De Novo CD44 Expression by Proliferating Mesangial Cells in Rat Anti-Thy-1 Nephritis

David J. Nikolic-Paterson, Zhao Jun, Gregory H. Tesch, Hui Y. Lan, Rita Foti, and Robert C. Atkins

ABSTRACT
CD44 is the major cell-surface receptor for hyaluronan, and cell-matrix interactions mediated by the CD44/hyaluronan receptor-ligand pair are involved in a variety of cellular functions, including cell migration. The aim of the study presented here was to examine the expression of CD44 and hyaluronan in the mesangial proliferative response in rat anti-Thy-1 nephritis. In normal rat kidney, CD44 is expressed by medullary tubules, some distal tubules and thick ascending limbs of Henle, dendritic-like cells around Bowman's capsule, and some interstitial cells. However, only occasional CD44⁺ cells were found within the glomerular tuft. In experimental nephritis, there was an early glomerular influx of CD44⁺ macrophages, which peaked on Day 4 after anti-Thy-1 antibody injection. A striking finding was de novo CD44 expression by mesangial cells. This CD44 expression was restricted to the transient period of mesangial cell proliferation as shown by double-staining with an antibody against the proliferating cell nuclear antigen. Immunohistochemistry staining also demonstrated hyaluronan deposition within segmental areas of proliferating CD44⁺ cells, suggesting a functional interaction between the CD44/hyaluronan receptor-ligand pair during mesangial cell proliferation. In vitro, rat mesangial cells were shown to express mRNA and protein for the 90-kd isoform of CD44. In addition, hyaluronan-dependent aggregation of CD44⁺ mesangial cells was specifically inhibited by an anti-CD44 antibody, demonstrating a functional interaction between hyaluronan and the CD44 expressed on the surface of rat mesangial cells. In conclusion, these data suggest that cell-matrix interactions mediated by the CD44/hyaluronan receptor-ligand pair are involved in mesangial cell proliferation in rat anti-Thy-1 nephritis.

Key Words: Hyaluronan, proliferation, OX-50

Mesangial cell proliferation is an important mechanism of glomerular injury in various forms of glomerulonephritis, leading to mesangial expansion, deposition of extracellular matrix, and, eventually, glomerular sclerosis (1). Although many studies have focused upon the cytokines that regulate mesangial cell proliferation and the synthesis of matrix proteins, relatively little is known of how mesangial cells interact with the extracellular matrix in vivo and how such interactions may regulate the migration and proliferation of mesangial cells. One possible mechanism by which mesangial cells may interact with the extracellular matrix is through the CD44/hyaluronan receptor-ligand pair.

CD44 is a Type I transmembrane glycoprotein expressed on the surface of many different cell types, including most leukocytes, some epithelium and endothelium, fibroblasts, keratinocytes, astrocytes, and glial cells (2). The CD44 molecule exists in a range of isoforms derived from alternative splicing of mRNA transcribed from a single gene (3). One of the major functions of CD44 is to act as a cellular receptor for hyaluronan, a component of the extracellular matrix (4). All of the known CD44 isoforms contain the hyaluronan-binding domain situated at the amino terminus of the extracellular region, but it is the 90-kd isoform of the molecule that has the highest binding affinity for hyaluronan (2,5). Many of the functions attributed to the CD44 molecule are based upon its specific interaction with hyaluronan (5). For example, CD44 expression plays an important role in metastasis of carcinoma cells and in the migration of leukocytes into lymphoid tissues (6,7). Thus, the aim of this study was to determine whether the CD44/hyaluronan interaction is involved in the mesangial proliferative response to glomerular injury. This was addressed by colocalization studies of CD44 expression and hyaluronan deposition in rat anti-Thy-1 disease—a model of mesangial proliferative nephritis.

METHODS

Anti-Thy-1 Disease
Male inbred Wistar rats (150 to 180 g) were obtained from Monash Animal Services. Disease was induced by a single intravenous injection of 5 mg/kg purified OX-7 immunoglobulin G (IgG) as previously described (8). Groups of five animals were killed on Days 1, 4, 8, 14, 21, and 28 after antibody injection. In addition, groups of three animals were killed on Days 6 and 10 to evaluate the kinetics of mesangial proliferation more precisely. A group of normal age-matched
male inbred Wistar rats served as a control (referred to as Day 0).

