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Embryonic Fibronectin Isoforms Are Synthesized in Crescents in Experimental Autoimmune Glomerulonephritis

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Crescents are a severe and stereotyped glomerular response to injury that occur in several forms of glomerulonephritis that progress to renal failure. The key pathogenetic step that leads to glomerular scarring is unknown, but fibronectin (FN), the clotting system, macrophages, and proliferating parietal epithelial cells are known to participate. This study was designed to determine whether FN is synthesized locally, and in what molecular isoform, and whether cytokines known to promote FN synthesis are present in the crescent. Rats immunized with bovine glomerular basement membrane develop cellular crescents by 14 days and fibrous crescents and glomerulosclerosis by 35 days. **In situ** ***hybridization was performed with oligonucleotides specific for sequences common to all FN isoforms (total FN) or sequences specific for the alternatively spliced segments (EIIIA, EIIIB, and V). Throughout the time period (14, 21, and 35 days) all crescents and glomerular tufts contained cells with strong ISH signals for total and V^+ mRNA, with the strongest signals present in large cellular crescents at day 21. In contrast, $EIIIA^+$ and $EIIIB^+$ mRNAs showed maximal abundance within sclerosing crescents at 35 days. Protein deposition of $EIIIA^+$, $EIIIB^+$, and V^+ FN isoforms was confirmed by immunofluorescence with segment-specific FN antibodies. Transforming growth factor-β and interleukin-1β, both known to promote FN synthesis, were found in cellular crescents (days 14 and 21) and were still present, but greatly diminished, in the sclerotic phase (day 35). In summary, $EIIIA^-$, $EIIIB^-$, and V^+ FN mRNA plasma isoforms predominate in cellular crescents, whereas in the fibrosing stage, mainly the oncofetal $EIIIA^+$, $EIIIB^+$, and V^+ isoforms are synthesized and accumulate. (Am J Pathol 1995, 147:965–978)***

Glomerular crescent formation is a stereotyped response of glomeruli to injury seen in severe forms of glomerulonephritis. Once crescents form they usually progress to glomerular scarring with resulting renal failure. Past studies have shown that crescents are composed of macrophages, proliferating parietal epithelial cells, and components of the clotting system, notably fibrin and fibronectin.[1–5] Inhibition of clotting[6] or leukocyte accumulation[3] has prevented the formation or progression of crescents. Little is known regarding the cellular source and molecular forms of fibronectin (FN) that are deposited during crescent formation[5] and their possible biological significance.

FN is an extracellular matrix protein that is involved in cell migration, attachment, differentiation, recruitment, and extracellular matrix assembly.[7] FN is encoded by a single gene that, by alternative splicing of the pre-mRNA in distinct segments (EIIIA, EIIIB, and V), gives rise to different FN isoforms (Figure 1).[7] The splicing pattern is tissue and cell specific. For example, the mature liver synthesizes FN isoforms either including or excluding the V region and usually excluding the EIIIA and EIIIB segments.[7] In contrast, $EIIIA^+$ and $EIIIB^+$ isoforms are

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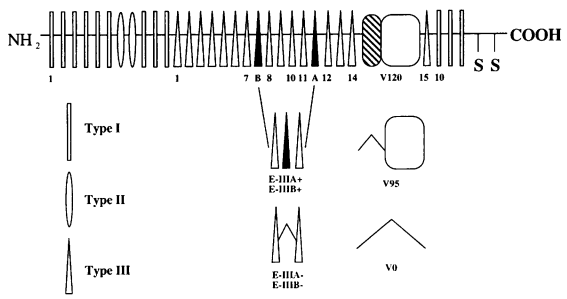

Figure 1. *Schematic of one subunit of fibronectin. The molecule (encoded by a single gene) is composed of a series of three different repeats, which are seperated in the gene by introns. The boxes/exons indicated (EIIIA, EIIIB, V) can either be included or excluded from mature FN mRNA or protein. In the rat, the V region is either completely spliced out (V0) or partially (V95) or entirely (V120) included. All combinations of EIIIA, EIIIB, and V are possible, allowing in the rat for 12 different variants. All other segments of the molecule are constant and can therefore be found in all fibronectin isoforms. Numbers under the symbols indicate the position of repeats.*

abundantly expressed during embryogenesis but to a much reduced extent in mature tissues[7–10] (J. Peters and R. O. Hynes, manuscript in preparation). The expression and splicing pattern of FN can be altered by transforming growth factor (TGF)-β and interleukin (IL)-1β. TGF-β has been found to increase FN synthesis in glomerular epithelial cell cultures[11] and to elevate FN mRNA levels, particularly mRNA copies including the alternatively spliced segments.[12,13]

IL-1β increases FN secretion in tissue cultures[14] and is co-deposited with FN in allograft vasculopathies.[15] IL-1β increases FN mRNA levels and protein synthesis in vascular smooth muscle cell cultures obtained from cardiac allografts.[16]

In this report we show in an anti-glomerular basement membrane (GBM) glomerulonephritis model in the rat that FN is synthesized within glomerular crescents, that parietal epithelial cells are capable of FN synthesis, and that the FN mRNA undergoes alternative splicing. Moreover, the synthesis of different FN isoforms follows temporally the local accumulation of TGF-β and IL-1β.

Materials and Methods

Anti-GBM Nephritis

The experimental protocol for the induction of crescentic glomerulonephritis with bovine GBM has been described previously.[3] Wistar Kyoto rats (150 to 190 g; Charles River Laboratories, Wilmington, MA) were housed in standard cages according to hospital and National Institutes of Health guidelines and had access to water and food *ad libitum*. Bovine GBM was prepared from fresh kidneys by a differential sieving method,[17] sonication, collagenase VII digestion (type VII collagenase; Sigma Chemical Co., St. Louis, MO) at 37°C for 16 hours, and subsequent centrifugation (14,500 $\times$ *g* for 30 minutes). Pellets were resuspended in citrate buffer (0.1 mol/L, pH 3.0). On day 0, experimental rats (n = 12) were immunized by a single subcutaneous injection of 50 mg of the GBM homogenate mixed with an equal volume of complete Freund's adjuvant (Sigma Chemical Co.). Control animals (n = 4) received a single subcutaneous injection of complete Freund's adjuvant.

Renal tissue samples were obtained 14, 21, or 35 days after sensitization. Portions to be examined by *in situ* hybridization (ISH) were fixed in 4% paraformaldehyde (4°C) for 3 hours, incubated overnight in 30% sucrose/phosphate-buffered saline (PBS) at 4°C, and frozen in OCT embedding medium (Miles Laboratories, Elkhart, IN). Tissue samples to be subjected to immunohistochemical analysis were embedded in OCT and fresh frozen. Tissue was stored at −80°C until sectioning. Cryostat 5-μm sections were placed on charged glass slides (probe on plus;Fisher Scientific, Fair Lawn, NJ) or on 3-aminopropyltriethoxy-silane-coated slides (Fisher) and stored at −80°C until use. Additional small tissue samples were fixed in 10% buffered formalin, paraffin embedded, and subsequently sectioned and stained with hematoxylin and eosin (H&E) or by the periodic acid-Schiff method.

