig. 3). The vessels displayed a smooth border in their vascular walls and often branched into smaller vessels, most of which drained into sinusoids (Fig. 1). In some places, the vessels drained directly into the terminal hepatic venules. To determine the origin of these neovessels, a bolus of fluorescein sodium was injected via the carotid artery. The fluorescent dye appeared first in the neovessels within 3 s following the injection and then became visible in sinusoids seconds later (Fig. 4). These results strongly suggest that the neovessels originate from the hepatic arterial system. This was further confirmed by clamping the common hepatic artery, which substantially decreased blood flow in the neovessels. Releasing of the clamp immediately restored the flow rate in the neovessels.

Response of Neovessels to Vasoconstrictors

To compare the responses of the neovessels and sinusoids to vasoactive substances, vasoconstrictors, α -adrenergic receptor agonist PE, and endothelin receptor B agonist IRL-1620 were used in sham and PPVL groups at 6 wk. PE, which has been previously

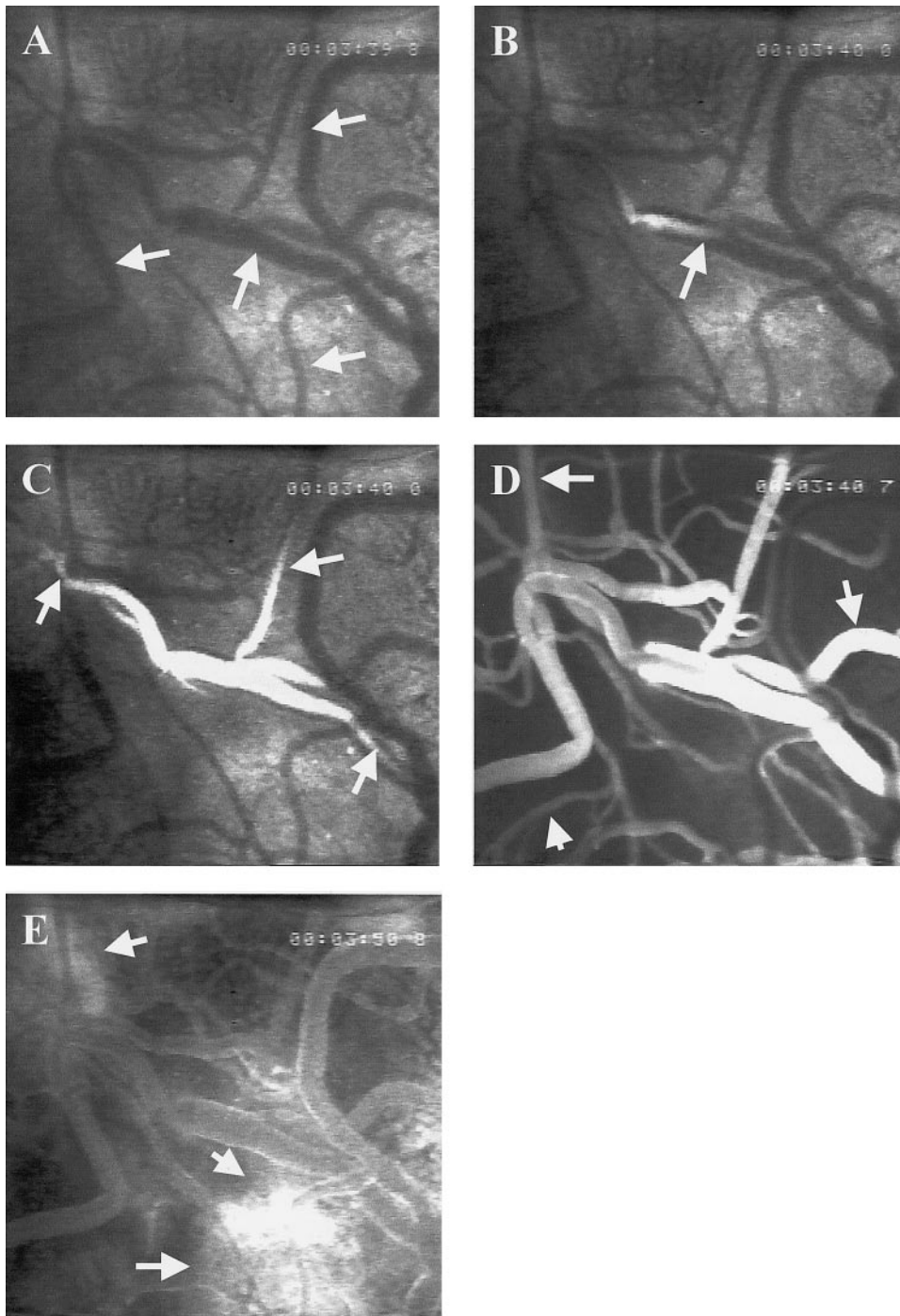


Fig. 4. Micrographs of fluorescent images demonstrating the origin of the neovessels in the PPVL liver (FITC epifluorescence; original magnification $\times 100$). A bolus of fluorescein sodium was injected via the carotid artery. Autofluorescence of the hepatic microcirculation is shown in A (arrows). The fluorescent dye first appeared in the neovessels within 3 s (B and C, arrows) and then quickly spread to the smaller branches (D, arrows) before it reached the sinusoids (E, arrows). The dye finally mixed with the sinusoidal flow coming from the portal vein seconds later (not shown).