**Antibodies**

The following mouse monoclonal antibodies (mAb) were used in this study:OX-1, anti-rat CD45, leukocyte common antigen (9);OX-7 (IgG1), anti-CDw90, Thy-1 antigen (10);OX-42 (IgG1), anti-CD11b, tissue macrophages (11);OX-50 (IgG1), anti-CD44 (12,13);NDGO1 (IgG1), anti-hyaluronan (14,15) (Serotec, Oxford, UK); PC10 (IgG2a), anti-PCNA (16) (Dakopatts, Glostrup, Denmark); IA4 (IgG2a), anti-a smooth muscle actin (Sigma Chemical Co., St. Louis, MO). Purified IgG of OX-1, OX-7, OX-42, and OX-50 mAb were conjugated with digoxigenin according to the manufacturer's instructions (Boehringer Mannheim GmbH, Mannheim, Germany). Peroxidase- and alkaline phosphatase-conjugated goat antirat IgG, peroxidase anti-peroxidase complexes, and mouse alkaline phosphatase anti-alkaline phosphatase complexes (APAAP) were purchased from Dakopatts. In addition, peroxidase-conjugated Fab( 1) fragments of sheep anti-digoxigenin were purchased from Boehringer Mannheim GmbH and alkaline phosphatase-conjugated sheep anti-mouse IgG2a from Zymed, South San Francisco, CA.

**Immunohistochemistry**

Tissues were cut into small blocks and either snap-frozen in liquid nitrogen or fixed in 3% paraformaldehyde-hysine-phenol-periodate (PLP), saturated with 7% sucrose in phosphate-buffered saline (PBS) and then snap-frozen.

To compare the immunohistochemistry staining patterns of OX-50, OX-7, and OX-1 mAb, serial 4-μm cryostat sections of snap-frozen tissue were post-fixed in 100% ethanol at 4°C for 10 min and washed in PBS. Sections were preincubated with 10% normal goat serum (NGS)/10% fetal calf serum (FCS) in PBS for 20 min, drained, incubated for 1 h with digoxigenin-conjugated mAb (5 μg/mL in 10% NGS/10% FCS in PBS) and washed in PBS. Endogenous peroxidase was inactivated by incubation in 0.3% H2O2 in methanol for 20 min. Monoclonal antibodies were then detected with peroxidase-conjugated Fab( 1) fragments of sheep anti-digoxigenin, washed, and developed with diaminobenzidine to produce a brown color.

Double immunohistochemistry staining was performed on 4-μm cryostat sections of PLP-fixed tissues. Sections were preincubated and labeled with digoxigenin-conjugated OX-50 or OX-1 mAb as described above and then developed with diaminobenzidine. Sections were then heated twice for 5 min each in 0.01 M sodium citrate (pH 6.0) in a microwave oven (2450 mHz, 800W) to denature bound antibody and thus prevent crossreactivity and to augment antigen retrieval as previously described (17). Sections were preincubated with 1% BSA in PBS for 20 min and then incubated for 60 min with NDOG1 or PC-10 in 1% BSA. After being washed, sections were labeled with alkaline phosphatase-conjugated goat anti-mouse antibody (to detect NDOG1) or alkaline phosphatase-conjugated rabbit anti-mouse IgG2a (to detect PC-10 or IA4 mAb), followed by mouse APAAP, developed with Fast Blue BB salt (Ajax Chemicals, Melbourne, Australia) to produce a blue color, and mounted in aqueous medium. Note that microwave treatment substantially in- creases antibody access to nuclear antigens, resulting in both increased sensitivity and nonspecific binding (17). Therefore, the PC-10 mAb was used at a 1-in-1000 dilution rather than the 1-in-50 dilution recommended by Dako, which results in nonspecific nuclear staining on microwave-treated sections.

