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

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Abstract

Mesangial cell proliferation is common in glomerulonephritis but it is unclear if proliferation is associated with any in vivo alteration in phenotype. We investigated whether mesangial cells acquire smooth muscle–like characteristics in a rat model of mesangial proliferative nephritis induced with antibody to the Thy-1 antigen present on mesangial cells. At day 3 glomeruli displayed *de novo* immunostaining for α-smooth muscle actin in a mesangial pattern, correlating with the onset of proliferation, and persisting until day 14. An increase in desmin and vimentin in mesangial regions was also noted. Immunoelectron microscopy confirmed that the actin-positive cells were mesangial cells, and double immunolabeling demonstrated that the smooth muscle actin-positive cells were actively proliferating. Northern analysis of isolated glomerular RNA confirmed an increase in α and β/γ actin mRNA at days 3 and 5. Complement depletion or platelet depletion prevented or reduced proliferation, respectively; these maneuvers also prevented smooth muscle actin and actin gene expression. Studies of five other experimental models of nephritis confirmed that smooth muscle actin expression is a marker for mesangial cell injury. Thus, mesangial cell proliferation in glomerulonephritis in the rat is associated with a distinct phenotypic change in which mesangial cells assume smooth muscle cell characteristics. (*J. Clin. Invest.* 1991. 87:847–858.) Key words: mesangial proliferative glomerulonephritis • actin • cytoskeleton

Introduction

The mesangial cell may have diverse functions. The mesangial cell provides structural support to the glomerulus via production of extracellular matrix components to form the mesangial matrix (reviewed in references 1–3). The mesangial cell may function as a proinflammatory effector cell, similar to the monocyte/macrophage, as in vitro studies have demonstrated that the mesangial cell is phagocytic, and is also capable of releasing oxidants, a basement membrane-degrading proteinase, prostaglandins, and cytokines including interleukin-1, tumor necrosis factor, interleukin-6, and platelet-derived growth factor (1–9). Mesangial cells also morphologically resemble smooth muscle cells in culture and can be shown to be contractile, a function that may modulate glomerular hemodynamics by controlling glomerular capillary surface area (2, 3).

The pathogenetic mechanisms for progressive glomerular injury in immune complex nephritis have been classically ascribed to the action of antibody, complement, and/or infiltrating leukocytes. Recently, a role for the mesangial cell in glomerular injury has been proposed, in which the mesangial cell has been hypothesized to become "activated" and assumes a proinflammatory phenotype as described in vitro (1–9). The glomerular diseases in which mesangial cells may be actively involved would likely include IgA nephropathy, membranoproliferative GN, variants of minimal change disease, and focal sclerosis; these are diseases in which mesangial cell proliferation is commonly observed. However, an increase in mesangial cell number does not necessarily mean that the mesangial cells have undergone a change in phenotype. Indeed, studies of mesangial cells in culture have failed to demonstrate a correlation between proliferative activity and the ability to acquire an "activated" phenotype, as demonstrated by the ability of the mesangial cell to release neutral proteinase or type IV collagen (10).

In this paper, we demonstrate that mesangial cells undergo phenotypic alteration in a rat model of mesangial proliferative glomerulonephritis (GN).[1] Specifically, mesangial cells acquire smooth muscle–like characteristics, with the *de novo* expression of α-smooth muscle actin protein, as well as increased expression of the type III intermediate filament proteins, desmin and vimentin. Both the α-smooth muscle gene and protein expression are associated with active cell proliferation and can be inhibited in rats in which proliferation was prevented by complement or platelet depletion. Finally, studies of five other models of GN demonstrate that the expression of α-smooth muscle actin is present only in those diseases associated with mesangial cell injury. Thus, α-smooth muscle actin is a marker of mesangial cell activation in GN in the rat.

Methods

Experimental protocol

Studies were performed to examine the expression of actin and the type III intermediate filament proteins, desmin and vimentin, in mesangial proliferative GN in the rat induced with anti-Thy 1 antibody. This model is associated with severe mesangiolysis (defined as dissolution of mesangial matrix with loss of mesangial cells [11]) followed by mesangial cell proliferation (12). Whereas the mesangial cell proliferation

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1. *Abbreviations used in this paper:* ATS, antithymocyte; CVF, cobra venom factor; GN, glomerulonephritis.

requires the presence of both complement (13) and platelets (14), the mesangiolysis is dependent only on complement (13).

Six groups of Wistar rats (Simonsen Laboratories, Gilroy, CA) (n = 6 in each group) were studied: (*a*) normal unmanipulated rats, (*b–d*) rats with anti-Thy 1 GN at 1, 3, and 5 d after disease induction, and (*e–f*) rats with anti-Thy 1 GN (day 3) that were either complement-depleted or platelet-depleted.

For each group of rats, biopsies were taken for histologic studies and then the rats were sacrificed, glomeruli isolated, and RNA extracted (see below). Actin expression was determined immunohistochemically with a panel of four monoclonal anti-actin antibodies, and actin mRNA was measured in glomerular RNA extracts by Northern analysis using a cDNA probe for the β/γ actin mRNA and with an oligonucleotide probe specific for α smooth muscle actin mRNA. Vimentin and desmin were also studied by immunohistochemistry on tissue sections with specific monoclonal antibodies (see below). These studies were correlated with the severity of the mesangiolysis and the degree of proliferation in each group (see below).

Additional studies included the determination of actin expression (immunohistochemically) in rats with anti-Thy 1 GN at days 8, 14, and 21 after disease induction, as well as actin expression in rats with five different experimentally-induced glomerular diseases (n = 2 in each group). To control for the possibility that the antiplatelet IgG could independently affect actin, desmin, or vimentin expression, antiplatelet IgG was administered to four normal rats, and kidneys were biopsied at 3 d for immunohistochemical studies.

Disease models

Anti-Thy 1 GN. Antithymocyte serum (ATS) was raised in a goat with repeated immunizations of Lewis rat thymocytes as previously described (14). An intravenous dose of 0.4 ml plasma/100 g body weight was used to induce disease.

Other models. Other GN models studied included diseases predominantly affecting: (*a*) the glomerular epithelial cell (i.e., aminonucleoside nephrosis and passive Heymann nephritis), (*b*) the glomerular basement membrane (GBM) (i.e., heterologous anti-GBM GN); (*c*) the mesangial cell (i.e., Habu snake venom GN), and (*d*) both the endothelial cell and mesangial cell (i.e., GN induced with concanavalin A (con A) and anti–con A antibody). The methods used to establish these diseases have been previously published (15, 16), and were not modified with the exception of Habu snake venom GN, in which the rats received a single i.v. dose of Habu snake venom (2 mg) (Quality Venoms, Punta Gorda, FL).

Quantitation of proliferation and mesangiolysis

For each biopsy, 20 glomeruli were sequentially examined and the mean number of proliferating cells, the total number of cells, and the degree of mesangiolysis was determined. Glomerular cross-sections containing only a minor portion of the glomerular tuft (< 20 discrete capillary segments/cross-section) were not included in the analysis. *Cellularity* was expressed as the total number of nuclei per glomerular cross-section in tissue sections stained with the periodic acid Schiff reagent. *Proliferation* was expressed as the number of cells per glomerular cross-section that were positive for the proliferating cell nuclear antigen (PCNA)/cyclin as determined by immunocytochemistry (14). PCNA/cyclin is an auxiliary protein to DNA polymerase-δ (17); expression is restricted to late G_1, S, G_2, and M phases of the cell cycle (18). *Mesangiolysis* was graded semiquantitatively using the following scale (19):

0 No mesangiolysis.

1+ Segmental mesangiolysis affecting occasional (< 25%) glomeruli.

2+ Focal (25–50%) involvement of glomeruli with affected mesangial areas showing global lucency with occasional disruption; generally good preservation of the underlying mesangial stalk architecture.