shown to induce vasoconstriction in arterial vasculatures and the portal vein but not in sinusoids (23, 29), resulted in a transient but substantial decrease in blood flow in the neovessels when injected as a bolus via the carotid artery (Fig. 5A). The vascular diameter of those vessels decreased from the baseline value of 44.0 ± 4.5 to 25.8 ± 2.0 μm ($P < 0.01$) on PE injection (Fig. 5B). Sinusoidal blood flow also diminished transiently, but no changes in sinusoidal diameters were observed with PE injection. In contrast, PE injection from the portal vein did not change the diameter of the neovessels (Fig. 5C).

In separate experiments, IRL-1620, an endothelin receptor B agonist, was infused through the portal vein in a concentration of $5 \text{ pmol} \cdot \text{min}^{-1} \cdot 100 \text{ g body wt}^{-1}$ for 20 min. IRL-1620 did not significantly change PP in the PPVL group, but it induced marked increase of PP in the sham group (Fig. 6A). Neither the MAP nor the average sinusoidal diameter showed significant difference in either group following infusion of IRL-1620 (Fig. 6, B and C). IRL-1620, previously shown to induce an increase in PP and heterogeneous responses of sinusoidal flow in normal rats (1), induced massive sinusoidal flow shutdown in some areas of the PPVL

