Technical Advance

Cationic Colloidal Silica Membrane Perturbation as a Means of Examining Changes at the Sinusoidal Surface during Liver Regeneration

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By employing the cationic colloidal silica membrane density perturbation technique, we examined growth factor receptor and extracellular matrix (ECM) changes at the sinusoidal surface during rat liver regeneration 72 hours after 70% partial hepatectomy (PHx). At this time after PHx, hepatocyte division has mostly subsided, while sinusoidal endothelial cell (SEC) proliferation is initiating, resulting in avascular hepatocyte islands. Because of the discontinuous nature of the surface of liver SEC, ECM proteins underlying the SEC, as well as SEC luminal membrane proteins, are available to adsorption to the charged silica beads when the liver is perfused with the colloid. Subsequent liver homogenization and density centrifugation yield two separate fractions, enriched in SECs as well as hepatocyte basolateral membrane-specific proteins up to 50-fold over whole liver lysates. This technique facilitates examination of changes in protein composition that influence or occur as a result of SEC mitogenesis and migration during regeneration of the liver. When ECM and receptor proteins from SEC-enriched fractions were examined by Western immunoblotting, urokinase plasminogen activator receptor, fibronectin, and plasmin increased at the SEC surface 72 hours after PHx. Epidermal growth factor receptor, plasminogen, SPARC (secreted protein, acidic and rich in cysteine, also called osteonectin or BM40), and collagen IV decreased, and fibrinogen subunits and c-Met expression remained constant 72 hours after PHx when compared to control liver. These results display the usefulness of the cationic colloidal silica membrane isolation protocol.

They also show considerable modulation of surface components that may regulate angiogenic processes at the end stage of liver regeneration during the reformation of sinusoids. (*Am J Pathol* 1999, 155:1487–1498)

Regeneration of the rat liver after 70% partial hepatectomy (PHx) is a well-studied system for controlled regrowth of tissue. After surgical resection, hepatocytes undergo DNA synthesis between 24 and 48 hours, followed in progression by DNA synthesis of ductular epithelia at 36–48 hours, Kupffer and stellate cells at 48 hours, and finally, sinusoidal endothelial cells (SECs) at 96 hours.^{1–6} At 72 hours after PHx, after the major hepatocyte proliferative phases have subsided, there are areas of avascularized parenchymal islands within the liver lobule. This occurs because the hepatocytes terminate their proliferation well before the SECs begin to proliferate.⁴ To restore the functional architecture of the liver, the parenchymal islands must become neovascularized. These areas remain incompletely vascularized until 6–8 days after PHx, when the division of the SECs is followed by motility events in which they infiltrate and vascularize the parenchymal islands.⁴ As a result, liver regeneration after 70% PHx provides an interesting system for the investigation of the initiation as well as abrogation of normal, controlled angiogenesis.

Angiogenesis *in vivo* is described as occurring in four distinct stages⁷ that can be extrapolated to the liver after PHx. Stage 1 of angiogenesis is initiated by proteolytic digestion of the extracellular matrix (ECM) adjacent to the endothelial cells (ECs). In many systems this is often

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called "priming" or "competency," because subsequent DNA synthesis and cell division does not appear to occur in the absence of matrix degradation.⁸ Stage 2 is the proliferation of "competent" ECs, followed by Stage 3, in which the ECs migrate into the avascular space to form continuous, aligned cords of cells. Stage 4 is the formation of patent vessels by the ECs, thus completing the angiogenic process and restoring normal vascularization to the parenchymal islands. All four stages are believed to be controlled, either directly by induction of proliferation and motility or indirectly by up-regulation of the activity of ECM proteases, breakdown of ECM components, ECM deposition, and an increase or decrease of specific peptide growth factors and/or their cognate receptors, including hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor α and β (TGF- α , TGF- β), vascular endothelial growth factor (VEGF), and acidic and basic fibroblast growth factor (acidic FGF and basic FGF).⁹

Methods for examining angiogenesis routinely employ combinations of *in vitro* (cell culture) and *ex vivo* (explants, angiogenesis assays) studies that often display a limited representation of the events occurring during the revascularization process in an intact animal or organ. Studies of angiogenic events using total organ systems *in situ* often resort to analysis of the endothelial cells via biochemical studies in which the entire organ is homogenized. This approach is often not very successful when the protein of interest is present in low abundance or when a specific protein is common to a variety of cell types. Here we present a procedure that allows for the examination of proteins at the sinusoidal surface that may be involved during vascularization within the intact regenerating liver. Fractions enriched 25–50-fold in SEC membrane as well as hepatocyte basolateral membrane markers can be rapidly obtained in high yield by noncovalently derivatizing the luminal surfaces of the SECs. The subsequent "freezing" of membrane components by cationic colloidal silica coating inhibits diffusion, endocytosis, and proteolysis at the SEC surface at distinct time points during the regenerative process. As a result, loss of specific proteins as a direct result of cell isolation procedures can be minimized.