In Situ *Hybridization*

ISH and oligonucleotide synthesis were performed as described previously.[18] Oligonucleotide probe sequences were chosen from previously published rat FN gene sequences.[19–21] Eight probes were synthesized on a DNA synthesizer (Applied Biosystems, Foster City, CA). The probes represented sense and corresponding antisense sequences of distinct exon segments of the FN gene (Figure 1). The term total denotes a sequence in the 10th/11th type I repeat near the carboxy terminal that does not undergo alternative mRNA splicing and is therefore common to all FN mRNA isoforms (GAC CCT TAC ACG GTT TCC CAT TAC GCC GTT GGA GAG GAA TGG GAG CGG). V denotes a sequence from the alternatively spliced V region of FN. The probe either detects the entirely included V region (composed of 360 bp/120 amino acids, V-120) or the partially included segment (composed of 285 bp/95 amino acids, V-95; CCT GGC ACA TCC CAC CAA CAA CCC AGT GTT GGG CAA CAA ATG ATC). EIIIA denotes a sequence from the alternatively spliced EIIIA segment

(GAG GAC ACG GCA GAG CTG CAC GGC CTC AGG CCG GGT TCT GAG TAC ACA). EIIIB denotes a sequence from the alternatively spliced EIIIB segment (AGG GCT GGA ACC CGG CAT TGA CTA TGA CAT CAG CGT TAT CAC TCT CAT).

On the day of hybridization, sense and corresponding antisense probes were 3′-end labeled with a terminal deoxynucleotidyl transferase (VWR Scientific-IBI) using ^{35}S-dATP (New England Nuclear, Dupont, Boston, MA) to a specific activity of 8 $\times$ 10^8 to 18 $\times$ 10^8 cpm/μg. The probes were mixed with hybridization solution (50% formamide, 4X standard saline citrate (SSC), 1X Denhardt's solution, 1% Sarkosyl, 0.02 mol/L $NaPO_4$, pH 7, 10% dextran sulfate, 150 μg/ml yeast tRNA, 150 μg/ml salmon sperm DNA, and 100 mmol/L dithiothreitol) to a final count of 1 $\times$ 10^3 cpm/μl. Hybridization with sense and antisense probes for each examined time point and animal was performed overnight at 42°C in a humidified chamber. Post-hybridization washes were performed in 1X SSC and 0.1X SSC, respectively, followed by dipping in NTB-2 emulsion (Kodak, Rochester, NY). Slides were stored in light-tight boxes at 4°C for 60 days. Slides were developed in D-19 (Kodak) for 5 minutes, fixed in fixer without hardener (Kodak) for 5 minutes, counterstained with H&E, dehydrated, and mounted with Permount (Fisher Scientific)

An embryonic rat fibroblast cell line (Rat-1) expressing all FN isoforms[22] served as positive hybridization control and was hybridized with all sense and antisense probes parallel to the tissue sections in each experimental run.

Immunofluorescence

Indirect immunofluorescence was performed with four segment-specific FN antibodies. Diluted ascites from a mouse hybridoma (BR 5.3) producing antibodies recognizing a constantly expressed epitope lying within the sequence encompassing the 7th through the 15th type III repeats of rat Fn[23] was used to detect all FN isoforms (total). To detect the V segment, polyclonal rabbit antiserum raised to a fusion protein containing the V95 segment of rat Fn,[24] and therefore recognizing both V120 and V95 spliced isoforms, was immunopurified on a rat plasma FN column as described (J. Peters and R. O. Hynes, manuscript in preparation). IST-9, a mouse monoclonal antibody raised to the human ED-A segement of Fn[25] (MAS 521b, Accurate Chemical and Scientific Corp., Westbury, CT) was used to detect EIIIA$^+$ forms. To detect EIIIB$^+$ isoforms of FN, rabbit antibodies raised to a fusion protein and immunopurified as described were employed.[23]

Immunofluorescence staining was performed according to standard protocols[26] except for the recently characterized EIIIB antibody,[23] which required an initial digestion step (see below). Unfixed fresh-frozen tissue sections were air dried overnight and briefly rehydrated in PBS. Sections were incubated with the primary antibodies at room temperature for 60 minutes in a humidified chamber (or with PBS as negative control for nonspecific binding of the secondary antibodies), washed in PBS, incubated with secondary fluorescein-conjugated antibodies (Jackson Immunoresearch Laboratories, West Grove, PA), washed again, and finally mounted with Aqua Mount (Lerner Laboratories, Pittsburgh, PA).

Direct antibody recognition of the EIIIB segment is normally blocked by *N*-linked carbohydrate side chains, masking target epitopes.[23] Therefore, fresh-frozen tissue sections to be stained with the EIIIB antibody were digested before antibody incubation with 7500 U/tissue section of *N*-glycanase (PNGase F, New England Biolabs, Beverly, MA) at 37°C overnight, followed by washes in PBS before EIIIB antibody incubation as described above. Sections treated with the PNGase F reaction buffer (supplied by manufacturer) but without the enzyme and followed by primary and secondary antibody incubations served as negative controls.

Immunoperoxidase

Immunoperoxidase staining followed standard procedures.[27] The antibodies used were (1) anti-TGF-β (DK-20-PB, R&D Systems, Minneapolis, MN), an affinity-purified polyclonal rabbit anti-pig antibody, which detects TGF-β_1 and to a lesser degree TGF-β_2 isoforms (description from the manufacturer), as found in the latent complexes[28]; (2) anti-IL-1β (Cytokine Sciences, Boston, MA), a polyclonal rabbit anti-rat antibody purified by chromatography; and (3) ED-1 (Accurate Chemical and Scientific Corp.), a monoclonal mouse anti-rat antibody, detecting a cytoplasmic antigen in rat monocytes/macrophages and dendritic cells.[29]

Previously sectioned fresh-frozen tissue, stored at −80°C, was air dried overnight and briefly (10 minutes) fixed in acetone, followed by blocking of endogenous peroxidase in 3% H_2O_2/methanol (containing 1% Triton X-100; for IL-1β only). Primary antibodies were applied to the sections at 4°C overnight. The next day, sections were incubated with a biotinylated secondary antibody (Vector Laborato-

ries, Burlingame, CA) and developed in 3-amino-9-ethylcarbazole (Aldrich Chemical Co., Milwaukee, WI). Sections were fixed in 2% paraformaldehyde for 10 minutes, counterstained with hematoxylin, and mounted in Glycergel (DAKO, Carpinteria, CA).