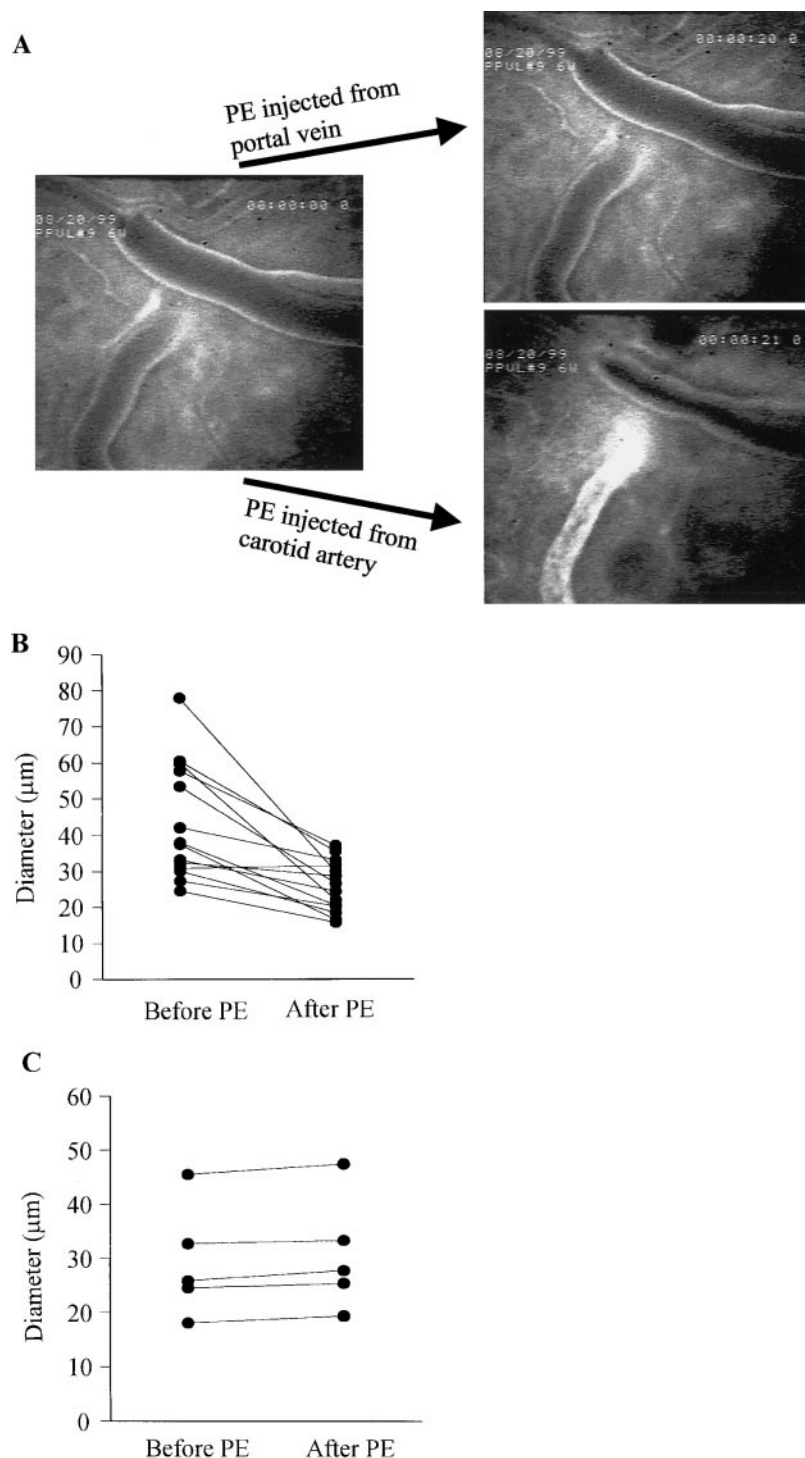


Fig. 5. Response of the neovessels to phenylephrine (PE) injection. *A*: micrographs of representative images showing the response of the neovessels to PE. *B* and *C*: graphic plots of the changes in diameter of the individual neovessels following PE injection via carotid artery (*B*; $P < 0.01$ before vs. after PE) and portal vein (*C*).

livers, which was manifested with the decreased number of perfused sinusoids (Fig. 6*D*). However, blood flow in the neovessels remained unaltered. No change in vascular diameter was detected in these vessels either. In the areas where the sinusoidal shutdown of blood flow occurred, the neovessels were seen to supply blood flow to the neighboring acini, which was revealed by the clear staining of injected fluorescein sodium (Fig. 7). This observation strongly suggests that the neoves-

sels provide an important route for blood flow compensation under conditions in which the portal blood flow is deprived.

Effect of Portal Vein Clamping

To evaluate the relative contribution of hepatic artery blood flow in feeding the sinusoids following PPVL, the portal vein was temporarily clamped while