3+ Most glomeruli affected with extensive disruption of mesangial areas, but with preservation of the underlying glomerular tuft architecture.

4+ Virtually all glomeruli affected, frequently with complete dissolution of the mesangial areas, usually in association with microaneurysm formation.

Complement depletion

Rats were depleted with cobra venom factor (CVF) (Naja naja kaounthia, Diamedix Corp., Miami, FL) (30 U) i.p. in three divided doses beginning 24 h before injection of ATS and then received an additional 10 U i.p. daily until sacrifice. Serum C3 levels were measured by radial immunodiffusion and a value of < 10% baseline was considered complement depleted (19). We have recently demonstrated that CVF treatment does not affect circulating platelet counts or in vitro aggregation of platelets to ADP or collagen (19).

Platelet depletion

Platelet depletion was performed with a goat anti–rat platelet IgG as previously described (14, 20). Rats received an i.p. dose of 8 mg/100 g body weight with repeat doses of 2–3 mg/100 g given i.v. at 24-h intervals. Thrombocytopenia (platelet count < 25,000/mm^3) was achieved in all rats at the time of injection of ATS and until sacrifice 3 d later. We have previously reported that platelet depletion of rats with anti-Thy 1 GN does not affect glomerular deposition of anti-Thy 1 IgG, circulating leukocyte counts, or plasma CH50 titers relative to controls (14).

Immunohistochemical staining for actin, desmin, vimentin, and the Proliferating Cell Nuclear Antigen (PCNA)/cyclin

Sections of methyl Carnoy's fixed tissue were deparaffinized with Histoclear (National Diagnostics, Highland Park, NJ) and graded ethanols, blocked with 0.3% hydrogen peroxide and 0.1% sodium azide, and washed with PBS (138 mM NaCl, 2.7 mM KCl, 3.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3) containing 0.1% BSA (Sigma Chemical Co., St. Louis, MO) and 0.01% Triton X-100. The tissue was then incubated with one of the primary murine monoclonal antibodies (see below), and subsequently processed using a strep-avidin-biotin immunoperoxidase method (21) with 3,3'-diaminobenzidine (with nickel chloride enhancement) as the chromogen. Sections were counterstained with methyl green.

A variety of murine monoclonal anti-actin antibodies were used for immunoperoxidase staining, including anti-αSM-1 (21) (gift of G. Gabbiani), CGA-7 (22), HHF-35 (23), and C4 (24) (gift of J. Lessard). The specificities of these antibodies are shown in Table I. Desmin was detected with D33 (Dako Corp., Santa Barbara, CA), vimentin with murine monoclonal antibody M725 (Dako Corp.), and PCNA/cyclin with murine IgM monoclonal antibody 19A2 (American Biotech Inc., Plantation, FL) (25). For all biopsies a negative control consisted of substituting the primary antibody with an irrelevant murine monoclonal antibody.

Tissue sections were also double labeled for both PCNA/cyclin and muscle actin. Sections were first stained for PCNA/cyclin with 19A2, a murine IgM monoclonal antibody, using an indirect immunogold procedure (Janssen Life Science Products, Piscataway, NJ) with a class-

Table I. Anti-actin Antibodies Used for Immunoperoxidase Staining

Actin isoforms	Anti-αSM-1 (21)	CGA-7* (22)	HHF-35 (23)	C4 (24)
Smooth muscle α-actin	+	+	+	+
Smooth muscle γ-actin		+	+	+
Straited muscle α-actin			+	+
Cardiac muscle α-actin			+	+
Cytoplasmic β-actin				+
Cytoplasmic γ-actin				+

* Does not detect all smooth muscle α-actin-positive cells.

specific anti-IgM antibody conjugated with 5-nm gold particles (Janssen Life Science Products) followed by detection of muscle actin with HHF35 (a murine IgG) utilizing biotinylated rabbit anti-mouse IgG_1 (Zymed, San Francisco, CA), strepavidin immunoperoxidase, and aminoethylcarbazole as the chromogen (Vector Laboratories, Inc., Burlingame, CA). Negative controls included the omission of 19A2, in which case no staining with immunogold was noted, and omission of HHF-35, in which no cells were stained with the red aminoethylcarbazole reagent.

Tissue was also double immunostained for monocyte-macrophages and α-smooth muscle actin. Sections were incubated with the murine monoclonal IgG_1 antibody, ED1 (Bioproducts for Science, Indianapolis, IN) which is specific for rat monocyte-macrophages (26) followed by a subclass-specific biotinylated rabbit anti-mouse IgG_1 antibody (Zymed) and strep avidin-alkaline phosphatase and color reagent. Sections were then incubated with anti-α-sm-1, an IgG_2 murine antibody, followed by a mouse anti-IgG conjugated with 5 nm gold particles (Janssen Life Science Products) as detailed above. Specificity of staining was demonstrated by omission of either of the primary antibodies.

Actin staining was graded semiquantitatively as follows:

0 Negative.

1+ Segmental staining in mesangial areas.

2+ Staining outlines the mesangial stalk.

3+ Staining of the mesangial stalk with focal areas of nodularity.

4+ Staining of the entire glomerulus with diffuse areas of nodularity.

Routine histology

Tissue for light microscopy was fixed in methyl Carnoy's solution, dehydrated in graded ethanols, and embedded in paraffin. 3-μm sections were stained with the periodic acid Schiff reagent. Tissue for transmission electron microscopy (EM) was processed as previously described (19).

Immunoelectronmicroscopy (Immuno-EM)

Tissue was perfusion fixed with periodate-lysine-paraformaldehyde (PLP) (27), then immersion fixed in PLP for 4 h, and transferred to PBS. After dehydration with graded ethanols, the tissue was incubated in LR white resin (Polysciences, Inc., Warrington, PA) and embedded in gelatin capsules, and cured overnight at 50°C. Thin sections were mounted on coated nickel grids, blocked with 10% normal goat serum, rinsed with PBS, incubated with HHF-35 or a control irrelevant monoclonal antibody overnight at 4°C, rinsed with PBS, followed by incubation with goat anti-mouse IgG conjugated to gold (10-nm particles) (Sigma Chemical Co.). Grids were treated with uranyl acetate and then examined using a Phillips 410 electron microscope.

Glomerular RNA preparation

Rats were sacrificed under ether anesthesia and the kidneys removed, decapsulated, and glomeruli isolated by differential sieving (20) at 4°C using PBS prepared with diethyl pyrocarbonate (DEP)-treated water. Isolated glomeruli were lysed with acid guanidinium thiocyanate containing 2-mercaptoethanol and 10% sarcosyl, and extracted with phenol and chloroform-isoamyl alcohol according to the method of Chomczynski and Sacchi (28). Contaminating DNA was removed by treatment with RQ1 DNase (Promega Biotec, Madison, WI) according to the manufacturer's instructions. The RNA was precipitated at −70°C with ethanol (2.5 vol) and redissolved in 10 mM Tris-HCl pH 7.4, 5 mM EDTA (TE buffer). ~ 25–40 μg glomerular RNA was obtained per rat with a purity as assessed by OD_{260}/OD_{280} ratio of 1.7 or higher.

Gel electrophoresis and transfer to membranes

The RNA from each group of animals was pooled and aliquots were denatured with formamide, formaldehyde, and heat (65°C × 10 min) and electrophoresed through 1% agarose gels containing 2.2 M formaldehyde/0.2 M MOPS pH 7.0 (15 μg/lane as measured by OD_{260}); transferred to a nylon filter (Zeta probe, Bio-Rad Laboratories, Inc., Richmond, CA) overnight by capillary blotting, and baked at 80°C for 2 h (29). Good resolution and integrity of the 28S and 18S ribosomal RNA bands was apparent when examined under UV light in the presence of ethidium bromide.