Microscopy

Coded tissue sections were examined under brightfield, darkfield, or immunofluorescence illumination with Zeiss microscopes and recorded with Kodak Ektachrome Elite 400 daylight films for immunofluorescence or Kodak Ektachrome 64T tungsten color reversal film for brightfield or darkfield photography.

Quantification

The intensity of the immunohistochemical stainings and ISH signals (grain density) was evaluated on a scale from 0 (negative) to ++++ (strong). Such analysis of the hybridization signal was limited to studies in which probes were applied in parallel in the same run to tissue sections representing all examined time points. All probes were labeled to similar specific activities.

Results

Morphology

At 14 and 21 days after immunization, glomeruli of experimental animals showed a marked increase in cellularity in glomerular tufts and under Bowman's capsule. Cellular crescents developed under Bowman's capsules in the vast majority of glomeruli, first seen on day 14 as small early crescents and on day 21 as large cellular crescents, involving either part or all of the circumference. Parietal epithelial cells, mononuclear inflammatory cells, scattered polymorphonuclear leukocytes, and fibrin were components of the crescents. The interstitium showed a focal mononuclear cell infiltrate, associated with very focal tubular injury and atrophy (data not shown).

At 35 days after immunization, nearly all glomeruli (>90%) were affected by crescent formation, many of which were circumferential, and undergoing fibrous transformation. Mononuclear inflammatory cells and fibrin were still present in crescents. A few glomeruli were globally sclerosed. The interstitium displayed a patchy mononuclear cell infiltrate with focal fibrosis and widespread tubular injury. Kidneys of control animals (complete Freund's adjuvant injection only) showed no morphological changes at the examined time points (data not shown).

In Situ *Hybridization for Fibronectin*

Control Kidneys

Weak hybridization signals for total and V^+ FN mRNA were detected over the endothelium of small arteries/arterioles; no signals were detectable with probes for EIIIA and EIIIB (data not shown). Glomeruli were negative with all probes (Figure 2).

Experimental Group
Total and V^+ FN mRNA Expression

mRNA expression for total FN and the alternatively spliced V region were very similar in the animals with anti-GBM nephritis, indicating that most of the locally synthesized FN isoforms were V^+. The results for these two forms of FN are therefore discussed together (Table 1).

In glomeruli, total and V^+ FN mRNAs were detected in all crescents with a peak expression level (+++) during the development of large cellular crescents (day 21, Figure 3). Less mRNA was seen in small crescents (day 14, ++; Figure 2) and in the late fibrous crescents (day 35, ++; Figure 4). Glomerular tufts also showed hybridization signals during cellular crescent formation on days 14 and 21 (++; Figures 2 and 3). Later, when fibrous crescents developed at day 35, only very occasional weak hybridization signals were detected in glomerular tufts (Figure 4). Parietal epithelial cells (flat cells lining Bowman's capsule) displayed V^+ and total FN mRNA focally from day 14 through 35 (Figure 2).

Tubular epithelial cells expressed FN mRNA focally in areas of injury (Figure 5). This was first detected on day 21 and more extensively on day 35 (approximately 25% of injured epithelial cells). These tubular cells were associated either with interstitial fibrosis, thickening of the tubular basement membrane, or epithelial cell sloughing into the tubular lumens. The sloughed epithelial cells displayed especially strong hybridization signals (data not shown). Frequently, even in areas of marked tubular

Figure 2. *ISH with an antisense probe detecting all fibronectin isoforms (total).* **A:** *No specific ISH signals are detected over normal glomeruli of control animals. Grains noted represent nonspecific background signals also seen with a sense probe. H&E counterstain; magnification, ×160.* **B:** *Corresponding darkfield.* **C:** *At 14 days after immunization, diffuse ISH signals are noted over developing crescents and glomerular tufts. Note the signals found over parietal epithelial cells not involved in crescent formation* (long arrow). *H&E counterstain; magnification, ×160.* **D:** *Corresponding darkfield.*

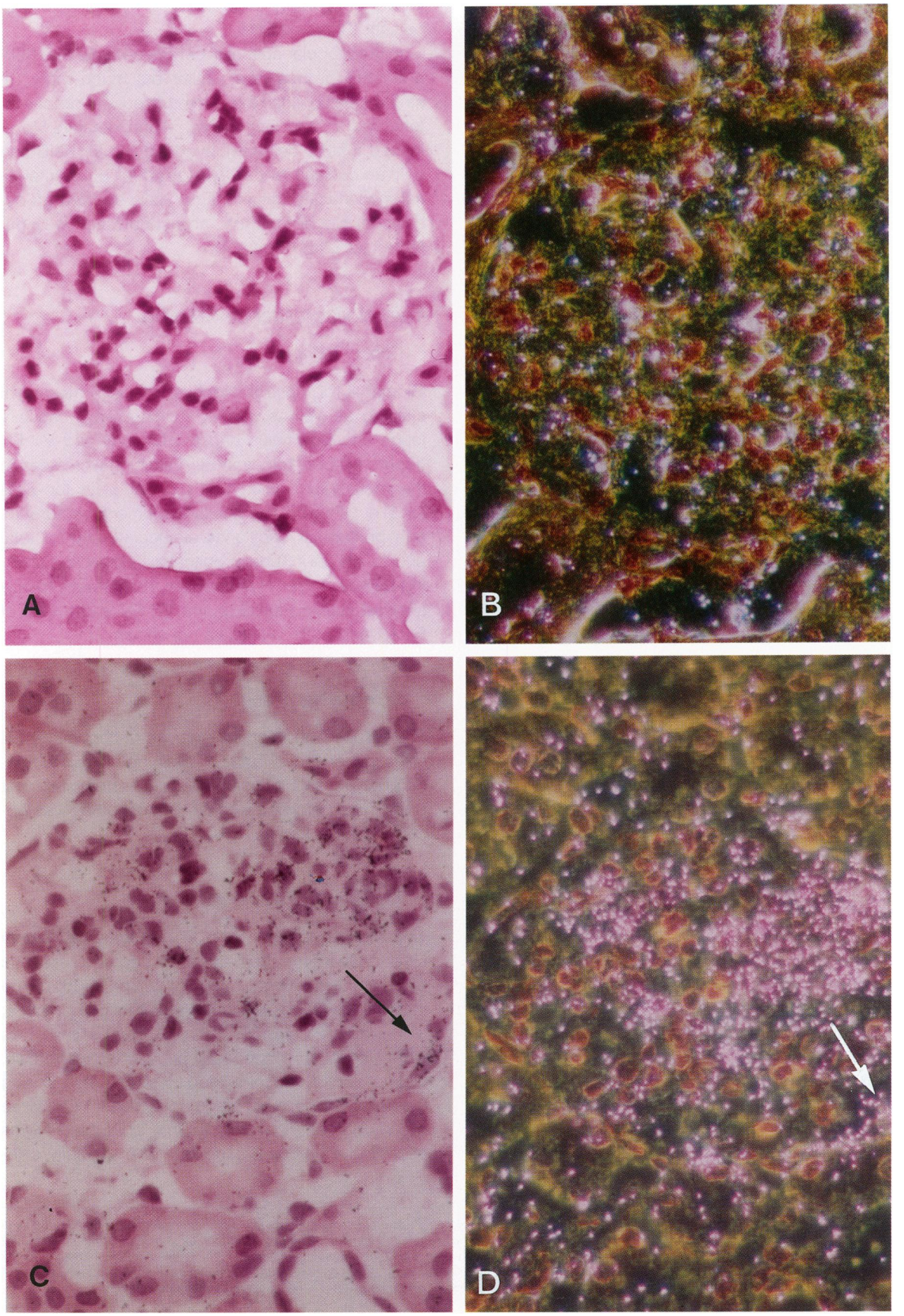
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Table 1. *Assessment of* in Situ *Hybridization Signals in Glomeruli*