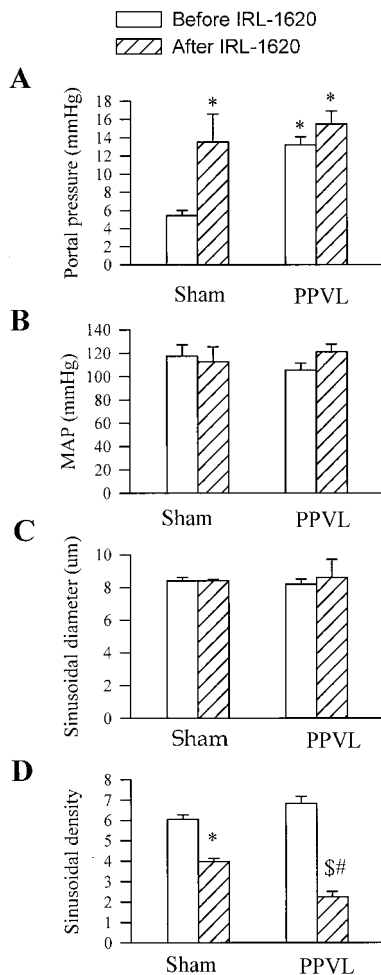


Fig. 6. Comparison of hemodynamic changes between sham-operated and PPVL rats after IRL-1620 injection. IRL-1620 was injected at a rate of $5 \text{ pmol} \cdot \text{min}^{-1} \cdot 100 \text{ g body wt}^{-1}$ for 20 min. A: portal venous pressure; B: mean arterial pressure (MAP); C: sinusoidal diameter; D: sinusoidal density. Data are means \pm SE. * $P < 0.05$ vs. sham before IRL. \$ $P < 0.05$ vs. PPVL before IRL. # $P < 0.05$ vs. sham after IRL.

the sinusoidal flow was monitored. PI decreased only by 9.5% after clamping the portal vein in the PPVL group, whereas the sham group showed a decrease by 71.2%, suggesting an important compensatory role for the hepatic artery system following PPVL (Fig. 8).

SEM of Hepatic Vascular Casts

To confirm that this vascular remodeling is not limited to the surface of the liver, SEM of corrosion casts of hepatic vessels was performed. Extensive growth of tortuous vessels was observed around the portal vein at the level of the hepatic hilum following PPVL (Fig. 9C). A greater number of hepatic arterial vessels were found surrounding the intrahepatic portal veins with numerous branches twisted, clustered, and disorganized in the PPVL group (Fig. 9D). In contrast, the hepatic arteries in sham showed fewer, relatively straight, and orderly vessels next to the portal vein (Fig. 9, A and B). These tortuous and clustered vessels

of the hepatic artery branches were seen to a different degree in all three corrosion casts of the PPVL group.

