Disruption of glomerular cell-cell and cell-matrix interactions in hydrocarbon nephropathy

Adrian Nanez, Napoleon F. Alejandro, M. Hadi Falahatpisheh, J. Kevin Kerzee, John B. Roths, and Kenneth S. Ramos. Disruption of glomerular cell-cell and cell-matrix interactions in hydrocarbon nephropathy. Am J Physiol Renal Physiol. 289: F1291–F1303, 2005. First published July 5, 2005; doi:10.1152/ajprenal.00107.2005.—Environmental chemicals play an etiological role in greater than 25% of idiopathic glomerular diseases. The present studies were conducted to define mechanisms of renal cell-specific hydrocarbon injury. Female rats were given 10 mg/kg benzo(a)pyrene (BaP) once a week for up to 16 wk. Progressive elevations in total urinary protein, protein/creatinine ratios, and microalbuminuria were observed in rats treated with BaP for up to 16 wk. The nephropathic response involved early reductions in mesangial cell numbers and fibronectin levels by 8 wk, coupled to transient increases in podocyte cellularity. Changes in podocyte numbers subsided by 16 wk and correlated with rebound increases in mesangial cell numbers and fibronectin levels, along with increased α-smooth muscle actin and Cu/Zn superoxide dismutase and fusion of podocyte foot processes. In culture, mesangial cells were more sensitive than podocytes to hydrocarbon injury and expressed higher levels of inducible aryl hydrocarbon hydroxylase activity. Naïve mesangial cells exerted a strong inhibitory influence on podocyte proliferation under both direct and indirect coculture conditions, and this response involved a mesangial cell-derived matrix that selectively inhibited podocyte proliferation. These findings indicate podocytes are involved in hydrocarbon nephropathy in rats involving disruption of glomerular cell-cell and cell-matrix interactions mediated by deposition of a mesangial cell-derived growth-inhibitory matrix that regulates podocyte proliferation.

defenses seen shortly after hydrocarbon exposure (43). The biological effects of BaP are often mediated by cytochrome P-450 (CYP) enzymes that catalyze the formation of reactive oxidative intermediates (39).

Human exposure to aromatic hydrocarbons is associated with a higher risk of renal dysfunction and renal cancer (5). Renal dysfunction on chronic exposure to aromatic hydrocarbons is secondary to glomerular injury and often associated with proteinuria. Other sites of the nephron including the proximal tubule are not affected (23), suggesting that region-selective patterns of protein expression influence pathogenetic outcomes. Subchronic oral dosing of male rats with BaP induces renal cast formation (29). Repeated challenge of glomerular cells in culture with BaP and related aromatic hydrocarbons induce phenotypic changes (2) and altered mitogenic signaling (44). If these alterations occur in vivo, progressive loss of renal glomerular function would be expected.

Glomerular diseases arise as a consequence of a primary or secondary response to injury. Primary glomerular diseases constitute a heterogeneous collection of disorders in which glomeruli are the predominant structure involved. Primary glomerular disorders include IgA nephropathy, IgM nephropathy, mesangio-proliferative glomerulonephritis, crescentic glomerulonephritis, membrano-proliferative glomerulonephritis, focal segmental glomerulosclerosis, membranous glomerulopathy, minimal change disease, and thin basement membrane disease. Secondary glomerular diseases include lupus nephritis, postinfectious glomerulonephritis, systemic vasculitides, diabetic nephropathy, amyloidosis, light-chain nephropathy, HIV nephropathy, Alport’s syndrome, and drug-induced nephropathy. Glomerulopathies are the most common causes of end-stage renal disease worldwide (24).

The present studies were conducted to evaluate mechanisms of hydrocarbon (i.e., BaP) nephropathy in female Sprague-Dawley (SD) rats. Rats are routinely employed as models to evaluate nephropathy, with renal injury often exhibiting strain, sex, and age dependence (4, 48). Evidence is presented here that BaP nephropathy involves changes in glomerular cellularity, mesangial cell activation, and loss of selective permeability. The nephropathic response is associated with pronounced disruption of cell-cell and cell-matrix interactions.