FN isoforms	Day	Crescents	Glomerular tufts
Total and V	Control	NA	0
	14	++ (diffuse)	++ (diffuse)
	21	+++ (diffuse)	++ (diffuse)
	35	++ (diffuse)	± (focal)
EIIIA and EIIIB	Control	NA	0
	14	+ (focal)	± (focal)
	21	+ (focal)	± (focal)
	35	+ to ++ (diffuse)	0

NA, not applicable; control, animals injected with CFA only. Glomerular involvement was judged according to criteria used in renal pathology as focal <60% and diffuse >60% of all glomeruli. The intensity of the ISH signal was evaluated according to the grain density observed and categorized as ± (weak) to ++++ (strong).

injury, only scattered cells displayed hybridization signals. In contrast, surrounding tubular cells of similar light microscopic appearance remained negative. Mononuclear inflammatory cells in the interstitium focally expressed total and V^+ FN (data not shown).

EIIIA$^+$ and EIIIB$^+$ FN mRNA Expression

In general, ISH signals for EIIIB tended to be stronger than those for EIIIA at all experimental time points despite similar specific probe activities.

In glomeruli, at days 14 and 21, expression of FN mRNA isoforms encoding the EIIIA and EIIIB segments was much more focal than for total and V^+ FN mRNAs (Figure 3). Only small foci (+) of EIIIA$^+$ and EIIIB$^+$ cells were found in cellular crescents and these foci were confined to 50 and 60% of all crescents, respectively. Positive cells (especially expressing EIIIB mRNA) were rarely encountered in glomerular tufts. Moreover, the intensity of the hybridization signals remained unchanged during the development of cellular crescents. In contrast to the cellular crescentic stage, hybridization signals were much more widespread in fibrosing crescents (65 and 90% of all glomeruli showed EIIIA (+) and EIIIB (+ to ++) expression, respectively (Figure 4). Areas of periglomerular fibrosis and glomerular sclerosis all showed EIIIA and EIIIB expression on day 35. No signal was detected in glomerular tufts. Parietal epithelial cells rarely showed weak hybridization signals for EIIIA and EIIIB.

Injured tubular epithelial cells hybridized with EIIIA and EIIIB probes in a similar but much more restricted fashion than those hybridized with probes for total and V (data not shown). Approximately 5% of injured tubular cells displayed signals for EIIIB and only an occasional cell hybridized for EIIIA. Interstitial mononuclear inflammatory cells showed an occasional signal for EIIIA; no hybridization was detected for EIIIB (Figure 4).

Immunofluorescence Staining

Control Kidneys

Glomeruli of control kidneys showed mesangial staining as well as weaker staining along capillary loops and Bowman's capsule for total and V^+ FN (Figure 6). Only traces of EIIIA staining were seen in the mesangium. Glomeruli did not stain for EIIIB (data not shown). The tubular basement membranes and peritubular capillaries stained weakly for total and V^+ FN only. Tubular epithelial cells lacked staining by any of the FN antibodies. Vessel walls focally showed staining for total and V^+ FN and traces of EIIIA$^+$ and EIIIB$^+$ FN (data not shown).

