

Sinusoidal Ultrastructure Evaluated During the Revascularization of Regenerating Rat Liver

KATHRYN E. WACK,^{1,2} MARK A. ROSS,¹ VASTHY ZEGARRA,^{1,3} LAURA R. SYSKO,¹ SIMON C. WATKINS,¹ AND DONNA BEER STOLZ¹

Sinusoidal endothelial cell (SEC) porosities were compared between the periportal (zone 1) and pericentral (zone 3) regions of the rat liver during regeneration following partial hepatectomy (PHx). SEC porosities and fenestration diameters were measured in control livers, as well as at 5 minutes, 24, 48, 72, 96, 120 hours, and 14 days following PHx. Bimodal maximums in both porosity and fenestration diameters were observed in both zones at 5 minutes and 5 days following PHx. SEC porosities increased significantly in both zones 1 and 3 within 5 minutes following PHx, but the increase was maintained only in zone 1 at 24 hours after resection. Following the initial rise, both zones displayed a gradual decrease to less than half their porosity values at 72 hr post-PHx. After 72 hours, porosities increased to over control levels and remained elevated until 14 days after PHx. The decrease in porosity at 72 hr post-PHx is accompanied by ultrastructural changes within the sinusoid at this time. Vascular corrosion casting and transmission electron microscopy (TEM) show sinusoid compression resulting from increased hepatic plate widths due to hepatocyte proliferation in the absence of SEC proliferation. Also at this time, we observed many SEC completely enveloped by stellate cells. The zonal variations observed for porosities throughout regeneration did not correlate with changes in laminin, collagen I and IV, or fibronectin deposition within the space of Disse. Taken together, the data reveal that SEC are dynamic regulators of porosity that respond rapidly and locally to environmental zonal stimuli during liver regeneration. (HEPATOLOGY 2001;33:363-378.)

Following chemical or mechanical injury, the liver has the extraordinary capacity to regenerate back to its normal mass within a short time. During regeneration, the same physiologic functions required by the organism must be performed with less metabolic "equipment," and therefore the liver must continuously adapt its metabolic output to its rapidly changing architecture. As a result of tissue loss, the concentrations of many factors, including growth factors, cytokines, and proteases, rise in the blood and liver to promote temporal and spatial proliferation and migration of the various cells to efficiently reconstitute the liver mass (reviewed by Michalopoulos¹ and Fausto²). Following 70% partial hepatectomy (PHx) in the rat, hepatocyte DNA synthesis peaks abruptly at 24 hours after PHx, and essentially terminates DNA synthesis by 72 hours following resection.³ Sinusoidal endothelial cells (SEC), the open, fenestrated, discontinuous endothelial cells that line the vessels supplying the parenchymal plates, do not initiate DNA synthesis until 48 to 72 hours after resection, peaking at 4 d post-PHx, but continue to proliferate at least until 8 days following PHx.³⁻⁵ This cellular order of proliferative events results in the formation of avascular hepatic islands throughout the liver lobule.⁶ Subsequent proliferation and migration of the sinusoidal endothelium into the avascular hepatic islands, which is strikingly similar to angiogenic events described in solid tumor growth, is suspected to be driven by the up-regulation of various angiogenic growth factors, including vascular endothelial growth factor synthesized by hepatocytes.^{7,8}

Surprisingly, very little is known about the ultrastructural changes at the sinusoidal surface during the regeneration and revascularization events that accompany compensatory hyperplasia. The fenestrated SEC, which lack the typical basement membrane extracellular matrix (ECM) components and structure, regulate uptake of chylomicrons and lipoproteins, as well as a variety of solutes such as growth factors, hormones and proteases between the blood and hepatocytes via the space of Disse.⁹ Fenestrations are thought to regulate flow of these agents by modulating porosity throughout the sinusoidal wall. Therefore, fenestrations are not passive sieves, but are most probably very dynamic structures whose size and density have been shown to be affected by physical factors such as portal pressure and shear stress^{10,11}; soluble substances such as alcohol,¹² serotonin,¹³ endotoxin,¹⁴ and nicotine¹⁵; and cytoskeletal disruption agents like cytochalasin B,^{16,17} as well as the composition of the ECM.^{18,19}

Given the fact that SEC fenestrations can modulate their diameters in response to a variety of factors and events, we sought to examine fenestration size and sinusoidal porosity index with respect to ultrastructural changes taking place within the regenerating lobule. Here, we document the

Abbreviations: PHx, 70% partial hepatectomy; SEC, sinusoidal endothelial cells; ECM, extracellular matrix; SEM, scanning electron microscopy; TEM, transmission electron microscopy; PBS, phosphate-buffered saline; PBG, PBS supplemented with 0.5% bovine serum albumin and 0.15% glycine; BM, basement membrane.

From ¹Cell Biology and Physiology, Center for Biologic Imaging, University of Pittsburgh Medical School, Pittsburgh, PA; the ²Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA; and ³Department of Biology, Washington and Jefferson College, Washington, PA.

Received April 24, 2000; accepted November 28, 2000.

Supported by grants from the NIH National Cancer Institute (CA 76541) and the American Liver Foundation (to D.B.S.). D.B.S. is an American Liver Foundation Liver Scholar 1997-2000.

K.E.W. and V.Z. are Summer Undergraduate Research Program fellowship recipients 1998-1999 at the Cell Biology and Physiology Department, University of Pittsburgh Medical School, Pittsburgh, PA.

Address reprint requests to: Donna Beer Stolz, Ph.D., Cell Biology and Physiology, Center for Biologic Imaging, BST-South 221, University of Pittsburgh Medical School, Pittsburgh, PA 15261. E-mail: dstolz+@pitt.edu; fax: 412-648-8330.

Copyright © 2001 by the American Association for the Study of Liver Diseases.

0270-9139/01/3302-0008\$35.00/0

doi:10.1053/jhep.2001.21998

changes in fenestration diameters and porosity of SEC in the periportal (zone 1) versus pericentral (zone 3) sinusoids during regeneration, from 5 minutes to 14 days following 70% PHx. Our results indicate that sinusoidal porosity of the periportal region is modulated independently from that of the pericentral area. This suggests that metabolic zonation and cell-cell interactions, but not exclusively portal pressure and ECM gradients along the periportal-pericentral hepatic axis, may play a critical role in regulation of sinusoidal porosity during liver regeneration.

MATERIALS AND METHODS

Reagents used were obtained from Sigma Chemical Company, St. Louis, MO, unless noted otherwise.

Animals and Surgery

All animals were treated in accordance with the guidelines of the Institutional Animal Use and Care Committee at the University of Pittsburgh School of Medicine and the NIH. Male Fisher 344 rats (NCI, Frederick, MD), averaging 200–225 g, were 70% partially hepatectomized under Metophane inhalation anesthesia as described previously.²⁰ All animals were allowed standard rat chow and water *ad libitum* and maintained in a 12-hour light-dark schedule. Control (unmanipulated) rats and rats at designated time points following surgery (5 minutes, 24, 48, 72, 96, 120 hours, and 14 days) were anesthetized with 300 μ L per 200 g intraperitoneal nembutal, and livers were processed as described below.

Liver Perfusion and Processing for Ultrastructural Analysis

For both scanning electron microscopy (SEM) and transmission electron microscopy (TEM), normal liver, or liver at specific time points following PHx, was cleared of blood by perfusion with phosphate-buffered saline (PBS) at 3 mL/min through the inferior vena cava as described by Seglen.²¹ Fifty milliliters of 2.5% glutaraldehyde in PBS was then perfused through the inferior vena cava at the same rate. The livers were then quickly removed and immersed in 2.5% glutaraldehyde for 2 days at 4°C. At this point, small samples of livers were reserved and processed for TEM as described previously.²² Thick sections (300 nm) for light microscopy were heated onto glass slides and stained with filtered 0.5% aqueous toluidine blue and rinsed with water. For SEM, perfused fixed livers were sliced into approximately 3-mm-thick samples. After three 15-minute washes with PBS buffer, the samples were postfixed in 1% aqueous OsO₄ for 6 hours at room temperature. After 5 additional 15-minute washes in PBS buffer, the samples were dehydrated with a graded series of 30% to 100% ethanol, and then critical-point-dried using an Emscope CPD750 critical point drier (Kent, England). The samples were then sputter-coated with gold/palladium, viewed under a Hitachi 2460N scanning electron microscope, and images were taken with the interactive Quartz PCI (Hitachi, version 3, Mountainview, CA) image system.

SEM and Light-Microscopy Sampling and Image Analysis

Regions up to 100 μ m in diameter around the portal triads were considered to be part of zone 1, and regions up to 100 μ m in diameter around the central veins were considered to be part of zone 3.²³

Fenestrations. At least 30 high-magnification images from 2 to 3 different rat livers were captured that resulted in a total of at least 1,500 separate fenestration readings for each zone at each time point, including control livers. Images were captured at low magnification (300 $\times$) showing the zonal regions and high-magnification images (20,000–25,000 $\times$) of the interior of sinusoids showing various clusters of fenestrations scattered freely or arranged in sieve plates. Fenestrations were considered to have diameters ranging from 20 to 300 nm, and those larger were considered to be “gaps,” probably resulting from the fusion of 2 or more fenestrations, or areas between cells. Fenestration diameters in each image were measured using NIH Im-

age analysis, version 1.62. The statistical significance in comparisons of fenestration diameters between zones 1 and 3 for each time point was evaluated by InStat (version 3.0, GraphPad Software, San Diego, CA) using the unpaired Student *t* test. Statistical significance was determined at *P* < .05.

Porosity. Twenty-four to 33 SEM images were taken for each time point and each zone at 10,000 $\times$ to 15,000 $\times$. Porosity was determined using MetaMorph Imaging System (Version 4.5, Universal Imaging System, West Chester, PA) by evaluating inclusive threshold on predetermined areas. Values were analyzed for statistical relevance using InStat statistical software.

Plate-Width Determinations. Toluidine blue thick-section images captured at 400 $\times$ were used to evaluate hepatic plate widths in both zones 1 and 3 of control and regenerating livers. At least 5 to 11 separate images from each zone and time point were examined. Plate widths were measured using the MetaMorph Imaging System.

Vascular Corrosion Casting

Vascular casts of normal and regenerating liver were prepared for SEM analysis. Livers were cleared of blood with PBS and perfused with Batson's #17 methyl methacrylate prepared according to the manufacturer's directions (Polysciences, Warrington, PA). Resin was allowed to polymerize at 50°C for 1 hour *in situ*, and then the liver was removed and tissue was digested by immersion in several changes of 20% KOH at 50°C for several days. Cleaned casts were rinsed several times in distilled water, air-dried, mounted on aluminum stubs, and sputter-coated with gold/palladium before viewing on a JEOL T300 scanning electron microscope at 20 kV.