DNA probes and hybridization conditions

Two DNA probes were used: (*a*) a 1.3-kb Pst I fragment of bovine γ-actin cDNA isolated from the plasmid pBA-1 (30) was used to detect the 2.1-kb transcripts of β/γ actin mRNA, and (*b*) an oligonucleotide (AGTGCTGTCCTCTTCTTCACACATA) complementary to a sequence unique to the human α-smooth muscle actin mRNA (31) was used to detect the 1.6-kb transcript of rat α-smooth muscle actin. The β/γ-actin probe was labeled with (α-^{32}P)-dCTP by random primer extension and the oligonucleotide probe was end-labeled with (γ-^{32}P)-ATP and T_4 polynucleotide kinase. The labeled oligomer was further purified by electrophoresis on a 15% polyacrylamide gel and elution from the gel slice into TE buffer.

For the general β/γ-actin probe, prehybridization was performed by incubating the membranes in a solution containing 5× SSPE (1× SSPE is 0.18 M NaCl, 0.01 M $NaPO_4$, 0.001 M EDTA, pH 7.7), 5× Denhardt's solution, 50% formamide, 0.1% SDS, and 100 μg denatured salmon sperm DNA for 2 h at 42°C, followed by addition of ^{32}P-labeled probe (final concentration, 10^6 cpm/ml) and continued incubation overnight at 42°C. The membranes were then washed with 6× SSPE, 0.1% SDS three times at room temperature, followed by two washes in 1× SSPE, 0.1% SDS at 42°C. For the oligonucleotide probe, prehybridization was performed with 1% SDS, 5× SSC (1× SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 7.0), 50 mM Tris, pH 7.5, 1× Denhardt's, and 20 μg/ml poly (*A*) at 42°C, followed by hybridization in the same buffer, to which 2×10^6 cpm/ml of ^{32}P-labeled oligonucleotide probe had been added, for an additional 16 h at 42°C. Washes were in 1× SSC, 0.1% SDS at room temperature, and 0.2× SSC, 0.1% SDS at 37°C, at 40°C, and at 42°C, each for 10 min. After the washes, filters were exposed to Kodak X-O MAT film with enhancing screens at −70°C for 30.5 and 22.5 h, and the resulting autoradiograms read by linear densitometry.

Quantitation of mRNA

A comparison of the amount of glomerular mRNA for β/γ actin and for α-smooth muscle actin between control and diseased groups was made by densitometry. Comparison was facilitated by loading each lane of the gel with equivalent amounts of RNA (15 μg) as measured by OD_{260}. Examination of the gel under UV light and of the filter (after transfer) by UV shadowing confirmed similar amounts of RNA in each lane. However, to correct for any minor differences in amount of RNA on the filter, hybridization was performed with a specific bovine 280-bp cDNA probe to 28S ribosomal RNA (a gift of Dr. Helene Sage and Dr. Luisa Iruela-Arispe). Hybridization was performed under conditions and probe concentrations which gave a linear relationship between densitometry signal and amount of RNA loaded (range tested, 2.5–15 μg RNA, $r^2 = 0.99$). Utilizing the densitometry readings obtained with the cDNA probe for 28S ribosomal RNA, the densitometry signals for the actin mRNAs were normalized to reflect equivalent total RNA in each lane. Values for the different actin mRNAs were then expressed relative to the amount of mRNA observed in normal glomerular RNA preparations (which were arbitrarily assigned the value of 1).

Statistical analysis

Values are expressed as the mean±SE. The statistical analysis of the multiple groups was performed using the one-way analysis of variance (ANOVA). Simultaneous multiple comparisons between groups were performed with modified *t* statistics using Bonferroni's method (32).

Results

Description of mesangial proliferative GN due to anti-Thy 1 antibody (ATS) and the effect of platelet and complement deple-

Table II. Comparison of Cellularity, Proliferation, and Mesangiolysis in Normal Rats, Rats with Anti-Thy 1 GN (ATS-treated Rats), and in Complement-depleted (CVF) or Platelet-depleted Rats (APS) with Anti-Thy 1 GN

Group	Total cells*	PCNA/cyclin + cells*	Mesangiolysis
Normal	76.5±1.8	0.9±0.2	0
ATS day 1	67.5±0.4‡	1.2±0.1	2.5±0.3‡
ATS day 3	95.7±2.4‡	21.8±2.7‡	1.7±0.5§
ATS day 5	106.8±2.7‡	20.1±0.8‡	0.7±0.2
CVF/ATS day 3	80.9±2.0‖	1.4±0.2‖	0‖
APS/ATS day 3	73.4±2.4‖	7.9±1.7‖	2.0±0.2‡

* Number of cells per glomerular cross-section, calculated from the mean number of cells in 20 glomerular cross-sections from six rats in each group. ‡ $P < 0.001$ relative to normal; § $P < 0.005$ relative to normal; ‖ $P < 0.001$ relative to ATS day 3.

tion. The injection of ATS into normal rats resulted in acute mesangiolysis that was associated with a decrease in total glomerular cellularity at 24 h (i.e., day 1) (Table II). This was followed by a rebound in total glomerular cellularity at 3 and 5 d in association with actively proliferating (i.e., PCNA/cyclin +) cells (Table II). Previous studies have demonstrated that the majority (i.e., > 80%) of these proliferating cells are glomerular in origin (14).

An important role for complement (13) and for platelets (14) in anti-Thy 1 GN was confirmed in this study (Table II). Complement depletion completely prevented both mesangiolysis and cell proliferation (Table II). In contrast, platelet depletion (platelet counts < 25,000/mm^3) did not prevent mesangiolysis, and reduced but did not abolish cell proliferation (Table II).

Expression of α-smooth muscle actin in anti-Thy 1 GN. Tissues were initially stained by immunoperoxidase with HHF-35, a monoclonal antibody that recognizes the muscle actin isoforms (Table I). Normal glomeruli do not express detectable amounts of muscle actin, although actin can be readily observed in the afferent and efferent arterioles (Fig. 1). In contrast, substantial amounts of muscle actin could be demonstrated in glomeruli of rats with anti-Thy 1 GN (Fig. 1). Actin expression in glomeruli was first noted 3 d after injection of ATS, and often appeared to radiate out from the hilus along the mesangial stalks. At day 5 muscle actin expression was extensive, often concentrated in a "nodular" pattern in mesangial areas, and occasionally involving the entire glomerulus (Fig. 1). The expression of muscle actin was transient, being barely detectable at day 14, and absent by day 21 ($n = 2$).

To further clarify the type of actin being expressed, sections were stained with three other anti-actin antibodies (Table I) (Fig. 2). C4, an actin antibody that recognizes all six actin isoforms, demonstrated a similar staining pattern as HHF-35, with the exception that small amounts of actin (1+) could be detected in normal glomeruli in mesangial regions. CGA-7, a monoclonal antibody that recognizes α and γ smooth muscle actins, resulted in negative glomerular staining despite a very positive staining of blood vessels (Fig. 2). However, CGA-7 does not bind all cells that bear α-smooth muscle actin (22, 23), possibly because it may recognize a smooth muscle specific-epitope that has undergone a posttranslational modification. This was further suggested in this study by the observation that anti-αsm-1, a monoclonal antibody specific for α-smooth muscle actin, gave identical results as HHF-35 (Fig. 2).