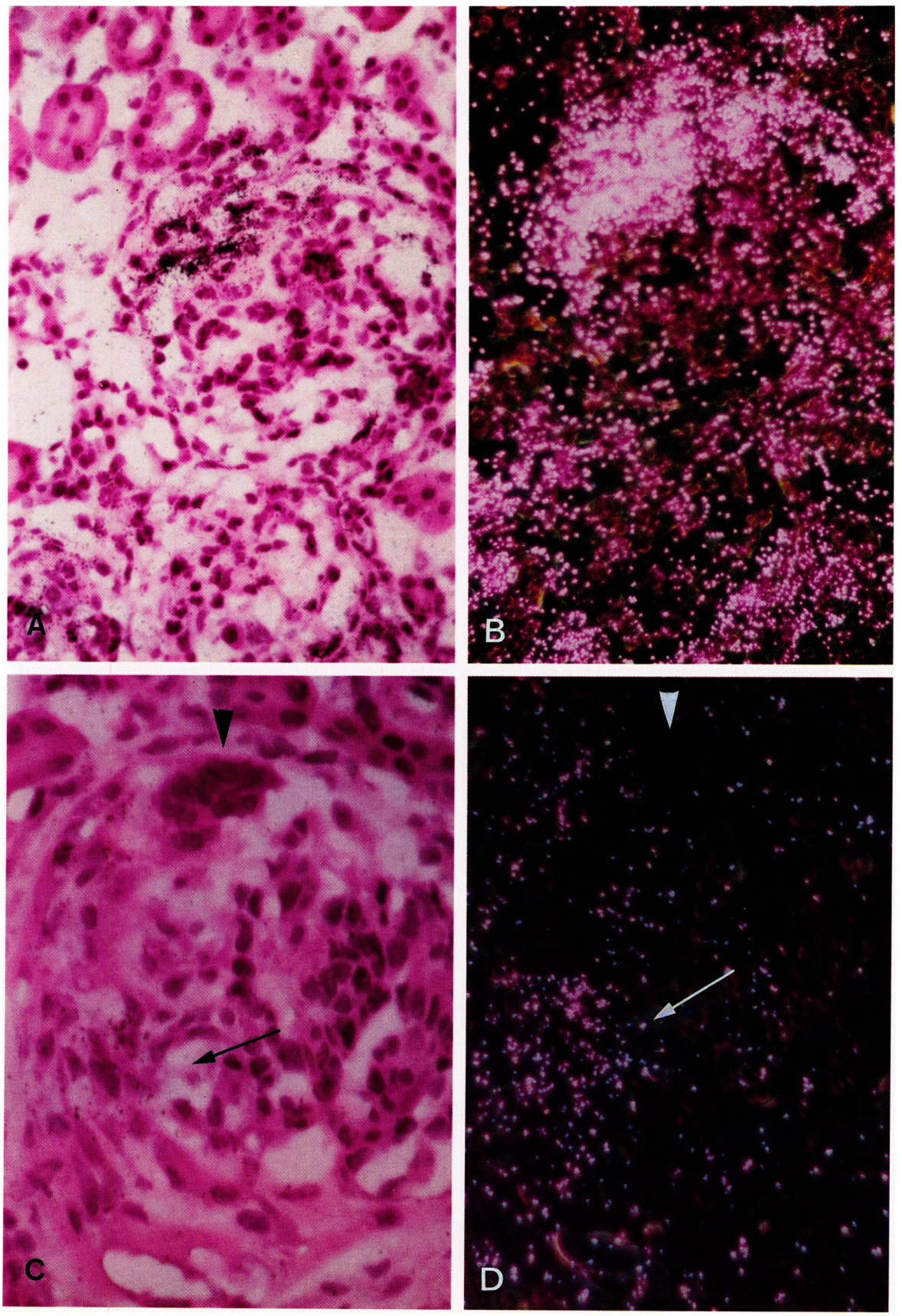
Experimental Group

The results with segment-specific antibodies to FN are summarized in Table 2. Staining with all FN antibodies was in a diffuse meshwork pattern; the most pronounced staining with all FN antibodies was observed in crescents.

Total and V^+ Deposition

The staining for total and V^+ FN was again very similar. Over time, from the early cellular crescentic stage to the late fibrotic stage, total and V^+ FNs were increasingly deposited in all crescents (++, day 14; +++, days 21 and 35; Figure 6). Total and V^+ FNs were also located in areas of periglomerular fibrosis. Glomerular tufts accumulated these FNs less. During the cellular crescentic stage, total and V^+ FNs were slightly increased. At day 35, when abundant FN was seen in the fibrous crescents and in globally scle-

Figure 3. *At 21 days after sensitization, large cellular crescents had developed.* A: *Strong ISH signals are detected over crescents with antisense probes detecting the alternatively spliced V segment. H&E counterstain; magnification, ×100.* B: *Corresponding darkfield.* C: *Antisense probe detecting the alternatively spliced EIIIB segment demonstrates signals only segmentally over a cellular crescent* (long arrow). *The intensity of the ISH signal is less than compared with that in Figure 3, A and B* (*also note the difference in the magnification*). *No signal is seen over the multinucleated giant cell* (arrowhead), *which is presumably of macrophage origin. H&E counterstain, magnification, ×160.* D: *Corresponding darkfield.*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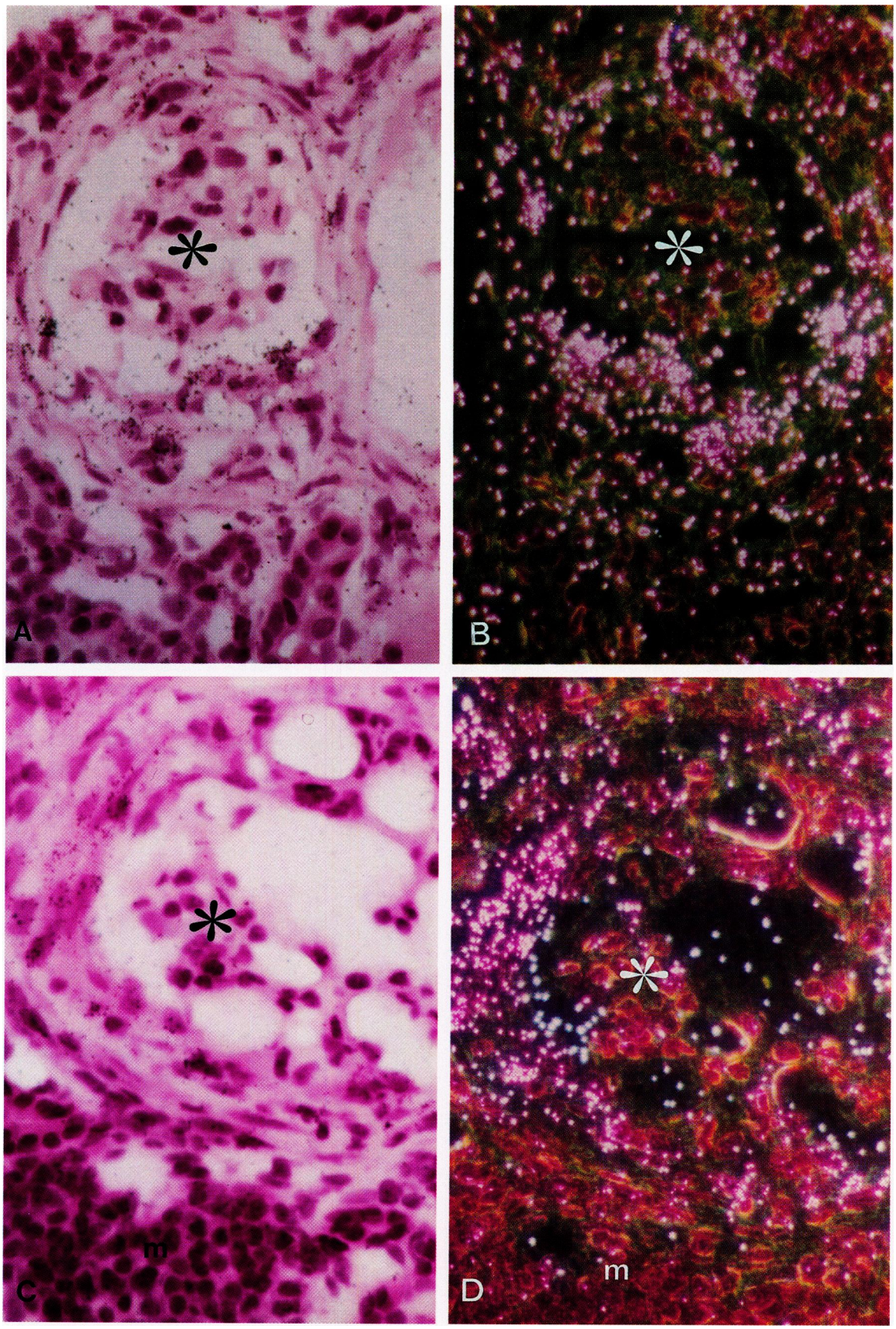
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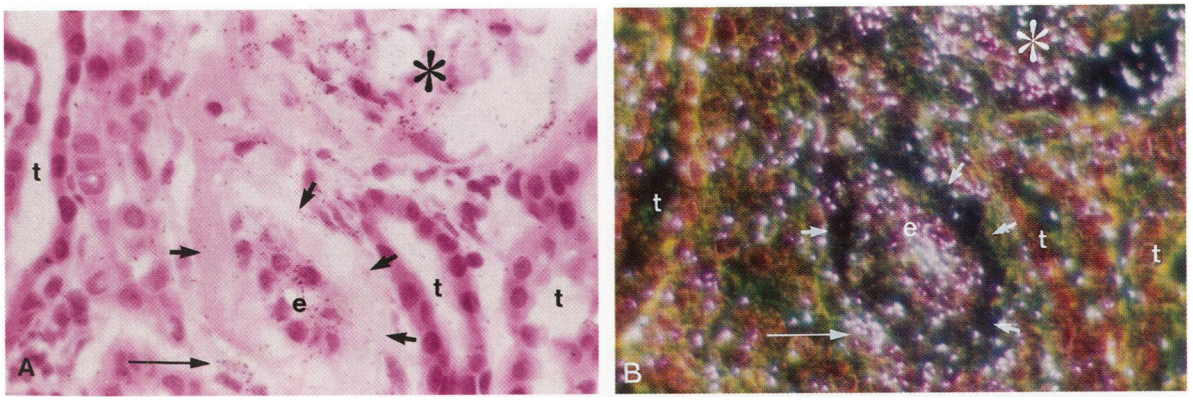