The specificity of NDOG1 mAb for hyaluronan was checked by incubating tissue sections for 1 h at 37°C with 50U/mL testicular hyaluronidase in 20 mM sodium phosphate, 77 mM sodium chloride, 0.01% BSA, pH 7.0, and thereafter performing immunohistochemistry staining. Hyaluronidase pretreatment specifically abolished the staining observed with the NDOG1 mAb, but not that seen with other mAb.

**Assessment of Immunohistochemistry Staining.** The diffuse nature of OX-50 immunostaining on PLP-fixed tissue sections made it difficult to score individual CD44+ cells within segmental lesions. Therefore, CD44 expression was semiquantitatively assessed by scoring the approximate number of cells stained with the OX-50 mAb per glomerular cross-sections (gcs) as follows: 0, no positive cells; 1, 1 to 25% positive cells; 2, 25 to 50% positive cells; 3, 50 to 75% positive cells; 4, more than 75% positive cells. Twenty glomeruli were scored under high power for each animal and the data is presented as mean ± SD.

The number of total OX-1+ leukocytes and proliferating leukocytes (OX-1+PCNA+) per gc, including double-labeled cells, was scored on sections labeled with both OX-1 and PC-10 mAb. Labeled cells were scored under high power in 20 glomerular cross-sections per animal and the data presented as mean ± SD.

The relationship between CD44 expression and glomerular cell proliferation was assessed on sections double-labeled with OX-50 and PC-10 mAb. The number of PCNA+ cells within CD44-positive and CD44-negative glomerular areas were counted. Twenty glomerular cross-sections were scored under high power per animal. The results are expressed as the mean number of PCNA+ cells within the CD44-positive and CD44-negative areas/gcs ± SD.

Hyaluronan deposition in glomeruli was assessed on tissue sections labeled with the NDOG1 mAb. Glomeruli were scored as being positive or negative for NDOG1 mAb staining. Fifty glomeruli were scored for each animal and the results are expressed as the mean percentage of glomeruli exhibiting hyaluronan deposition ± SD.

**Mesangial Cell Culture**

A well-characterized mesangial cell line (1097) isolated from Sprague-Dawley rats (18-20) was used between Passages 20 and 30. Cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) with 10% heat-inactivated FCS, 10 mM N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified 5% CO2 atmosphere at 37°C.

**Northern Blot Analysis**

Mesangial cells were grown in 175-cm2 tissue-culture flasks, harvested by trypsinisation, and lysed in guanidine thiocyanate. Total cellular RNA was prepared by acid-phenol-chloroform extraction (21), and Northern blotting, following a standard method, was then performed (22). In brief, RNA samples were denatured with glyoxal and dimethylsulphoxide, size-fractionated on 1.2% agarose gels, transferred to nylon membranes, washed, and hybridized with [32P]RNA synthesized by in vitro transcription from a 1800-base pair BamHII-EcoRI fragment of a plasmid containing the rat CD44 cDNA. Membranes were hybridized overnight at 42°C with a CD44 digoxigenin-labeled cDNA probe in 5 × SSPE (1 × SSPE: 0.15 M NaCl, 10 mM NaH2PO4, 1 mM EDTA, pH 7.0), 50% formamide, 0.2 mg/ml herring sperm DNA, 0.2 mg/ml yeast RNA, 0.5% sodium dodecyl sulfate (SDS), 5 × Denhardt’s solution, 1%
blocking agent (Boehringer Mannheim GmbH). After hybridization, membranes were washed finally in 0.2 x standard saline citrate/0.1% SDS at 42°C. Bound probes were detected by chemiluminescence on Kodak X-ray film (Eastman Kodak, Rochester, NY) using alkaline phosphatase-conjugated Fab fragments of sheep anti-digoxigenin antibody with AMPPD substrate according to the manufacturer's recommended protocol (Boehringer Mannheim GmbH). CD44 cDNA was amplified from rat spleen by reverse transcription-polymerase chain reaction (RT-PCR; see below) and then random-primed in the presence of digoxigenindUTP using the DIG-High Prime kit (Boehringer Mannheim GmbH).