Ultrastructural studies were performed to confirm the light microscopy findings. Transmission EM in a rat with anti-Thy 1 GN 8 d after disease induction demonstrated large cells in mesangial regions with abundant mitochondria, rough endoplasmic reticulum, and intermediate and microfilaments. In some cells microfilament bundles with interspersed electron dense regions could be found. Immuno-EM with HHF-35 demonstrated numerous gold particles within these mesangial cells,

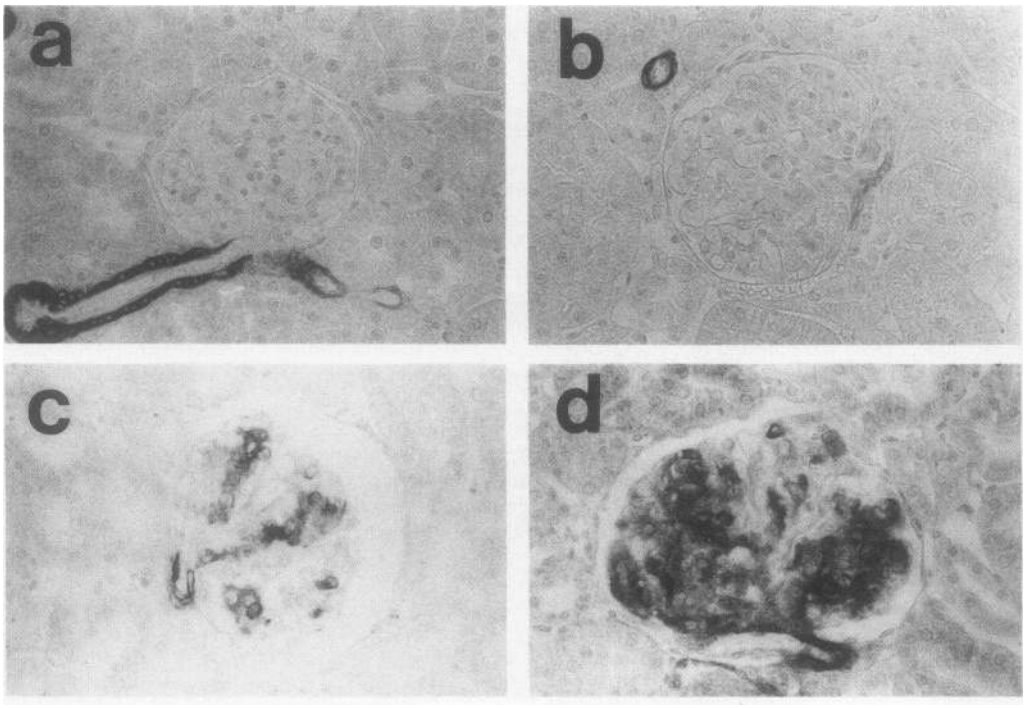


Figure 1. Muscle actin expression in mesangial proliferative glomerulonephritis due to anti-Thy 1 antibody. Glomeruli were stained with HHF-35 by immunoperoxidase for muscle actins. Whereas muscle actin is not detectable in glomeruli of a normal rat (*a*) or in a rat 1 day (*b*) after induction of GN, by day 3 (*c*) muscle actin can be detected along the mesangial stalk, becoming more diffuse and nodular by day 5 (*d*). Smooth muscle cells in the walls of small arteries and arterioles are uniformly strongly positive. (Immunoperoxidase with methyl green counterstain, 400×).

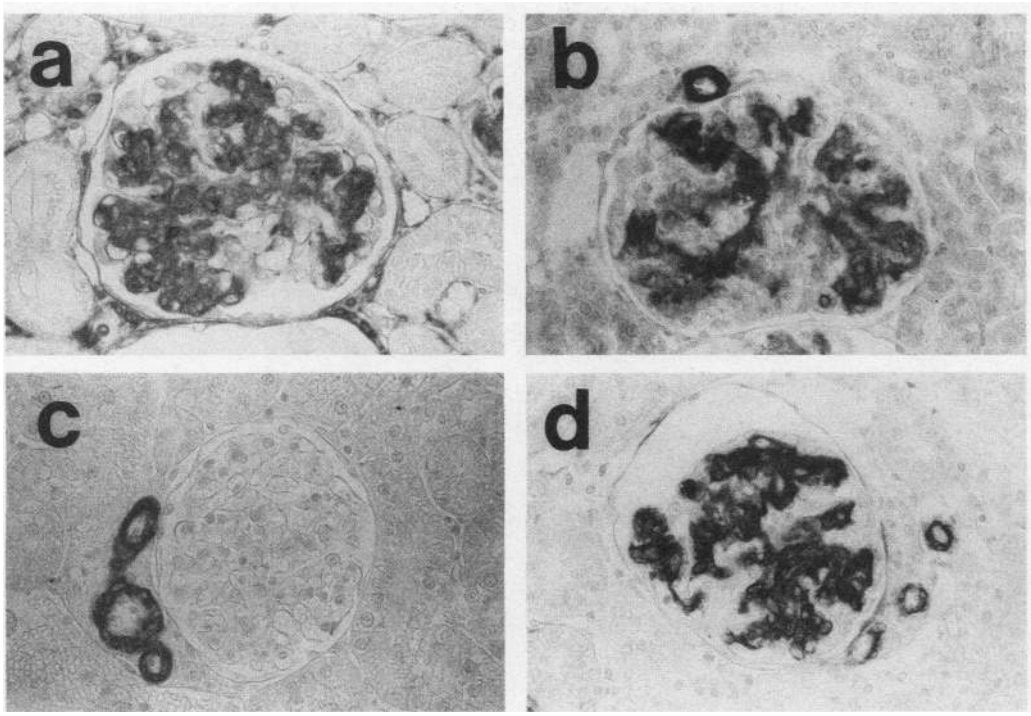


Figure 2. Actin expression in mesangial proliferative GN at day 5 as assessed with different anti-actin antibodies. Glomeruli from a rat with anti-Thy 1 GN (day 5) were stained by immunoperoxidase with the following antibodies: the panactin antibody, C4 (*a*); the anti–muscle actin antibody, HHF-35 (*b*); the anti–smooth muscle actin antibody, CGA-7 (*c*); and the anti-α-smooth muscle actin antibody, anti-αsm-1 (*d*). Actin can be detected in glomeruli with all of the antibodies except for CGA-7. As in Fig. 1, vascular smooth muscle uniformly showed strong reactivity with all of the anti-actin antibodies (immunoperoxidase with methyl green counterstain, 400×).

thus confirming the presence of muscle actin (Fig. 3). Normal glomeruli had substantially less HHF-35 reactivity in mesangial areas, although it was of interest that both normal and diseased glomeruli had reactivity of HHF-35 within the epithelial cell foot processes (Fig. 3 *C*). The density of gold particles within the foot processes of diseased and normal animals appeared equivalent. Specificity of the immuno-EM was shown by substituting an irrelevant control monoclonal antibody for HHF-35.