Immunofluorescence Microscopy

Livers were harvested at designated times in one of two ways, depending on immunoreactivity of antibodies under specific fixation conditions. For one set, harvested livers were immediately frozen in liquid nitrogen. In another set, livers were perfused-fixed in 2% paraformaldehyde in PBS after clearing livers with PBS. Fixed livers were immersed in 2.3 mol/L sucrose in PBS overnight at 4°C, and then frozen in liquid nitrogen-cooled 2-methylpentane. Livers were stored at –80°C until sectioned. Livers were sectioned at 5 μ m at –18°C and affixed to charged (Superfrost/Plus, Fisher, Pittsburgh, PA) or gelatin-subbed slides (0.5% gelatin, 0.05% chromium potassium sulfate in ddH₂O). Antibodies to collagen I, collagen IV, laminin, and fibronectin were purchased from and applied to specifically fixed livers as described in Table 1. Tissue was rinsed 3 times in PBS, rinsed 3 times in PBS containing 0.5% bovine serum albumin, 0.15% glycine (PBG buffer [PBS supplemented with 0.5% bovine serum albumin and 0.15% glycine]) blocked in 20% nonimmune goat, or donkey serum in PBG buffer for 30 minutes at room temperature. Primary antibodies diluted in PBG buffer were added to sections at dilutions described in Table 1. Sections were washed 5 times in PBG buffer, and then fluorescently tagged secondary antibodies (Table 1), diluted in PBG buffer, were added to the sections for 1 hour at room temperature. Tissue was washed 3 times in PBG buffer, 3 times in PBS, and then nuclei were stained using 0.01% Hoechst dye (bis Benzimide) in ddH₂O for 30 seconds. Following a wash in PBS, tissue was cover-slipped using gelvatol (23 g poly[vinyl alcohol] 2000, 50 mL glycerol, 0.1% sodium azide to 100 mL PBS) and viewed on a Nikon Eclipse epi-fluorescence microscope (Melville, NY). Digital images were obtained on a Sony CCD camera (Tokyo, Japan) using Optimas image acquisition software with a frame grabber board. Collages were prepared using Photoshop (Version 5.5, Adobe Systems, Inc., San Jose, CA).

RESULTS

Porosity and Fenestration Diameter. Livers were isolated without manipulation (control) for 5 minutes, 24, 48, 72 hours, 4, 5, or 14 days following 70% PHx, and then processed for SEM. Porosity index (the area of sinusoidal surface covered by fenestrations and gaps), and mean fenestration diameter (trans-

TABLE 1. Immunofluorescence Staining Protocols

Primary Antibody	Source	Liver Treatment		Secondary Antibody Dilution [‡]
		Liver Fixation*	Postsection Fix [†]	
Rabbit anti-rat collagen I 1:100, 2 hours (goat serum block)	Chemicon, Cat# AB755P, Temecula, CA	None	−20°C Methanol Gelatin-subbed slides	Goat anti-rabbit Cy3 1:3,000
Mab anti-collagen IV 1:100, 2 hours (goat serum block)	Biodesign International Cat# MC4-HA, Kennebunk, ME	None	−20°C Methanol Gelatin-subbed slides	Goat anti-mouse Cy3 1:3,000
Rabbit anti-rat fibronectin 1:100, 2 hr (goat serum block)	Gibco-BRL, Cat# 12113-05, Grand Island, NY	2% Paraformaldehyde-perfused	None Charged slides	Goat anti-rabbit Cy3 1:3,000
Goat anti-γ1-laminin 1:100 overnight 4°C (donkey serum block)	Santa Cruz Biotechnology, Cat# SC-609, Santa Cruz, CA	2% Paraformaldehyde-perfused	None Charged slides	Donkey anti-goat Cy3 1:500

Conditions of liver tissue section staining for fluorescence microscopy.

*None: liver snap-frozen in liquid nitrogen following harvest; 2% paraformaldehyde-perfused: Liver cleared of blood using PBS, then perfused-fixed with 2% paraformaldehyde in PBS.

[†]Treatment following sectioning. Sections placed onto gelatin-subbed slides, then fixed in −20°C methanol. Charged slides used for fixed tissue.

[‡]All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, West Grove, PA, and incubated for 1 hour at room temperature.

cytotic pores with diameters between 20 and 300 nm), were evaluated in zone 1 and zone 3. Table 2 shows average porosity index as well as the mean fenestral diameter for each time point at each zone in control rats, as well as at designated times following PHx. These measurements are illustrated graphically in Fig. 1 and show parallel trends in both fenestration diameter and porosity index in each zone. In control rats, the mean fenestral diameter and porosity index in zone 1 was significantly smaller than those observed in zone 3. Striking porosity increases were observed at 5 minutes and remained elevated in zone 1 until 48 hr post-PHx. Fenestrations and porosities steadily decreased until 72 hr, at which time they were observed to be significantly smaller than controls in both zones. Fenestrations in both zones recovered to near-preresection levels by 14 days, but porosity index remained above normal levels. Representative SEM micrographs for most time points are shown in Fig. 2. Many fenestrations are arranged into distinct collections called sieve plates, but many single fenestrations were also observed, indicating that fenestrations need not be arranged into groups. At 5 min and 24 hr post-PHx, a greater number of intraendothelial gaps were present than in control, resulting in the increased porosity measurements documented in Fig. 1 and Table 2.

Figure 3 shows the percent distribution of fenestration size as a function of diameter range. Fenestration diameters were distributed into a typical gaussian pattern, when evaluated at 20-nm steps between 0 to 140 nm. Larger fenestrae, between 140 to 300 nm, were grouped into 2 ranges: 140-200 and 200-300, as they were rare. Gaps are included in the porosity index measurements, because it is unclear if these larger openings are actually the result of fusion between several smaller fenestrations or entire sieve plates.

Consistent with the observation that the zone 1:zone 3 ratio of both porosity and fenestration diameter was greater than 1 at 5 min and 24 hr post-PHx (Table 2), we expected that chylomicron remnants would have greater access to the space of Disse and hepatocytes in zone 1, resulting in greater amount of lipid inclusions in these hepatocytes. Figure 4 shows that the significant increase in zone 1 porosity was indeed accompanied by a parallel increase in lipid accumulation in periportal hepatocytes, but not those situated within the pericentral area of the lobule. The lipid nature of the inclusions was verified by Oil Red O staining (data not shown).

We were intrigued by the striking decrease in and fenestral diameters at 72 hr post-PHx. It is known that, at this time,

TABLE 2. Zonal Porosity and Fenestration Measurements

Time After PHx	No. of Rats	Zone 1		Zone 3		Z1:Z3 Ratio	
		Porosity*	Fenestration Mean Diameter [†] (μm)	Porosity*	Fenestration Mean Diameter [†] (μm)	Porosity*	Fenestrations
Control	3	6.81 ± 0.50	76.57 ± 1.40	11.63 ± 0.94	97.92 ± 1.45	0.59	0.87
5 min	2	22.20 ± 1.26	112.26 ± 1.80	19.99 ± 1.57	91.44 ± 1.94	1.12	1.20
24 hr	3	15.56 ± 1.17	122.05 ± 1.77	11.28 ± 0.58	107.17 ± 1.78	1.37	1.17
48 hr	3	11.57 ± 0.72	93.93 ± 1.17	12.47 ± 0.74	99.88 ± 1.57	0.93	1.00
72 hr	3	3.79 ± 0.43	69.00 ± 0.99	5.10 ± 0.43	76.00 ± 1.25	0.74	0.90
4 days	3	10.44 ± 0.98	88.36 ± 1.37	15.49 ± 1.02	89.70 ± 1.39	0.67	0.92
5 days	3	13.06 ± 1.12	92.73 ± 1.67	17.56 ± 1.16	114.43 ± 1.94	0.74	0.83
14 days	2	9.56 ± 0.99	81.17 ± 1.20	15.36 ± 1.02	93.42 ± 1.29	0.62	0.87

NOTE. Zone 1 measurements were taken from images captured 100 μm from the portal triad area. Zone 3 measurements were taken from images captured 100 μm from the central vein. Each measurement given as mean ± SEM. See also Fig. 1 for statistical significance.

*Porosity measurements were assessed as the total percent open surface area occupied by both fenestrations (diameter less than 300 nm) and intraendothelial cell gaps (diameters greater than 300 nm). For each time point and zone, n = 24-33 images taken at 10,000× to 15,000×.

[†]Fenestrations were determined to be transcytotic pores equal to or less than 300 nm. For each time point and zone, n ≥ 1,500 measurements of fenestration diameters analyzed from 30 images taken at 20,000-25,000×.

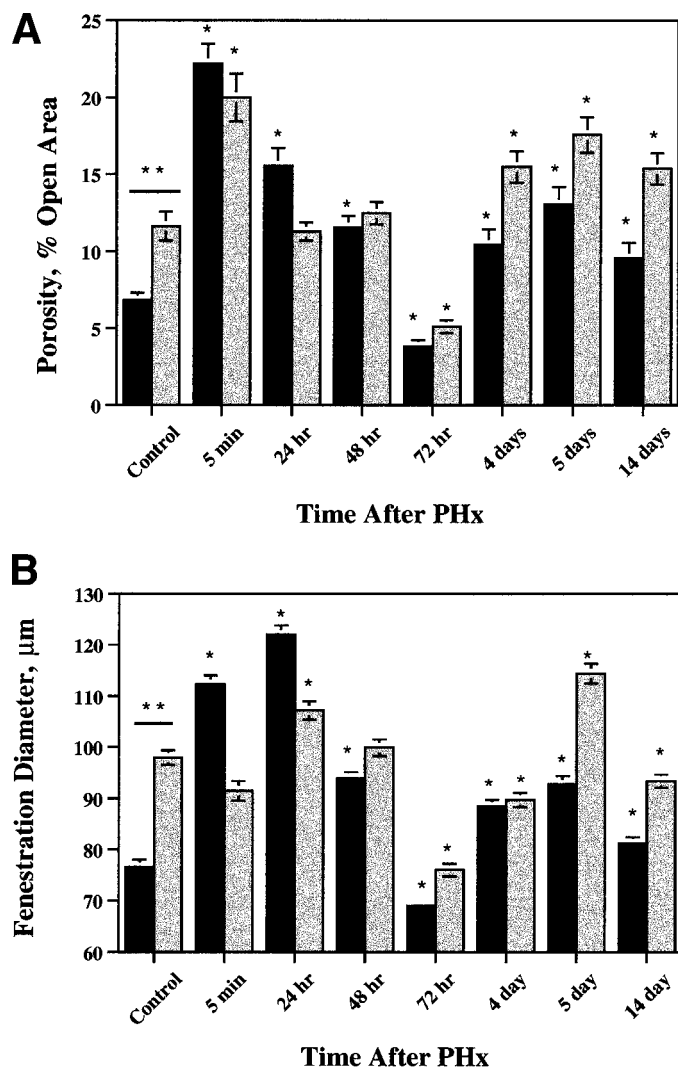


FIG. 1. Graphic representation of mean porosity index (A) and fenestration diameters (B) in zone 1 and zone 3 during liver regeneration after PHx (see also Table 2). (A) In nonmanipulated control liver, the porosity is significantly greater in zone 3 vs. zone 1 (** $P < 0.0001$). At 5 minutes, both zones show a significant increase in porosity over control ($P < .0001$) and a significant decrease over control at 72 hours ($P < .0001$). *Significant change from control zone ($P < .05$). (B) Fenestration diameters essentially parallel the trends seen in the porosity index (A). In control liver, the mean fenestration diameter is significantly greater in zone 3 vs. zone 1 (** $P < .0001$). *Significant change from control zone ($P < .05$). (See also Fig. 2 and Table 2.) (■) Zone 1; (▒), zone 3.

hepatocyte proliferation has subsided and SEC proliferation has just initiated.¹ It follows that at 72 hours following PHx, the hepatocyte:SEC ratio is at its highest and hepatocytes are not arranged in typical plate-like architecture, bounded on either basolateral side by SEC. Instead, areas of hepatocyte islands without a blood supply are abundant throughout the lobule. We examined the general vascular morphology of sinusoids by low-magnification SEM surveys and vascular casting at 72 hr post-PHx. As shown by SEM in Fig. 5A and 5B, sinusoids approaching avascular islands become increasingly compressed, whereas sinusoids in nonregenerating livers maintain a relatively consistent lobular distribution, density, and sinusoidal width. These areas, when visualized by vascular corrosion casting, showed lower sinusoid density at 72 hr post-PHx than in nonmanipulated controls (Fig. 5C vs. 5D). These sinusoids also appeared wider and flatter than those in

control livers. The sinusoids often end abruptly, indicating loss of vessel continuity along the portal-central axis where the proliferative hepatic islands have interrupted, or severely constricted, sinusoidal blood flow. At higher magnification (Fig. 5F), vascular growth, as evidenced by sinusoidal casts terminating in "points" and blind ends, was observed growing toward the avascular sites. Often, the sinusoidal casts in these areas were extremely flattened, compared with controls (Fig. 5E).