RT-PCR
cDNA was produced from 5 μg total cellular RNA per sample using oligo-dT (15) primer and Superscript II reverse transcriptase (Gibco BRL, Gaithersburg, MD) for 50 min at 42°C. The cDNA was amplified in the PCR using Hot Start 100 tubes (Molecular Bioproducts, San Diego, CA) with 0.5U Taq polymerase in 50 mM KCl, 1.5 mM MgCl₂, 200 μM dNTP-mix, 50 pmol oligonucleotide primers, 20 mM Tris-HCl, pH 8.4, for 35 cycles (94°C for 60 s, 55°C for 90 s, 72°C for 120 s, with a 300-s first step at 94°C). Oligonucleotide primers used to amplify rat CD44 were as follows: 5' GCCAG-GCTTCAACAAACC 3' (Exon 2) and 5' ACTCCACTGTCTTC-ATTCCC 3' (Exon 17) on the basis of the published rat CD44 sequence (6). The PCR products were analyzed by agarose gel electrophoresis and restriction enzyme digestion.

Flow Cytometry
Mesangial cells were harvested by trypsinisation and grown for 18 h to 10 mL of RPMI/10% FCS in a 50 mL flask at 37°C with continuous agitation. Cells were washed, resuspended in ice-cold 1% FCS/0.01% NaN₃ in PBS, incubated with OX-50 or an irrelevant isotype control mAb for 60 min, washed three times, incubated with fluorescein-conjugated sheep anti-mouse immunoglobulin (Silenus, Melbourne, Australia), washed again, and analyzed on an EPICS V (Beckton-Dickson, Oxnard, CA). For each sample, 10,000 viable cells (gated by forward light scatter) were counted.

Western Blot Analysis. Cultured mesangial cells and rat splenic lymphocytes stimulated for 3 days with 5 μg/mL concanavalin A were harvested, washed extensively with cold PBS, and 1 to 5 x 10⁶ cells were lysed by incubation in 1 mL of cell lysis buffer (1% Nonidet P-40, 25 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 5 mM iodacetamide, 1 mM phenylmethysulfonyl fluoride, pH 8.0) for 30 min on ice. Samples were centrifuged at 14,000 g for 5 min to pellet debris. The supernatant was mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, boiled for 5 min, and samples were then electrophoresed on a 10% SDS polyacrylamide gel. Proteins were transferred onto Hybond-ECL nitrocellulose membrane (Amersham International) with a Biorad Transblot cell (BioRad Laboratories, Hercules, CA) at 1 amper overnight. The membrane was blocked in PBS containing 5% skimmed milk powder, 1% FCS, 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO), and then incubated for 1 h with 2 μg/mL of purified OX-50 diluted in the above buffer. After being washed, the membrane was incubated with a 1:20,000 dilution of peroxidase-conjugated goat anti-mouse IgG in PBS containing 1% normal goat serum and 1% FCS. The blot was then developed using the ECL detection kit to produce a chemiluminescent signal that was captured upon X-ray film according to the manufacturer's instructions (Amersham International).

Mesangial Cell Aggregation Assay
The assay of mesangial cell aggregation was based upon the method of Green et al. (23). To ensure CD44 expression, rat mesangial cells were harvested by trypsinisation and cultured overnight in 20 mL RPMI/10% FCS at 37°C in siliconized 50-mL polypropylene tubes in a rotary incubator. Cells were harvested by centrifugation and CD44 expression confirmed by flow cytometry. The remaining cells were resuspended in 10% FCS, PBS, 0.02% EDTA. Any cell clumps present at this stage were removed by allowing the solution to stand for 10 to 15 min and then separating them under the microscope. Cells were then diluted to 4 x 10⁵/mL and 900 μL of cells were added to 1.5-mL Eppendorf tubes. Ten microliters of different mAb (5 mg/mL), bovine testicular hyaluronidase (2 mg/mL; Sigma Chemical Co.) or PBS were added to cells and incubated for 30 min at room temperature on a rotator at 16 rpm. Next, a 100-μL addition of either 1 mg/mL hyaluronan (bovine testis, Sigma Chemical Co., St. Louis, MO) or PBS was made and cells were mixed on a rotator for 30 min at 78 rpm. The numbers of cell aggregates/mL in cell suspensions were then quantitated using a haemocytometer. Each sample was counted four times and aggregates were scored as clumps of three or more nondividing cells. Results are expressed as mean ± SD of three experiments and compared with the number of cell aggregates/mL in the control sample (cells alone), which was normalized to 100%.