Figure 5. *ISH signals over injured/atrophic proximal tubular epithelial cells (e) surrounded by a thickened basement membrane* (small arrows). *Note that adjacent interstitial spindled cells are also positive* (long arrow). *Other tubular cross-sections (t) seen in the picture do not display ISH signals. A crescent undergoing sclerosis is seen in the right upper corner* (asterisk). A: *At 35 days after sensitization, ISH with a V antisense probe. H&E counterstain; magnification, ×160.* B: *Corresponding darkfield.*

rosed glomeruli, glomerular tufts showed little total and V^+ staining (similar to the mesangial staining seen in control kidneys). Bowman's capsule showed focal/segmental total and V^+ FN deposition at all examined time points (+ to ++). Tubules/tubular basement membranes focally contained total and V^+ FN on days 21 and day 35 (data not shown).

EIIIA$^+$ and EIIIB$^+$ Deposition (Figure 6)

Accumulation of the EIIIA$^+$ and EIIIB$^+$ isoforms was also most pronounced in areas of crescentic remodeling, where it paralleled the deposition of total and V^+ forms.

EIIIA$^+$ and EIIIB $^+$ FNs accumulated in the same glomerular crescents as demonstrated by adjacent serial 5-μm sections. However, the crescentic area in which EIIIA$^+$ and EIIIB$^+$ isoforms were deposited was smaller than that seen for total FN (data not shown).

No EIIIB$^+$ FN was detected in glomerular tufts (days 14, 21, and 35), whereas EIIIA$^+$ FN was found focally/segmentally in glomerular tufts underneath developing cellular crescents (+, days 14 and 21). Little EIIIA$^+$ FN (±) was detected in tufts on day 35. EIIIA$^+$ FN was found in Bowman's capsule in a similar pattern to total and V^+ FN (focal/segmental, + to ++ at all examined time points). EIIIB$^+$ isoforms were not detected. Tubules/tubular basement membranes stained focally for both alternatively spliced isoforms (+ to ++, days 21 and 35, data not shown).

Immunoperoxidase Staining

IL-1β and TGF-β

Antibodies to IL-1β and TGF-β stained cellular crescents in different patterns at days 14 and 21. IL-1β was seen in a cytoplasmic pattern (Figure 7), whereas TGF-β predominantly stained in a more intense, broad, fibrillar extracellular fashion (++ to +++), surrounding cells in the crescents (Figure 7). Only traces of IL-1β and TGF-β were seen on day 35 in scarred crescents. Parietal epithelial cells underlying scarred crescents stained in a focal/segmental fashion for TGF-β (++ to +++). In glomerular tufts only a very occasional IL-1β- or TGF-β-positive cell was found. Tubular epithelial cells as well as the corresponding tubular basement membranes focally (± to +) stained in areas of injury for both cytokines (data not shown). Control kidneys displayed an occasional cell staining for IL-1β or TGF-β in glomeruli (±). Tubules did not stain.

ED-1

ED-1-positive cells (macrophages) were found from the early cellular crescentic stage (day 14) to the fibro-crescentic stage (day 35) as a constant component of glomerular crescents and glomerular tufts (Figure 7). In addition, macrophages were also part of the interstitial inflammatory cell infiltrate. Only a very rare ED-1-positive cell was seen in glomeruli of control kidneys.

Figure 4. *At 35 days after sensitization, scarring occurred in cellular crescents.* A: *Antisense probe detecting the alternatively spliced V segment demonstrates ISH signals over crescents. Although no signal is found over the glomerular tuft pictured* (asterisk), *occasional weak glomerular signals were observed. The crescentic signal intensity is less than compared with that in Figure 3, A and B. H&E counterstain; magnification, ×160.* B: *Corresponding darkfield.* C: *Antisense probe detecting the EIIIB segment shows similar intensity and distribution in a crescent at day 35 as compared with the V probe* (asterisk *marks the negative glomerular tuft*). *No signals are detected over interstitial mononuclear cells (m). H&E counterstain; magnification, ×160.* D: *Corresponding darkfield.*