To display the utility of the cationic colloidal silica membrane perturbation technique in examining changes at the sinusoid surface coincident with regeneration, we chose to compare the expression of growth factor receptors as well as extracellular matrix proteins present at the SEC surface in normal, unmanipulated livers and in livers 72 hours after PHx, when the SEC/hepatocyte ratio is at its lowest. It is known that extracellular matrix has profound effects on the development, growth, differentiation, and pathobiology of the liver.¹⁰ Especially scarce is information describing the revascularization of the liver by SECs after PHx and its regulation by ECM components. It has been elegantly shown in a number of investigations that ECM has profound effects on SEC morphology *in vitro* that are applicable to their physiology *in situ*.^{11,12} Because liver SECs are fenestrated and discontinuous, ECM components within the space of Disse, the area occupying the space abluminal to the sinusoidal endo-

thelial cell and basolateral to the hepatocyte, are also available to derivatization and isolation by the cationic silica. As such, this protocol allows for intimate examination of the ECM constituents, as well as the appearance of specific ECM precursors and/or breakdown products, that may regulate proliferation and/or motility processes. Also relevant to the angiogenic process is the role of growth factors and their receptors. Because we can separate SEC luminal and hepatocyte basolateral membranes from the other membrane populations, we can examine the quantity of specific receptors on these surfaces relative to other liver fractions during regeneration.

Here we describe modulation of ECM products and surface receptors at the sinusoidal surface 72 hours after 70% PHx in the rat, a time point just preceding peak proliferation of SECs during the revascularization phase of regeneration. We have focused on components of the plasminogen/fibrinogen system because many proteins within this family have been implicated in the control of angiogenesis in other systems.^{9,13} More importantly, because plasminogen, fibrinogen, fibronectin, and urokinase plasminogen activator receptor (u-PAR) have been shown to be involved in liver regeneration,^{14,15} they were likely to contribute to the revascularization process in the later stages of the regenerative process. Our results show temporal and spatial changes in several major liver ECM proteins as well as growth factor receptors at 72 hours after PHx. Because of the modulation of expression at the sinusoidal surface, these changes may facilitate the angiogenic progression within the liver coincident with regeneration.

Materials and Methods

All reagents were obtained from Sigma Chemical Company (St. Louis, MO) unless indicated otherwise.

Animals and Surgery

All animals were treated using approved procedures in accordance with the guidelines of the Institutional Animal Use and Care Committee at the University of Pittsburgh School of Medicine and the National Institutes of Health. Male Fisher 344 rats (National Cancer Institute, Frederick, MD), averaging 200–225 g, were 70% partially hepatectomized under Metophane inhalation anesthesia, as described previously.¹⁶ Before surgery, animals were allowed standard rat chow and water *ad libitum* and maintained on a 12-hour light-dark schedule. At specific times after surgery, animals were anesthetized with 500 μ g/200 g IP injection of Nembutal, and livers were processed as described below. Control livers were obtained from Metophane- or Nembutal-anesthetized nonmanipulated animals.

In Situ Silica SEC Membrane Isolation Techniques

The luminal SEC membranes were isolated by an *in situ* membrane density perturbation technique originally used

Figure 1. Flow chart depicting the isolation scheme of sinusoidal surface proteins (pellet 2) and hepatocyte basolateral membranes (light membrane fraction), using the cationic colloidal silica membrane perturbation technique. The Nycodenz percentage (w/v) in each step gradient is labeled within each tube to help identify specific liver fractions. Fractions with asterisks indicate those characterized by Western immunoblotting in Figure 4.

for examination of the membrane polarity of ECs *in vitro*¹⁷ and luminal membrane protein composition of lung microvascular ECs *in vivo*.¹⁸ Normal liver or liver 72 hours after PHx, was cleared of blood with 2-(*N*-morpholino)ethanesulfonic acid (MES)-buffered saline (MBS: 20 mmol/L MES, 150 mmol/L NaCl, pH 5) containing broad-spectrum protease/phosphatase inhibitors (4 mmol/L diisopropylfluorophosphate, 1 mmol/L sodium orthovanadate, and 10 μ g/ml each pepstatin, leupeptin, E64) at 4°C, using a peristaltic pump with a flow rate of 5 ml/min. Cationic colloidal silica (1% colloidal suspension in MBS, prepared as described previously¹⁹) was then perfused through the liver for 2 minutes, after which the liver was perfused with MBS (pH 6.0). The cationically coated SEC membrane was then overcoated with 1 mg/ml polyacrylic acid (Aldrich, St. Louis, MO) in MBS to restore the net negative charge of the SEC membrane. After one more wash with MBS, the liver was perfused with lysis buffer (LB) (25 mmol/L HEPES, 250 mmol/L sucrose, pH 7.4) containing a panel of broad-spectrum protease inhibitors, including 4 mmol/L diisopropylfluorophosphate; 1 mmol/L sodium orthovanadate and EDTA; and 10 μ g/ml pepstatin, leupeptin, E64, and 1, 10, phenanthroline. The liver was excised, minced into tiny pieces, and thoroughly homogenized by 100 strokes of a Dounce homogenizer. The separation protocol described in detail below is also shown in the Figure 1 flow chart. The lysate was mixed with an equal volume of 1.03 g/ml Nycodenz (prepared as 10 g in 5.5 ml LB) and then layered onto a 0.5-ml cushion of 70% (w/v) Nycodenz in LB. The tube was topped off with LB and then separated in a swinging bucket rotor (SW60Ti; Beckman) at 20,000 $\times g$ for 20 minutes, after which several layers of liver fractions were visibly discernible. The SEC luminal membranes, because of their increased density (>1.37 mg/ml), sedimented through the 70% Nycodenz and formed a glassy pellet at the bottom of the tube.