RESULTS
CD44 Expression in Normal Rat Kidney

Immunoperoxidase staining of cryostat tissue sections using the OX-50 mAb showed CD44 expression by several cell types in the tubulointerstitium: medullary tubules, some distal tubules and thick ascending limbs of Henle, dendritic-like cells around Bowman's capsule, and some interstital cells. However, only occasional CD44⁺ cells were observed within the glomerular tuft (Figure 1a).

Figure 1. Detection of CD44 expression in rat anti-Thy-1 nephritis by single and double immunohistochemistry. (a) OX-50 mAb staining of normal rat kidney, showing CD44 expression (brown) by a distal tubule and cells around Bowman's capsule with few CD44⁺ cells within the glomerular tuft; (b) Day 6 of anti-Thy-1 nephritis, showing a segmental pattern of OX-50 mAb staining (brown) in two glomeruli; (c) serial section to that in (b), showing that the segmental areas of CD44⁺ cells are also labeled with the OX-7 mAb (brown); (d) double staining of Day 6 of anti-Thy-1 nephritis, showing that many CD44⁺ cells (brown) within a segmental lesion are PCNA⁺ (blue nuclear stain); (e) double staining of Day 10 of anti-Thy-1 nephritis, showing a marked reduction in glomerular CD44⁺PCNA⁺ cells, although there is still increased CD44 expression in Bowman's capsule and tubules, and (f) double staining of Day 8 of anti-Thy-1 nephritis showing colocalization of CD44⁺ cells (brown) and hyaluronan deposition (blue) within the mesangium and in Bowman's capsule. Original magnification, ×250 (b,c,e) and ×400 (a,d,f).
Anti-Thy-1 Nephritis

A transient mesangial proliferative nephritis was induced in rats by a single injection of OX-7 IgG as previously described (8). After antibody and complement deposition within the glomerulus on Day 1, there was profound mesangiolysis and loss of glomerular cellularity and structure. An early glomerular influx of macrophages was evident over Days 1 to 6, which was followed by a period of mesangial cell proliferation over Days 6 to 10. This proliferative phase led to development of mild glomerular hypercellularity on Days 8 to 14, which resolved by Day 28. Mesangiolysis caused glomerular injury in terms of a transient proteinuria that peaked on Day 4 (74 ± 20 mg/24 h in normal rats; P < 0.05).

CD44 Expression by Proliferating Mesangial Cells in Anti-Thy-1 Nephritis

Immunoperoxidase staining of tissue sections using digoxigenin-conjugated OX-50 mAb demonstrated a marked increase in glomerular CD44 expression on Days 6 and 8 of rat anti-Thy-i nephritis (Figure 1b). Semi-quantitation of glomerular CD44 expression is shown in Figure 2. The large number of CD44+ mesangial cells seen on Days 6 and 8 were identified as proliferating mesangial cells on the basis of the following criteria.

First, labeling of serial tissue sections with digoxigenin-conjugated OX-50 and OX-7 mAb revealed a very similar pattern of segmental mesangial staining (Figures 1b and c), which was quite distinct from that observed with the OX-1 mAb (not shown). Second, antibody labeling of serial sections with OX-7 and PC-10 mAb showed that, on the basis of PCNA expression, the focal areas of OX-7+ mesangial cells contained many proliferating cells. Third, double antibody staining demonstrated that most glomerular proliferating (PCNA+) cells also expressed the CD44 antigen (Figure 1d). Quantitation of double-stained sections showed a peak of glomerular cell proliferation on Days 6 and 8 and that most (71 to 80%) of these proliferating cells expressed the CD44 antigen (Figure 3). Fourth, staining of serial sections showed a very similar segmental staining pattern for OX-50 and anti-smooth muscle actin mAb (not shown). Fifth, mesangial cells lost CD44 expression at the same time that glomerular cell proliferation was downregulated (Figures 1e and 3).

