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Sequential Expression of Cellular Fibronectin by Platelets, Macrophages, and Mesangial Cells in Proliferative Glomerulonephritis

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Fibronectin (Fn) regulates cell migration, proliferation, and extracellular matrix formation during embryogenesis, angiogenesis, and wound healing. Fn also promotes mesangial cell migration and proliferation* in vitro *and contributes to extracellular matrix formation and tissue remodeling during glomerular disease. In this study, we examined, by immunohistochemistry and* in situ *hybridization, the temporal glomerular localization and cellular sources of Fn in Habu snake venom (HSV)-induced proliferative glomerulonephritis. Early HSV-induced glomerular lesions consisted of microaneurysms devoid of resident glomerular cells and filled with platelets, leukocytes, and erythrocytes. Over the course of the disease, mesangial cells migrated into the lesions, proliferated, and formed a confluent cellular mass. Fn was present in lesions beginning at 8 hours, with highest intensity at 72 hours and diminishing at 2 weeks after HSV. Staining for Fn at 8 and 24 hours after HSV was attributed to platelets and macrophages.* In situ *hybridization and phenotypic identification of cell types within lesions revealed macrophages as the predominant source of cellular Fn mRNA at these times. At 48 hours after HSV, Fn mRNA was expressed in proliferating mesangial cells in addition to macrophages. Most cells in lesions at 72 hours after HSV were mesangial, at a time when expression of Fn mRNA peaked. Cellular expression for Fn mRNA and translated protein declined at 2 weeks after HSV. These studies support the hypothesis that Fn, derived from platelets and macrophages, provides a provisional matrix involved with mesangial cell migration into glomerular lesions. Fn produced by mesangial cells might contribute to the formation of a stable extracellular matrix. (Am J Pathol 1994, 145:585–597)

Fibronectin (Fn) is a multifunctional extracellular matrix glycoprotein thought to play an important role in embryogenesis, angiogenesis, and cell remodeling in tissue repair and wound healing.[1–3] Fn has a variety of biological properties that may be important in the mediation of the above events through cell-matrix interactions involving cell-cell adhesion, adhesion to connective tissue components, cell migration, proliferation, and differentiation.[1–6]

A variety of cell types synthesize Fn, including mesenchymal cells, epithelial, and endothelial cells[1,3,6] and inflammatory cells including monocyte/macrophages[7–9] and megakaryocytes (secretory product in platelets).[10] During embryogenesis or wound healing, expression of Fn is often abundant and occurs along tracts associated with cell migration and proliferation.[1–6] Fn also forms an important part of normal extracellular matrix composition in a variety of organs and is frequently overexpressed during wound healing and in disease states including atherosclerosis,[11] pulmonary fibrosis,[12] and in various glomerulopathies.[13–17] During injury or wound healing, Fn can be derived from the plasma (pFn) as an exudate from injured blood vessels or synthesized directly by cells (cFn) in the local microenvironment.[1–3] The structure of these Fns is very similar, but through alternative gene splicing up to 12 distinct forms of Fn can be synthesized in the rat, each with potentially different specific functions.[18,19] Fn gene

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transcript can be alternatively spliced in three regions (EIIIA, EIIIB, and V). EIIIA and EIIIB are excluded in hepatocytes that produce pFn, whereas Fn, synthesized by cultured cells and platelets, is a mixture of forms with and without these domains.[18,19]

Fn is a normal component of glomerular extracellular matrix and is localized primarily around mesangial cells in the mesangial matrix and to a lesser extent in the glomerular basement membrane.[20–25] Localization of Fn is accentuated in a variety of experimental[26–32] and clinical forms[13–17] of glomerular diseases involving matrix expansion. The source of Fn has been controversial and both pFn and cFn derived from cells in the local microenvironment may play a role in cell function during remodeling over the course of glomerular injury and glomerulosclerosis. Currently, most studies have examined Fn localization in glomerular injury using polyclonal antibodies to Fn that recognize Fn in general but do not distinguish between pFn and cFn.[14–17,26–31] Northern analysis of mRNA encoding Fn in kidney or isolated glomeruli from diseased renal tissue has identified an increase in expression endogenous Fn[26,28,31] but the cellular sources of Fn synthesis have not been specifically identified.

Because Fn has been implicated in a number of facets of cell remodeling after injury and has been shown to stimulate mesangial cell migration[33,34] and mesangial cell spreading and proliferation[35] *in vitro*, we were interested in examining the course of cFn localization by immunohistochemistry and cellular sources of mRNA encoding Fn in glomerular lesions in an accelerated model of proliferative glomerulonephritis induced by Habu snake venom (HSV). This model has previously been shown to have cell migration and proliferation as features of tissue remodeling during the disease process.[36–38]

Materials and Methods

Induction of Glomerulonephritis

Glomerular lesions were induced in male Sprague-Dawley rats (Charles River, Willmington, MA) weighing 200 to 250 g, as previously reported.[36] Briefly, the rats were unilaterally nephrectomized and 24 hours later were injected with HSV (*Trimeresurus flavoviridis*; Sigma Chemical Co., St. Louis, MO) at a dose of 3 mg/kg intravenously. At 8 (n = 4), 24 (n = 4), 48 (n = 4), and 72 (n = 4) hours and 2 weeks (n = 4) after injection of HSV, the rats were sacrificed and slices of renal cortex obtained and immersed in 10% neutral-buffered formalin for subsequent processing, paraffin embedding, and light microscopical evaluation. Additional slices of cortex were snap-frozen in liquid nitrogen for subsequent immunoperoxidase identification of cell types and the presence of cFn within glomerular lesions. Separate frozen slices of renal cortex were obtained for *in situ* hybridization.

Characterization of Cell Types Within Glomerular Lesions

The principal cell type in HSV-induced glomerular lesions was previously determined to be mesangial in origin, however, macrophages were also present in early lesions.[36–38] Cell types in glomerular lesions were identified by immunoperoxidase histochemistry using the following primary antibodies (20 μg/ml) to cell-specific phenotypic markers. Mesangial cells: mouse monoclonal antidesmin (Dako Corporation, Carpinteria, CA), mouse monoclonal anti-rat Thy-1.1, clone OX 7 (Accurate Chemical & Scientific Corp., Westbury, NY), and mouse monoclonal anti-α smooth muscle actin, clone IA4 (Sigma Chemical Company, St. Louis, MO). Monocytes and macrophages: mouse monoclonal anti-rat myeloid cell, clone ED-1 (Sero-Tech, Bioproducts for Science, Inc., Indianapolis, IN). Blocking treatments and incubation with biotin-labeled secondary antibodies were as previously described.[37] Sections were developed using 3-amino-9-ethylcarbozole as substrate and counterstained with hematoxylin.

Quantitation of Macrophages Within Glomerular Lesions

Because macrophages have been observed to express Fn mRNA and secrete a provisional matrix in healing wounds[9] and macrophages might similarly contribute Fn to glomerular lesions, the number of macrophages present within lesions throughout the course of HSV-induced glomerulonephritis was determined. Sections stained with ED-1 antibody, as above, were examined for positive cells and the number of macrophages per glomerulus recorded. Approximately 30 glomeruli/section were analyzed.