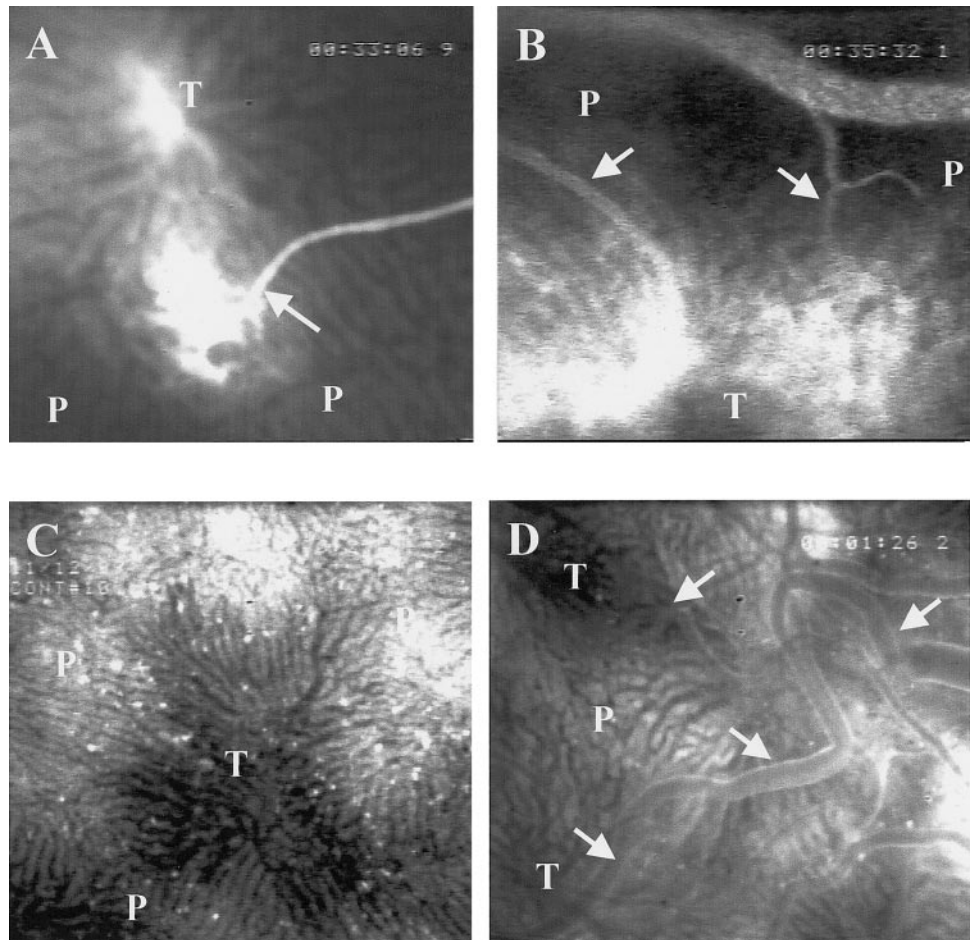
DISCUSSION

The liver receives its blood supply from both the portal vein and the hepatic artery. Although the portal venous flow comprises $\sim 75\%$ of the total blood flow volume to the liver, the liver does not control the volume of portal blood flow. Intrahepatic regulation in total hepatic blood flow is thus mediated by regulation of hepatic arterial blood flow via the HABR (14). HABR increases hepatic artery blood flow in response to acutely decreased portal venous flow. Kawasaki et al. (11) reported a significant increase of hepatic arterial flow (436% increase compared with sham) in the acute PPVL model. This increase in hepatic arterial flow could well be induced by HABR. However, studies have been limited to the acute response of hepatic arterial flow after the change of portal venous flow, and no previous reports have shown the chronic effects of portal venous flow reduction on the hepatic arterial system. In particular, the functional and morphological changes in the hepatic vasculature, especially at microvascular levels, following PPVL are unknown. In the present study, we report for the first time the observation of neovascularization from the hepatic arterial system that takes place to accommodate the permanent reduction of the portal venous flow following PPVL in rats. We therefore propose that this vascular remodeling serves as an important mechanism in regulating total hepatic blood flow under conditions in which the portal venous blood flow is chronically compromised.

In this model of PPVL-induced portal hypertension, we did not detect significant changes in sinusoidal perfusion when compared with the sham-controlled animals. The sinusoidal density, sinusoidal diameter, RBC velocity in the sinusoids, and calculated sinusoidal volumetric flow and PI showed no significant difference between sham-operated and PPVL rats 2 wk (data not shown) or 6 wk after PPVL. No differences in the enzyme levels of LDH and ALT were observed between the two groups 2 wk after the operation. These data raise the essential question: what mechanisms exist to compensate for the blood flow loss due to the PPVL procedure, ultimately maintaining the integrity of sinusoidal perfusion and preventing the hepatocyte injury? The answer lies, at least partially, in neovascularization initiating from the hepatic arterial system.

Using in vivo intravital fluorescence microscopy, we were able to observe extensive neovascularization in the livers that were subjected to PPVL for 2 or 6 wk. There was no significant difference in frequency of the neovessels observed following PPVL at either 2 or 6 wk. The neovessels were easily identifiable in 35.1% of PPVL rats that were subjected to the procedure (Fig. 1). Because of the difficulty of maneuvering the liver for microscopic viewing, only a small area of the left lobe was viewed in each rat. In the PPVL rats in which

Fig. 7. Micrographs of epifluorescent images of the neovessels that feed into sinusoids. A: blood flow in the neovessels shown by injection of fluorescein sodium via the carotid artery is well maintained and supplies to the sinusoids of the acinus, whereas the surrounding acini showed flow shutdown after injection of IRL-1620 in PPVL rats. B: 2 feeding vessels maintaining blood flow of 2 adjacent acini after the same treatment as in A. C: heterogeneous microcirculatory perfusion in sham-operated rats after IRL-1620, with flow shutdown in several acini (dark areas). D: the normal sinusoidal perfusion without IRL-1620 in PPVL rats. T, terminal hepatic venule; P, portal areas; arrows, neovessels. Original magnification, $\times 200$ for A and B, $\times 100$ for C and D.