CD44 Expression by Other Cell Types in Anti-Thy-1 Nephritis

The marked upregulation of glomerular CD44 expression seen in rat anti-Thy-1 nephritis (Figure 2) was not restricted to just glomerular mesangial cells. Two other glomerular cell types—filtrating macrophages and podocytes—exhibited CD44 expression during the period after OX-7 IgG injection and before the development of mesangial cell proliferation. Immunoperoxidase staining with the OX-1 mAb demonstrated a marked leukocyte infiltrate on Day 1 of anti-Thy-1 nephritis, which peaked on Day 4 and declined thereafter (Figure 4). The leukocytic infiltrate was composed predominantly of OX-42+ macrophages (not shown). Labeling of serial tissue sections found that the infiltrating OX-1+ leukocytes exhibited strong CD44 expression (not shown). Consistent with previous studies of this model (24,25), a population of proliferating glomerular leukocytes (OX-1+PCNA+ cells) was identified in double-stained sections. The number of OX-1+PCNA+ proliferating leukocytes peaked on Day 4 of the disease, which was also the peak in total leukocyte accumulation, and declined thereafter (Figure 4).

An interesting observation was the presence of many proliferating CD44+ glomerular cells on Day 1 of anti-Thy-1 nephritis, a time at which there was a
Days after OX-7 injection

Figure 4. Glomerular leukocyte infiltration and leukocyte proliferation in rat anti-Thy-1 nephritis. The number of total OX-1+ leukocytes (closed bars) and proliferating leukocytes (OX-1+PCNA+ cells, open bars) per glomerular cross-section were counted in double-stained tissue sections. Data are shown as mean ± SD. * P < 0.01 compared with normal animals (Day 0, control group) by an unpaired t-test.

marked loss of mesangial cells and only a relatively small population of proliferating leukocytes. Histological examination found that these CD44+PCNA+ cells were podocyte-like cells in terms of their morphology and positioning within the collapsed glomerular tuft (not shown). In addition, CD44 expression was also upregulated on Bowman’s capsule parietal epithelial cells (Figure 1).

Although the immunologic insult in anti-Thy-1 nephritis is restricted to the glomerulus, mild tubulointerstitial injury is evident in this disease, which comprises occasional tubular necrosis, a tubular proliferative response, and periglomerular leukocyte infiltration (8,26). In the study presented here, there was an increase in the number of cortical tubules expressing the CD44 antigen and a marked increase in the intensity of OX-50 immunostaining of tubules. The increased tubular CD44 expression was seen from Day 4 to Day 14 and declined thereafter.

Hyaluronan Deposition in Anti-Thy-1 Nephritis

Hyaluronan deposition was assessed by immunohistochemistry. No antibody staining was detected in normal rat kidney. However, there was strong immunostaining of deposited hyaluronan in anti-Thy-1 nephritis. Although it was not possible to determine when hyaluronan deposition first occurred (because of the remaining OX-7 IgG within the glomerulus), hyaluronan deposition was detected in 52% of glomeruli on Day 6 of anti-Thy-1 nephritis. Staining of hyaluronan colocalized with CD44 expression within segmental mesangial lesions and in Bowman’s capsule (Figure 1f). Glomerular hyaluronan staining remained strong until Day 14, after which it declined through to Day 28 (Figure 5). The specificity of antibody staining was demonstrated by the ability of hyaluronidase pretreatment to abolish detection of hyaluronan in diseased kidneys (not shown).

Figure 5. Glomerular hyaluronan deposition in rat anti-Thy-1 nephritis. Hyaluronan deposition was assessed by semi-quantitative scoring of mAb-labeled tissue sections as described in the Methods section (note that Days 1 and 4 could not be scored because of the remaining OX-7 IgG in the glomerulus). Data are shown as mean ± SD.