Glomerular Localization of cFn

Glomerular localization of cFn was assessed using a mouse monoclonal antibody specific for cFn (clone FN-3E2; Sigma). Immunoperoxidase localization of cFn was examined in frozen sections as described above using anti-cFn antibody and biotinylated rat anti-mouse IgM (Zymed, South San Francisco, CA) as second antibody followed by avidin-biotin complex

amplification (Vector Laboratories, Burlingame, CA). Controls consisted of mouse IgM ascites or phosphate-buffered saline (PBS) in place of primary antibody. The antibody recognizes the 240-kd band of cFn using immunoblotting assays and does not cross-react with pFn, as stated by the manufacturer. Also, we determined a lack of the antibody to stain hepatocytes (principal source of pFn) in normal liver using the same immunodetection methods used in these experiments. Adsorption of the anti-cFn antibody with rat plasma Fn at 5 and 10 times antigen excess did not alter the intensity or distribution of staining in glomerular lesions, indicating the specificity of the antibody to cellular forms of Fn. The specificity of the antibody to domain(s) (EIIIA or EIIIB) unique to cellular Fn have not been determined.

In Situ *Hybridization*

Preparation of Riboprobes

A 270-bp segment of Fn complementary DNA subcloned into pGEM-2 plasmid (generously provided by Richard O. Hynes, Massachusetts Institute of Technology) was used for the generation of labeled riboprobes to detect cellular localization of Fn transcript within glomerular lesions. The antisense transcript (complementary to mRNA) corresponds to a region (C) in the type 1 repeats near the COOH-terminal end of Fn common to all known forms of mRNA.[6] A 438-bp cDNA fragment in pGEM-3 representing murine lysozyme (generously provided by Livingston Van De Water, Harvard University, reference 9) and a 130-bp cDNA segment in pGEM-1 (generously provided by Giulio Gabbiani, University of Geneva)[39] representing α-smooth muscle cell actin were used to generate riboprobes for *in situ* phenotypic identification and verification of macrophages and mesangial cells within lesions. All experiments were performed simultaneously with labeled sense riboprobe (anticomplementary to mRNA) as a negative control.

In vitro transcription of cDNA for [^{35}S]-labeled antisense and sense riboprobes was performed using a Riboprobe system II kit (Promega, Madison, WI), according to the manufacturer's instructions. Briefly, 300 μC of [^{35}S]uridine-5′-(a-thio)-triphosphate (1300 Ci/mmol; New England Nuclear, Boston, MA) was added to a 20 μl reaction mixture containing 0.5 mmol/L each of adenosine-, guanosine-, cytosine-5′triphosphate, 10 mmol/L dithiothreitol, 20 U RNasin ribonuclease inhibitor, 500 ng linearized plasmid, and 15 U of either SP6 or T7 RNA polymerase using a transcription buffer supplied with the kit. The reaction mixture was incubated for 60 minutes at 40 C then the DNA template removed by digestion with 0.5 U RNase-free DNase, followed by removal of unincorporated nucleotides by phenol/chloroform extraction and ethanol precipitation. RNA probes (specific activity approximately 4×10^6 cpm/μl) were stored at −70 C and used within 3 days.

Tissue Preparation

Frozen sections (6 μ) were cut and collected onto aminosilane-glutaraldehyde-treated slides, dried for 30 seconds on a hot plate at 80 C, and fixed for 20 minutes in 4% paraformaldehyde in 0.01 M PBS, pH 7.4. The sections were washed twice in PBS, dehydrated through a graded series of ethanols, air-dried, and immediately used for *in situ* hybridization.

Tissue Hybridization

In situ hybridization procedures involving prehybridization, hybridization, and removal of nonspecifically bound probe were performed using the protocol of Hogan et al[40] as adapted by Milani et al.[41] Air-dried sections were treated with O.2 N HCl for 20 minutes followed by 5-minute washes with H_2O. The sections were digested with proteinase K (1 μg/ml) for 10 minutes, washed in PBS, 0.1 M glycine in PBS, and fixed for 20 minutes in 4% paraformaldehyde in PBS. Sections were then washed in PBS for 5 minutes and acetylated in a freshly prepared solution of acetic anhydride diluted 1:400 in 0.1 M triethanolamine, pH 8, for 10 minutes. The sections were washed in PBS for 5 minutes, dehydrated in graded ethanols, and air-dried for subsequent hybridization. Twenty-five microliters of hybridization mixture containing 50% formamide, 10% dextran sulfate, 10 mmol/L dithiothreitol, 0.1 M Tris-HCl, pH 7.5, 0.1 M $NaPO_4$, 0.3 M NaCl, 50 mmol/L EDTA, 1× Denhardt's solution, 0.2 mg/ml yeast tRNA, and 2×10^5 cpm of [^{35}S]-labeled riboprobe was applied to each section and covered with a siliconized coverslip. Hybridizations were performed in a sealed humid chamber for 18 hours at 50 C. Excess probe was removed by washing for 4 hours at 50 C in 0.1 M Tris-HCl, pH 7.5, 0.1 M $NaPO_4$, 0.3 M NaCl, 50 mmol/L EDTA, 1× Denhardt's solution, and 10 mmol/L dithiothreitol.

Slides were digested for 30 minutes at 37 C with 20 μg/ml of RNase A in 0.1 M Tris-HCl, pH 7.5, 1 mmol/L EDTA, and 0.5 M NaCl to decrease nonspecific background activity. After a 30-minute wash at 37 C in the same buffer without the enzyme, sections were rinsed in 2× standard saline citrate, 0.1% sodium dodecyl

sulfate for 30 minutes each, dehydrated in graded ethanols containing 0.3 M ammonium acetate, and air-dried. Sections were immersed in the dark in Kodak NTB-2 photographic emulsion (Eastman-Kodak, Rochester, NY) diluted with equal parts of 0.6 M ammonium acetate and prewarmed to 45 C. The slides were kept in the dark, allowed to air dry, then exposed for a maximum of 2 weeks. The emulsion was developed and stained with hematoxylin and eosin for subsequent light microscopical analysis.

Results

Morphology

Glomerular pathological alterations after injection of HSV agreed with previously published studies.[36–38,42] Early (8 and 24 hours) lesions were characterized by capillary dilatation and ballooning resulting in microaneurysms filled with platelet aggregates, leukocytes, erythrocytes, and plasma proteins (Figure 1a). The microaneurysms develop into prolifera-