When the initial silica pellet was gently resuspended 1:1 with 1.03 g/ml Nycodenz solution and recentrifuged over a 70% Nycodenz cushion, a purer SEC membrane pellet was obtained, in addition to a "light fraction" en-

riched in hepatocyte basolateral membrane that forms at the 50%/lysis buffer interface. The final silica membrane pellet was washed twice in 1.5 ml of LB in a microfuge tube at 14,000 $\times g$ for 10 minutes.

The entire process took about 2 hours and consistently yielded 0.19–0.25% recovery of protein, resulting in up to 38 mg of protein as the SEC membrane fraction of a control liver weighing approximately 20 g before PHx, and 0.05–0.08% SEC protein recovery from livers processed 72 hours after PHx. The drop in yield was expected because 72 hours after resection, the SEC/hepatocyte ratio is lower than in the normal liver.

Electron Microscopy

Livers perfused with silica as described above were perfusion-fixed with 2.5% glutaraldehyde in phosphate-buffered saline (PBS) after the final MBS wash. Livers were removed from the animals and immersed in the same fixative for two additional days at 4°C. Alternatively, samples obtained from the fractionation procedure were fixed in 2.5% glutaraldehyde in PBS as a pellet after isolation. Several 1-mm³ cubes were removed from the liver, washed three times in PBS, and then postfixed in 1% OsO₄, 1% K₃Fe(CN)₆ for 1 hour. After three PBS washes, the tissue was dehydrated through a graded series of 30–100% ethanol, 100% propylene oxide, and then infiltrated in 1:1 mixture of propylene oxide:Polybed 812 epoxy resin (Polysciences, Warrington, PA) for 1 hour. After several changes of 100% resin over 24 hours, tissue was embedded in molds and cured at 37°C overnight, followed by additional hardening at 65°C for two more days. Ultrathin (60 nm) sections were collected on 200-mesh copper grids and stained with 2% uranyl acetate in 50% methanol for 10 minutes, followed by 1% lead citrate for 7 minutes. Sections were viewed with a JEOL JEM 1210 transmission electron microscope at 80 or 60 kV.

Gel Electrophoresis

Proteins isolated from liver fractions were processed as described. Briefly, silica was removed from derivatized membrane pellets by probe sonicating pellets (Branson Sonifier) in a small volume of 2% sodium dodecyl sulfate (SDS) in LB followed by heating of the suspension at 100°C for 5 minutes. Silica was removed from solubilized proteins by centrifugation at 14,000 $\times g$ for 15 minutes. The supernatant was retained as the SEC protein fraction, and the pellet was discarded. The light membrane fraction enriched in hepatocyte basolateral membrane was collected and diluted with several volumes of LB, sedimented at 20,000 $\times g$, and then washed in LB and resedimented twice. The final pellet was solubilized in 2% SDS in LB as described for the SEC pellet. Twenty micrograms of protein of each sample, as determined by bicinchoninic acid protein assay, were loaded into each lane, resolved on discontinuous SDS polyacrylamide gels (12, 10, or 8% where designated), and then electrophoretically transferred at 250 mA in 25 mmol/L Tris, 192 mmol/L glycine, 0.01% SDS overnight at room tempera-

ture to Immobilon-PVDF membrane (Millipore, Bedford, MA). Even transfer of proteins was determined by reversible staining with Ponceau Red S Solution before blocking.

Blots were blocked with 5% nonfat dry instant milk (5% Milk Blotto) in TBST (20 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 0.1% Tween-20) for at least 1 hour at room temperature or at 4°C overnight. Blotting proceeded with primary antibodies for 2 hours at room temperature or overnight at 4°C in 5% milk Blotto. Blots were washed three times in 1% milk in TBST (1% Blotto). Secondary antibodies were applied in 1% Blotto for 1 hour at room temperature. Blots were washed three times in 1% Blotto, then once in TBST before development on X-ray film, using enhanced chemiluminescence reagents (Amersham, Arlington Heights, IL).