Figure 6. Mesangial cell expression of CD44 mRNA. (a) Detection of a single mRNA species in cultured rat mesangial cells and normal rat kidney by Northern hybridization of total cellular RNA by using a CD44 cDNA probe. The position of 28S (upper) and 18S (lower) rRNA bands are indicated. (b) Ethidium bromide-stained agarose gel showing detection of a single DNA product from rat mesangial cells and normal rat spleen by RT-PCR using CD44 oligonucleotide primers. Molecular weight markers are shown (1114/489, 404, etc.; DNA molecular weight marker VIII, Boehringer Mannheim GmbH).

Cultured Rat Mesangial Cells Express the 90 kDa CD44 Isoform

A well-characterized rat mesangial cell line was shown to express a single species of CD44 mRNA of approximately 3.4 kilobase by Northern blot analysis (Figure 6a). Analysis of mesangial cell CD44 mRNA by RT-PCR using primers that span the alternatively spliced region of the molecule detected a single band of approximately 600 base pair, consistent with the predicted 603-base pair product for the 90-kd form of the CD44 molecule (Figure 6b). The specificity of the
CD44 Expression and Mesangial Proliferation

Figure 7. Mesangial-cell surface expression of the CD44 protein. (a) Flow cytometry showing OX-50 mAb-labeling of cell-surface CD44 protein in cultured rat mesangial cells (bold line) compared with an irrelevant control antibody (dashed line). (b) Western blot analysis using the OX-50 mAb detects three distinct CD44 bands from concanavalin-A stimulated rat spleen cells and a single CD44 band from cultured rat mesangial cells. Molecular weight markers are shown.

RT-PCR product was also demonstrated by restriction enzyme analysis (not shown). Mesangial cell expression of the CD44 protein was shown in two ways. Cell-surface CD44 expression was demonstrated by flow cytometry using the OX-50 mAb (Figure 7a). This expression was exquisitely sensitive to trypsin, as a 2-min treatment with 0.25% trypsin removed all CD44 antigen from the cell surface, whereas the expression of other antigens (such as major histocompatibility complex Class II and intracellular adhesion molecule-1) were unaffected (not shown). Western blot analysis of mesangial cell lysates by using the OX-50 mAb identified a single band of roughly 90-kd (Figure 7b). This contrasts with the multiple isoforms of CD44 detected in concanavalin-A stimulated rat spleen cells and a single CD44 band from cultured rat mesangial cells. Molecular weight markers are shown.

Hyaluronan Induces CD44-Dependent Mesangial Cell Aggregation In Vitro

A cell aggregation assay was used to investigate the CD44/hyaluronan interaction in cultured rat mesangial cells. Previous studies have shown that hyaluronan-induced fibroblast aggregation is cation-independent (23), therefore, an assay of mesangial cell aggregation was performed in the presence of EDTA. Under these conditions, the presence of 100 μg/mL hyaluronan induced a 3.5-fold increase in mesangial cell aggregation over that seen with cells alone (Figure 8). This hyaluronan-induced mesangial cell aggregation was dependent upon both CD44 expression and the presence of hyaluronan as shown by the complete inhibition of aggregation by preincubation of cells with OX-50 mAb or hyaluronidase, but not with either of two control antibodies (Figure 8). The addition of the OX-50 mAb to mesangial cells which were already attached to, and growing upon, hyaluronan-coated wells showed no effect on cell proliferation (data not shown). However, these experiments were inconclusive and further studies are required to determine the functional role of CD44 expression in the growth of cultured mesangial cells upon hyaluronan.