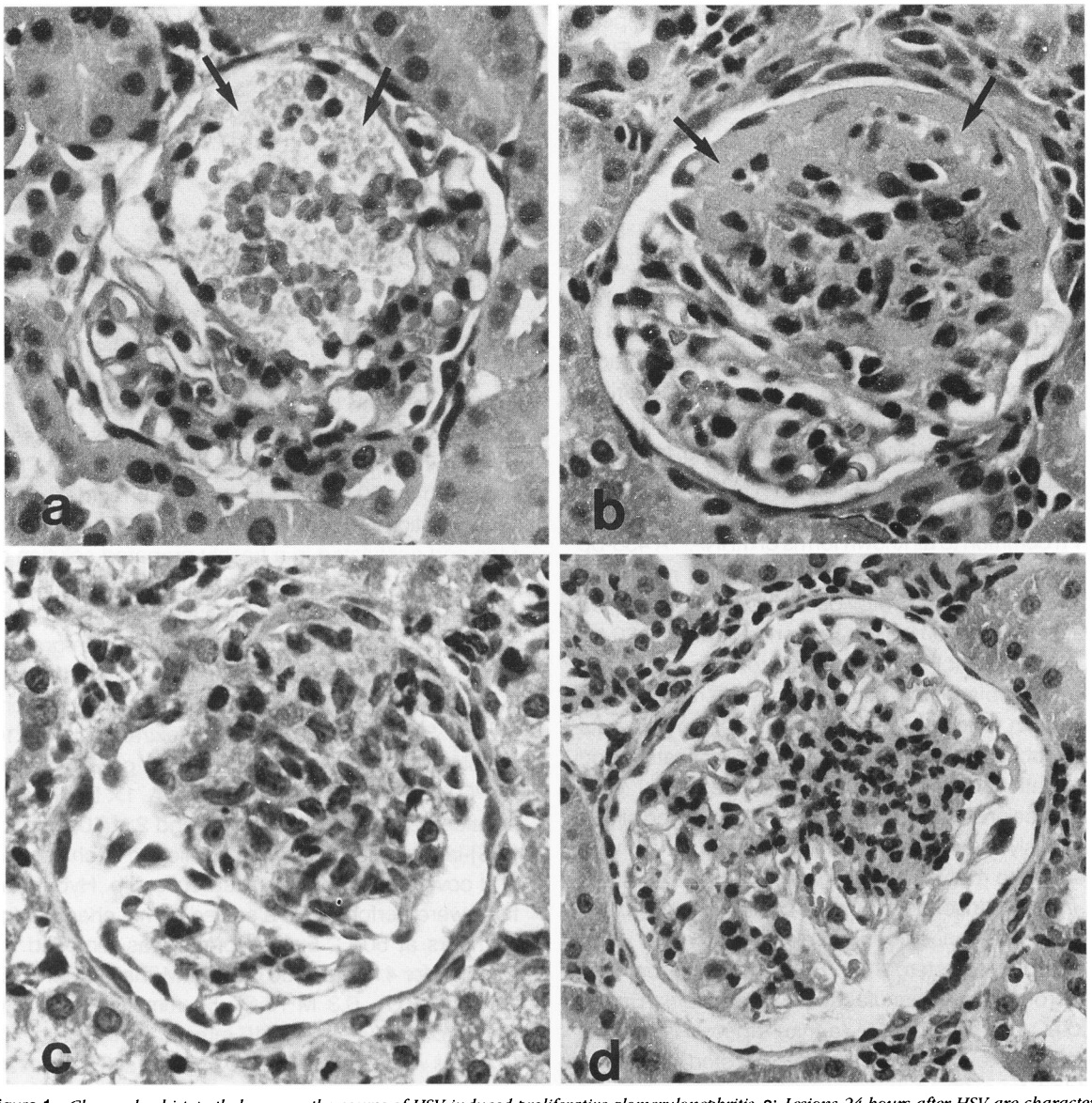


Figure 1. *Glomerular histopathology over the course of HSV-induced proliferative glomerulonephritis.* a: *Lesions 24 hours after HSV are characterized by microaneurysms (arrows) filled with platelet aggregates, leukocytes, erythrocytes, and plasma proteins (×600).* b: *Lesions (arrows) 48 hours after HSV contain numerous cells throughout the central aspects of the microaneurysm (×550).* c: *At 72 hours after HSV, lesions contain a confluent mass of cells forming a micronodule (×550).* d: *Micronodules at 2 weeks after HSV contain numerous cells with expanded extracellular spaces (×440). Sections stained with hematoxylin and eosin.*

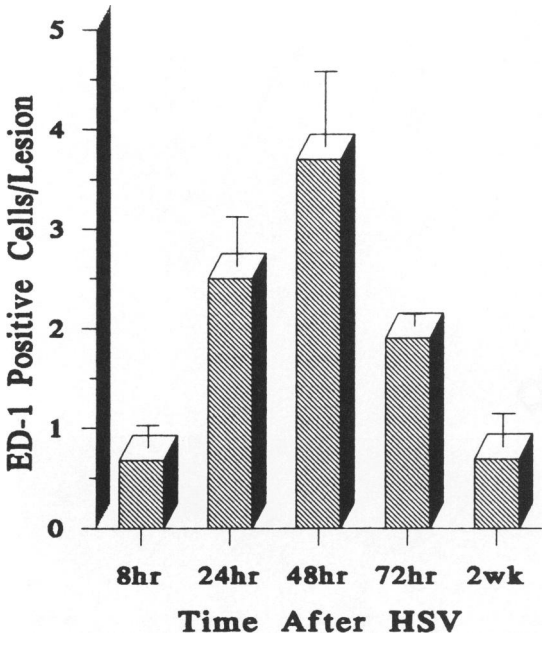


Figure 2. *Macrophage infiltration in glomerular lesions over the course of HSV-induced glomerular disease. The number of macrophages (detected by immunoperoxidase staining for ED-1) peaked at 48 hours and diminished at 72 hours and 2 weeks after HSV.*

tive lesions by 48 hours, characterized by the presence of numerous individual cells or clusters of cells surrounded by platelets and blood elements (Figure 1b) followed by development of micronodular lesions consisting of a confluent mass of proliferative cells. Most lesions at 72 hours and all lesions at 2 weeks after HSV were of the micronodular type (Figure 1, c and d). Spaces between cells in lesions at 2 weeks after HSV were expanded (Figure 1d).

Cell Types Within Glomerular Lesions

These studies agree with previous reports that showed a sequential presence of macrophages and mesangial cells within glomerular lesions.[37] Macrophages, identified by ED-1 staining, were observed in the central aspects of microaneurysms, increasing in number from 8 to 48 hours after HSV, declined at later periods and were uncommon in micronodular lesions (Figures 2 and 3). Mesangial cells, identified by Thy-1.1 surface antigen (Figure 4), were observed at the margins of lesions 24 hours after HSV (Figure 4a), followed by the proliferation of these cells at 48 and 72 hours filling the lesions forming micronodules consisting of confluent masses of cells (Figures 4, b-d). By 48 hours and after, cells in lesions acquired expression of α-smooth muscle actin, a specific marker of smooth muscle cells, and activated mesangial cells *in vivo*[43] (Figure 4, c and d). Tissue stained with PBS or nonimmune IgG in place of primary antibody was negative.

Cell types were verified by *in situ* hybridization using lysozyme antisense riboprobe to detect macrophages throughout the time course of the disease (Figure 3a) and α-smooth muscle cell actin to detect mesangial cells in advanced lesions (Figure 4d). Expression of lysozyme mRNA showed the same spatial and temporal distribution of macrophages within lesions as detection of ED-1 surface antigen by immunoperoxidase histochemistry. As with immunodetection of α-smooth muscle cell actin protein, expression of α-actin mRNA by *in situ* hybridization identified mesangial cells only in advanced lesions (48 hours and after) (Figure 4d). Control tissue hybridized with

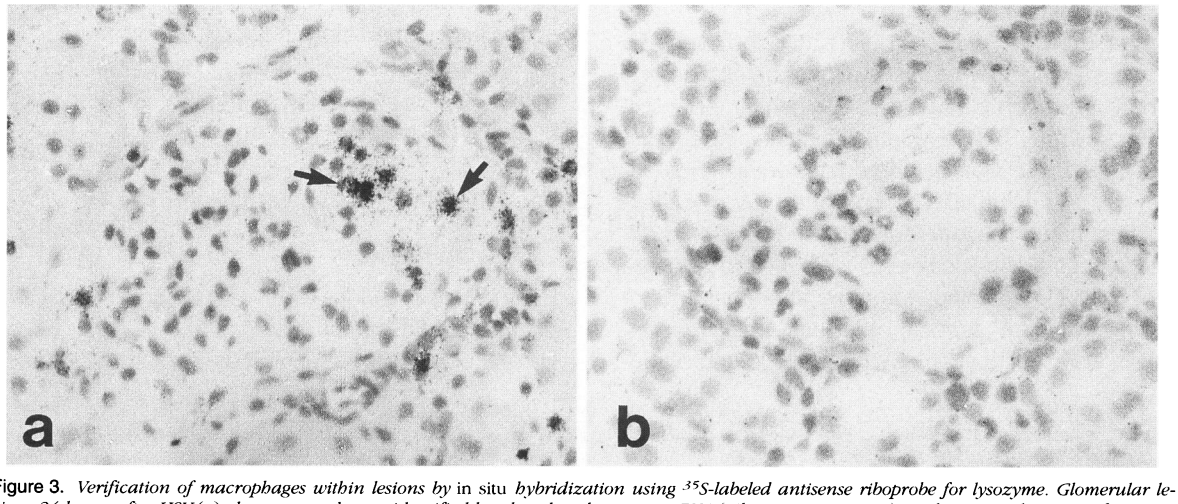


Figure 3. *Verification of macrophages within lesions by* in situ *hybridization using ^{35}S-labeled antisense riboprobe for lysozyme. Glomerular lesions 24 hours after HSV* (a) *show macrophages, identified by abundant lysozyme mRNA (silver grains, arrows), within central aspects of microaneurysms. Tissue hybridized with ^{35}S-labeled sense probe as a control is negative in glomerular lesions* (b). *Hematoxylin counter stain (×350).*