To correlate the observations of the flattening of the sinusoidal spaces with known temporal proliferative data of hepatocytes and SEC, we investigated the changes in hepatic plate width in both zones during the regenerative response. Figure 6 and Table 3 show that hepatic plate widths do indeed increase significantly in both zones at 24 hr post-PHx, at the time when hepatocytes have peak DNA synthesis. Hepatic plate-width averages remain highly elevated over controls up to the time, when SEC begin their peak proliferative wave at 4 d post-PHx. Additionally, there is a large increase in the frequency of large clusters of hepatocytes (widths of 30–60 μm) from the average plate widths of 20 to 30 μm between 24 hr and 4 d postresection. Interestingly, plate widths remain significantly larger than those in control zones even at 14 days after PHx.

Next, we evaluated the relationship among SEC and other nonparenchymal cells and hepatocytes at 72 hr post-PHx by TEM. At this time, as also observed previously by other techniques (Figs. 4, 5, and 6), hepatocytes are not arranged in typical plate-like fashion, but are in groups of several cells in between flattened sinusoids (Fig. 7). Interestingly, SEC often appear to be closely associated with another cell type within the space of Disse, presumably stellate-type cells (Fig. 7B). At higher magnification (Fig. 7D), the SEC often appear nearly completely enveloped on the basolateral side by a stellate-type cell, making numerous close cell-cell interactions along the periphery. This phenomenon, while seen frequently at 72 hr post-PHx in all zones of the lobule, was not observed with regularity at any other time point following PHx. High magnification of the areas of close contact show that there is deposition of matrix material between the cells in these areas (Fig. 7E and 7F). During the regeneration period, deposition of true basement membrane ECM ultrastructure was not observed in the space of Disse. This would be recognized as an increase in continuous electron-dense material in close apposition to the basolateral membrane of the SEC. Additionally, these stellate-type cells do not appear to have many lipid inclusions, typical of stellate cells, but show fibrous or flocculent material within vesicular structures (Fig. 7D, insert), and may represent a more intermediate cell stage between resting and activated stellate cells. When comparing similar-sized sinusoids in zone 1, the control SEC show many gaps and fenestrations, while those SEC in close association with stellate-type cells show numerous small fenestrations, but very few gaps. This observation is consistent with the SEM fenestration and porosity data presented in Figs. 1–3.

Given that production of true basement membrane within the sinusoid was not apparent at any time during regeneration (data not shown), we evaluated the presence of various ECM proteins in the space of Disse at 24, 72, and 96 hours following PHx using immunofluorescent labeling. We then compared the expression of these proteins with those in control livers to determine if the expression levels of one particular matrix

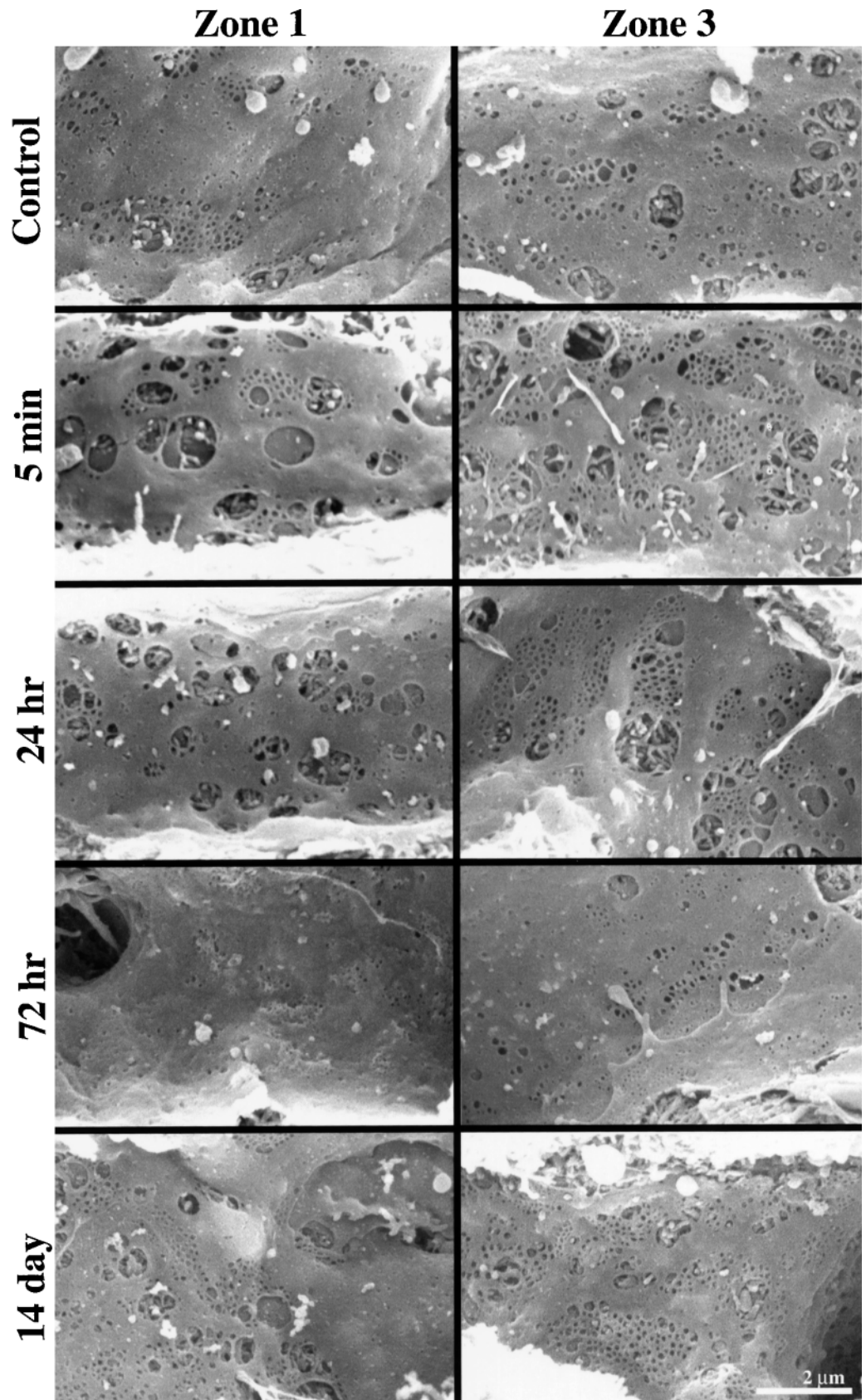


FIG. 2. Representative micrographs of zone 1 and zone 3 SEC fenestrations and intraendothelial cell gaps in control and regenerating liver (see also Fig. 1 and Table 2).

protein vary temporally and spatially with the changes in fenestration diameters observed in Figs. 1-3. Others have reported that ECM composition can influence fenestration diameter^{18,19} and that presented a valid argument for evaluation

of ECM protein deposition in this study. Because porosity increases dramatically in the first 24 hours after PHx, survey of resident ECM proteins within the space of Disse may provide evidence for matrix control of SEC porosity *in vivo*. Fig-

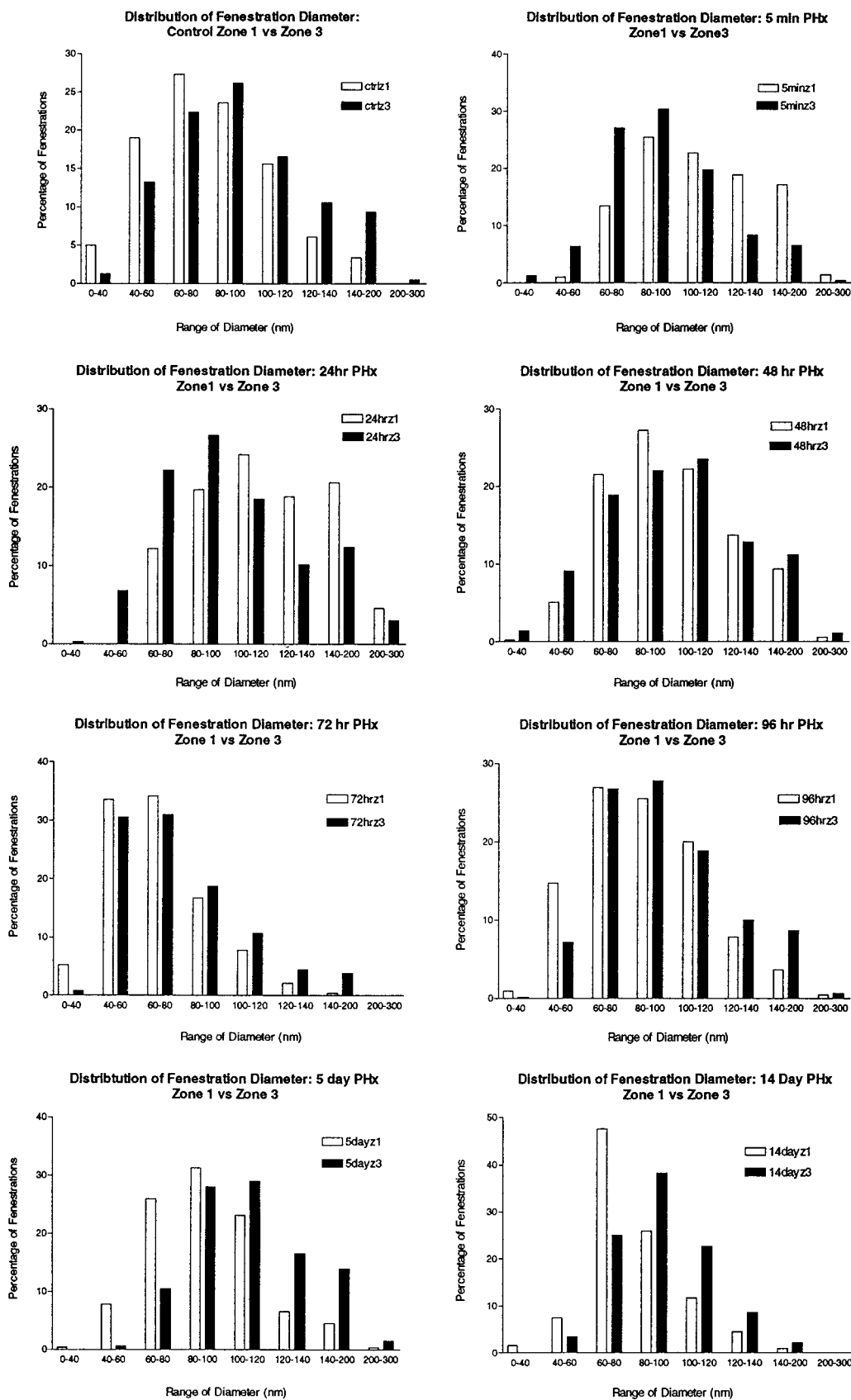


FIG. 3. Histogrammic examination of the ranges of fenestration diameter distributions between zones at specific times during regeneration. Fenestrations show a gaussian size distribution at all times examined.