Antibodies used in this study include rabbit anti-serum to asialoglycoprotein receptor (a gift of Dr. Kurt Drickamer, University of Oxford, Oxford, UK), monoclonal anti-SE-1 (supplied by Dr. Katsuhiko Enomoto, Akita University, Akita, Japan), rabbit anti-collagen IV (a gift of Dr. Peter Amenta, Robert Wood Johnson Medical School, New Brunswick, NJ), rabbit anti-caveolin 1 (Transduction Laboratories, Lexington, KY), goat anti-plasminogen and rabbit anti-rat u-PAR (ADI, Greenwich, CT), goat anti-fibrinogen (ICN, Costa Mesa, CA), goat anti-rat fibronectin (Gibco-BRL, Gaithersburg, MD), anti-SPARC/ossteonectin (Hematological Technologies, Essex Junction, VT), sheep anti-human EGF receptor (Upstate Biotechnology, Lake Placid, NY), rabbit anti-mouse c-Met (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-actin (Chemicon, Temecula, CA), and rabbit anti-rat albumin conjugated to horseradish peroxidase (Cappel/Organon Teknika, Durham, NC). Secondary antibodies against sheep, mouse, rabbit, and goat conjugated to horseradish peroxidase were purchased from Sigma.

Results

Isolation of Sinusoidal Luminal Membrane Domains

The cationic colloidal silica membrane perturbation technique has been shown previously to allow rapid isolation of apical membranes from cells in culture¹⁷ or luminal membranes from continuous, tight endothelium from tissues such as lung *in vivo*.¹⁸ Because liver sinusoids are lined with discontinuous, fenestrated endothelium, it was critical to assess the ability of the silica pellicle to be restricted to the sinusoidal surface and not to diffuse into the underlying space of Disse, onto the hepatocyte basolateral membrane. We determined the distribution of the silica adsorbed to the luminal surface in the perfused liver by transmission electron microscopy. As shown in Figure 2, A and B, perfusion of normal liver with cationic silica causes the formation of a dense pellicle that uniformly covers the surface of the SEC luminal membrane. It is important to note that even though the SECs are a discontinuous, fenestrated endothelium, in normal liver

very little silica is observed in areas other than the luminal membrane. This suggests that ECM proteins cover the "exposed" hepatic basolateral domain, extending from and surrounding the space of Disse, protecting areas even though they are not directly covered by SECs. Collagen fibrils are seen to be in contact with the silica pellicle in Figure 2, A and C, indicating that silica adsorbs to exposed ECM molecules within the space of Disse. Because the luminal surface has a net negative charge at the time of perfusion, the cationic colloidal silica forms a strong ionic but noncovalent bond to this surface, regardless of its protein composition. As expected, large vessels with continuous endothelium are also uniformly coated with the silica, but the luminal aspect of the bile duct epithelium and bile canaliculi are not labeled (Figure 2B), indicating that the perfused silica is restricted to surfaces only in contact with the blood.

Isolation of the derivatized SEC membranes, as well as proteins available to adsorption by the silica particles, was performed by taking advantage of the high density characteristics of the silica-derivatized membranes. The silica-coated membranes have a density greater than 1.37 g/ml and readily sediment through the 70% Nycodenz cushion at 20,000 × *g*, leaving underivatized membranes at the 50% Nycodenz/lysis buffer interface (Figure 1). Ultrastructural examination of the initial silica pellet reveals long, linear sheets of silica-coated membranes, but also collagen fibrils and underivatized membranes (Figure 3A). In fact, isolated silica membrane fractions in the initial silica pellet are shown to contain fibrillar collagens when examined by transmission electron microscopy (Figure 3A). At higher magnification, the derivatized membranes retain numerous associated caveoli attached to the membrane (Figure 3B). We further purified SEC pellet 1 membranes by gently resuspending the initial pellet in an equal volume of 1.03 g/ml Nycodenz and sedimented the solution one additional time through 70% Nycodenz at 20,000 × *g*. After this treatment, shorter membrane sheets were visible, and the overall purity of the SEC membrane was visibly increased (Figure 3C). The residual material that did not sediment was found at the 50% Nycodenz:lysis buffer interface and consisted mainly of uncoated membranes and collagen fibrils (Figure 3D).

Biochemical analysis of these fractions for specific markers indicated the cell of origin component in each fraction. During examination for the rat SEC-specific antigen SE-1,²⁰ a 25-fold enrichment in this protein was seen in SEC pellet 1, and a 50-fold enrichment was seen in the final purified SEC pellet when compared to perfused liver lysates (Figure 4A). The hepatocyte basolateral membrane marker asialoglycoprotein receptor (ASGP-R) was enriched in the light membrane fraction relative to both perfused liver and final SEC pellet, indicating that a substantial amount of the uncoated membranes seen in Figure 3A (corresponding to SEC pellet 1) originated from the basolateral hepatocyte membranes lining the space of Disse. Additional information from the fractions was determined by blotting with caveolin 1, a