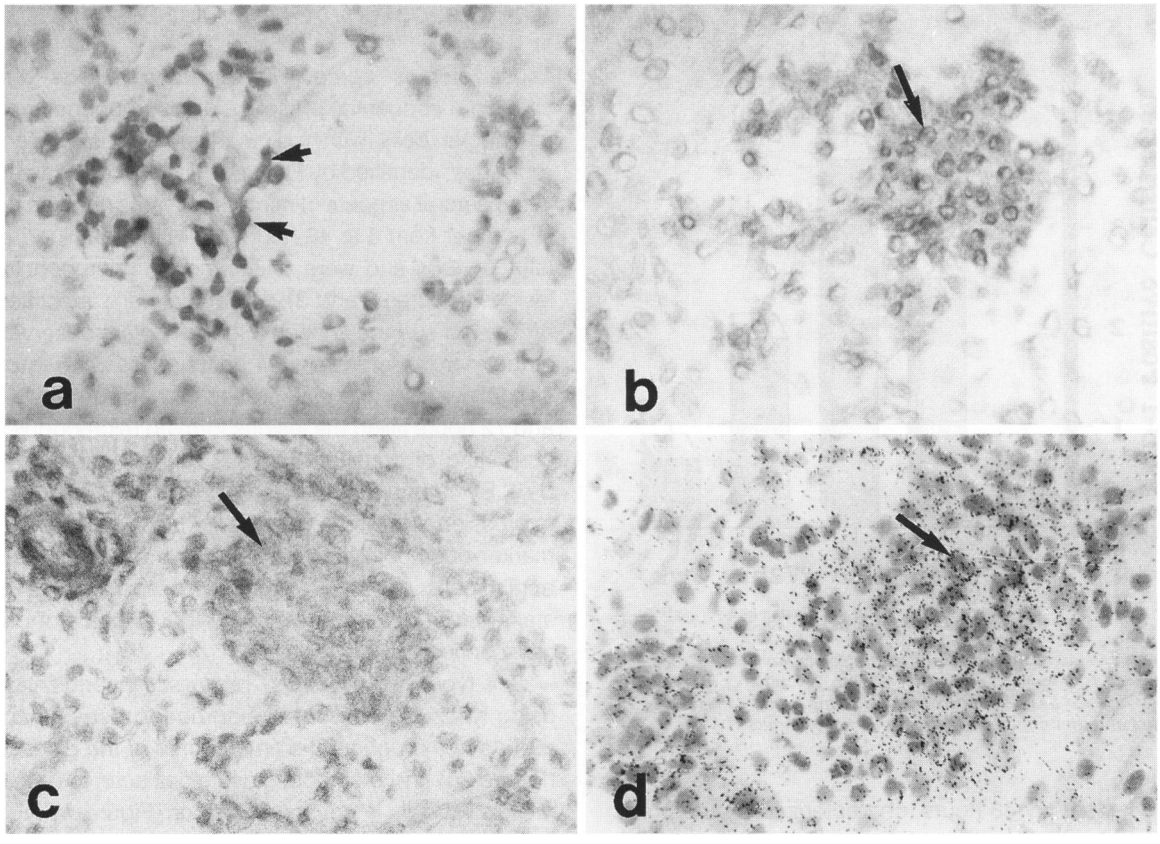


Figure 4. *Localization of mesangial cells (detected by Thy-1.1 antigen and α-smooth muscle cell actin) over the course of HSV-induced glomerular injury. At 24 hours after HSV* (**a**)*, Thy-1.1 is present in mesangial cells at the margins of lesions (short arrows)* (×*380*)*. Lesions at 72 hours after HSV* (**b**) *show Thy-1.1 staining throughout micronodules (long arrows)* (×*380*)*. Mesangial cell origin of cells within micronodules (long arrows) at 72 hours after HSV is verified by staining for α-smooth muscle cell actin protein by immunoperoxidase* (**c**) (×*480*) *and by mRNA expression by* in situ *hybridization* (**d**) (×*380*)*. Immunoperoxidase staining with 3-amino-9-ethylcarbazole as substrate* (**a-c**)*. Hematoxylin counter stain* (**a-d**).

labeled riboprobes transcribed in the sense orientation were negative (Figure 3b).