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Table 1. Molecular markers used to characterize cellular identity and nephropathic response profiles

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<td>WT1</td>
<td>Podocytes</td>
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<td>Thy-1.1</td>
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<td>Fibronectin</td>
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<tr>
<td>EGF-cadherin</td>
<td>Glomerular epithelial/podocyte identity</td>
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METHODOLOGY

Animals. Female SD rats (175–225 g) were purchased from Harlan (Indianapolis, IN) and placed under standard housing conditions. Female rats were used to obviate the influence of gender-related nephropathy in male rats (6, 34, 35). This form of nephropathy is a gender-specific alteration of renal function associated with accumulation of hyaline droplets containing α2-μ-globulin. It occurs only in adult male rats because α2-μ-globulin is synthesized exclusively by male rats (53). The care and handling of animals were conducted in accord with National Institutes of Health guidelines and approved by the Texas A&M University and University of Louisville Animal Care and Use Committee.

Chemicals. BaP (>98% purity) was purchased from Sigma (St. Louis, MO). Medium chain triglyceride (MCT) oil was from Mead Johnson (Evansville, IN). Fetal bovine serum (FBS) was from Atlanta Biologicals (Norcross, GA). RPMI 1640 was from Gibco/BRL (Grand Island, NY). FITC-labeled anti-mouse IgG and WT-1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). EGF-cadherin antibody was from Transduction Laboratories (Lexington, KY). Thy-1, L1, fibronectin, and CD3 mouse monoclonal antibodies were from BD PharMingen (Palo Alto, CA), Neo Markers (Freemont, CA), Oncogene (San Diego, CA), and US Biological (Swampscott, NS), respectively. Goat serum, Vector Antigen Unmasking Solution, Mayer’s hematoxylin, and the avidin/biotin blocking kit were from Vector Laboratories (Burlingame, CA). Citrus solv was from Fisher (Pittsburgh, PA). Prolong Antifade mounting media, biotinylated secondary antibody, SYBB green, and Texas Red secondary antibody were from Molecular Probes (Eugene, OR). Factor VIII-RA rabbit polyclonal antibody was from Innovagen (San Ramon, CA). Pepsin was from Biogenex (San Ramon, CA). Tritiated thymidine was from ICN Biomedicals (Costa Mesa, CA). [32P]DCTP was from New England Nuclear (Boston, MA). Scintillation fluid was from Packard Instrument (Meriden, CT). Storm was from Molecular Dynamics, 4-hydroxyxenon-4 (HNE), and Cu/Zn superoxide dismutase antibodies were kindly provided by Dr. A. Bhattacharj (University of Louisville, Louisville, KY). All other chemicals were from Sigma.

Chemical treatments in vivo. Rats were injected intraperitoneally with BaP (10 mg/kg) in MCT oil or with MCT oil once a week for 8 or 16 wk. Animals were euthanized by lethal injection of 120 mg/kg pentobarbital sodium.

Kidney harvests. Kidneys were fixed by perfusion with sterile PBS followed by 4% paraformaldehyde (PFA) at a rate of 6 ml/min (57). One-half of the left and right kidneys was immersed in 4% PFA for 4 h and dehydrated as described (58).

Blood and urine chemistries. Blood was collected via cardiac puncture and 24-h urine in metabolic cages. BUN and plasma creatinine, urine total protein, and creatinine levels were monitored in all animals.

Morphometric analysis. Hematoxylin and eosin sections (5 μm) were examined using brightfield light microscopy. Computer-assisted analysis was performed on at least 30 glomeruli from each rat. Hematoxylin-positive cells were normalized to glomerular area. Images were captured using an Olympus Vanox research microscope equipped with a 3-CCD Camera (model Del-750) and analyzed with the NIH Image v1.60 public domain program developed at the National Institutes of Health and available at http://zippy.nimh.nih.gov/pub/nih-image.

Electron microscopy. The second half of the kidney was processed for electron microscopy as described by Lees et al. (33).