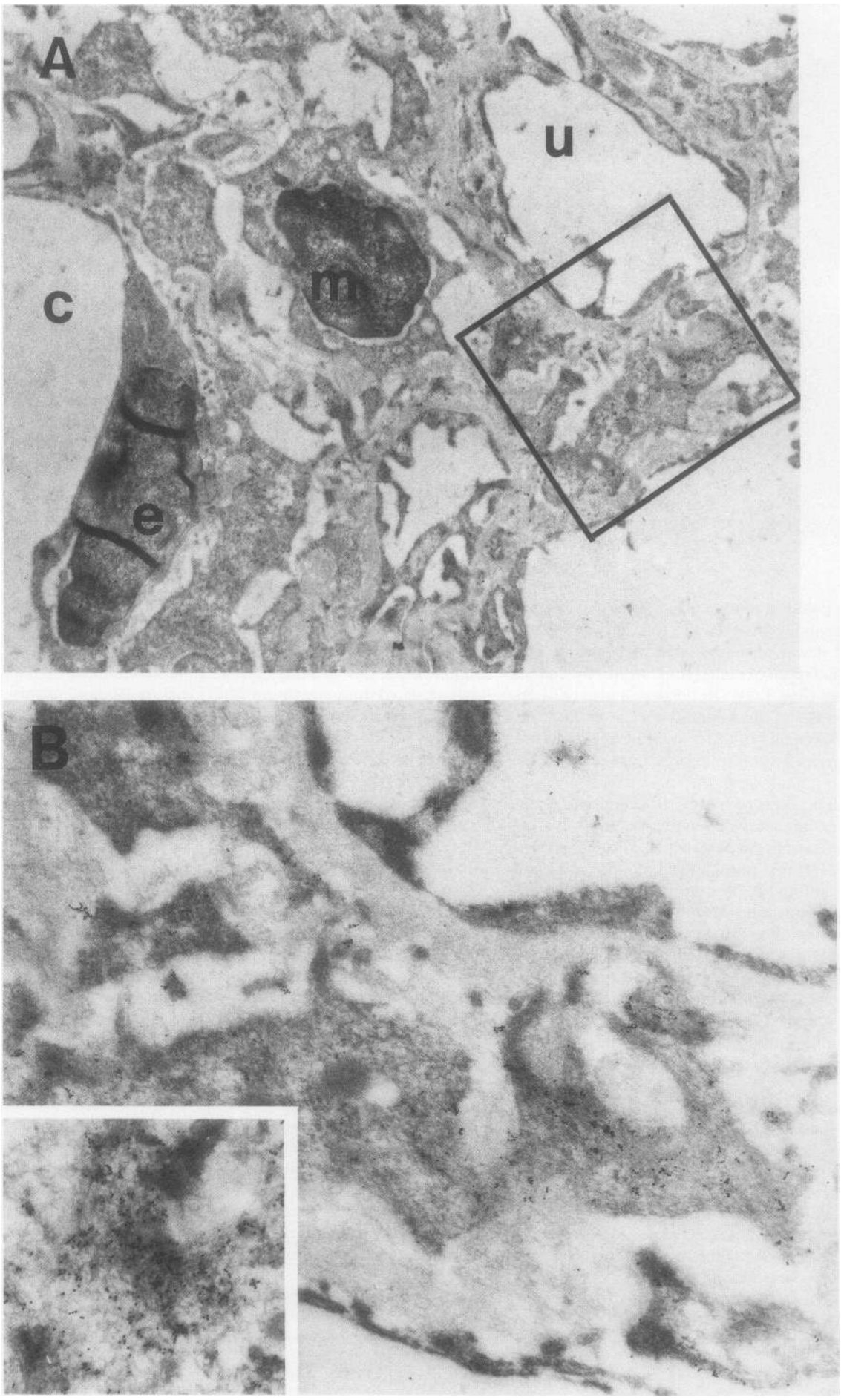
α-Smooth muscle actin expression is associated with proliferation. To determine if the muscle actin expression was associated with cell proliferation, double-labeling of tissue sections was performed using HHF-35 (muscle actin marker) and 19A2 (anti-PCNA/cyclin antibody). The double-labeled preparations unequivocally demonstrated that the majority of proliferating cells were also expressing muscle actin (Fig. 4).

Furthermore, measures to prevent or reduce cell proliferation, such as complement depletion and platelet depletion, respectively, were associated with either a prevention or reduction in muscle actin staining (Table III) (Fig. 5). Similar degrees of reduction in staining pattern were noted with the monoclonal antibody specific for α-smooth muscle actin (i.e., anti-α-sm-1).

Actin gene expression in anti-Thy 1 GN. Northern analysis was performed with glomerular RNA from normal rats and rats with anti-Thy 1 GN to determine if the expression of actin mRNA paralleled the immunohistochemical findings. Hybridization with the general actin probe, pBA1, demonstrated that the 2.1-kb transcripts for β/γ actin mRNAs were markedly elevated in rat glomerular RNA collected 3 and 5 d after induction of anti-Thy 1 GN (Fig. 6 *A*). On prolonged exposure with the general actin probe there appeared to be a second band of 1.6 kb consistent with α actin (data not shown). To determine if this band represented α-smooth muscle actin mRNA, a separate hybridization was performed with an oligonucleotide probe complementary to a sequence unique to human α-smooth muscle actin mRNA. Hybridization with the oligonucleotide probe proved the 1.6-kb transcript to be α-smooth muscle actin, and demonstrated a similar temporal pattern as that observed for β/γ actin mRNA (Fig. 6 *B*). At day 3 expression was maximal with an 11-fold increase in β/γ actin mRNA and a 6.5-fold increase in α-smooth muscle actin mRNA as assessed by linear densitometry (after normalizing for the quantity of 28S RNA in each lane) (Table III). Both the α and β/γ actin mRNA expression were markedly reduced in complement-depleted and platelet-depleted rats with anti-Thy 1 GN (Fig. 6, Table III).

Identification of the cell type expressing α-smooth muscle actin. The cells bearing the muscle actin phenotype were primarily in mesangial locations, were actively proliferating, and by immuno-EM appeared to be mesangial cells. To exclude the possibility that the smooth muscle actin positive cells were not infiltrating monocyte-macrophages, we performed double immunolabeling with an antibody to rat monocytes and macrophages (ED-1) and with an antibody to α-smooth muscle actin. The great majority of smooth muscle actin-positive cells (> 90%) were unreactive with the macrophage marker. In areas of prominent hypercellularity, it was occasionally difficult to distinguish cells that may have been positive for both smooth muscle actin and for ED1 as opposed to cells of one type overlying another. However, the vast majority of the smooth muscle actin-positive cells and ED-1 positive cells were distinct from each other. These results are consistent with previous observations that human leukocytes (23, 33) as well as rat peritoneal macrophages (data not shown) are negative for smooth muscle actin when immunostained with HHF-35 or anti-αsm-1 antibodies.

Expression of the type III intermediate filament proteins, desmin and vimentin, in anti-Thy 1 GN. Tissue sections were also stained for the intermediate filament proteins, desmin and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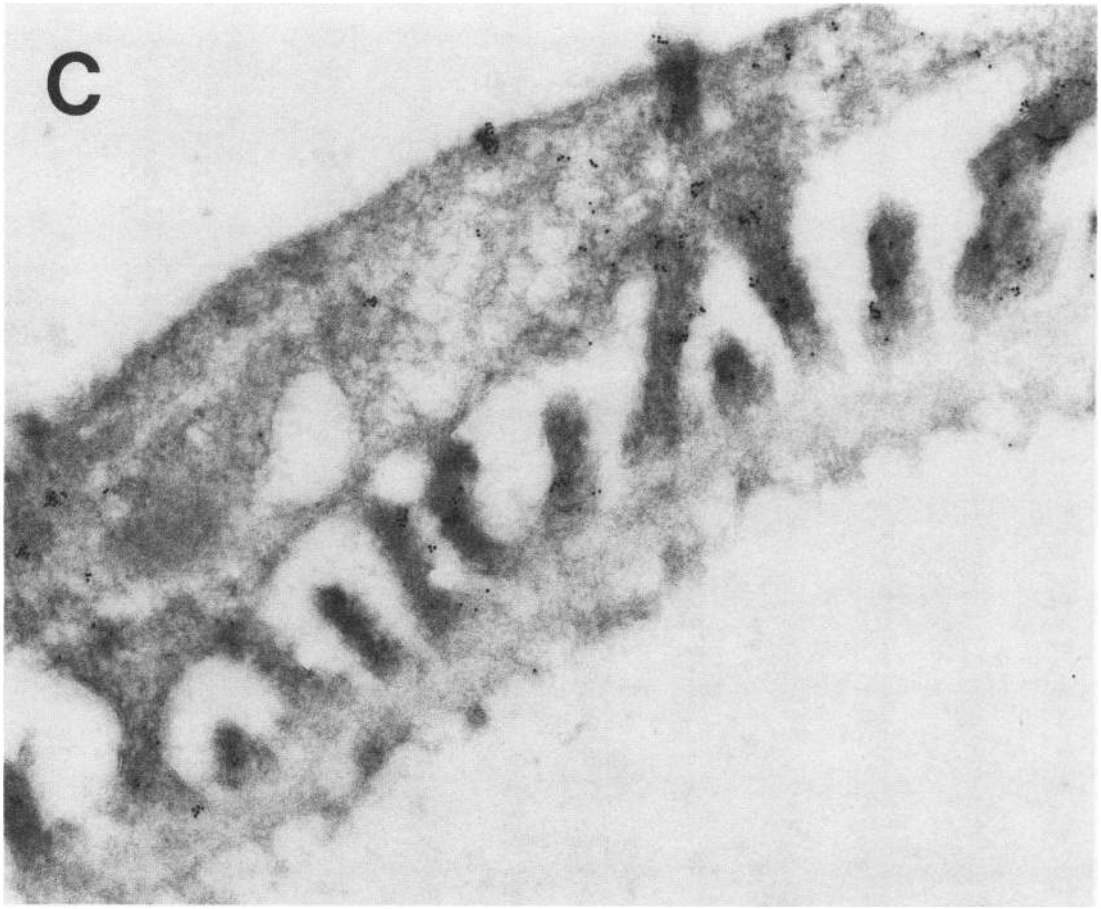