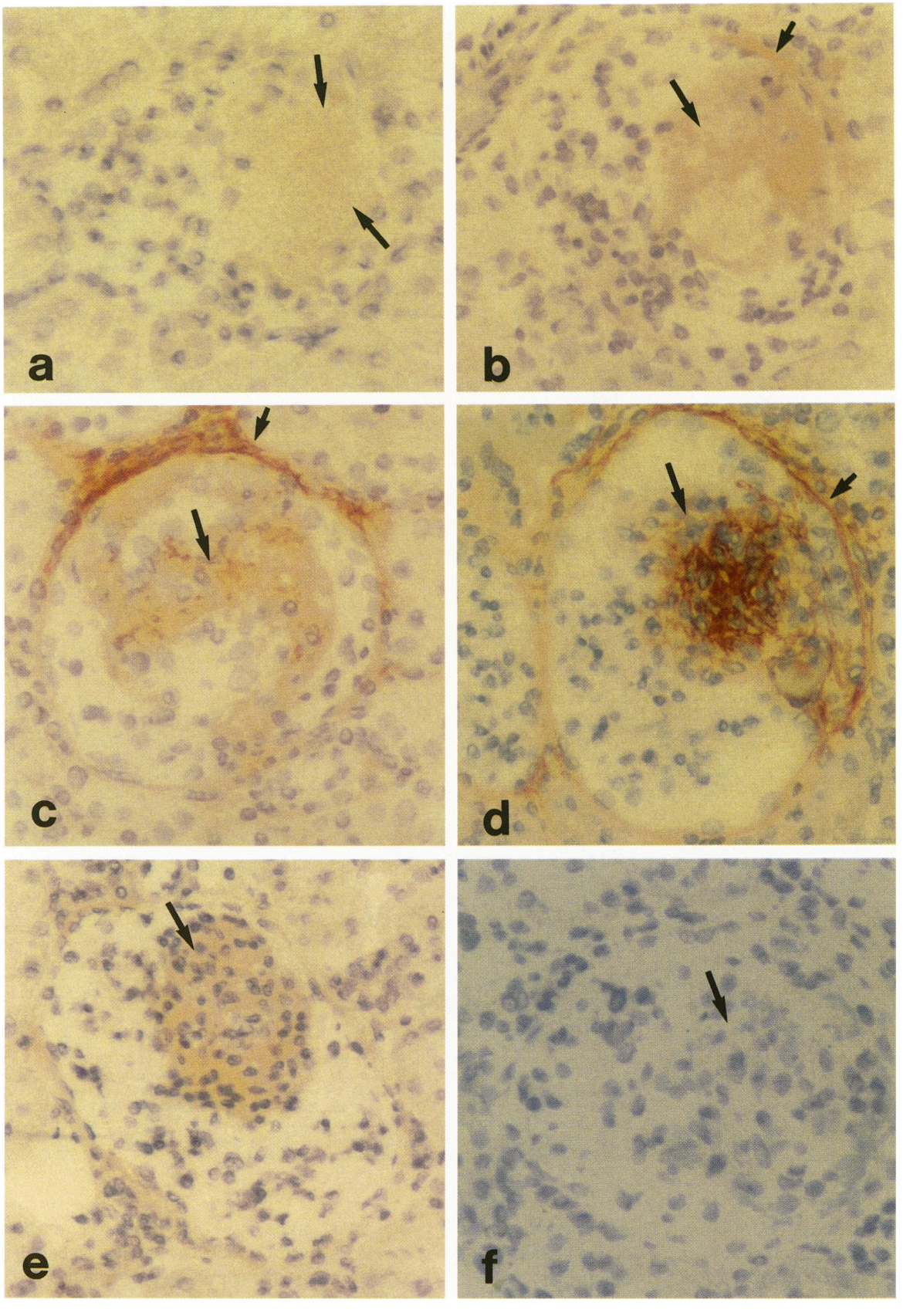
Immunolocalization of cFn

Immunodetection of cFn protein paralleled the distribution and intensity of mRNA signal detected by *in situ* hybridization (see below). Glomerular lesions at 8 hours after HSV showed only faint staining for cFn associated with platelet aggregates within microaneurysms (Figure 5a). By 24 hours after HSV, cFn staining was more intense (Figure 5b) and appeared diffuse in central aspects of lesions. At 48 and 72 hours after HSV, cFn staining was more abundant and localized in central aspects of lesions, peaking in intensity at 72 hours after HSV (Figure 5, c and d). Localization of cFn in glomerular lesions at 2 weeks was less intense than at 72 hours after HSV (Figure 5e). In many glomeruli, the parietal epithelium of Bowman's capsule and adjoining interstitial space showed enhanced staining for cFn, particularly in aspects of the epithelium immediately adjacent to glomerular lesions (Figures 5, b-e). Controls stained with PBS or nonimmune IgM in place of primary antibody were negative (Figure 5f).

Expression of Fn mRNA by In Situ *Hybridization*

Early (8 hours) lesions were generally devoid of cells expressing Fn mRNA (Figure 6a). However, occasional cells within the central aspects of microaneu-

Figure 5. *Immunoperoxidase localization of cFn protein throughout the course of HSV-induced glomerulopathy.* (**a**)*: Platelets (arrows) in glomerular lesions 8 hours after HSV stain weakly for cFn. cFn immunolocalization within microaneurysms (long arrows) at 24* (**b**) *and 48* (**c**) *hours is progressively more intense and corresponds with an increase cellularity within glomerular lesions. Intensity of staining for cFn is most intense at 72 hours after HSV* (**d**)*. By 2 weeks after HSV* (**e**)*, the intensity of cFn staining diminishes. Enhanced immunostaining for cFn is shown in Bowman's capsule immediately adjacent to glomerular lesions at 24, 48, and 72 hours after HSV* (**c, d,** *short arrows). A control section (72 hours after HSV) exposed to mouse IgM in place of primary anti-cFn antibody is negative for reaction product within glomerular structures* (**f**)*. The 3-amino-9-ethylcarbazole substrate, hematoxylin counter stain* (**a-c, f** ×*400;* **d** *and* **e** ×*330*).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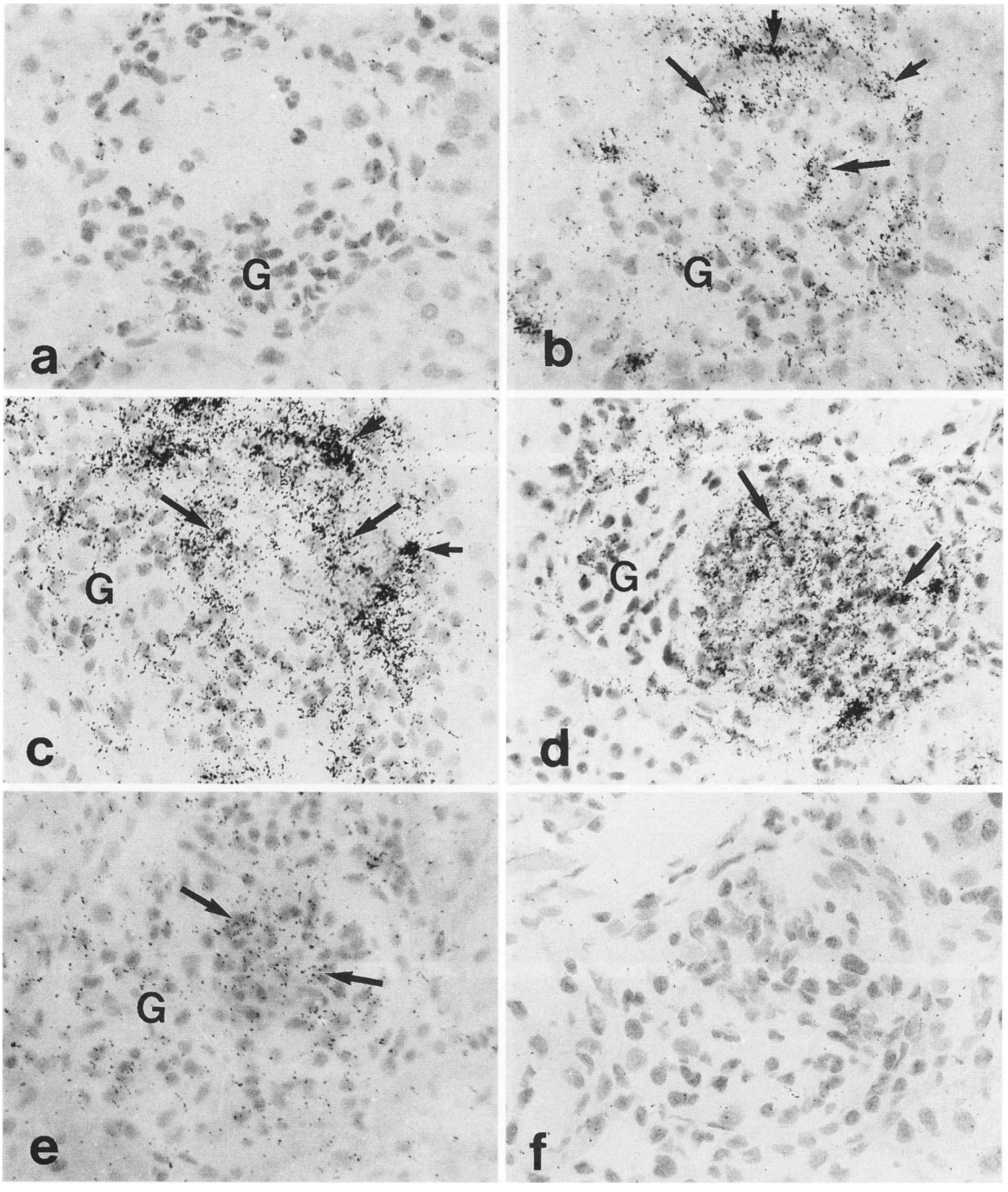

Figure 6. In situ *detection of cFn mRNA in cells within glomerular lesions (long arrows) and intact glomerular capillary tufts (G) over the course of HSV-induced proliferative glomerulonephritis.* (**a**): *Most glomerular lesions at 8 hours after HSV are negative.* (**b**): *Glomerular lesions 24 hours after HSV show several cells within the central aspects of glomerular lesions (long arrows) expressing Fn mRNA; cells in Bowman's capsule (short arrows) also express message for Fn.* (**c**): *Glomerular lesions at 48 hours after HSV show an increase in cells expressing Fn mRNA within the microaneurysm (long arrows) and Bowman's capsule.* (**d**): *The most intense signal for Fn mRNA was observed in micronodules (arrows) at 72 hours, diminishing in intensity at 2 weeks* (**e**) *and corresponding to the staining intensity of translated protein by immunohistochemistry.* (**f**): *Control sections hybridized with riboprobe transcribed in the sense orientation are negative (^{35}S-labeled sense riboprobe for Fn, 72 hours after HSV). Hematoxylin counterstain (×420).*

rysms expressed Fn message. These cells were identified as mononuclear cells or macrophages based on the temporal location of macrophage markers (ED-1 and lysozyme) by immunohistochemistry and *in situ* hybridization, respectively, and absence of resident glomerular cells in this region at this time. Neutrophils (identified by their distinct nuclear morphology) did not express Fn mRNA.