Immunohistochemistry. Five-micrometer paraffin sections were de-waxed in Citrus solv and rehydrated in a graded alcohol series. A listing of markers used to characterize cellular identity and nephropathic response is provided in Table 1. Thy-1, L1, fibronectin, and CD3 mouse monoclonal antibodies (5, 4, 4, and 20 μg/ml, respectively) were applied in 10% horse serum, while the WT-1 rabbit polyclonal antibody (4 μg/ml) was applied overnight at 4°C in a solution of 0.3% Triton X-100 and 10% goat serum. Slides processed for measurements of WT-1 antigen were treated under pressure with Vector Antigen Unmasking Solution, while L1 antigen detection required trypsin digestion (1 mg/ml for 10 min at 37°C). Specimens were treated with an avidin/biotin blocking kit. Thy-1, L1, fibronectin, and CD3 primary antibodies were bound with a horseradish peroxidase anti-mouse rat absorbed biotinylated secondary antibody (1.6 μg/ml), and the WT-1 primary antibody was bound with a goat anti-rabbit biotinylated secondary antibody (0.5 μg/ml). All secondary antibodies were amplified with ABCelite, developed with DAB or Vector Red, and counterstained with Mayer’s hematoxylin. For detection of Factor-8 antigen, 4-μm paraffin sections were dewaxed in Citrus solv, rehydrated in a graded alcohol series, and digested in pepsin 2.5 mg/ml. Factor VIII-RA rabbit polyclonal antibody (20 μg/ml) was applied overnight at 4°C in a solution of 0.3% Triton X-100 and 7% goat serum followed by incubation with a fluorescent, goat anti-rabbit Texas Red secondary antibody (2 μg/ml). Slides were counterstained in DAPI (0.3 μg/ml) and mounted using Prolong Antifade mounting media.

Mesangial cell numbers and fibronectin were quantified by measuring Thy-1 and fibronectin integrated intensity normalized to glomerular area. Podocyte numbers were quantified by counting WT-1/hematoxylin-positive cells and normalizing to glomerular area. Endothelial cell numbers were quantified by counting round, perinuclear factor 8 staining, and normalized to glomerular area. For measurements of WT-1-positive cells, five images of each renal quadrant were captured (total 20 images per animal) for at least seven animals per group per time point. WT-1-positive and -negative cell counts and glomerular area (6.49 pixels/μm) were acquired using ImageJ which is available at http://rsb.info.nih.gov/ij/download.html. For Thy-1, Factor-8, 4-HNE, and Cu/Zn superoxide dismutase measurements, five images were examined in two renal quadrants for a total of 10 images per animal from at least seven animals per group per time.
point. Factor 8-positive cells and DAPI-positive nuclei along with glomerular area were quantified using ImageJ. Thy-1 and fibronectin intensity was analyzed using KS300 (Zeiss). Nuclear counts and glomerular area were quantified (0.154 μm²/pixel). In all experiments, specimens processed without primary antibodies were used as negative controls.

For measurements of α-smooth muscle actin, tissue sections were washed in PBS three times (2 min each) and digested for 5 min with 0.1% trypsin. α-Smooth muscle actin monoclonal antibody clone 1A4 (1 μg/ml) was placed on tissue samples in a humidifier chamber at 37°C for 2 h. Sections were then incubated in a humidifier chamber for 20 min at room temperature with an alkaline phosphatase-conjugated streptavidin (5 μg/ml). The chromogen, fast red supplemented with levamisole (2 μM), was incubated for 10 min at room temperature. Slides were counterstained with hematoxylin and mounted with GVA mount. A horseradish peroxidase-conjugated streptavidin (5 μg/ml) was added for 30 min at room temperature. All slides were examined using brightfield microscopy (Olympus Vanox or Zeiss). Negative controls consisted of PBS or preimmune serum in lieu of primary antibody.

SDS-PAGE. Urine samples were processed as described by Marshall and Williams (37) for the isolation and separation of urinary proteins on a 10% SDS-PAGE gel. Albumin sum density was measured as the band corresponding to the bovine serum albumin standard and total protein was measured as the sum density of all bands on the gel.