Figure 2. Representative electron micrograph of normal rat liver perfused with cationic colloidal silica as described in Materials and Methods. Silica (Si), visible as electron-dense particles evenly coating the SEC surface, does not penetrate into the space of Disse (SD) or to the hepatocyte basolateral membrane via fenestrations in the SEC (arrowheads); however, it adsorbs to collagen fibrils (Col) that are in the space of Disse beneath SEC fenestrations. L, lumen of sinusoid. Bar = 5 μ m. **A:** Silica (Si) adsorbs to the surface of large vessel endothelium (PV, portal vein; HA, hepatic artery) but not to bile duct apical membranes or hepatic basolateral membranes. H, hepatocyte. Bar = 5 μ m. **B:** High magnification of silica (Si) adsorbing to collagen fibrils exposed through a gap in the SEC (*). Fenestrations are also covered by silica (arrowheads), but little silica is seen to have entered the space of Disse (SD) to adhere to the hepatocyte basolateral membrane (H). Bar = 100 nm.

Figure 3. **A:** Transmission electron micrograph of SEC pellet 1, showing isolated silica-adsorbed membrane sheets (Si), collagen fibrils, and noncoated membranes (Mem). **B:** Higher power examination of an isolated silica-adsorbed membrane sheet (Si) with attached caveolae (Cav). **C:** Electron micrograph of purified SEC membranes (derived from the original high-speed sedimentation as seen in Figure 3A) after a second centrifugation step. **D:** Ultrastructural examination of the light membrane fraction (hepatocyte basolateral membranes) derived from SEC pellet 1 after an additional centrifugation step, showing intact collagen fibrils, uncoated membranes (Mem), and a minor amount of short silica-coated membrane fragments (Si). Bar = 1 μ m in all panels.

membrane-specific protein enriched in endothelial cell membranes. Caveolin 1 was found in abundance in both silica fractions (pellet 1 and final SEC pellet) but not in the hepatocyte basolateral membrane fraction. This information, in conjunction with the ASGP-R data, indicates that the light membrane fraction derived from SEC pellet 1 was enriched in basolateral hepatocyte membranes because hepatocytes do not normally express caveolin.²¹

Other membrane-associated proteins were examined in the various fractions (Figure 4B). Actin was found in all fractions, including those enriched in SEC and hepatocyte basolateral membranes, indicating that subcortical cytoskeletal-membrane protein associations known to occur in silica-isolated endothelial cell membranes *in vitro* were maintained during the *in situ* isolation procedure.¹⁷ Albumin is synthesized by hepatocytes and moves through hepatocytes and endothelial cells as a serum carrier protein. It was routinely seen in all fractions at essentially similar signal levels when examined by Western immunoblotting.

Examination of Sinusoidal Protein Changes during Regeneration

The belief that architectural remodeling accompanies regeneration of the liver after 70% PHx stems from the observation that parenchymal cells undergo several rounds of replication, whereas the SECs, at this time point, are mitotically quiescent.^{1,4} This results in hepatic islands devoid of vascularization at approximately 72 hours after PHx. It is not until approximately 4 days after PHx that SECs undergo peak DNA synthesis and subsequently migrate into the avascular hepatic islands, eventually reforming the normal sinusoidal architecture of the liver lobule. These events had been postulated to begin at 72 hours after PHx. Therefore, we wished to compare the ultrastructure of regenerating liver sinusoids at 72 hours after resection with that of normal liver, to evaluate potential changes relevant to revascularization of the liver during regeneration. This would also serve to verify that the silica remained at the SEC surface in the regenerating

Figure 4. (A) Biochemical characterization of isolated fractions as determined by Western immunoblotting. Isolated proteins were resolved on 12% discontinuous reducing gels, blotted to PVDF membrane, and probed for the designated proteins as described. Fractions are identified in the isolation scheme by asterisks in Figure 1. Sinusoidal endothelial cell-specific antigen SE-1 is enriched 25-fold in SEC pellet 1 and 50-fold in SEC pellet 2, indicating enrichment of the SEC membrane in these fractions. ASGP-R, a hepatocyte basolateral membrane-specific protein, is found enriched in the light membrane fraction. Caveolin 1 is enriched in the SEC pellets but is not found in the light membrane fraction, further verifying the identity of the light and SEC fractions. (B) Subcortical actin and albumin association with total liver lysates and isolated fractions. Albumin is overloaded in the whole liver lane relative to other samples because of its presence in the serum.

sinusoid. Figure 5 displays a representative pair of low-magnification transmission electron micrographs that compare silica-perfused livers from control and regenerating livers 72 hours after resection. Readily obvious in control livers (Figure 5A) is the typical plate formation of the hepatocytes, bounded on either side by sinusoids that are 5–10 μm in diameter.²² In stark contrast, sinusoids in the regenerating liver 72 hours after PHx are highly compressed (2–3 μm in diameter) between groups of 8–12 small hepatocytes (Figure 5B). Ultrastructural examination was also performed 72 hours after PHx on livers that were not perfused with silica, and the sinusoids were difficult to identify, given the extent of extreme compression within the lobule (data not shown). Thus, under these conditions, electron-dense silica serves as a marker for sinusoid placement.