By 24 hours after HSV, the expression of Fn message increased (Figure 6b), associated with the increase in the number of macrophages within lesions. Mesangial cells at margins of lesions tended not to express Fn message above that observed in the mesangium in intact glomerular capillary tufts. However, occasional positive cells were identified with enhanced amounts of message compared with adjacent mesangium in the intact glomerular tuft.

At 48 hours after HSV, the number of cells expressing Fn mRNA within central aspects of lesions increased, correlating with the increase in macrophages and mesangial cells within lesions and with the increased expression of translated protein detected by immunoperoxidase histochemistry (Figures 5c and 6c). Cells at the interface between the intact capillary tuft and glomerular lesions frequently expressed Fn mRNA. The most abundant message was detected 72 hours after HSV, at a time when lesions became filled with a confluent mass of mesangial cells and expression of Fn protein was maximal (Figures 5d and 6d). By 2 weeks after HSV, signal for Fn mRNA and translated protein declined (Figures 5e and 6e) but remained above basal levels in adjacent capillary tufts.

At 24 hours after HSV and subsequent intervals, signal for Fn mRNA was frequently observed in the parietal epithelium lining Bowman's capsule (Figure 6). As with immunohistochemical detection of cFn, the most abundant mRNA message in Bowman's capsule was observed directly adjacent to the lesion (Figure 5), suggesting enhanced expression mediated by substances diffusing from the contents of the lesion.

Discussion

These studies show that cFn localized within glomerular lesions throughout the course of HSV-induced proliferative glomerulonephritis. cFn in glomerular lesions can potentially be derived from several sources: platelets, infiltrating leukocytes, and resident glomerular cells including mesangial, epithelial, and endothelial cells. With the aid of immunohistochemistry and *in situ* hybridization, we showed differential and sequential cellular sources of Fn over the course of HSV-induced glomerular injury. Fn was first derived from platelets followed by macrophages then mesangial cells. Glomerular epithelial or endothelial cells did not appear to contribute to the localization of Fn within glomerular lesions.

Platelets have been shown to contain a type of Fn distinct from pFn and similar to Fn purified from fibroblasts in that they demonstrate the V and EIIIA spliced regions that are not associated with pFn.[18,19] In this study, the contribution of platelet Fn detected by immunoperoxidase histochemistry in glomerular lesions was minimal compared with that of macrophages and mesangial cells. In early glomerular lesions (8 hours after HSV) immunolocalization of cFn was observed to be weak and restricted to platelets in the central aspects of microaneurysms. We did not, however, detect Fn mRNA in platelet aggregates by *in situ* hybridization. The inability to detect Fn mRNA in platelet aggregates might reflect published data showing detectable levels of mRNA by Northern analysis encoding abundant platelet proteins such as PF4 and actin but only trace amounts of mRNA encoding thrombospondin and no detectable levels of platelet Fn mRNA.[44] The low copy number of platelet Fn mRNA in platelets is probably below the sensitivity of *in situ* hybridization, despite the immunodetection of translated platelet secretory product.

The first cellular source of increased expression of Fn mRNA during the early stages of the disease process (8 and 24 hours) appeared to be blood borne monocytes/macrophages. Occasional cells in the central aspects of glomerular lesions at 8 hours after HSV were observed to contain Fn mRNA, however, most glomeruli were negative for cellular expression of Fn mRNA. The Fn-positive cells were deduced to be macrophages based on the immunodetection of the phenotypic marker ED-1, the monocyte-specific expression of lysozyme mRNA by *in situ* hybridization, and the lack of cells expressing mesangial markers desmin and Thy-1.1 antigen in central aspects of lesions at this time.[37,38] Also, a marked enhancement in cFn protein within lesions at 24 hours corresponded with an increase in the number of cells (macrophages) containing Fn mRNA. As observed at 8 hours after HSV, Fn mRNA in cells within lesions at 24 hours after HSV was determined to be derived from macrophages based on the phenotypic markers and the absence of mesangial cells from central aspects of glomerular lesions at this time.

Blood monocytes generally do not express Fn mRNA, however, mature or activated mononuclear phagocytes such as alveolar and peritoneal macrophages derived from patients with idiopathic pulmonary fibrosis[45] or peritoneal exudates[46] express abundant Fn mRNA message. Also, previous studies by Brown et al[9] reported an enhanced expression of Fn in macrophages during cutaneous wound healing, the first *in vivo* description of expression of Fn mRNA by macrophages as a response to acute injury. Our studies provide additional evidence that activated

macrophages have the ability to synthesize Fn *in vivo* in a setting of glomerular injury.

By 48 hours after HSV, the population of cell types within the central aspects of microaneurysms changed to include mesangial cells in addition to macrophages. Our studies also showed that expression cFn protein in glomerular lesions increased at 48 hours and staining was most intense 72 hours after HSV. Mesangial cells expressed abundant message and cFn protein during and after the proliferative phase (48 and 72 hours) in repopulating glomerular lesions. The most abundant staining for cFn protein and mRNA by *in situ* hybridization occurred 72 hours after HSV at a time when mesangial cells were the predominant cell population within glomerular lesions and the macrophage population within glomerular lesions declined. These findings closely parallel observations by Brown et al[9] showing that macrophages are the first cells in wound healing to express increased amounts of Fn mRNA followed by the assumption of the role of expression of Fn by fibroblasts at later stages of repair. In our studies, mesangial cells (like fibroblasts in wound healing) assumed the expression of abundant Fn mRNA after glomerular infiltration of macrophage and their expression of Fn.

The exact roles of Fn deposition in wound healing and glomerular injury and how they might differ from pFn are poorly understood. However, cFn may have specific roles that facilitate cellular function in terms of cell migration, proliferation, and formation of extracellular matrix. Fn is chemotactic for a variety of cell types *in vitro* including endothelial, vascular smooth muscle, and epithelial cells.[1–6,33,34] *In vivo* observations in embryos show that Fn is present in large amounts associated with cell migratory pathways.[1,2,4,5] Also, Fn synthesis and deposition in cutaneous wound healing suggests that it may have a role in promoting cell migration in developing granulation tissue.[1,2,6,9] Similarly, mesangial cell migration in early glomerular lesions might in part be regulated by Fn derived from platelets and macrophages.