Figure 3. Immuno-EM of a glomerulus of a rat with anti-Thy 1 GN at day 8. A low-power view is shown in *A* (12,300×). At higher magnification, (*A, boxed area*), HHF-35 reactive material can be demonstrated in mesangial cells (*black dots*) (34,000×) often along microfilaments (see *inset*, 50,000×) (*B*). Staining of the glomerular epithelial cell, especially within the foot processes, with HHF-35 is also observed (*C*) (50,000×). (*c*, capillary lumen; *e*, endothelial cell; *m*, mesangial cell; *u*, urinary space).

vimentin. In normal rat glomeruli desmin was distributed primarily in a mesangial pattern (Fig. 7 *a*). Occasional podocytes were also desmin-positive. After injection of ATS, there was a loss of desmin staining at 24 h, in association with the severe mesangiolysis. However, by day 3 mesangial staining for desmin was restored, and by day 5 a marked expansion of desmin was noted in areas of mesangial hypercellularity (Fig. 7 *b*).

Vimentin, in contrast to desmin, was present primarily in glomerular visceral epithelial cells; staining of mesangial cells and glomerular endothelial cells was minimal in normal rat glomeruli (Fig. 7 *c*). In anti-Thy 1 GN vimentin staining was not altered until day 3 when it was focally increased in areas of mesangial hypercellularity. By day 5 the mesangial staining for vimentin was extensive (Fig. 7 *d*).

Rats with anti-Thy 1 GN that were complement-depleted had desmin and vimentin staining patterns that were identical to that seen in normal rats. In contrast, platelet-depleted rats with anti-Thy 1 GN had normal vimentin staining of the glomerular visceral epithelial cells but only minimal desmin staining in mesangial regions. The lack of desmin staining in platelet-depleted rats was likely due to the persistent mesangiolysis rather than to direct effects of the anti-platelet IgG on the glomerulus as desmin staining was not altered in rats without GN ($n = 4$) that were given equivalent doses of anti-platelet IgG.

Expression of α-smooth muscle actin in other glomerular diseases. Studies were performed to determine if muscle actins were expressed in other experimental glomerular diseases (Table IV). Immunohistochemical studies of glomeruli in diseases associated with glomerular epithelial cell injury (i.e., passive Heymann's nephritis and aminonucleoside nephrosis) or glomerular basement membrane injury (i.e., anti-GBM GN) were negative for muscle actin. However, in the model of mesangial cell injury induced with Habu snake venom, glomeruli that demonstrated mesangial hypercellularity expressed smooth muscle actin. Muscle actin could also be detected in mesangial areas in the immune complex GN due to concanavalin A (con A) and anti–con A antibody, which is a model of both endothelial and mesangial cell injury.

Discussion

α-Smooth muscle actin is one of six closely-related actin isoforms (see Table I). Unlike the β and γ cytoplasmic actins, which are present in most cells, the presence of α-smooth muscle actin has been interpreted as a marker for smooth muscle–derived tissues (34). In adult vascular smooth muscle, α-smooth muscle actin is the predominant actin isoform (35) and has been proposed to have a major contractile function (36). Recently, this actin isoform has also been observed in myoepithelial cells, myofibroblasts in pathologic tissues, and in some skeletal muscle tumors (37, 38). α-Smooth muscle actin has also been reported in chicken embryo fibroblasts and certain rodent fibroblast cell lines (39, 40), suggesting that these cell

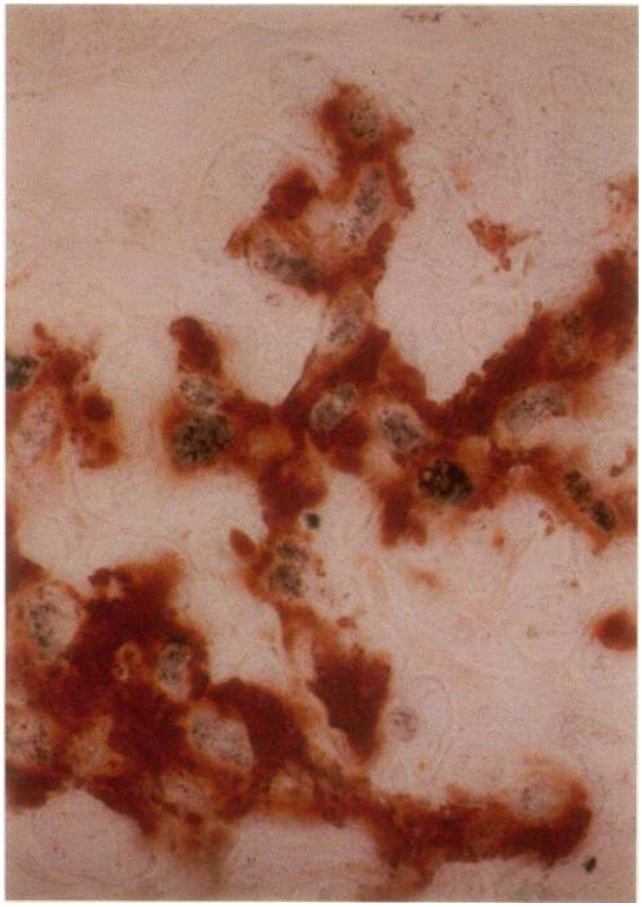

Figure 4. Double immunolabeling of a glomerulus from a rat with mesangial proliferative GN. Proliferating cells (PCNA/cyclin +) are identified by the dark nuclear stain and muscle actin-positive cells are orange/red. It is evident that most of the proliferating cells are also positive for muscle actin. (Immuno-gold and immunoperoxidase double label, 630×.)

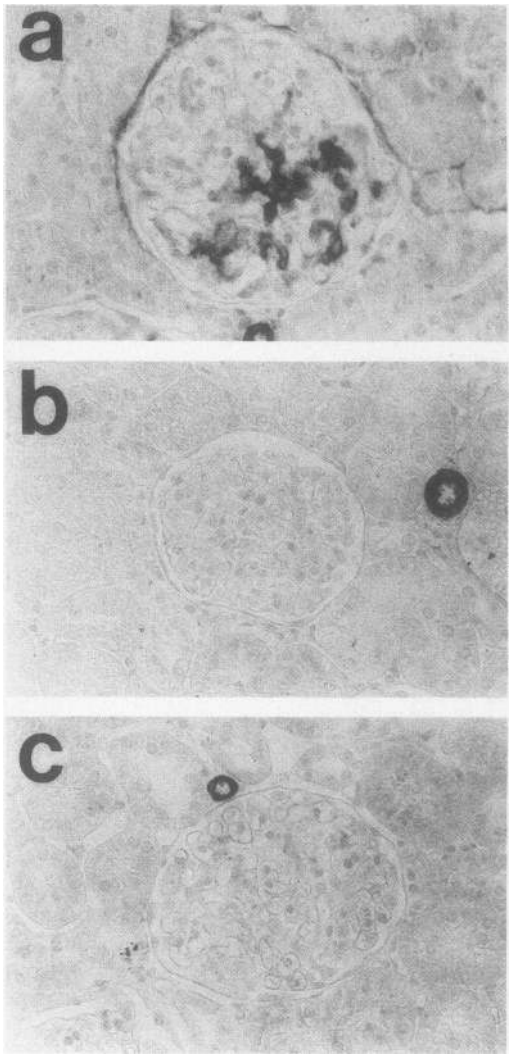

Figure 5. Effect of complement or platelet depletion on actin expression in mesangial proliferative GN due to anti-Thy 1 antibody, day 3. Control rats with anti-Thy 1 GN develop significant mesangial staining of smooth muscle actin, as detected by immunoperoxidase with HHF-35 antibody (*a*). In contrast, complement-depleted rats (*b*) or platelet-depleted rats (*c*) with anti-Thy 1 GN have either no or minimal actin expression, respectively. (Immunoperoxidase with methyl green counterstain, 400×).

lines have some phenotypic characteristics of smooth muscle cells.