Comparative biochemical analysis of membrane fractions isolated from normal *versus* regenerating liver verified that the silica membrane isolation procedure is also an efficient method for isolating luminal surface components from regenerating liver. Figure 6 compares cell-specific membrane markers SE-1 and ASGP-R in SEC pellet 2 (SEC membrane) and the light membrane fraction (hepatocyte basolateral membrane) from normal and regenerating liver. SE-1 is found exclusively in the SEC membrane fraction, and ASGP-R is found in the light

membrane fraction originating from SEC pellet 1, although its expression is decreased in the hepatocyte basolateral membrane at 72 hours compared to control. This regeneration-related change in ASGP-R is consistent with previous reports.²³ Very little caveolin 1 expression was detected in the light membrane fraction/hepatocyte basolateral membrane and remained constant in the SEC membrane before and after PHx. Actin association with isolated membrane fractions fluctuated slightly among fractions.

To assess potential growth factor involvement at the sinusoidal surface, we evaluated the expression of EGF-R and c-Met (HGF receptor), as both receptors have ligands known to be intimately involved in liver regeneration in addition to angiogenesis. Additionally, we examined the receptor for urokinase plasminogen activator (u-PAR), which is involved in both HGF activation and plasmin generation. As shown in Figure 7, EGF-R expression was greatly reduced at the sinusoid surface and moderately reduced in the hepatocyte basolateral membrane after PHx. HGF receptor c-Met remained stable at the SEC surface but decreased within the hepatocyte basolateral membrane. Similar to c-Met, u-PAR exhibited decreased association with the hepatocyte basolateral membrane fraction 72 hours after PHx; however, it was increased in the SEC pellet.

We were interested in using the silica membrane isolation procedure to examine ECM protein changes at the sinusoidal surface during regeneration (Figure 8). It is known that ECM components elicit significant positive and negative influences in angiogenic events^{24–26} as well as in liver regeneration.^{4,15} Fibrinogen and fibronectin are both ECM and circulating proteins that are synthesized by the liver and are potential regulators of growth and remodeling. At 72 hours after PHx, an increase in fibrinogen subunits α (67 kD), β (56 kD), and γ (47 kD) was seen in the hepatocyte basolateral membrane fraction, whereas no change in these subunits was noted at the SEC surface during regeneration. An increase in the perfused liver lysate at 72 hours indicates the increased expression of non-membrane-associated fibrinogen subunits by hepatocytes, most likely in response to the surgical insult. Also worth noting is the presence of a high-molecular-mass fibrinogen fragment at 200 kD in all fractions examined in regenerating livers and a 100-kD fragment found in perfused liver and hepatocyte basolateral membrane. On the other hand, fibronectin does not fluctuate at the hepatocyte basolateral membrane, but increases nearly threefold at the SEC surface in response to PHx. Likewise, collagen IV association with hepatocyte basolateral membranes does not change, but decreases at the SEC surface after PHx. SPARC (secreted protein, acidic and rich in cysteine, also called osteonectin or BM40) was found to be highly enriched at the sinusoidal surface relative to whole liver lysates, where its expression diminished by 50% at 72 hours after PHx. Plasminogen expression was decreased at the sinusoidal surface after PHx, but its active enzymatic product, plasmin, was increased in the SEC fraction, indicating a probable activation in the context of u-PAR (see Figure 7). A similar increase in plasmin was

Figure 5. Comparison of normal and regenerating liver at 72 hours after 70% PHx. **(A)** Normal liver displays typical plate arrangements of hepatocytes bounded on either side by SEC. Surfaces of SEC are evenly coated with silica, with very little colloid seen on hepatocyte basolateral membranes. **(B)** Three days after PHx, sinusoids are completely compressed as a result of several rounds of hepatocyte division in the absence of SEC proliferation. The space of Disse is not visible under these conditions. S, sinusoid; H, hepatocyte; SD, space of Disse.

seen in the hepatocyte basolateral membrane fraction at this same time.

Discussion

Here we describe a technique that allows for rapid isolation, fractionation, and spatiotemporal characterization of proteins lining the liver sinusoid. The cationic colloidal

silica membrane perturbation technique allows the isolation of a variety of proteins available to derivatization via perfusion through the blood supply. In the context of angiogenesis during liver regeneration, ECM proteins can be assessed because of the discontinuous nature of the SECs, allowing access of the cationized silica particles, 50–70 nm in diameter, through fenestrations whose diameter ranges from 80 to 130 nm, as well as through gaps between the SECs.²² The changes in protein ex-

Figure 6. Biochemical characterization and comparison of cell-specific markers in isolated membrane fractions by Western immunoblotting. Isolated proteins were resolved on 12% discontinuous reducing gels, blotted to PVDF membrane, and probed for the designated proteins as described. Marker protein expression as determined for membrane fractions described in Figure 4 was maintained at 72 hours after PHx, indicating that the protocol is valid during the regenerative process.