We have shown a progressive sequence of events from early mesangial cell margination to cell proliferation in HSV-induced proliferative glomerulonephritis.[38] The early microaneurysms are filled with platelet aggregates, neutrophils, and macrophages but devoid of mesangial cells. As the lesions progress mesangial cells marginate along the periphery of the glomerulus-lesion interface before cell proliferation. Cell margination was retarded and proliferation blocked by prior platelet depletion, suggesting that mesangial cells migrate from intact glomerular structures toward a milieu replete with platelet secretory products.[36,38] A number of platelet secretory products might potentially influence cell migration *in vivo*.[33] We have shown that mesangial cells migrate *in vitro* in response to platelet releasate in general and specifically to platelet-derived growth factor and platelet Fn but not to platelet factor 4, epidermal growth factor, or transforming growth factor-α or -β.[34,47] Also, platelet Fn was determined to be a potent constituent of platelet releasate responsible for cell migration by the ability to inhibit migration with arg-gly-asp-ser tetrapeptide.[34]

Fn might also be involved in cell proliferation during tissue remodeling and wound healing.[1–3] A variety of cell types, including mesangial cells, proliferate in response to Fn.[1–3,35] As in the case for cell migration, Fn localizes at sites of proliferation in embryogenesis and wound healing.[1–6] Mesangial cells have been shown to proliferate in response to Fn,[35] however, a role for Fn in cell proliferation in glomerular disease remains to be determined. Because of the abundance of Fn mRNA and translated protein throughout the course of development of proliferative lesions, a case for cFn either as an autocrine or paracrine growth factor for mesangial cell hyperplasia cannot be ruled out.

Fn has been shown to be a constitutive extracellular matrix protein comprising the glomerular basement membrane and mesangial matrix. Glomerular mesangial cells have the ability to synthesize Fn in culture.[21,48,49] Endothelial and epithelial cells in general also synthesize Fn and glomerular epithelial and endothelial cells have integrin receptors for Fn.[51,52] However, in our *in situ* hybridization studies, glomerular epithelial or endothelial cells did not express Fn mRNA. On the other hand, mesangial cells in glomerular tufts showed small amounts of Fn mRNA that increased slightly in HSV-treated rats. Mesangial cell synthesis of cFn within glomerular lesions peaked at 72 hours, indicating that the largest amount of Fn synthesized during glomerular injury, at least in this model, is derived from mesangial cells. These studies corroborate reports in other models of glomerular disease that increased amounts of Fn in extracellular matrix is derived from mesangial cells.[26–32]

Equally important, however, is the finding of Fn synthesis by macrophages in a glomerular disease setting. Macrophages are a frequent cellular component in a variety of clinical[50] and experimental[28,29,31,32,53,54] glomerular diseases and have recently been associated with fibrosis. In several models, Fn mRNA obtained from diseased kidney cortex has been shown to be elevated around the time of macrophage influx into glomerular structures.[28–30,32] Increased extracellular matrix production in these and other models of renal fibrosis has

been attributed to a direct role of release of transforming growth factor-β from intrinsic glomerular cells and macrophages[28,29,31,32,53,54] followed by synthesis of extracellular matrix by resident glomerular cells. Our studies indicate that macrophages can in addition contribute to increased extracellular matrix by directly secreting this extracellular matrix protein at sites of injury. This becomes even more important when one considers that cFn has been suggested to provide a provisional extracellular matrix and forms a foundation for further laying down of additional extracellular matrix proteins such as collagen and laminin.[55,56]

Previous studies in our laboratory showed that mesangial cell proliferation subsided 72 hours after HSV at a time when immunodetection of collagen increased.[37] We have also shown that expression of transforming growth factor-β mRNA by Northern analysis is most abundant at 48 hours and persists at 72 hours after HSV, coinciding with the immunolocalization of collagen type IV. This study shows that mesangial cell synthesis of Fn was also most abundant at 48 and 72 hours after HSV and agrees with other *in vitro* and *in vivo* experiments, indicating that transforming growth factor-β is a potent promoter of mesangial cell synthesis of extracellular matrix.[28,29,31–33]

These studies have shown that cFn localization and cell synthesis follows a sequential pattern similar to that described in wound healing in general. Glomerular localization of cFn is first derived from platelets, followed by macrophages then mesangial cells. Early localization of cFn from platelets and macrophages occurs at a time associated with mesangial cell migration and before mesangial cell proliferation. Mesangial cell synthesis of cFn occurred primarily at a time when mesangial cells are proliferating and synthesizing extracellular matrix. The precise roles of cFn on mesangial cell behavior in the pathogenesis of this model of proliferative glomerulonephritis remain to be elucidated.

These studies do not address which specific forms of Fn are expressed by macrophages and mesangial cells over the course of the disease process. Because alternatively spliced regions of Fn might influence cell behavior,[5,6,9,18,19] studies are underway to examine the cellular expression of the different variants of Fn over the course of HSV-induced glomerulonephritis.

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References

1. Ruoslahti E: Fibronectin and its receptors. Annu Rev Biochem 1988, 57:375–413
2. Hynes RO: Integrins: versatility, modulation, and signaling in cell adhesion. Cell 1992, 69:11–25
3. Grinnell F: Fibronectin and wound healing. J Cell Biochem 1984, 26:107–116
4. Dufour S, Duband J-L, Kornblihtt AR, Thiery JP: The role of fibronectins in embryonic cell migrations. Trends Genet 1988, 4:198–203
5. Ffrench-Constant C, Hynes RO: Patterns of fibronectin gene expression and splicing during cell migration in chicken embryos. Development 1988, 104:369–382
6. Ffrench-Constant C, Van De Water L, Dvorak HF, Hynes RO: Reappearance of an embryonic pattern of fibronectin splicing during wound healing in the adult rat. J Cell Biol 1989, 109:903–914
7. Alitalo K, Hovi T, Vaheri A: Fibronectin is produced by human macrophages. J Exp Med 1980, 151:602–613
8. Yamauchi K, Martinet Y, Crystal RG: Modulation of fibronectin gene expression in human mononuclear phagocytes. J Clin Invest 1987, 80:1720–1727
9. Brown LF, Dubin D, Lavigne L, Logan B, Dvorak HF, Van De Water L: Macrophages and fibroblasts express embryonic fibronectins during cutaneous wound healing. Am J Pathol 1993, 142:793–801
10. Plow EF, Birdwell C, Ginsberg MH, Byers V, Taylor L, Braisier A: Identification and quantitation of platelet-associated fibronectin antigen. J Clin Invest 1979, 63: 540–543
11. Shekhonin BV, Tararak EM, Samokhin GP, Mitkevich OV, Mazurov AV, Vinogradov DV, Vlasik TN, Kalantarov GF, Koteliansky VE: Visualization of apo B, fibrinogen/fibrin, and fibronectin in the intima of normal human aorta and large arteries and during atherosclerosis. Atherosclerosis 1990, 82:213–226
12. Limper AH, Roman J: Fibronectin. A versitile matrix protein with roles in thoracic development, repair and infection. Chest 1992, 101:1663–1673
13. Gould VE, Martinez-Lacabe V, Virtanen I, Sahlin KM, Schwartz MM: Differential distribution of tenascin and cellular fibronectins in acute and chronic renal allograft rejection. Lab Invest 1992, 67:71–79
14. Ikeya M, Nagase M, Honda N: Intraglomerular distribution of fibronectin in primary glomerular diseases. Clin Nephrol 1985, 24:53–59
15. Oomura A, Nakamura T, Arakawa M, Ooshima A, Isemura M: Alterations in the extracellular matrix components in human glomerular diseases. Virchows Arch A Pathol Anat 1989, 415:151–159
16. Linder E, Miettinen A, Tornroth T: Fibronectin as a marker for the glomerular mesangium in immunohistology of kidney biopsies. Lab Invest 1980, 42:70–75

