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.

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Sinusoidal Ultrastructure Evaluated During the Revascularization of Regenerating Rat Liver

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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 stress10,11; soluble substances such as alcohol,12 serotonin,13 endotoxin,14 and nicotine15; and cytoskeletal disruption agents like cyclophosphamideB16,17 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.

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changes in fenestration diameters and porosity of SEC in the periporal (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 periporal 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 periporal-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. 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 mg/L 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. 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. 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 an 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 μm in diameter around the portal triads were considered to be part of zone 1, and regions up to 100 μm in diameter around the central veins were considered to be part of zone 3. 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 (300X) showing the zonal regions and high-magnification images (20,000–25,000X) 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 Image 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 10,000X to 15,000X. 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 400X 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 μm 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 glycero, 0.1% sodium azide to 100 mL PBS) and viewed on a Nikon Eclipse epifluorescence 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 5.3, 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-
diameters analyzed from 30 images taken at 20,000-25,000 fold magnification. For each time point and zone, n = 100 measurements documented in Fig. 1 and Table 2.

We were intrigued by the striking decrease in fenestral porosity and fenestral diameter on the pericentral area of the lobule. The lipid nature of the hepatocytes was indeed accompanied by a parallel increase in lipid accumulation in both zones. Fenestrations in both zones recovered to near-normal levels by 14 days, but porosity index remained elevated in zone 1 until 48 hr post-PHx. Fenestrations or entire sieve plates.

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 perportal 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 fenestral diameters at 72 hr post-PHx. It is known that, at this time,
hepatocyte proliferation has subsided and SEC proliferation has just initiated. 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. 7). 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
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\textsuperscript{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 \textit{in vivo}. Fig.

![Image of micrographs showing fenestrations and intraendothelial cell gaps in control and regenerating liver (see also Fig. 1 and Table 2).]
Figure 8 examines periportal (zone 1) regions for variations in the expression of collagen I, collagen IV, fibronectin, and laminin (γ1 subunit, also known as B2 subunit) in nonresected livers and livers 24 hr post-PHx. Laminin γ1–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 (×200); lower 4 panels, higher magnifications of top panels (×400).
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 preresection sizes (Figs. 1, 2). In general, the deposition of all 4 proteins was
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. 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. In

Table 3. Zonal Hepatic Plate Width Measurements

<table>
<thead>
<tr>
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<th>Plate Width (μm)</th>
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<tbody>
<tr>
<td>Z1</td>
<td>18.53 ± 0.35</td>
</tr>
<tr>
<td>Z3</td>
<td>19.13 ± 0.47</td>
</tr>
<tr>
<td>Z1</td>
<td>25.64 ± 0.52</td>
</tr>
<tr>
<td>Z3</td>
<td>22.94 ± 0.41</td>
</tr>
<tr>
<td>Z1</td>
<td>29.13 ± 0.62</td>
</tr>
<tr>
<td>Z3</td>
<td>25.77 ± 0.61</td>
</tr>
<tr>
<td>Z1</td>
<td>20.62 ± 0.28</td>
</tr>
<tr>
<td>Z3</td>
<td>22.49 ± 0.26</td>
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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.)
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

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. 5D, 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).
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 hepatocytes proliferate very early, and SEC very late in the regenerative response,38 areas of avascularity would develop within the liver. Such increases in plate width could affect 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. 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 ×200.)](image-url)

![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 ×200.)](image-url)
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. 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, 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 impared 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.

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. Upon activation, or during proliferation, they often lose their lipid droplets. 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. 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. 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, perhaps losing much of their lipid in the process. 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 process via close exchange of growth, matrix, and chemotactic factors. 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 and are known to influence SEC fenestrations both in vitro and in vivo. Previously, it was shown that ECM degradation occurs in the sinusoids immediately following PHx. 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. 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. 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 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, collagen IV, fibronectin, and laminin have been shown to display differential deposition within sinusoids at various times. Laminin increases before 6 hr post-PHx and remains elevated until 3 days after PHx (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. 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. 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. 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 endothel-
lum 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.\textsuperscript{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.\textsuperscript{18,19} Additions that are required for BM formation have been shown to breakdown products, as well as a variety of soluble agents. Likewise, undisclosed soluble molecules, including proteases, growth factors, and adrenegens, that rise in the blood immediately following PHx may also influence dilatation of fenestrations during the first 24 hours following resection,\textsuperscript{1,2,4,3,44,48} 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.

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REFERENCES

3. Grisham JW. A morphologic study of deoxyribonucleic acid synthesis and cell proliferation in regenerating rat liver; autoradiography with thymidine-H\textsuperscript{3}. Cancer Res 1962;22:842-849