ure 8 examines periportal (zone 1) regions for variations in the expression of collagen I, collagen IV, fibronectin, and laminin ($\gamma 1$ subunit, also known as B2 subunit) in nonresected livers and livers 24 hr post-PHx. Laminin $\gamma 1$ -specific

antibodies were used because this subunit is present in all laminin isoforms in liver.^{5,6} Collagen I and IV and fibronectin do not appear to increase or decrease dramatically in the sinusoids from control levels at 24 hr post-PHx. Laminin is

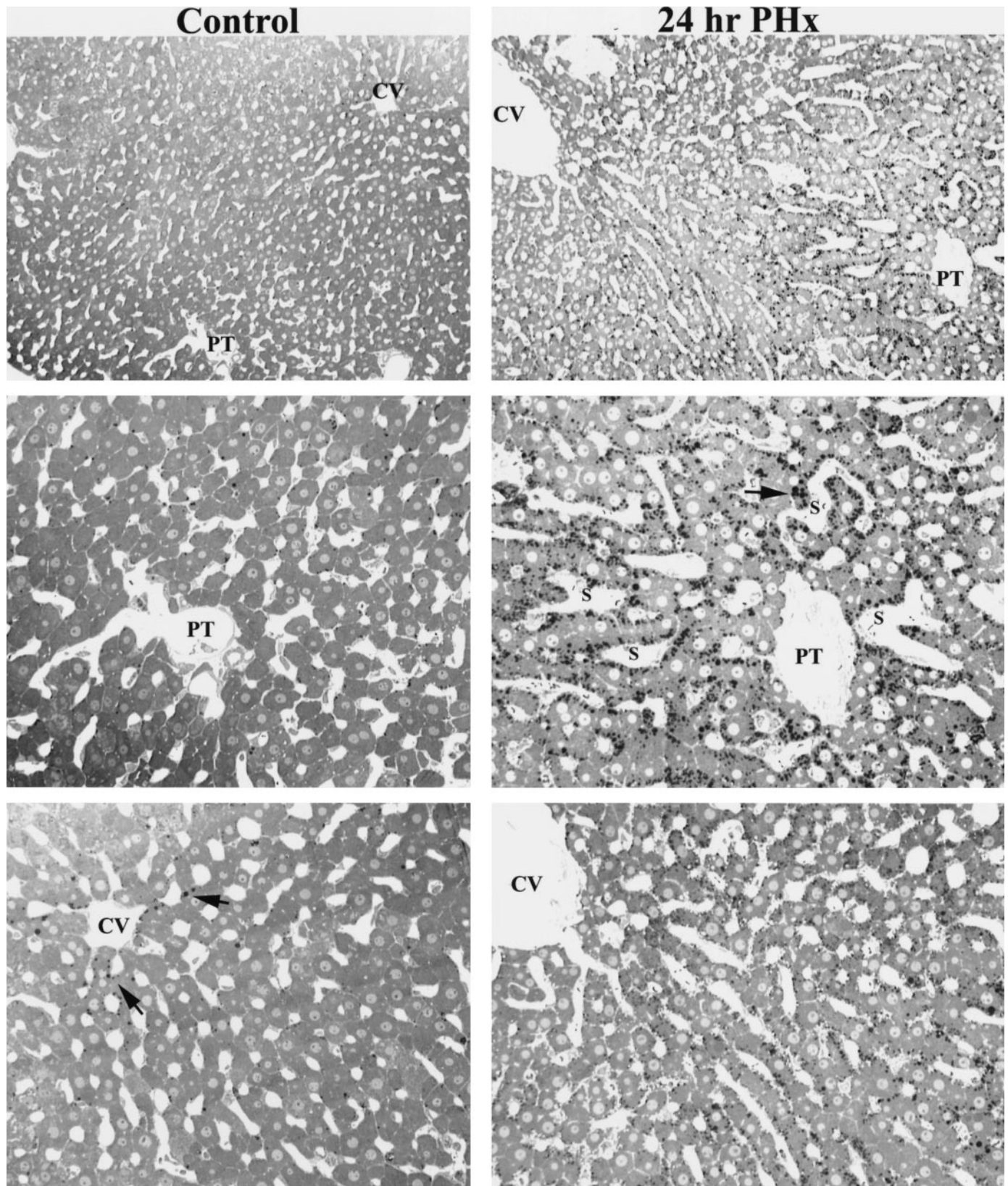


FIG. 4. A gradient of lipid droplet (arrows) accumulation along the portal-central axis is apparent at 24 hr post-PHx. Light micrographs of toluidine blue-stained thick sections of TEM-processed 24 hr post-PHx livers show large lipid droplets in close apposition to basolateral domains of hepatocytes around the portal triads in zone 1 (portal triad [PT]), with little deposition apparent in pericentral zone 3 (central vein [CV]). At this time, porosity and fenestration diameters are greater in zone 1 than in zone 3 (see Figs. 1 and 2 and Table 2). In control livers, where fenestrations are normally larger around zone 3, more lipid accumulation is evident. *Top panels*, low magnification ($\times 200$); *lower 4 panels*, higher magnifications of top panels ($\times 400$).

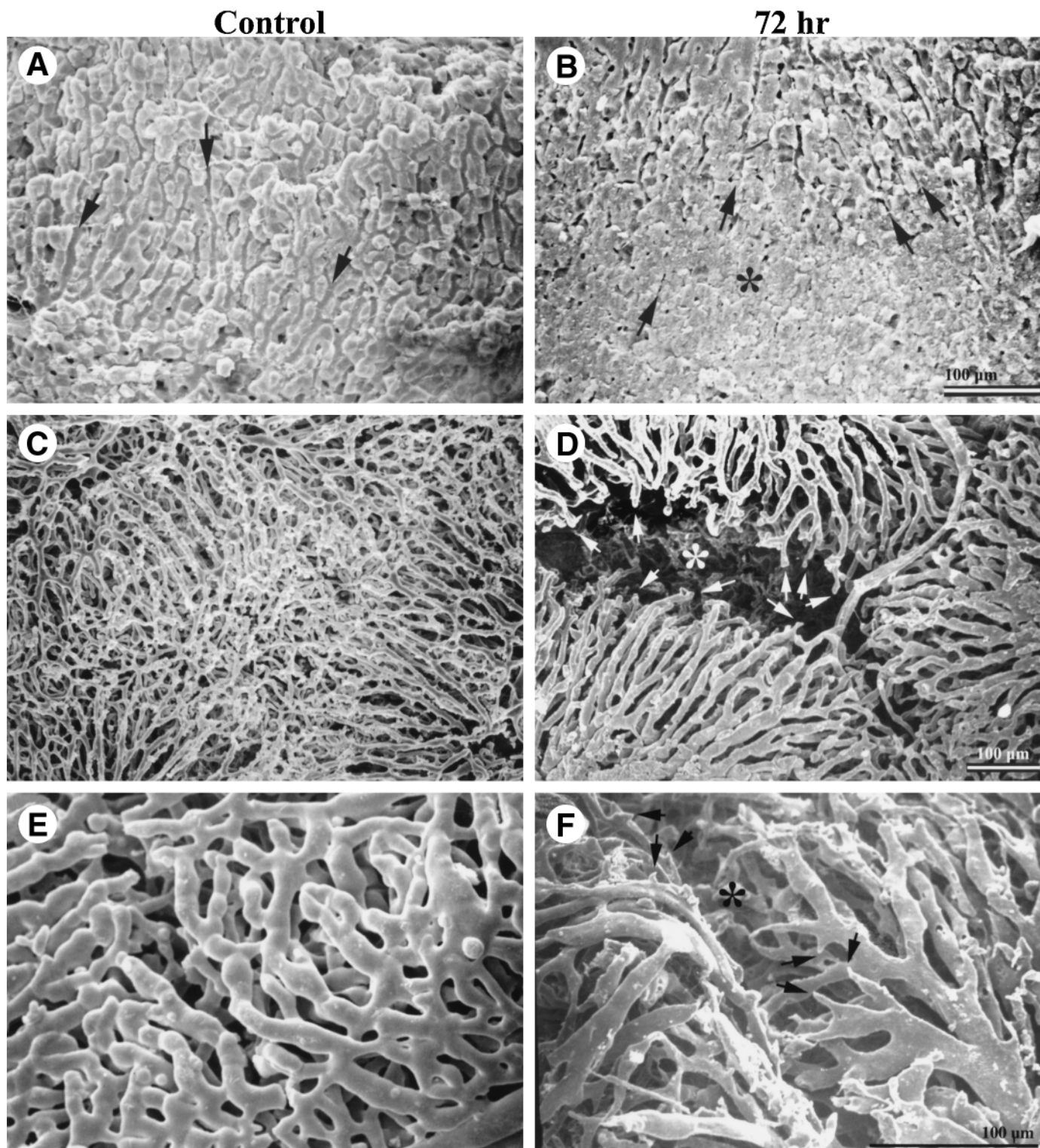


FIG. 5. SEM survey of control (A) and regenerating rat livers 72 hr post-PHx (B). Arrows indicate sinusoids that are of consistent width in control livers, but are narrowed as they approach avascular hepatic islands (*). Vascular casts of control (C, E) and regenerating rat livers 72 hr post-PHx (D, F). Control casts show sinusoids of relatively even width, while regenerating livers display voids in the casts (D and F, *) that correspond to large avascular hepatic islands, often 50 to 70 μm in width (B). Sinusoid termini adjacent to the voids display blind and pointed ends (arrows) indicative of vessel growth into the avascular hepatic islands.

increased at this time, although the staining appears diffuse along the entire sinusoid.