Figure 7. Comparison of receptor proteins in isolated liver membrane fractions in control and regenerating livers 72 hours after PHx, as evaluated by Western immunoblotting. Proteins were resolved on 10% reducing gels, transferred to PVDF, and probed with specific antibodies as described.

Figure 8. Comparison of various extracellular matrix molecules in liver membrane fractions, in control and regenerating livers 72 hours after PHx, as evaluated by Western immunoblotting. Proteins were resolved on 10% reducing gels, transferred to PVDF, and probed with specific antibodies as described.

pression within enriched fractions during the various stages of liver regeneration and angiogenesis can be ascertained using Western blotting techniques such as those we describe here. The cationic colloidal silica membrane perturbation technique has been implicated as a useful way to examine changes accompanying angiogenic processes in tumors;²⁷ here we show its utility in examining normal, controlled revascularization processes during liver regeneration.

To date, very little is known about events at the sinusoidal surface during liver regeneration. It is known that expression of growth factor receptors on the cell surface serves to regulate the ability of cells lining the sinusoid to respond to a set of growth factors at specific times during the regenerative response. Therefore, identifying surface expression of receptors on SEC should provide insight into the possible mechanisms and growth factors involved in controlling mitogenesis and motility coincident with revascularization. Furthermore, recent research has indicated that modulation of ECM, in conjunction with cell surface and growth factor receptor proteins, is critical to both angiogenesis⁹ and liver regeneration.^{4,5,15,17,28}

ECM has the ability to regulate gene expression,²⁹ motility, mitogenesis, morphogenesis, and specific liver cell functions *in vitro*.^{30–33} *In situ* examination of the ECM components at the sinusoidal surface during liver regeneration after PHx should help to elucidate the relevance of ECM to revascularization within the liver during normal tissue remodeling.

The ability of specific cells within the liver to differentially respond to growth factors during the regenerative process can be ultimately fine-tuned by the surface expression of growth factor receptors. This type of regulation may be part of the reason that the SECs remain mitotically quiescent during the first 72 hours after PHx, whereas hepatocytes undergo several rounds of division, despite the fact that the two cell types are known to respond to some of the same mitogens. Endothelial cells^{34–36} and hepatocytes^{32,37} are known to elicit a mitotic and mitogenic response to both EGF or TGF- α and HGF. Furthermore, protein concentration and/or mRNA levels of these growth factors have been shown by many laboratories to increase in the liver after PHx,^{5,6,38} suggesting that they play a pivotal role in the initiation and progression of liver regeneration. We investigated the expression of the EGF receptor (the shared receptor for both TGF- α and EGF) and the HGF receptor, c-Met, in whole livers, and the fractions enriched in SEC membranes and hepatocyte basolateral membrane (Figure 7). A decrease in the expression EGF receptor protein in all fractions was observed at 72 hours after PHx when compared to control livers. Decreases in EGF receptor have been documented after PHx by evaluating EGF binding sites in intact liver.^{39,40} In agreement with the published data, our results indicate that EGF receptor is down-regulated at 72 hours after 70% resection in the hepatocyte basolateral membrane as well as in the SEC population.

HGF is a very potent hepatotrophic mitogen,⁴¹ but it has also been implicated in the induction of angiogenesis.^{34,35} Under the conditions described here, HGF receptor (c-Met) expression within the intact liver decreases at 72 hours after 70% PHx. This appears to be attributable to its down-regulation at the hepatocyte basolateral membrane, because preresection levels in the SEC membrane fraction are maintained or even elevated after PHx, whereas the hepatocyte basolateral fraction is reduced. General down-regulation of c-Met coincident with liver regeneration after 70% PHx has been shown by using Western immunoblotting of whole liver lysates⁴² as well as by deducing HGF binding sites.⁴³ Although our whole liver data correlate well with these findings, we now show, however, that c-Met expression is probably differentially regulated within these two cell populations, correlating well with its dual role as hepatocyte mitogen and angiogenesis factor.

Urokinase plasminogen activator receptor (u-PAR) is also thought to play a distinct role in both liver regeneration¹⁴ and angiogenesis.¹³ Like c-Met, u-PAR displays bimodal membrane distribution at different times after PHx. In resting livers, u-PAR is expressed at higher levels in the hepatocyte basolateral membrane than on the SEC surface. At 72 hours after PHx, while the total amount of

u-PAR expression is unchanged, u-PAR is increased at the SEC surface but diminished in the hepatocyte basolateral membrane. These data, in conjunction with the plasminogen data described below, indicate that up-regulation of the major components making up the fibrinolytic system occurs before SEC peak DNA synthesis at 96 hours after PHx and subsequent SEC migration into avascular hepatic islands. Conversely, these receptors are down-regulated in the hepatocyte fraction after their rounds of proliferation, physiologically lowering its ability to respond to HGF activated in the context of u-PAR.¹⁴