no neovessels were seen in the limited viewing area, no attempt was made to search for the neovessels in other areas or other lobes. However, it was clear that the phenomenon of neovascularization we observed was specific for the PPVL rats because no neovessels were

identified in the sham-operated rats unless severe adhesions occurred on the surface of the observed liver lobe. As seen in Fig. 1, the neovessels were clearly distinguished from sinusoids, with smooth vascular walls. The size of the vessels ranged from 8 to 60 μm in diameter. The blood flow velocity was extremely fast, with RBC velocity $>2,000 \mu\text{m/s}$ even in the smaller vessels (Fig. 3). The morphological and functional properties of those neovessels clearly demonstrated characteristics of arterioles. Injection of fluorescein sodium via the carotid artery showed the appearance of the fluorescent dye first in the neovessels (within 3 s) and then in sinusoids ~ 8 – 10 s later. These results provide strong evidence that these neovessels originate from the hepatic arterial system and not from the portal vein because the fast appearance of the dye in these neovessels following intra-arterial injection indicates a direct traveling route from the aorta to the neovessels through the hepatic artery. On the other hand, the delayed appearance of the dye in the sinusoids can be explained by the extra traveling distance of the splanchnic circulation and the portal vein. This was further confirmed by clamping the common hepatic artery, which substantially decreased blood flow in those vessels. Releasing the clamp immediately restored the blood flow rate in the neovessels.

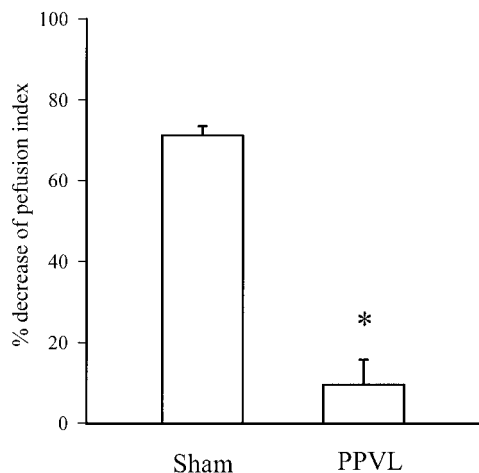


Fig. 8. The effect of portal vein clamping on hepatic microcirculation. Microcirculatory blood perfusion in sham and PPVL livers was measured as perfusion index using intravital microscopy and expressed as % decrease from baseline. Data are means \pm SE. * $P < 0.05$ vs. sham.