Figure 9 shows representative zonal survey panels of staining for collagen I, collagen IV, laminin, and fibronectin in the

sinusoids at 72 and 96 hr post-PHx. At 72 hr post-PHx, fenestrations and porosity index are the smallest in both zones, while at 96 hours, they return to closer to prerection sizes (Figs. 1, 2). In general, the deposition of all 4 proteins was

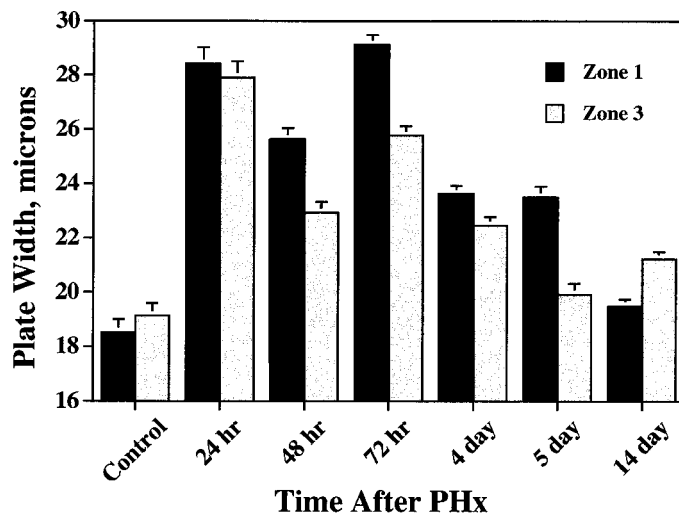


FIG. 6. Examination of intersinusoidal hepatic plate widths in regenerating rat livers. A significant increase in hepatic plate width in both zones 1 and 3 is observed after the first round of hepatocyte proliferation (24 hours) in the absence of SEC proliferation. This significant increase in plate width in both zones is maintained until 3 to 4 d post PHx, after which it gradually approaches, but does not revert back to, preresection plate widths. In general, zone 1 plates are larger than those in zone 3 in regenerating liver (24 hours to 5 day) as opposed to proliferatively quiescent livers (control, 14 days). At every time point, significant increases ($P < .05$) are seen over corresponding control zones. (See also Table 3.)

primarily concentrated around the large vessels occupying zones 1 and 3. In these surveys, levels of collagen I and IV appear to remain constant during the time periods examined. Laminin, which is also normally concentrated around large vessels, also showed a substantial increase in deposition at 72 hr post-PHx throughout the lobule at the sinusoidal surface. By 96 hours, however, the sinusoidal staining had decreased, but the periportal and pericentral laminin remained higher than in control livers. Fibronectin staining was evenly distributed throughout the lobule and did not appear to change in the sinusoids when comparing control and 72 hr post-PHx, although the staining around large vessels was notably increased. Fibronectin staining pattern at 72 hours also highlights the sinusoid dilatation observed by vascular casting (Fig. 5D and 5F). By 96 hours, a visible decrease in fibronectin staining was observed in the sinusoids, when compared with the control and 72-hour time point, while maintaining the large vessel staining observed at 72 hours.

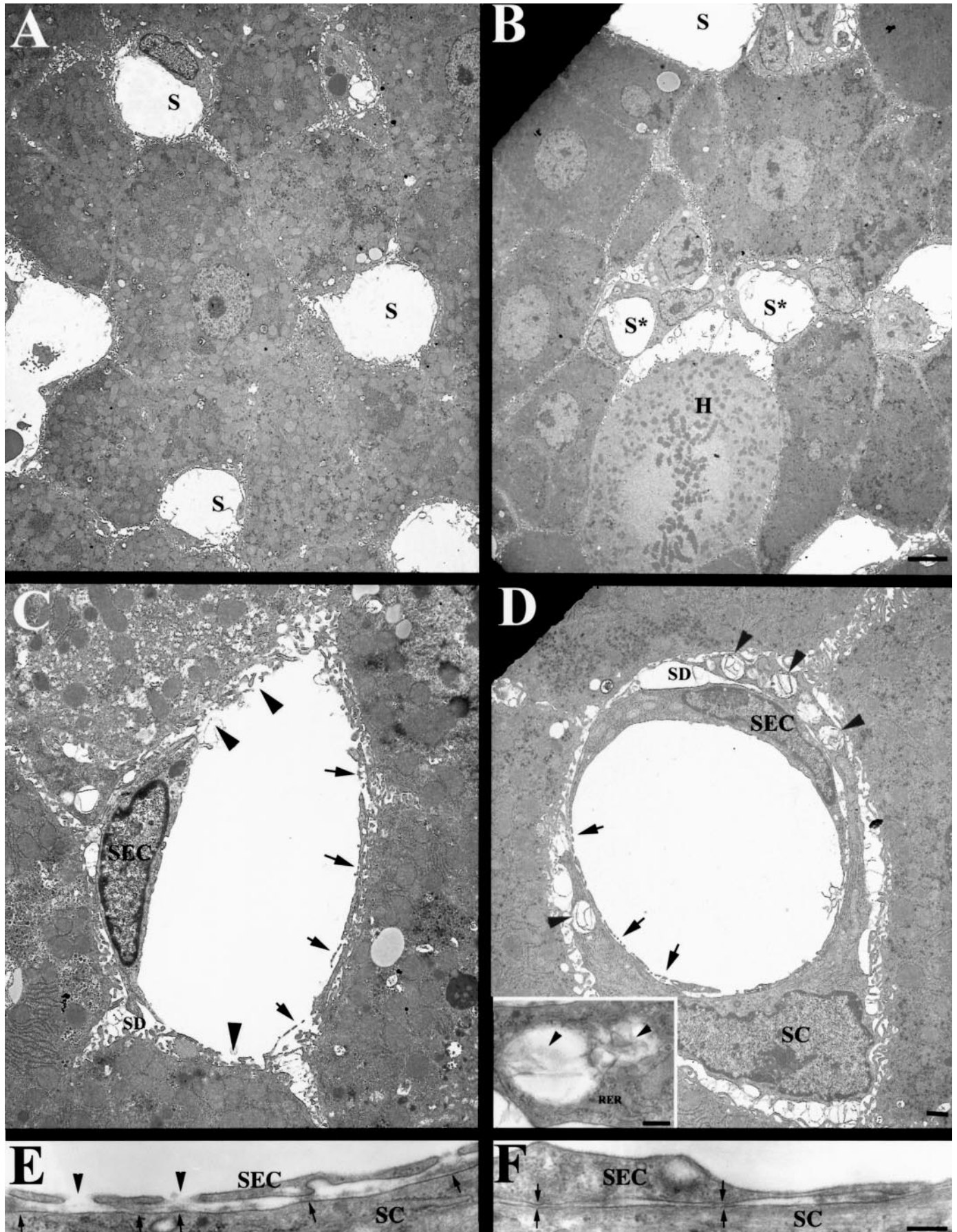
DISCUSSION

Zonal Porosity Changes During Regeneration. The undiaphragmed, fenestrated endothelium of the liver sinusoid provide a variably permeable barrier between the blood and parenchyma. In this capacity, the fenestrations can modulate liver metabolism by regulating the traffic of an innumerable variety of soluble agents, including growth factors, cytokines and proteases, and particulates such as lipoproteins and chylomicron remnants. This is believed to be accomplished by adjusting the diameter and/or number of fenestrae via an actin-based contraction system.^{13,16,17} Compelling data from several laboratories have documented the exquisite zonal variation of sinusoidal porosity as a function of fenestration diameter and number along the portal-central axis of the lobule.^{9,23-25} In

TABLE 3. Zonal Hepatic Plate Width Measurements

	Control			24 hr			48 hr			72 hr			4 day			5 day			14 day		
	Z1	Z3		Z1	Z3		Z1	Z3		Z1	Z3		Z1	Z3		Z1	Z3		Z1	Z3	
Average (μ m)	18.53	19.13		28.42	27.91		25.64	22.94		29.13	25.77		20.62	22.49		22.61	20.12		19.49	21.23	
SEM	0.35	0.47		0.62	0.61		0.52	0.41		0.58	0.35		0.31	0.33		0.59	0.40		0.38	0.25	
Median (μ m)	18.65	20.00		25.11	25.04		24.86	20.00		24.41	25.00		21.01	20.04		20.12	18.06		20.00	20.00	
Range (μ m)	10.00-35.00	9.95-35.43		9.29-71.06	10.00-75.00		10.10-76.00	10.00-51.80		11.50-75.70	10.00-72.90		8.00-60.00	9.10-73.60		7.50-60.00	7.00-60.00		7.30-41.00	7.5-53.00	
n	294	203		300	400		288	401		550	635		770	787		341	590		570	866	
Plate-width frequencies (%)																					
0-20 μ m	56.61	48.28		19.06	23.00		23.96	35.91		27.82	25.35		33.12	42.95		38.71	57.12		45.79	42.73	
20-30 μ m	34.92	39.41		44.48	39.00		46.53	37.91		37.45	44.41		43.25	38.25		32.55	26.10		47.02	45.38	
30-40 μ m	7.46	11.33		22.41	17.50		19.10	16.96		11.82	18.90		15.32	11.56		16.42	7.80		6.14	9.35	
40-50 μ m	—	—		9.36	12.75		6.94	7.98		9.27	8.82		5.06	4.70		6.74	5.42		0.70	0.58	
50-60 μ m	—	—		2.01	4.25		1.74	0.50		7.45	1.57		2.73	2.03		3.23	3.90		—	2.19	
60-70 μ m	—	—		1.34	1.50		0.35	—		4.18	0.31		—	0.13		—	—		—	—	
70-80 μ m	—	—		0.33	0.50		—	—		0.91	0.16		—	—		—	—		—	—	

NOTE: Evaluation of zonal intersinusoidal hepatic plate widths during regeneration. Measurements were made on toluidine blue-stained thick sections (300 nm) of tissues prepared for TEM as described in Materials and Methods (see also Fig. 6). The lower part of the table describes frequencies of specific plate widths, showing a shift to more areas with larger intersinusoidal diffusional distances at 24 to 72 hours post-PHx.



vitro and *in vivo* studies have also shown that fenestrae can modulate their diameter in response to soluble agents as well as a variety of environmental conditions, including changes in shear stress and portal pressure, and pathological ECM deposition.¹⁰⁻¹⁹

We sought to investigate the trends in porosity expression in the SEC within and between lobule zones throughout the revascularization process following PHx. We believed that such changes may play a critical role in the sieving function of the sinusoids and contribute to the progression of the regenerative process. Furthermore, porosity changes are most likely in response to modulation of the local SEC environment. Changes with respect to local environments, including vessel architecture, hepatic plate widths, cell-cell interactions, and ECM deposition in the space of Disse during liver regeneration, have not yet been thoroughly investigated during the regenerative and revascularization processes. The fenestration sizes documented here for each zone correspond well with several published reports on rat liver SEC, indicating that zone 1 (we report 76.57 nm) fenestrations are smaller than those in zone 3 (we report 97.92 nm),^{23,24} but others describe the opposite.^{9,26,27} The reasons for these discrepancies are unclear, but strain, age and weight of rat, metabolic differences among the animals used, preparation of tissue, as well as sampling techniques in these studies may contribute to variation in mean fenestration size. Inclusion of "gaps"—those pores larger than 300 μm in diameter—as fenestrae could skew the mean diameter to the larger size, especially if there are more "gaps" in zone 1 than zone 3. Our mean porosity measurements also vary from previously published reports,^{11,23} but otherwise correlate well in that porosity is greater in zone 3 than in zone 1.^{9,23}

Previous work has suggested that direction of perfusion, either anterograde or retrograde to inherent blood flow, can influence fenestration size.²³ However, the zone 1:zone 3 ratio remains consistent when comparing the fenestration diameters prepared by perfusion fixation in either direction. In the present study, we perfused-fixed in retrograde fashion at a very low flow rate (3 mL/min), resulting in the zone 1 fenestrae smaller than those in zone 3 in control livers, and porosity measurements higher in zone 3 than 1. Low flow rate was used to assure that excessive pressures would not influence fenestration size.