DISCUSSION

The results of this study suggest that mesangial cell-matrix interactions mediated by the CD44/hyaluronan receptor-ligand pair play an important role in the mesangial cell response to glomerular injury in rat anti-Thy-1 nephritis. This postulate is based upon the following data. First, de novo CD44 expression by mesangial cells was very tightly regulated, paralleling exactly the period of mesangial cell proliferation. This contrasts with the much longer period during which CD44 expression was upregulated by tubular epithelial cells. Second, there was marked deposition of hyaluronan in the glomerulus and this colocalized within areas of proliferating CD44+ mesangial cells. Third, mesangial cells in culture were shown to express the 90-kd form of CD44 on the cell surface. The ability of hyaluronan to induce CD44-dependent cell aggregation in vitro demonstrated that the CD44 antigen expressed by mesangial cells can interact functionally with hyaluronan. Although these observations provide strong evidence that the CD44/hyaluronan interaction is involved in mesangial-cell proliferative response, we are currently attempting to prove the functional significance of this interaction by blocking the CD44 molecule in this disease model.
Cell-matrix interactions probably play a crucial role in the various stages of the mesangial response in this disease model: cell migration, cell proliferation, matrix deposition, and termination of the proliferative response. In Habu snake venom-induced glomerular injury, a model analogous to anti-Thy-1 nephritis, it has been shown that mesangial cell migration precedes proliferation and that this migration appears to involve mesangial cell interaction with newly deposited fibronectin (28,29). It is well known that the proliferation of mesangial cells in culture is profoundly affected by the nature of the matrix molecules on which the cells are grown (30–32). There is a good correlation between increased matrix deposition and mesangial hypercellularity in anti-Thy-1 nephritis (28,33), and other experimental models of glomerulonephritis (34). This relationship is exemplified by the finding that maneuvers that inhibit or promote mesangial cell proliferation in anti-Thy-1 nephritis also modulate matrix deposition (34). The association between matrix deposition and mesangial cell proliferation has many parallels with the process of wound healing. Hyaluronan deposition is a hallmark of the wound-healing response and is usually associated with the presence of many CD44+ cells, such as fibroblasts. For example, strong CD44 expression is evident on migrating keratinocytes in areas of marked hyaluronan deposition in skin wounds, whereas hyaluronan deposition together with macrophage accumulation is evident during glomerular crescent formation in rat anti-GBM glomerulonephritis (35,36). Also, a role for CD44 in matrix assembly and cell migration has been demonstrated in vitro in which CD44 expression by transfected COS cells enables them to form a pericellular matrix in the presence of endogenous hyaluronan and proteoglycan (37).

An unexpected finding was the inability of the NDOG1 mAb to detect hyaluronan within normal rat kidney. Nishikawa et al. (36), demonstrated the presence of hyaluronan in the interstitial tissue of the papilla and in the adventitia of large vessels in normal rat kidney by using a hyaluronic acid-binding protein and a soluble form of the CD44 molecule. There are two possible explanations for the apparent discrepancy. First, immunohistochemistry staining with the NDOG1 mAb is not sensitive enough to detect hyaluronan within sections of normal kidney. Second, the hyaluronan epitope recognized by the NDOG1 mAb could be masked within the structure of normal basement membrane, but is available within the matrix deposited during mesangial proliferation.

Glomerular macrophage infiltration is another feature of anti-Thy-1 nephritis in which the CD44 molecule may also be involved. Although the function of infiltrating macrophages in this disease model is not clear, it has been shown that CD44 expression is required to enable leukocyte recruitment into sites of inflammation in nonlymphoid tissues (38,39). Glomerular macrophages may be involved in removal of the cellular debris caused by mesangial lysis and/or they may participate in the process of mesangial cell proliferation. A clear association between glomerular macrophage infiltration and mesangial hypercellularity has been established in experimental glomerulonephritis (40–42), and macrophages are known to synthesize mesangial cell growth factors such as fibroblast growth factor-2 (FGF-2) and platelet-derived growth factor-B. Interestingly, it has been shown that macrophages can express a proteoglycan-containing form of CD44 that is capable of binding FGF-2 and heparin-binding epidermal growth factor (43). This may be of relevance in macrophage delivery of mesangial cell growth factors.

In conclusion, this study suggests that cell-matrix interactions mediated by the CD44/hyaluronan receptor-ligand pair are involved in the process of mesangial cell proliferation in rat anti-Thy-1 proliferative nephritis. These observations open up a new avenue of investigation into the regulation of the mesangial cell response to glomerular injury.

REFERENCES