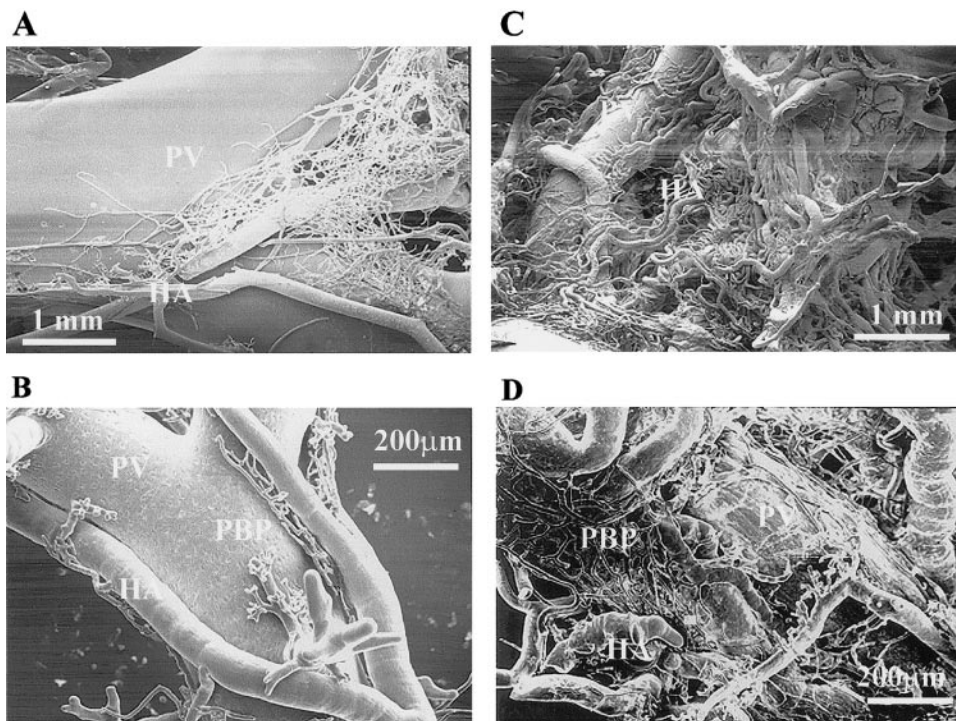


Fig. 9. Scanning electron micrographs of hepatic vascular casts. Abundant curly neovessels were observed in the PPVL group at the hepatic hilum (C) compared with the sham group (A). These changes were also observed in the intrahepatic vessels with a diameter of $\sim 300\mu\text{m}$ (B, sham; D, PPVL). Original magnification: A and C, $\times 22$; B, $\times 90$; D, $\times 100$. PV, portal vein; HA, hepatic artery; PBP, peribiliary plexus.

Since the limitation of intravital microscopy in observing the hepatic microcirculation only allows us to view the peripheral region of the liver, we performed additional experiments in which we examined the hepatic vascular corrosion casts under SEM. Substantial neovascularization from the hepatic arterial vessels observed on the hepatic vascular casts by SEM (Fig. 8) confirmed that the vascular remodeling also occurred around the smaller portal vein (diameter $\sim 300\mu\text{m}$) as well as at the hilum of the liver in the PPVL group. These results suggest that the occurrence of vascular remodeling is not limited to the surface of the liver.

To determine the functional importance of these neovessels, PE and IRL-1620 were administered through the carotid artery and the portal vein, respectively. PE, an α -adrenergic receptor agonist, which has been shown previously to induce vasoconstriction in the arterial vasculature and portal vein but not in sinusoids (1), resulted in transient but substantial constriction and decrease in blood flow in the neovessels. In contrast, the diameter of the neovessels was not changed following PE injection via the portal vein even though the sinusoidal flow was substantially decreased. These results demonstrated the functional similarity of the neovessels to arterioles. It is conceivable that those neovessels play an important role in regulating the hepatic blood flow. It was observed that those vessels make connections with more than one acinus and their branches directly drained into sinusoids and the terminal hepatic venules in some cases. Their functional importance was exemplified by the experiment using IRL-1620, an endothelin receptor B agonist. We have previously shown that the compound produces heterogeneous hemodynamic responses in

the hepatic microcirculation depending on activation of receptor B subtypes (1). In the present study, we showed that IRL-1620 induced a significant decrease in sinusoidal flow in both sham and PPVL rats without changing the average sinusoidal diameter (Fig. 6). Interestingly, the sinusoidal flow was substantially diminished in some acini with infusion of IRL-1620 via the portal vein, whereas the blood flow in the neovessels was well maintained and essentially fed the flow-deprived acini. These observations suggest that the neovessels contribute to the regulation of the hepatic microvascular perfusion, and neovascularization following PPVL may represent a novel mechanism of regulation of the hepatic blood flow under conditions in which the portal venous flow is chronically deprived. The difference seen in the number of perfused sinusoids between sham and PPVL after IRL-1620 can most likely be attributed to the fact that the neovessels only supply the sinusoids they directly feed, whereas the areas without the supply by the neovessels suffer a greater loss of flow perfusion.

The notion that the hepatic artery system compensates and becomes an important blood supply to the liver after permanently depriving the portal venous flow has been previously reported by McCuskey et al. (18). This is further supported by the present study, in which the sinusoidal perfusion was compared between sham and PPVL groups while portal flow was temporarily stopped by clamping the portal vein. The sinusoidal perfusion decreased only by 9.5% in PPVL rats, whereas it decreased by 71.2% in sham when the portal flow was occluded (Fig. 8). These results clearly demonstrate the importance of the hepatic blood flow as the dominant supply to the liver following PPVL. It is

likely that the hepatic arterial system becomes the primary blood supplier for the sinusoids following PPVL by remodeling the existing vasculature and/or generating neovessels.