Over the course of regeneration, we observed that fenestration diameters and porosities resident in each zone change independently, suggesting that the local environments may also change independently. This is not surprising because there exist considerable gradients and heterogeneity along the portal-central hepatic axis, involving hepatocyte metabolism,²⁸ cell composition,²⁸⁻³⁰ space of Disse ECM deposition,^{30,31} and sinusoidal architecture.³² The most striking

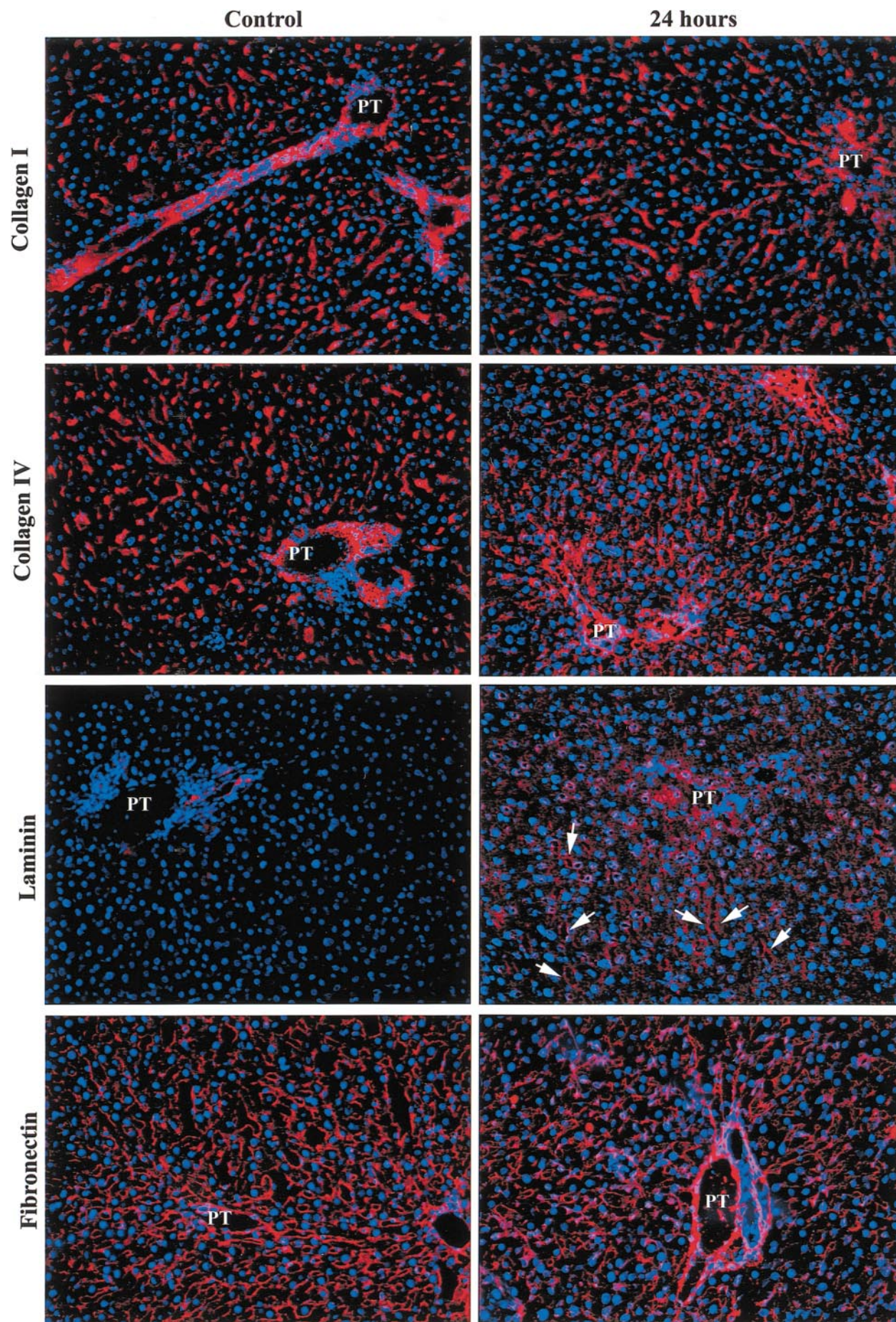
change occurs at 5 minutes following PHx, when porosity and fenestration diameters in zone 1 increase significantly over control. The reasons for such a rapid modulation may reflect changes in a number of criteria immediately after surgical removal of two thirds of the liver, including increases in blood pressure and shear stress, ECM degradation, and changes in the concentrations of soluble effectors as the result of PHx.

Immediately after 70% PHx, intrasinusoidal pressure greatly increases as a result of an increased blood flow velocity and shear stress along the sinusoidal walls.^{10,33-35} At this time, we observed that fenestration diameter and porosity increases significantly in zone 1. Fenestration diameter does not increase as markedly in zone 3, and this could be a result of the pressure differences reported between the 2 zones.^{10,33} Rapid increases in portal pressures are suspected to contribute to fenestration diameters, because artificially high perfusion pressures have been shown to greatly enlarge fenestrations potentially damaging sieve plate structures.³⁶ Increased portal pressure resulting from 70% PHx has been shown to persist at least until 72 hr after resection.^{10,34,35,37} Our results indicate that increased portal pressure is probably not the only effector of porosity, because increased porosity was not maintained throughout the reported period of elevated pressure (Figs. 1-3). For example, at 72 hr post-PHx, when recorded portal pressures were significantly higher than controls,^{34,37} we observed the smallest fenestrations and lowest porosity index in both zones during the revascularization process.

Consistent with the larger porosity index in zone 1 at 24 hours after PHx, increased accumulation of lipid droplets was evident in hepatocytes residing in the periportal area (Fig. 4). This correlation has been shown previously as a general phenomenon in the midlobular region of the sinusoid within 24 hours of resection.¹¹ Here, we report that a gradient of lipid deposition is spatially and temporally related to the increased fenestration size along the portal-central axis. Under conditions of increased fenestration size in zone 1, chylomicron remnants are not excluded from hepatocytes by the SEC. This finding has functional significance in that increased permeability in zone 1 during the first 24 hours may contribute to the fact that DNA synthesis of hepatocytes is initiated in that area of the lobule before those in zone 2 or 3.³

Ultrastructural Examination of Sinusoids During Regeneration. We evaluated ultrastructural characteristics of the sinusoid that may contribute to the porosity and fenestral diameter reduction at 72 hr post-PHx. Low-magnification SEM surveys of the lobule indicate areas that are nearly devoid of vascularization at this time (Fig. 5B). Sinusoids compress to a fraction of their original width as they approach these avascular hepatic islands. Additional information on sinusoid structure was obtained using vascular casting techniques on regenerating sinusoids (Figure 5C-5F). Control liver casts show a finely spaced meshwork of vascularization (Fig. 5C). Casts obtained from regenerating livers 72 hr post-PHx show fewer, but flattened, sinusoids that abruptly end, leaving large areas often greater than 70 μm^2 without casting resin (Fig. 5D). We suspect that these voids in the cast represent the avascular hepatic islands, because they often have a similar size to those seen in the SEM surveys (Fig. 5B). On closer inspection, the termini of the casts have pointed or blind ends moving into the void, indicating areas of SEC growth and migration during the revascularization of the islands (Fig. 5F). This cast morphology has been shown to represent neovascularization during wound healing and angiogenesis accompanying tumor

FIG. 7. Ultrastructural examination of normal (A, C) and regenerating rat livers 72 hr post-PHx (B, D). Sinusoids (S) in regenerating livers (*) are often surrounded by stellate-type cells (SC) that have vesicles filled with flocculent material (insert D, arrowheads) often open to their basolateral side (arrowheads). Regenerating SEC surrounded by SC have smaller fenestrations and fewer gaps (D, arrows) than those in normal, nonenveloped SEC (C, arrows). Arrowheads in (C) designate gaps. SD, space of Disse. (H) shows hepatocyte in mitosis. Higher magnification of areas of close contact between SEC and SC at 72 hr post-PHx (E, F) indicate that ECM components may provide the basis for the interaction between these cells (arrows). Bar = 5 μm in (A, B); 1 μm in (C, D); (D insert) = 200 nm. Bar in (F) = 200 nm for (E) and (F).