17. Yamamoto T, Nakamura T, Noble NA, Ruoslahti E, and Border WA: Expression of transforming growth factor B is elevated in human and experimental diabetic nephropathy. Proc Natl Acad Sci, USA 1993, 90:1814–1818
18. Paul JI, Schwarzbauer JE, Tamkun JW, Hynes RO: Cell-type-specific fibronectin subunits generated by alternative splicing. J Biol Chem 1986, 261:12258–12265
19. Schwarzbauer JE: Alternative splicing of fibronectin: three variants, three functions. Bioessays 1991, 527–533
20. Weiss MA, Ooi BS, Ooi YM, Engvall E, Rouslahti E: Immunofluorescent localization of fibronectin in the human kidney. Lab Invest 1979, 41:340–347
21. Oberley TD, Mosher DF, Mills MD: Localization of fibronectin within the renal glomerulus and its production by cultured glomerular cells. Am J Pathol 1979, 96:651–662
22. Madri JA, Roll FJ, Furthmayr H, Foidart JM: Ultrastructural localization of fibronectin and laminin in the basement membranes of the murine kidney. J Cell Biol 1980, 86:682–687
23. Courtoy PJ, Kanwar YS, Hynes RO, Farquhar MG: Fibronectin localization in the rat glomerulus. J Cell Biol 1980, 87:691–696
24. Houser MT, Scheinman JI, Basgen J, Steffes MW, Michael AF: Preservation of mesangium and immunohistochemically defined antigens in glomerular basement membrane isolated by detergent extraction. J Clin Invest 1982, 69:1169–1175
25. Laitinen L, Vartio T, Virtanen I: Cellular fibronectins are differentially expressed in human fetal and adult kidney. Lab Invest 1991, 64:492–498
26. Tamaki K, Okuda S, Ando T, Iwamoto T, Nakayama M, Fujishima M: TGF-B1 in glomerulosclerosis and interstitial fibrosis of adriamycin nephropathy. Kidney Int 1994, 45:525–536
27. Yoneyama T, Nagase M, Ikeya M, Hishida A, Honda N: Intraglomerular fibronectin in rat experimental glomerulonephritis. Virchows Arch B Cell Pathol 1992, 62:179–188
28. Goyal M, Wiggins R: Fibronectin mRNA and protein accumulation, distribution, and breakdown in rabbit anti-glomerular basement membrane disease. J Am Soc Nephrol 1992, 1:1334–1342
29. Floege J, Alpers CE, Burns MW, Pritzl P, Gordon K, Couser WG, Johnson RJ: Glomerular cells, extracellular matrix accumulation, and the development of glomerulosclerosis in the remnant kidney model. Lab Invest 1992, 66:485–497
30. Floege J, Johnson RJ, Gordon K, Yoshimura A, Campell C, Iruela-Arispe L, Alpers CE, Couser WG: Altered glomerular extracellular matrix synthesis in experimental membranous nephropathy. Kidney Int 1992, 42:573–585
31. Ding G, Pesek-Diamond I, Diamond JR: Cholesterol, macrophages, and gene expression of TGF-β_1 and fibronectin during nephrosis. Am J Physiol 1993, 264: F577–F584
32. Yamamoto T, Noble NA, Miller DE, Border WA: Sustained expression of TGF-B1 underlies development of progressive kidney fibrosis. Kidney Int 1994, 45:916–927
33. Barnes JL: Platelets in renal disease. In Immunopharmacology of the Renal System: Handbook of Immunopharmacology. Edited by Tetta C. London, Academic Press, 1993, pp 87–118
34. Barnes JL, Hevey KA: Glomerular mesangial cell migration: response to platelet secretory products. Am J Pathol 1991, 138:859–866
35. Simonson MS, Culp LA, Dunn MJ: Rat mesangial cell-matrix interactions in culture. Exp Cell Res 1989, 184: 484–498
36. Barnes JL: Glomerular localization of platelet secretory proteins in mesangial proliferative lesions induced by Habu snake venom. J Histochem Cytochem 1989, 37: 1075–1082
37. Barnes JL, Abboud HE: Temporal expression of autocrine growth factors corresponds to morphologic features of mesangial proliferation in habu snake venom-induced glomerulonephritis. Am J Pathol 1993, 143: 1366–1376
38. Barnes JL, Hevey KA, Hastings RR, Bocanegra RA: Mesangial cell migration precedes proliferation in Habu snake venom-induced glomerular injury. Lab Invest 1994, 70:460–467
39. Kocher O, Gabbani, G: Analysis of alpha-smooth-muscle actin mRNA expression in rat aortic smooth-muscle cells using a specific cDNA probe. Differentiation 1987, 34:201–209
40. Hogan B, Costantini F, Lacy E: In Manipulating the Mouse Embryo: A Laboratory Manual. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory, 1986, pp 228–242
41. Milani S, Herbst H, Schuppan D, Stein H, Surrenti C: Transforming growth factors β_1 and β_2 are differentially expressed in fibrotic liver disease. Am J Pathol 1991, 139:1221–1229
42. Cattell VE, Bradfield JWB: Focal mesangial proliferative glomerulonephritis in the rat caused by Habu snake venom: a morphologic study. Am J Pathol 1977, 87:511–524
43. Johnson RJ, Iida H, Alpers CE, Majesky MW, Schwartz SM, Pritzl P, Gordon K, Gown AM: Expression of smooth muscle cell phenotype by rat mesangial cells in immune complex nephritis. α-smooth muscle actin is a marker of mesangial cell proliferation. J Clin Invest 1991, 87:847–858
44. Sottile J, Mosher DF, Fullenweider J, George JN: Human platelets contain mRNA transcripts for platelet factor 4 and actin. Thromb Haemost 1989, 62:1100–1102
45. Rennard SI, Hunninghake GW, Bitterman PB, Crystal RG: Production of fibronectin by the human alveolar macrophage: mechanism for the recruitment of fibroblasts to sites of tissue injury in interstitial lung diseases. Proc Natl Acad Sci USA 1981, 78:7147–7151

46. Goldstein CS, Garrick RE, Polin RA, Gerdes JS, Kolski GB, Neilson EG, Douglas SD: Fibronectin and complement secretion by monocytes and peritoneal macrophages in vitro from patients undergoing continuous ambulatory peritoneal dialysis. J Leukocyte Biol 1986, 39:457–464
47. Barnes JL, Hevey KA: Glomerular mesangial cell migration in response to platelet-derived growth factor. Lab Invest 1990, 62:379–382
48. Ishimura E, Sterzel RB, Budde K, Kashgarian M: Formation of extracellular matrix by cultured rat mesangial cells. Am J Pathol 1989, 134:843–855
49. Cosio FG, Sedmak DD, Nahman NS Jr: Cellular receptors for matrix proteins in normal human kidney and human mesangial cells. Kidney Int 1990, 38:886–895
50. Atkins RC, Holdsworth SR, Hancock WW, Thomson NM, Glasgow EF: Cellular immune mechanisms in human glomerulonephritis: the role of mononuclear leukocytes. Springer Semin Immunopathol 1982, 5:269–296
51. Adler S: Characterization of glomerular epithelial cell matrix receptors. Am J Pathol 1992, 141:571–578
52. Adler S, Eng B: Integrin receptors and function on cultured glomerular endothelial cells. Kidney Int 1993, 44:278–284
53. van Goor H, van der Horst MLC, Fiddler V, Grond J: Glomerular macrophage modulation affects mesangial expansion in the rat after renal ablation. Lab Invest 1992, 66:564–571
54. Mosquera JA: Increase production of fibronectin by glomerular cultures from rats with nephrotoxic nephritis: macrophages induce fibronectin production in cultured mesangial cells. Lab Invest 1993, 68:406–412
55. Ignotz RA, Massague J: Transforming growth factor-β stimulates the expression of fibronectin and collagen and their inorporation into the extracellular matrix. J Biol Chem 1986, 261:4337–4345
56. McDonald JA: Extracellular matrix assembly. Annu Rev Cell Biol 1988, 4:183–207