Although the existence of the hepatic artery-originated vessels following PPVL was clearly demonstrated in the present study, whether these vessels were newly synthesized via angiogenesis or simply the result of hypertrophy and expansion of the existing arterial vessels is still of debate. Previously, McCuskey et al. (18) reported that numerous arteriosinus twigs (branches of hepatic arterioles terminating in some of the sinusoids) were seen in rats with portacaval anastomosis, and they were observed to be greatly enlarged and contain higher rates of blood flow than the sinusoids. The neovessels seen in the present study following PPVL seem to be different from the arteriosinus twigs. They were not generally seen across the surface of the liver and tended to cluster in isolated areas. Moreover, we did not observe similar vessels in the sham-operated rats. Most recently, Ekataksin (6) showed an isolated artery that ramified and anastomosed extensively to form a capsular plexus in the liver of humans and other mammals. It could be argued that the vessels we observed in this study using intravital microscopy are vessels of the capsular plexus but are not neovessels generated through angiogenesis. The evidence available, however, would argue against that possibility. First of all, the capsular plexus fed by the isolated artery was only observed in mammals such as pig, ox, horse, and human and was not found in rats and mice according to the study of Ekataksin (6). Secondly, we not only observed the vessels in isolated areas of the liver surface in PPVL rats with intravital fluorescence microscopy but also observed the extensive network of the hepatic arterial vessels intrahepatically, particularly at the hilum, using SEM on the hepatic vascular cast. Furthermore, we have never seen the capsular arterial vessels in livers of normal and sham-operated rats. Therefore, the results of the current study favor the notion that the vessels we observed after PPVL are newly synthesized as a result of angiogenesis.

Neovascularization or angiogenesis is essential in a variety of conditions such as inflammation, hypoxia, increased shear stress, and wound healing (5, 8, 9, 19, 21, 25). In the liver, the development of solid tumors, chronic viral infection by hepatitis C virus, and cirrhosis have been proposed as inducing factors for angiogenesis (12, 17, 19, 20, 28). Neovascularization observed in the current PPVL model is likely to be induced by hypoxia resulting from diminished portal venous flow due to PPVL. Another possibility for neovascularization is the increased shear stress, which can be induced by the sudden increase of hepatic arterial flow due to HABR following PPVL (9, 10). Using a rat PPVL model, Sumanovski et al. (24) reported increased angiogenesis in the abdominal cavity using quantitative angiogenesis assay in vivo. However, to our knowledge, our present report is the first time that neovascularization in the liver after PPVL was re-

ported. This discovery may be of functional significance in several areas. First, understanding the mechanisms that control the angiogenesis derived from the hepatic arterial system following the chronic restriction of portal venous flow may help us understand the fundamental mechanisms of how the liver regulates its total blood flow under the chronic disease conditions that affect the portal venous flow. Second, the finding will help us learn more about prehepatic portal hypertension resulting from extrahepatic portal vein obstruction, a relatively rare disease occurring primarily in children. The information on neovascularization after PPVL will certainly allow us to gain more insight into pathological changes in the liver and how the liver responds to those changes under disease conditions. Finally, angiogenesis plays an essential role in the growth of tumors. Formation and development of the hepatic tumor requires extensive angiogenesis from the hepatic artery. The results reported here suggest that PPVL can be used as a simple useful animal model to conduct mechanistic studies of hepatic artery-derived angiogenesis associated with hepatic tumors.

In summary, we investigated the microhemodynamic and structural changes of microvessels in the rat liver following PPVL. Using intravital microscopy, we showed extensive neovascularization of the hepatic arterial system in the rat liver subjected to PPVL. The neovessels supplied blood flow directly to sinusoids or terminal hepatic venules. This phenomenon may represent a very important regulatory mechanism that maintains total hepatic blood flow under conditions in which the portal flow is chronically compromised. In addition, our results suggest that the rat model of PPVL can serve as a useful animal model to study hepatic artery-derived angiogenesis in hepatic tumors.

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