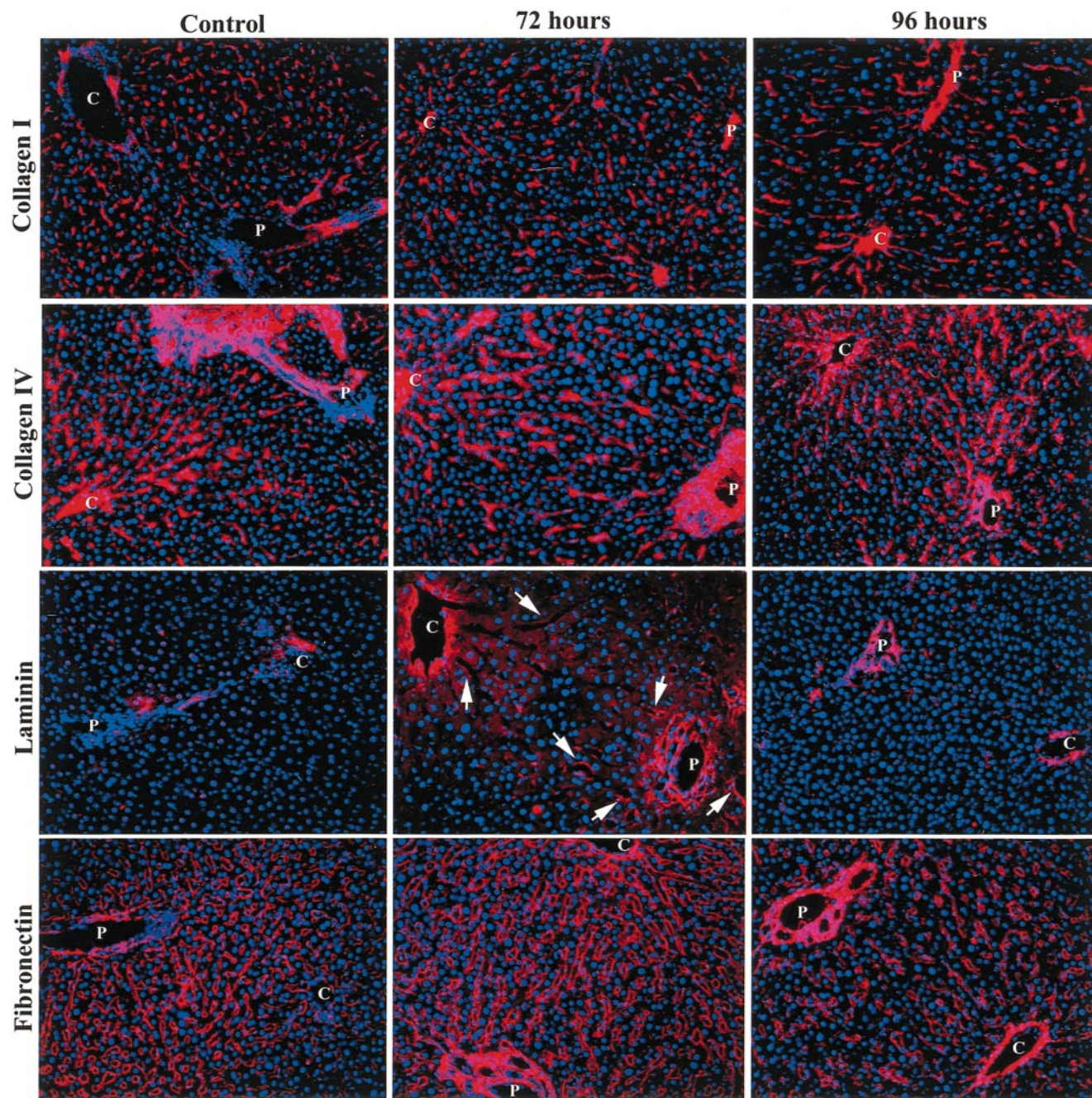


FIG. 9. Immunofluorescent examination of zones 1 and 3 collagen I and IV, laminin, and fibronectin deposition in control and regenerating livers 72 and 96 hours following PHx. Arrows indicate sinusoidal laminin deposition. Labeling prepared as described in Table 1. Red (Cy3 labeling) indicates ECM protein; blue indicates nuclei stained with Hoechst's dye. P, portal triad; C, central vein. (Original magnification $\times 200$.)

growth, and does not represent blockage in resin flow during the casting procedure.^{38,39}

One important aspect of sinusoidal architecture that may influence both regional pressure and porosity is hepatic plate width along the portal-central lobule axis. Because hepato-

cytes proliferate very early, and SEC very late in the regenerative response,¹ areas of avascularity would develop within the liver. Such increases in plate width could effect diffusion of materials between hepatocytes and SEC ultimately influence porosity. Our results indicate that plate width does not appear to correlate directly with zonal porosity changes, because significant increases in plate width in each zone occur between 24 to 72 hours (Fig. 6, Table 3).

Zonal changes in sinusoid width has been documented previously indicating that at 72 hr post-PHx, the width of zone 1 sinusoids decreases from control width by 32%, whereas the zone 3 sinusoid width remains constant.³⁷ Smaller, less-nu-

FIG. 8. Immunofluorescent examination of zone 1 collagen I and IV, laminin, and fibronectin deposition in control and regenerating livers 24 hours following PHx. Arrows indicate sinusoidal laminin deposition. Labeling prepared as described in Table 1. PT, portal triad. Red (Cy3 labeling) indicates ECM protein; blue indicates nuclei stained with Hoechst's dye. (Original magnification $\times 200$.)

merous sinusoids would result in increased intrasinusoidal pressures throughout the lobule. As a result of prior studies, others have concluded that increased intrasinusoidal pressure is responsible for fenestration dilatation.^{10,36} However, these increased pressures have been shown to persist immediately following PHx to 72 hours into the regeneration process. During this time, fenestrations range from the largest (zone 1, 24 hours) to the smallest (zone 3, 72 hours), and therefore do not correlate to elevated pressure values. At this point, we were interested in examining other events within the sinusoid at 72 hr post-PHx that may result in decreased porosity.

Little has been documented about interactions among SEC and other cells present in the sinusoid during liver regeneration and revascularization, and how they may regulate sinusoidal porosity. Because SEM surveys cannot yield detailed information regarding the identity or interactions between SEC and other cells, we undertook a TEM study to evaluate these potential interactions. Previously,^{5,6,22} and in this study, it has been shown that at 72 hr post-PHx, normal hepatic plate structure is not maintained following several waves of hepatocyte division in the absence of SEC proliferation (Figs. 5B and 6, Table 3). Large aggregates of avascularized hepatocytes are situated throughout the lobule. In areas adjacent to these avascular islands, we observed SEC in close association and often nearly completely enveloped by stellate-type, pericyte-, or myofibroblastic-related cells. As a result, the general porosity of the enveloped SEC was reduced (Fig. 7B and 7D), with smaller fenestrations and fewer gaps when compared with control SEC (Fig. 7A and 7C). Direct exchange between blood and parenchyma is impaired within these areas of the sinusoid, and fenestrae do not provide access to the underlying hepatocytes. Additionally, these enveloping cells possess a large number of vesicles (Fig. 7D), often open to the basolateral surface, that are filled with flocculent material. We suspect that this exudate may contain ECM components, synthesized and deposited to aid infiltration of SEC into the avascular islands, as suggested by Martinez-Hernandez et al.^{5,6}

The exact identity of these cells is in question, because they do not possess lipid-filled inclusions typical of stellate cells of resting liver. However, stellate cells are known for their ability to become activated and change phenotype under conditions of stress or injury.⁴⁰ Upon activation, or during proliferation, they often lose their lipid droplets.⁴¹ Additionally, biological markers for these cells also apparently change according to their level of activation or position within the lobule, making them difficult to follow through physiological or pathological responses.⁴⁰ Stellate cells can become myofibroblastic, contributing to extreme ECM deposition seen in pathological conditions of fibrosis and cirrhosis, resulting in the capillarization of the sinusoids.^{19,40} Furthermore, stellate cells are not a homogeneous population of cells and can express varying properties of pericytes, smooth muscle cells, as well as myofibroblastic cells. Few data are available on the activation state and morphology of the stellate cells of regenerating liver. During this time, they may not be totally quiescent, but also do not truly conform to an activated state. However, they do proliferate, peaking at 48 hr post-PHx,⁴² perhaps losing much of their lipid in the process.^{40,41} As a result, the stellate cell/SEC ratio is the greatest at 72 hr post-PHx, allowing for the greatest interaction between these two cell types at a critical period of neovascularization during regeneration following PHx. It is likely that such a ratio could facilitate the vascularization pro-

cess via close exchange of growth, matrix, and chemotactic factors.^{5,6} Clearly, more investigation is required to elucidate the identity, mechanism, and activation state of the stellate cell under these conditions and characterize its role in the revascularization process.

ECM Modulation in the Space of Disse Following PHx. ECM degradation and deposition have been shown to progress in the sinusoid during regeneration^{5,6} and are known to influence SEC fenestrations both *in vitro*¹⁸ and *in vivo*.¹² Previously, it was shown that ECM degradation occurs in the sinusoids immediately following PHx.⁴³ In these studies, periportal and pericentral fibrinogen decreased, and periportal fibronectin decreased at 5 minutes following PHx. This degradation of ECM correlates with the up-regulation of urokinase plasminogen activator enzymatic activity and increased expression of its receptor in the liver within 1 minute after PHx.⁴⁴ Reduction in sinusoidal fibrinogen expression within the sinusoid remained until at least 24 hr post-PHx. Such ECM degradation may be responsible for fenestration dilation, because excessive ECM or basement membrane (BM) deposition has been shown to capillarize SEC.^{18,19} However, the removal of ECM by proteolytic degradation has not been investigated as a potential mechanism for fenestration dilation. We sought to investigate ECM expression relative to control at 24 hr post-PHx to evaluate whether zonal changes in specific ECM proteins correlate to fenestration diameters at this time.

Investigations^{5,6,43-47} showed that several ECM components within the space of Disse undergo modulation throughout the regenerative process. While collagen I and III deposition do not appear to change dramatically,^{5,6,45} collagen IV, fibronectin,^{5,6,45,46} and laminin^{5,6,45} have been shown to display differential deposition within sinusoids at various times. Laminin increases before 6 hr post-PHx⁴⁵ and remains elevated until 3 days after PHx^{5,6,45,47} (as well as findings in this study). Laminin is a major constituent of BM, but formation of a bona fide BM involves deposition and cross-linking by other ECM proteins, notably entactin/nidogen.^{5,6} Because fenestrations are largest in zone 1 between 5 minutes and 24 hours, and decrease thereafter until 3 d post-PHx, when they are the smallest, we were interested in determining if zonal increases in the major ECM proteins of the space of Disse would mirror fenestration and porosity changes.

We observed no major changes in collagen I or IV, an increase in laminin, as well as a slight decrease in fibronectin deposition within zone 1 at 24 hours after PHx (Fig. 8). The change in fibronectin is observed as more diffuse staining as opposed to complete removal of the protein. Laminin deposition within the sinusoids represents the most consistent change of the ECM proteins examined at 24 hr post-PHx. Laminin is a component of BM formation, and pathologic overexpression in the liver is found during fibrosis and contributes to the capillarization of the sinusoids.¹⁹ Our results suggest that laminin deposition alone is not the causative agent for fenestration contraction, because the fenestrations are largest at 24 hours, when increases in laminin deposition are observed.^{5,6,45} At 72 hr post-PHx, during the revascularization stage of regeneration, fenestration diameters in both zones 1 and 3 are at the minimum. At this time, laminin is deposited within the space of Disse in both zones (Fig. 9). Other ECM proteins in the sinusoids do not appear to change significantly in either zone when compared with controls, but increased deposition of collagen IV, fibronectin, as well as laminin is evident around large vessels. Because the endothe-

lium lining the large vessels are not fenestrated, ECM constituents around large vessels do not directly influence fenestrations in the sinusoids. It has been reported that no single ECM protein can modulate SEC fenestration diameters *in vitro*.¹⁸ Our data indicate that specific ECM proteins are not regulating the SEC fenestration diameters in regenerating livers *in vivo*.

By evaluating porosity during regeneration, we expected to associate zonal changes with either specific ECM expression or reported intrasinusoidal pressures. Neither appear to solely correlate with fenestration size during the regenerative process. Given the data we present here, we suspect that there is a synergism among a large number of ECM proteins, ECM breakdown products, as well as a variety of soluble agents. Examples describing unique combinations of ECM components that are required for BM formation have been shown to greatly reduce SEC fenestration size and number.^{18,19} Additionally, at the point of initiation of SEC proliferation around 72 hours after PHx, cell-cell interactions may play a pivotal role in influencing the reduction of fenestrations via soluble agents. Likewise, undisclosed soluble molecules, including proteases, growth factors, and adrenergics, that rise in the blood immediately following PHx may also influence dilation of fenestrations during the first 24 hours following resection,^{1,2,43,44,48} as well as their subsequent decrease thereafter. We have undertaken a quantitative ultrastructural study that clearly has limitations in identifying such agents, but our results indicate the critical importance of evaluating these agents in the context of fenestration diameter regulation in future studies.