We next examined changes in the expression of various ECM molecules in normal and regenerating liver to determine whether specific proteins were independently regulated at the sinusoidal surface (Figure 8). The ECM within the space of Disse does not resemble typical basement membranes underlying vascular endothelium, which facilitates rapid molecular exchange between blood and hepatocytes.^{10,44} Using histological techniques, others have shown variations in ECM at the sinusoidal surface in both normal and regenerating livers, but because of the extreme attenuation of the SECs as well as the space of Disse, it was difficult to assess whether the molecules in question had access to the blood supply or were associated with either hepatocytes or SECs. Increased expression of fibronectin relative to control has been shown at 72 hours after PHx in total liver homogenates by Western immunoblotting techniques.^{15,45} Furthermore, increased deposition within the sinusoids at 72 hours after PHx was also shown by immunofluorescence.⁴⁶ We now show that fibronectin, the major ECM protein within the space of Disse,⁴ is up-regulated at the sinusoid surface but does not change in its association with the hepatocyte basolateral membrane at 72 hours after PHx.

Collagen IV, another major component of the space of Disse,⁴ shows the opposite response of fibronectin at 72 hours after resection, by increasing slightly in the hepatocyte basolateral membrane fraction but essentially disappearing from the SEC surface. This general decrease in collagen IV within the liver has been shown at 72 hours after PHx by others using immunofluorescence.⁴⁶ The differences in spatiotemporal deposition of fibronectin and collagen IV at 72 hours after PHx may indicate independent effects of these two ECM molecules on the SEC population at different times during regeneration. This is not an unexpected finding because fibronectin and collagen IV can differentially modulate hepatocyte adhesion, mitogenesis, and motility *in vitro* in response to a variety of growth factors.^{32,33,47} Nevertheless, our ability to dissect the deposition of these two molecules within the space of Disse *in vivo* lends credence to the previous *in vitro* results.

SPARC, also known as osteonectin or BM40, is known to be synthesized by endothelial cells as well as stellate (Ito) cells, a population of cells resident within the space of Disse.^{26,48} Although its function is unclear, SPARC has been implicated in the regulation of angiogenesis and wound healing in a variety of systems.^{26,49,50} SPARC decreases at the SEC membrane at 72 hours after PHx when compared to nonresected livers but is not seen in

any other fraction, indicating that its lack of abundance within the liver is compensated for by its extreme spatial specificity. Although SPARC mRNA has been shown in the liver and in isolated Ito cells,^{26,48} the exact location of SPARC deposition within the liver was unknown⁴⁴ but is elucidated here by the cationic colloidal silica membrane enrichment technique. Clearly, more work must be done to evaluate its role in liver during regeneration and revascularization.

Evaluation of ECM proteins modulated by the plasminogen activator system indicates that plasminogen is expressed at high levels at the sinusoidal surface in resting liver but is significantly dissipated 72 hours after PHx. However, an increase in the active zymogen, plasmin, is seen associated with the SEC surface at 72 hours compared to nonresected controls. Although very little plasminogen is seen to be associated with the hepatocyte basolateral surface of resting liver, an increase in both plasminogen and plasmin associated with the hepatocyte basolateral membrane at 72 hours after resection is observed. These results indicate that up-regulation of ECM proteolytic enzymes occurs before SEC mitogenesis and migration into avascular hepatic islands. Changes in plasminogen and plasmin have been shown to change significantly during the immediate early stages of liver regeneration.¹⁵ The data presented here allow us to dissect the relative quantities of each at the SEC and hepatocyte basolateral membrane during the regenerative process.

Other ECM molecules modulated by the fibrinolytic system were also evaluated. A high-molecular-mass fibrinogen fragment (~200 kd) was observed in both the SEC and hepatocyte basolateral membrane fractions at 72 hours after PHx, with only slight amounts seen in the SEC fraction of resting liver. An additional 100-kd fragment was observed in the perfused liver and associated with the hepatocyte basolateral membrane in regenerating liver. The significance of these fragments and their increased expression within the liver microenvironment at this time is unknown, but we suspect that they are fibrin degradation products (FDPs), generated by plasmin cleavage of fibrin polymers. FDPs are physiologically relevant to angiogenesis because cells have been shown to adhere to and spread on fibrin monomers and their degradation products better than to fibrinogen alone.⁵¹ Previously we have shown that modulation of the components of the fibrinolytic system is involved in the early stages of liver regeneration.^{14,15} As the fibrinolytic system is also involved in angiogenesis,¹³ it is most likely that the changes we now describe are also physiologically significant for liver revascularization.

In summary, using the cationic colloidal silica membrane perturbation protocol, we have developed a novel system with which to simultaneously dissect protein expression at the SEC surface as well as the hepatocyte basolateral membrane during liver regeneration and subsequent revascularization. We show that this technique allows characterization of surface changes that may accompany the neovascularization of avascular hepatic islands in the later stages of liver regeneration. We are currently using this technology for an in-depth inves-

tigation of mechanisms that may contribute to the angiogenic stage of liver regeneration at the sinusoidal cell surface.⁵²

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