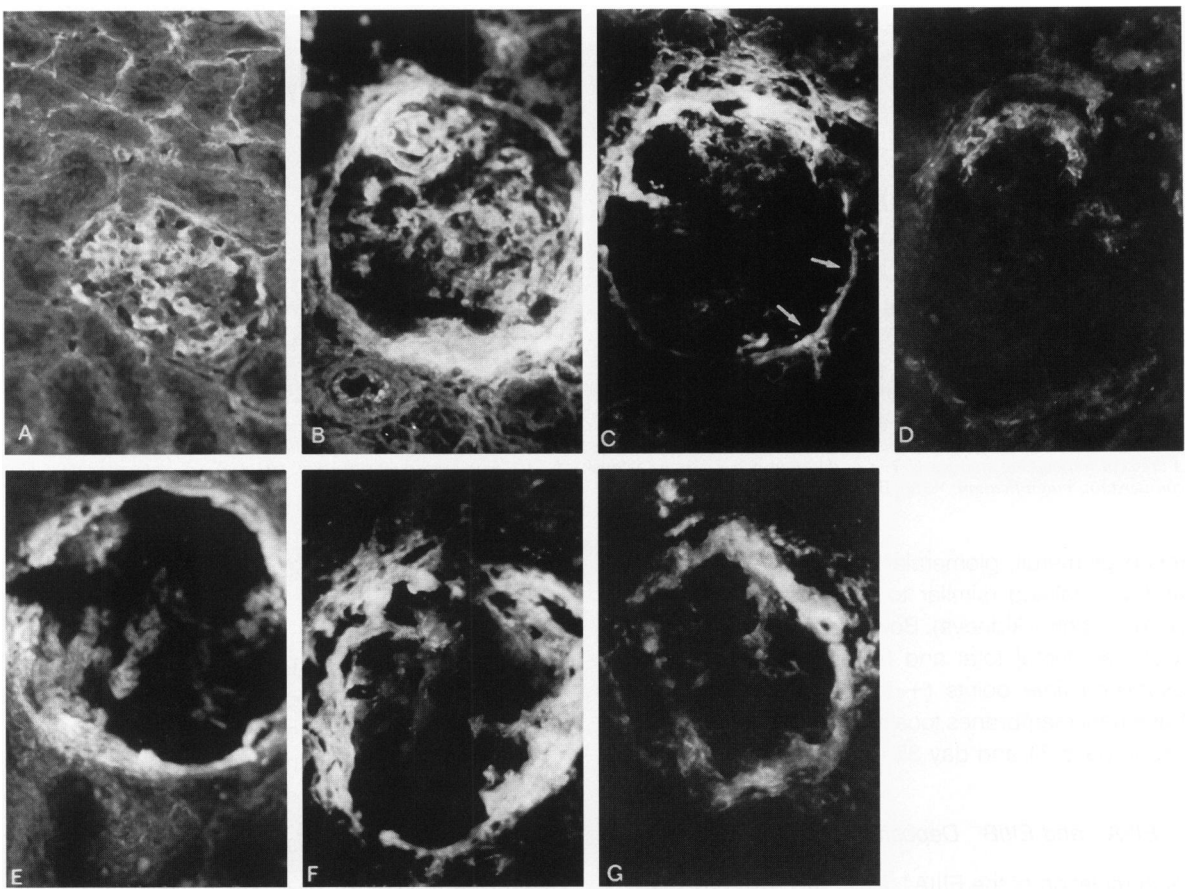

Figure 6. *Immunofluorescence staining of glomeruli.* **A:** *Glomeruli of control kidneys show staining of the mesangium, the GBM, Bowman's capsule, and peritubular capillaries/tubular basement membranes with an antibody detecting all FN isoforms (total). Magnification, ×80.* **B:** *At 21 days after sensitization, large cellular crescents had developed with marked FN deposition. Antibody detecting total FN; magnification, ×100.* **C:** *EIIIA;* **arrows** *indicating staining of Bowman's capsule. Magnification, ×100.* **D:** *EIIIB. Magnification, ×100.* **E:** *At 35 days after sensitization, FNs were still markedly accumulated in crescents. Total FN; magnification, ×100.* **F:** *EIIIA. Magnification, ×100.* **G:** *EIIIB. Magnification, ×160.*

Discussion

This study demonstrated local synthesis of total FN mRNA in cellular and fibrous crescents in early crescents 14 days after sensitization, with the highest levels seen in large cellular crescents at day 21. This is consistent with a previous report.[5] Analysis of the splicing pattern (EIIIA, EIIIB, and V regions) showed two major spatial and temporal patterns in crescents. mRNA expression of the V segment was similar to that of total FN, whereas expression of the EIIIA and EIIIB segments conformed to a second pattern. During the development of cellular crescents (days 14 and 21), relatively little EIIIA$^+$ or EIIIB$^+$ FN mRNA was found in crescentic glomeruli, whereas total and V$^+$ were abundantly expressed. By the time of fibrous crescent formation (day 35), EIIIA$^+$ and EIIIB$^+$ FN mRNAs were more extensively encountered in areas of fibrous remodeling, and their signals became similar to the signals for total and V$^+$ mRNAs. Injured tubular epithelial cells also expressed EIIIA$^+$ and EIIIB$^+$ mRNAs but only focally. Thus, crescent formation correlates with increased levels of FN

Table 2. *Immunofluorescence Staining for Different Fibronectin Isoforms in Glomeruli*

FN isoforms	Day	Crescents	Glomerular tufts/ mesangium
Total and V	Control	NA	+
	14	++	++
	21	+++	++
	35	+++	+
EIIIA	Control	NA	±
	14	++	+
	21	+++	+
	35	+++	±
EIIIB	Control	NA	0
	14	++	0
	21	+++	0
	35	+++	0

NA, not applicable; control, animals injected with CFA only, all examined time points. Immunofluorescence staining was linear. Staining intensity was evaluated in a semiquantitative fashion on a scale from ± (weak) to ++++ (strong).