Acknowledgment: The authors thank Fran Shagas, Mara Grove Sullivan, and Ana Bursick for their technical support, and Joe Suhan of Carnegie Mellon University for the generous use of the Hitachi scanning electron microscope. They also thank Dr. Dorothee M. Runge for critically reading the manuscript.

REFERENCES

1. Michalopoulos G, DeFrances M. Liver regeneration. *Science* 1997;276:60-66.
2. Fausto N, Webber E. Liver Regeneration. In: Arias IM, Boyer JL, Fausto N, Jakoby WB, Schachter DA, Shafritz DA, eds. *The Liver: Biology and Pathology*. 3rd ed. New York: Raven Press, 1994:1059-1084.
3. Grisham JW. A morphologic study of deoxyribonucleic acid synthesis and cell proliferation in regenerating rat liver; autoradiography with thymidine-H³. *Cancer Res* 1962;22:842-849.
4. Widmann JJ, Fahimi HD. Proliferation of mononuclear phagocytes (Kupffer cells) and endothelial cells in regenerating rat liver. *Am J Pathol* 1975;80:349-366.
5. Martinez-Hernandez A, Delgado FM, Amenta PS. The extracellular matrix in hepatic regeneration. Localization of collagen types I, III, IV, laminin, and fibronectin. *Lab Invest* 1991;64:157-166.
6. Martinez-Hernandez, Amenta P. The extracellular matrix in hepatic regeneration. *FASEB J* 1995;9:1401-1410.
7. Yamane A, Seetharam L, Yamaguchi S, Gotoh, N, Takahashi T, Neufeld G, Shibuya M. A new communication system between hepatocytes and sinusoidal endothelial cells in liver through vascular endothelial growth factor and FLT tyrosine kinase receptor family (flt-1 and KDR/Flk-1). *Oncogene* 1994;9:2683-2690.
8. Mochida S, Ishikawa K, Toshima K, Inao M, Ikeda H, Matsui A, Shibuya M, et al. The mechanisms of hepatic sinusoidal endothelial cell regeneration: a possible communication system associated with vascular endothelial growth factor in liver cells. *J Gastroenterol Hepatol* 1998;13(Suppl):1S-5S.
9. Wisse E, DeZanger RB, Charels K, Van Der Smissen P, McCuskey RS. The liver sieve: consideration concerning the structure and function of endothelial fenestrae, the sinusoidal wall and the space of Disse. *HEPATOLOGY* 1985;5:683-692.
10. Morsiani E, Aleotti A, Ricci D. Haemodynamic and ultrastructural observations on the rat liver after two-thirds partial hepatectomy. *J Anat* 1984;192:507-515.
11. Morsiani E, Mazzoni M, Aleotti A, Gorini P, Ricci D. Increased sinusoidal wall permeability and liver fatty change after two-thirds hepatectomy: an ultrastructural study in the rat. *HEPATOLOGY* 1995;21:539-544.
12. Horn T, Christoffersen P, Henriksen JH. Alcoholic liver injury: defenestration in noncirrhotic livers—a scanning electron microscopic study. *HEPATOLOGY* 1987;7:77-82.
13. Gattmaitan Z, Varticovski L, Ling L, Mikkelsen R, Steffan A-M, Arias IM. Studies on fenestral contraction in rat liver endothelial cells in culture. *Am J Pathol* 1996;148:2027-2041.
14. Dobbs BR, Rogers GWT, Xing H-Y, Fraser R. Endotoxin-induced defenestration of the hepatic sinusoidal endothelium: a factor in the pathogenesis of cirrhosis? *Liver* 1994;14:230-233.
15. Fraser R, Clark SA, Day WA, Murray FEM. Nicotine decreases porosity of the rat liver sieve: a possible mechanism for hypocholesterolaemia. *Br J Exp Pathol* 1988;69:145-350.
16. Steffan A-M, Gendrault J-L, Kirn A. Increase in the number of fenestrae in mouse endothelial cells by altering the cytoskeleton with cytochalasin B. *HEPATOLOGY* 1987;7:1230-1238.
17. Braet F, De Zanger R, Baekeland M, Crabbe E, Van Der Smissen P, Wisse E. Structure and dynamics of the fenestrae-associated cytoskeleton of rat liver sinusoidal endothelial cells. *HEPATOLOGY* 1995;12:180-189.
18. McGuire R, Bissell M, Boules J, Roll F. Role of extracellular matrix in regulating fenestrations of sinusoidal endothelial cells isolated from normal rat liver. *HEPATOLOGY* 1992;15:989-997.
19. Mori T, Okanoue T, Sawa Y, Hori N, Ohta M, Kagawa K. Defenestration of the sinusoidal endothelial cell in a rat model of cirrhosis. *HEPATOLOGY* 1993;17:891-897.
20. Higgins GM, Anderson RM. Experimental pathology of the liver. I. Restoration of the liver of the white rat following partial surgical removal. *Arch Pathol* 1934;12:186-202.
21. Seglen PO. Preparation of isolated rat liver cells. In: Prescott DM, ed. *Methods in Cell Biology*. New York: Academic Press, 1976:29-83.
22. Stolz DB, Ross MA, Salem HM, Mars WM, Michalopoulos GM, Enomoto K. Cationic colloidal silica membrane perturbation as a means of examining changes at the sinusoidal surface during liver regeneration. *Am J Pathol* 1999;155:1487-1498.
23. Vidal-Vanaclocha F, Barbera-Guillem E. Fenestration patterns in endothelial cells of rat liver sinusoids. *J Ultrastruc Res* 1985;90:115-123.
24. Shin Y-C. Reevaluation on the types and pattern of distribution of sinusoidal fenestrations in the lobule of normal rat liver. *Anat Rec* 1997;247:206-213.
25. Horn T, Henriksen JH, Christoffersen P. The sinusoidal lining cells in "normal" human liver. A scanning electron microscopic investigation. *Liver* 1986;6:98-110.
26. Motta P, Muto M, Fujita T. The vascular system. In: *The Liver. An Atlas of Scanning Electron Microscopy*. Tokyo: Igaku-Shoin, 1978:83-86.
27. Grisham JW, Nopanitaya W, Compagno J, Nagel AEH. Scanning electron microscopy of normal rat liver: the surface structure of its cells and tissue components. *Am J Anat* 1975;144:295-322.
28. Gebhardt R. Metabolic zonation of the liver: regulation and implications for liver function. *Pharmacol Ther* 1992;53:275-354.
29. Blouin A, Bolender RP, Weible ER. Distribution of organelles and membranes between hepatocytes and non-hepatocytes in rat liver parenchyma. A stereological study. *J Cell Biol* 1977;72:441-455.
30. Wisse E, Braet F, Luo D, De Zanger R, Jans D, Crabbe E, Vermoesen A. Structure and function of sinusoidal lining cells of the liver. *Toxicol Pathol* 1996;24:100-111.
31. Rojkind M, Greenwel P. The extracellular matrix of the liver. In: Arias IM, Boyer JL, Fausto N, Jakoby WB, Schachter DA, Shafritz DA, eds. *The Liver: Biology and Pathology*, 3rd ed. New York: Raven, 1994:843-868.
32. Reid LM, Fiorino AS, Sigal SH, Brill S, Holst PA. Extracellular matrix gradients in the space of Disse: relevance to liver biology. *HEPATOLOGY* 1992;15:1198-1203.
33. McCuskey RS, Reilly FD. Hepatic microvasculature: dynamic structure and its regulation. *Semin Liver Dis* 1993;13:1-12.
34. Sato Y, Koyama S, Tsukada, Katakeyama K. Acute portal hypertension reflecting shear stress as a trigger of liver regeneration following partial hepatectomy. *Surgery Today: The Japanese Journal of Surgery* 1997;27:518-526.
35. Niiya T, Murakami M, Aoki T, Murai N, Shimizu Y, Kusano M. Immediate increase of portal pressure, reflecting sinusoidal shear stress, induced liver regeneration after partial hepatectomy. *J Hepatobiliary Pancreat Surg* 1999;6:275-280.
36. Fraser R, Bowler LM, Day WA, Dobbs B, Johnson HD, Lee D. High perfusion pressure damages the sieving ability of sinusoidal endothelium in rat livers. *Br J Exp Pathol* 1980;61:222-228.

37. Gertsch P, Stipa F, Ho J, Yuen ST, Luk I, Lauder IJ. Changes in hepatic portal resistance and in liver morphology during regeneration: in vitro study in rats. *Eur J Surg* 1997;163:297-304.
38. Christofferson RH, Nilsson BO. Microvascular corrosion casting in angiogenesis research. In: Motta PM, Murakami T, Fujita H, eds. *Scanning Electron Microscopy of Vascular Casts: Methods and Applications*. Boston: Kluwer Academic Publishers, 1992:27-37.
39. Grunt TW. The fundamental vascular structures in epithelial cancer demonstrated in an experimental lung carcinoma. In: Motta PM, Murakami T, Fujita H, eds. *Scanning Electron Microscopy of Vascular Casts: Methods and Applications*. Boston: Kluwer Academic Publishers, 1992: 331-344.
40. Friedman SL. Cytokines and fibrogenesis. *Semin Liver Dis* 1999;19:129-140.
41. Geerts A, De Bleser P, Hautekeete ML, Niki T, Wisse E. Fat-storing (Ito) cell biology. In: Arias IM, Boyer JL, Fausto N, Jakoby WB, Schachter DA, Shafritz DA, eds. *The Liver: Biology and Pathology*, 3rd ed. New York: Raven, 1994:819-838.
42. Tanaka Y, Mak KM, Lieber CS. Immunohistochemical detection of proliferating lipocytes in regenerating rat liver. *J Pathol* 1990;160:129-134.
43. Kim T-Y, Mars WM, Stolz DB, Petersen BE, Michalopoulos GK. Extracellular matrix remodeling at the early stages of liver regeneration in the rat. *HEPATOLOGY* 1997;26:896-904.
44. Mars WM, Liu M-L, Kitson RP, Goldfarb RH, Gebauer MK, Michalopoulos GK. Immediate early detection of urokinase receptor after partial hepatectomy and its implications for the initiation of liver regeneration. *HEPATOLOGY* 1995;21:1695-1701.
45. Kato S, Otsu K, Ohtake K, Kimura Y, Yashiro T, Suzuki T, Akamatsu N. Concurrent changes in sinusoidal expression of laminin and affinity of hepatocytes to laminin during rat liver regeneration. *Exp Cell Res* 1992; 198:59-68.
46. Pujades C, Forsberg E, Enrich C, Johansson S. Changes in cell surface expression of fibronectin and fibronectin receptor during liver regeneration. *J Cell Sci* 1992;102:815-820.
47. Wewer UM, Engvall E, Paulsson M, Yamada Y, Albrechtsen R. Laminin A, B1, B2 S and M subunits in the postnatal rat liver development and after partial hepatectomy. *Lab Invest* 1992;66:378-389.
48. Cruise JL, Knechtle SJ, Bollinger RR, Kuhn C, Michalopoulos G. $\alpha 1$ -Adrenergic effects and liver regeneration. *HEPATOLOGY* 1987;7:1189-1194.