The current study demonstrates that expression of α-smooth muscle actin is upregulated in mesangial proliferative GN in the rat induced with anti-Thy 1 antibody. It is known that smooth muscle actin content may be controlled either at the level of transcription, translation, or protein turnover (41). In this study, Northern analysis of isolated glomerular RNA showed an increase in α-smooth muscle actin mRNA in rats with anti-Thy 1 GN, peaking at day 3; this corresponded with the appearance of α-smooth muscle actin-positive cells that were greatest at day 5. We also observed a marked increase in β/γ actin mRNA in diseased rats at days 3 and 5. It is not known whether this increase in β/γ actin mRNA represents an increase in cytoplasmic (i.e., nonmuscle β and γ actin) mRNA as opposed to an increase in γ-smooth muscle actin mRNA. These studies demonstrate that the upregulation of α-smooth muscle actin and β/γ actin expression in this setting may occur at the transcriptional level.

Table III. Muscle Actin (HHF-35 Reactivity) and Actin Gene Expression in Normal Rats and Rats with Anti-Thy 1 GN

Groups	Muscle actin*	α-sm actin mRNA‡	β/γ actin mRNA‡
Normal	0	1	1
ATS day 1	0	1	1.7
ATS day 3	2	6.5	11.0
ATS day 5	4	3.2	9.0
CVF/ATS day 3	0	1	1.1
APS/ATS day 3	tr	1.6	1.4

* Graded semiquantitatively from 0–4+. ‡ Densitometry reading normalized for equivalent 28S ribosomal RNA which was measured using a specific 28S cDNA probe.

The cells expressing smooth muscle actin appear to be mesangial cells. This is based on the observation that these cells were predominantly in mesangial locations and by the studies that demonstrated HHF-35 reactivity in mesangial cells by immuno-EM. The possibility that the cells expressing muscle actin represent infiltrating leukocytes was excluded by the double

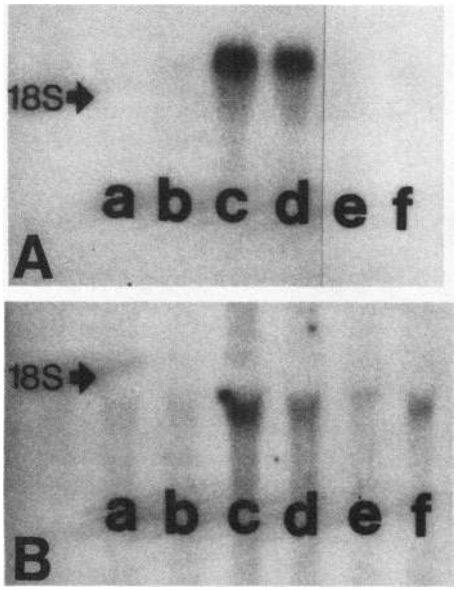

Figure 6. Northern analysis of glomerular RNA for actin gene expression. (*A*) Hybridization with the general actin probe, pBA1. (*B*) Hybridization with an oligonucleotide probe for human α-smooth muscle actin. General key: Lane *a*, normal rat glomerular RNA; lanes *b–d* contain glomerular RNA from rats with anti-Thy 1 GN at days 1, 3, and 5, respectively; lanes *e* and *f*, glomerular RNA from complement-depleted and platelet-depleted rats with anti-Thy 1 GN at day 3. All lanes contain 15 μg of RNA. Hybridization with the general actin probe, pBA1, demonstrates the 2.1-kb transcript for β/γ actin mRNA (*A*) and with the oligonucleotide probe reveals the 1.6-kb transcript for α-smooth muscle actin mRNA (*B*) in RNA samples from rats with anti-Thy 1 GN; this expression is blocked or reduced by complement or platelet depletion, respectively. The band detected by the oligonucleotide probe in *B* could be shown to comigrate with the 1.6-kb α-smooth muscle actin mRNA transcript present in rat aortic smooth muscle RNA (36) (data not shown).

immunolabeling experiments which demonstrated that the smooth muscle actin-positive cells and the monocyte-macrophages represented two populations. This is consistent with previous observations that human leukocytes (23, 33) as well as rat peritoneal macrophages (data not shown) are negative for smooth muscle actin. It is also unlikely that the increase in smooth muscle actin mRNA is being generated by the glomerular epithelial cell. Smooth muscle actin could not be demonstrated in glomerular epithelial cells by immunohistology. Although some immunoreactivity with the HHF-35 antibody could be demonstrated in the glomerular epithelial cell foot processes utilizing the more sensitive technique of immuno-EM, no differences in the level of antigen expression were noted in rats with anti-Thy 1 GN.

Mesangial cells have been previously considered smooth muscle–like (reviewed in references 2, 3). In vivo, the decrease in the ultrafiltration coefficient noted with the infusion of various vasoactive agents has been ascribed to modulation of the glomerular capillary surface area by active mesangial cell contraction (2, 3). In cell culture, mesangial cells are morphologically similar to smooth muscle cells and express the muscle-specific marker desmin (42), as well as the nonspecific isoforms of myosin (3) and actin (42). It is interesting that mesangial cells in culture show only weak immunostaining for smooth muscle actin (with HHF-35) despite strong staining with the pan-actin antibody (i.e., C4) (data not shown). Mesangial cells in vitro will also contract in response to various stimuli, although several minutes are required to demonstrate this effect (2, 3). It is tempting to speculate that the slow onset of contraction may relate to only low levels of smooth muscle actin being constitutively expressed by mesangial cells in culture.

Studies in vivo have also demonstrated that mesangial cells are continuous with and ultrastructurally similar to the lacis cells and smooth muscle cells of the juxtaglomerular apparatus (43). Mesangial regions of human glomeruli also stain with an antimyosin antibody (44, 45), although there is not any evidence that this is specific for smooth muscle cells. Our studies demonstrate that under pathological conditions (i.e., with mesangial proliferation) mesangial cells express α-smooth muscle actin and desmin, both muscle specific markers, thus providing additional evidence for a phenotypic similarity between smooth muscle cells and mesangial cells.

The second major finding in these experiments was the demonstration that smooth muscle actin expression was associated with active cellular proliferation. Cells bearing smooth muscle actin were not observed by light microscopy until day 3 of anti-Thy 1 GN, at a time when cellular proliferation was first observed. Double immunolabeling demonstrated that the proliferating cells were those that expressed muscle actin. Finally, prevention or reduction of the number of proliferating cells within the mesangium by complement depletion or platelet depletion, respectively, significantly reduced the number of cells expressing α-smooth muscle actin in the glomerulus, and the amount of α-smooth muscle actin and β/γ actin mRNA in glomerular RNA extracts.