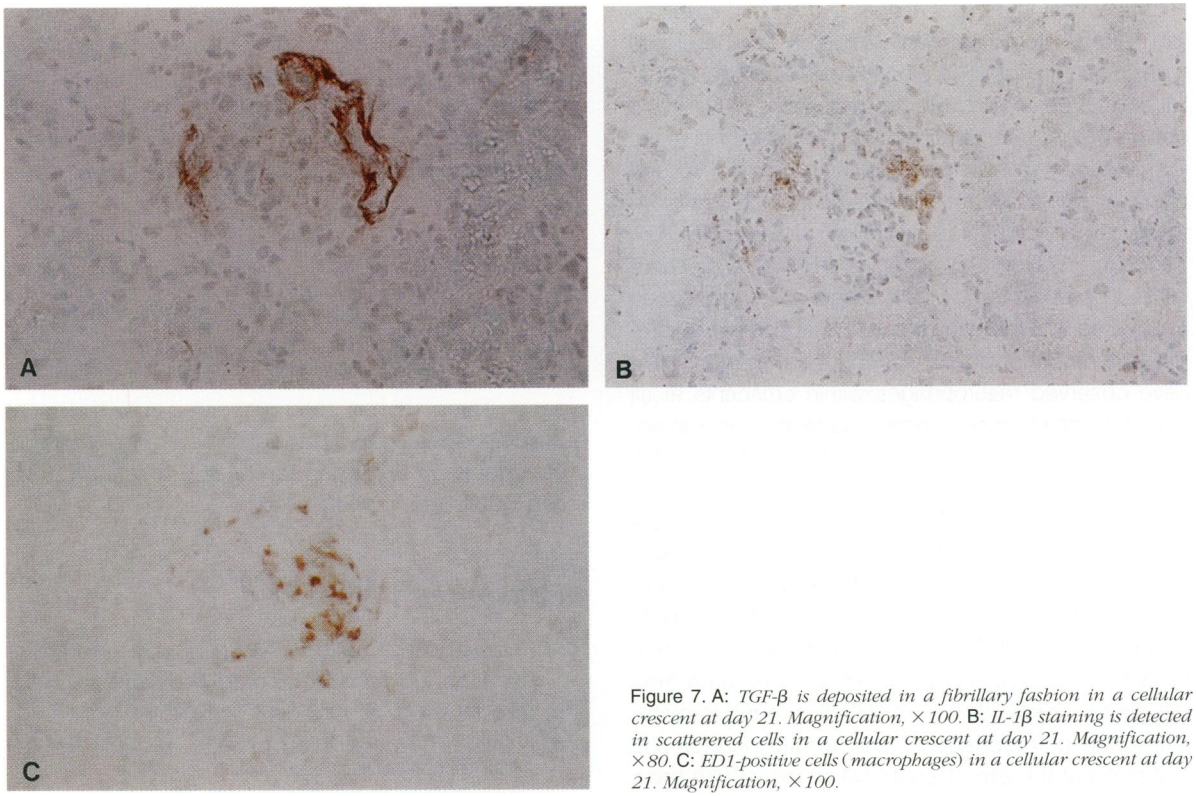

Figure 7. A: *TGF-β is deposited in a fibrillary fashion in a cellular crescent at day 21. Magnification, ×100.* B: *IL-1β staining is detected in scatterered cells in a cellular crescent at day 21. Magnification, ×80.* C: *ED1-positive cells (macrophages) in a cellular crescent at day 21. Magnification, ×100.*

mRNA, with an early predominance of EIIIA$^-$, EIIIB$^-$, V$^+$ transcripts during cellular crescent formation, and a greater abundancy of EIIIA$^+$, EIIIB$^+$, V$^+$ forms during fibrous remodeling. These data contrast with healing skin[30,31] and cornea wounds (V. Nickeleit, L. Zagachin, A.H. Kaufman, J.E. Dutt, C.S. Foster, and R.B. Colvin, manuscript in preparation) in which cellular EIIIA$^+$, EIIIB$^+$ isoforms predominate even in the early phases of tissue response.

FN synthesis was accompanied by protein accumulation in our anti-GBM glomerulonephritis model. Of interest, EIIIA$^+$ and EIIIB$^+$ protein isoforms accumulated in cellular crescents that showed little or no EIIIA$^+$ or EIIIB$^+$ FN mRNA. These discrepancies are most likely a result of the fact that the hybridization signals encountered for total and V$^+$ mRNAs were stronger than those for EIIIA$^+$ and EIIIB$^+$ transcripts. The latter two signals were much closer to the level of detection and therefore more likely to be affected by spatial fluctuations in mRNA levels. The immunofluorescence observation (with serial sections) that EIIIA$^+$ and EIIIB$^+$ protein isoforms are co-deposited in the same crescents, but in a smaller crescentic distribution pattern than total FN, also raises the possibility that only a subpopulation of cells synthesize EIIIA$^+$ and EIIIB$^+$ FN isoforms. Accumulation of FN including the EIIIA$^+$ and EIIIB$^+$ segments has also been reported in human glomerular crescents.[32] In this latter study[32] the BC-1 antibody[33], which was originally believed to detect the EIIIB segment specifically, was used. Subsequent studies, however, demonstrated that the BC-1 antibody does not react with the alternatively spliced EIIIB segment but instead recognizes the adjacent constant seventh FN type III repeat.[23]

A central event in the development of crescents is the transition from the cellular to the fibrous crescentic stage, ultimately leading to global sclerosis of glomeruli. The demonstration that the pattern of FN mRNA splicing is shifted toward inclusion of EIIIA and EIIIB segments during the transition to scarring is interesting as it may reflect a change to functionally different forms. Previous reports demonstrated embryonic-type EIIIA$^+$ and EIIIB$^+$ FN mRNAs in the granulation/scar tissue of excisional skin wounds.[30,31] Thus, this type of splicing pattern might be related to extracellular matrix assembly/fibroblast activity/fibrosis, and locally synthesized embryonic-type EIIIA$^+$ and EIIIB$^+$ FNs may serve a role in crescent formation associated with scarring.

Although we have not defined the cell of origin for the alternatively spliced FN mRNA during crescent formation, two major possibilities exist: epithelial cells and/or macrophages. We observed EIIIA$^+$, EIIIB$^+$, V$^+$

FN transcripts in parietal epithelial and tubular epithelial cells, ie, two functionally different epithelial cell types. Our findings regarding FN expression in epithelial cells is consistent with those of Clark et al,[34] who observed that skin keratinocytes synthesize FN in tissue culture. We also observed in a corneal wound healing model in the rat that corneal epithelial cells are focally expressing FN (V. Nickeleit, L. Zagachin, A.H. Kaufman, J.E. Dutt, C.S. Foster, and R.B. Colvin, manuscript in preparation). Macrophages have also been associated with cellular isotype FN deposition in a proliferative model of glomerulonephritis in the rat.[35] We have observed macrophages within crescents at all three time points in our model, suggesting that macrophages constitute a source of EIIIA$^+$, EIIIB$^+$, and V$^+$ FN synthesis.

We postulate that TGF-β and IL-1β enhance local FN synthesis. TGF-β stimulates extracellular matrix synthesis and alters the FN mRNA splicing pattern by increasing the inclusion of the alternatively spliced EIIIA, EIIIB, and V segments.[12,13,36] This growth factor appears to play a major role in promoting glomerulosclerosis.[37,38] TGF-β is found in platelets[39] and macrophages[40] and would therefore be expected at the site of acute tissue injury with fibrin exudation and macrophage accumulation. Indeed, TGF-β1 was evident in glomerular crescents. The TGF-β1 had a fibrillar extracellular staining pattern in cellular crescents (days 14 and 21), suggesting that the growth factor may be bound to other extracellular matrix proteins, such as thrombospondin,[41] fibrin, or FN.[42] Potentially, such binding could influence TGF-β activity by altering diffusion and dilution.[42] Increased TGF-β mRNA levels have been found in glomeruli of rats in a macrophage-dependent form of anti-GBM glomerulonephritis but not in a macrophage-independent form.[43] In a rabbit anti-GBM nephritis model it has been shown that most of the TGF-β is present in the active stimulatory form.[44]

Activated macrophages constitute one of the major sources of IL-1β[45,46] and it is therefore no surprise to find accumulation of IL-1β in cellular crescents as demonstrated here. The IL-1β was localized in individual cells, in contrast to the TGF-β1 pattern, compatible with a macrophage localization. IL-1β increases FN mRNA levels and protein synthesis in vascular smooth muscle cell cultures[16] and FN and IL-1β are co-deposited during early postcardiac transplant coronary arteriopathy in piglets.[15]

The synthesis and deposition of FN isoforms containing the EIIIA, EIIIB, and V segments in glomerular crescents may be a critical parameter in the development of these lesions, in particular the irreversible transformation from the cellular to the fibrous stage. Although the regulatory mechanisms of FN synthesis and mRNA splicing are poorly understood, TGF-β and IL-1β appear to be involved. This might have therapeutic consequences, as blocking of potentially stimulatory signals could alter FN synthesis.

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