In vascular smooth muscle, proliferation has been correlated with a loss of α-smooth muscle actin, and with an increase in β-actin synthesis (46, 47). This phenotypic change in actin expression is not an absolute prerequisite for cell proliferation (48), but does appear to be coupled to the cell cycle, occurring late in the G0/G1 phase (48, 49). Thus, the mesangial cell may be relatively unique in that α-smooth muscle actin is expressed during proliferation as opposed to being repressed.

An alternative hypothesis is that the appearance of cells bearing a smooth muscle phenotype in mesangial locations represents smooth muscle cells that migrate into the glomerulus, proliferate, eventually lose α-smooth muscle actin and "differentiate" into true mesangial cells. This would be more consistent with the known propensity for smooth muscle cells to lose α-actin with proliferation. This would also be consistent with our understanding of the developing nephron, in which mesangial cells have been proposed to originate from endothelial/mesangial cell precursors that invade the nephrogenic mesenchyme (50). This is further supported by recent in situ hybridization studies in fetal kidneys that demonstrate that renin-producing cells can be observed extending from the juxtaglomerular apparatus into the mesangium (51). The observation that platelets may mediate smooth muscle migration into injured intima of the carotid after balloon catheter injury (52) is consistent with the hypothesis that platelets may stimulate smooth muscle cell migration into glomeruli. The mechanism of cellular migration may involve platelet-derived growth factor, as this factor has been reported to be chemotactic for both smooth muscle cells (53) and mesangial cells (54).

We also examined the distribution of the intermediate filament proteins, desmin and vimentin, in normal and diseased glomeruli. Desmin has been regarded as a muscle differentiation marker (55) and has been reported to be present in vivo in mesangial cells of rats (56, 57) but not man (56, 58). Desmin

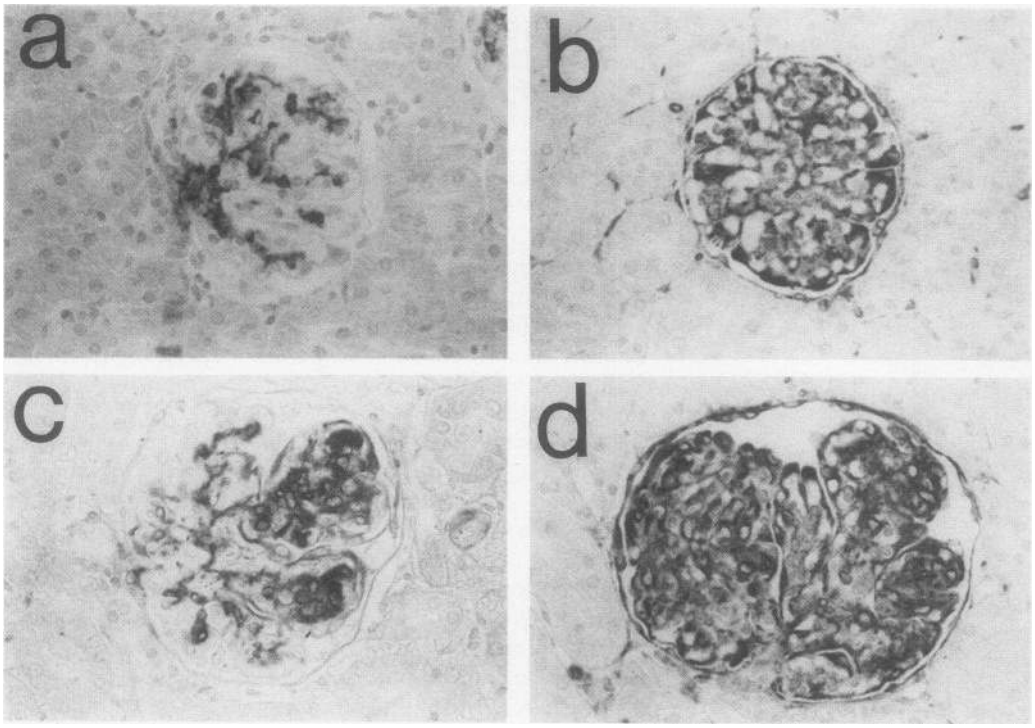

Figure 7. Desmin and vimentin staining in normal rats and rats with mesangial proliferative GN 5 d after injection of anti-Thy 1 antibody. In normal rats desmin is localized primarily to mesangial locations (*a*) whereas vimentin stains primarily glomerular visceral epithelial cells (*b*). In contrast, glomeruli from rats injected 5 d previously with ATS have an increase in staining for both desmin (*c*) and vimentin (*d*) especially in mesangial areas. The increase in both desmin and vimentin was confined to areas of mesangial cell proliferation, as demonstrated in *c*. (Immunoperoxidase with methyl green counterstain, 400×).

has also been reported to be either present (56), variably expressed (59), or absent (57) in vivo in glomerular epithelial cells in the rat. We noted that desmin was localized primarily in a mesangial distribution. However, occasional glomerular epithelial cells were also desmin-positive. In mesangial proliferative GN a marked increase in desmin in mesangial areas was noted; this was prevented in platelet- or complement-depleted rats in which proliferation was largely inhibited.

Table IV. Muscle Actin Expression (i.e., HHF-35 Immunostaining) in Different Experimentally-induced Glomerular Diseases

Site of injury	Model	Muscle actin (HHF-35)
Glomerular epithelial cell	Passive Heymann nephritis (day 5)	0
	Aminonucleoside nephrosis (day 14)	0
Glomerular basement membrane (GBM)	Anti-GBM disease (day 5)	0
Mesangial cell	Anti-Thy 1 GN (days 3–14)	2–4+
	Habu snake venom GN (day 5)	2+*
Endothelial cell and mesangial cell	Con A/anti-Con A GN (day 5)	1–2+

* Actin staining was only positive in glomeruli showing mesangial proliferative changes.

Vimentin is a general mesenchymal cell marker and is absent in most epithelial cells (55). However, both mesangial and glomerular epithelial cells have been reported to be vimentin-positive in vivo (56, 57). In our study, vimentin stained glomerular epithelial cells with only a trace staining of the mesangium. However, with proliferation, a marked increase in vimentin in mesangial regions was noted. An increase of vimentin in the mesangium has also been reported in membranoproliferative, diffuse proliferative, and mesangial proliferative GN in man (56).

Although we could not detect smooth muscle actin in glomerular epithelial cells by light microscopy, by immuno-EM muscle actin could be demonstrated at the base of the podocyte foot processes. This is consistent with a previous study in which both actin and meromyosin were localized by immuno-EM to the podocyte foot processes (using polyclonal antibodies) (60) and also with the observation that the podocyte foot processes are rich in microfilaments (61). The glomerular epithelial cell originates from nephrogenic mesenchyme but develops epithelial cell features via an inductive process (62). The observation that glomerular epithelial cells contain vimentin, muscle actin, and rarely, desmin support the concept (56) that these "epithelial" cells retain some mesenchymal/muscle cell features.

To determine if the acquisition by the mesangial cell of a smooth muscle phenotype was unique to anti-Thy 1 GN, we examined several other glomerular disease models. Models of glomerular disease associated with epithelial cell or basement membrane injury were not associated with smooth muscle actin expression. In contrast, diseases in which mesangial cell injury occurs, such as Habu snake venom GN (16) and con

A/anti-con A immune complex GN (20), were associated with smooth muscle actin expression by cells within mesangial regions. This suggests that smooth muscle actin expression may be a marker for mesangial cell injury in the rat.

The importance of the phenotypic change by mesangial cells in GN remains unknown. It is possible that these alterations may result in a mesangial cell with greater contractility. The concept that mesangial cell contraction may occur in GN is supported by the observation that several inflammatory substances known to mediate mesangial cell contraction in vitro (2) are likely to be present in GN, including platelet activating factor, thromboxane, and PDGF. Thus, it is possible that the acquisition of a smooth muscle phenotype may enable the mesangial cell to modulate and perhaps to contribute to the altered glomerular hemodynamics and pressures that are known to occur in GN.

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