Single-cell cloning. Single-cell clones (SCCs) were isolated from glomerular subcultures by limited dilution as described (1). SCC 1 and SCC 4E clones were identified as mesangial cells and podocytes, respectively, as described previously. SCC-1 cells exhibited a mes-
enchymal phenotype characterized by abundant expression of α-smooth muscle actin and absence of cadherin. Conversely, SCC-4E cells displayed an epithelial phenotype characterized by a polygonal, cobblestone morphology and high expression of cadherin. Reverse transcription-polymerase chain reaction. Total RNA (200 ng) was reverse transcribed with an oligo d(T) primer, and multiplex PCR was performed to amplify CYP1A1 and β-actin using a modified protocol (47). The thermoprofile used consisted of a denaturing temperature of 95°C for 1 min, an annealing temperature of 64°C for 1 min, and an extension temperature of 72°C for 1 min for 25 cycles. The PCR products were separated on a 1.2% 1× TAE agarose gel. Gels were stained with SYBR green [1:10,000 dilution in 1× TAE (pH 8.0)] for 30 min on a shaker at room temperature, and digital images were captured on the Storm.

Northern analysis. Total RNA (10 μg) was separated on a 1.2% 1× TAE agarose gel at 70 V. The membrane was washed in 6× SSC, UV cross-linked, and probed with CYP1B1 (1 × 10^6 cpm/ml), a generous gift from Dr. C. Jefcoate (University of Wisconsin,

Fig. 3. A: glomerular cellularity in rats challenged with BaP. Female SD rats were challenged with BaP (10 mg/kg ip) once a week for 8 or 16 wk. Hematoxylin and eosin-stained sections (5 μm) were evaluated using a brightfield light microscope (Olympus Vanox). Images were captured using NIH Image (v1.61) and are representative of 6 different animals. B: morphometric analysis of glomeruli from BaP-challenged rats. Hematoxylin and eosin-stained kidney sections (5 μm) were evaluated using a brightfield light microscope (Olympus Vanox). Images were captured using NIH Image (v1.61). Glomerular cellularity, glomerular tuft, Bowman’s capsule, and urinary space areas were quantified in 6 different animals. Statistical differences were evaluated using ANOVA and Scheffe’s post hoc test. *Significance at the P < 0.05 level.
overnight; 50 ng of CYP1B1 probe were synthesized using 5× Highprime (Roche Molecular Diagnostics) and 10 μg/ml [32P]dCTP for 1 h at 37°C. The membrane was then washed with a series of SSC and SDS solutions (6× SSC, 0.1× SDS for 20 min 2 times at 25°C; 6× SSC, 0.5× SDS for 20 min 2 times at 55°C). The final wash solution was 0.5× SSC, 0.5× SDS for 75 min at 55°C. The membrane was exposed to a phosphor screen for 24 h and scanned on the Storm.

**AHH and EROD assays.** The protocols used for these assays have been described previously (27). Media were aspirated and cells were scraped with extraction buffer (Tris-sucrose, pH 8.0), collected, and centrifuged at 4°C. The pellet was resuspended in Tris-sucrose buffer (pH 8.0).

**DNA synthesis.** The protocol for DNA synthesis was described previously (1). Briefly, cells were incubated with 1 μCi/ml of [3H]thymidine for 24 h and harvested with 5% trichloroacetic acid. Following washing, the acid-precipitable material was dissolved in NaOH (1 N), neutralized with HCl (1 N), and counted on a Tri-carb scintillation counter (Packard Instruments). Protein concentrations were measured by the method of Bradford (8).

**Coculture experiments.** Coculture experiments were conducted using SCC1 and SCC4E populations. The direct coculture system placed both cell types in the same culture well (microchamber slides (Lab-Tek)), with SCC4E seeded at 150 cells/mm² and SCC1 seeded at different cell densities (0, 75, 150, and 300 cells/mm²). Given their epithelial lineage, SCC4E were identified as E-cadherin-positive cells by immunofluorescence. The indirect coculture system seeded SCC4E on an insert (Transwell culture plates) at different cell densities. DNA synthetic rates were measured as described (1).

**Cross-plating experiments.** Subcultures of SCC1 or SCC4E were grown to confluence for 5 days. Cultures were trypsinized and immediately cross-plated onto SCC1 or SCC4E-deposited matrix at a density of 150 cells/mm² with [3H]thymidine for 24 h. DNA synthetic rates were measured as described (1).

**Statistics.** For in vivo experiments, a two-way paired Student's t-test was used to assess the statistical significance of clinical chemistry and morphometric differences between control and treatment groups (P < 0.05). A Wilcoxon rank sum test was used to determine the statistical significance in immunohistochemical experiments. Values always represent means ± SE. For in vitro evaluation, ANOVA in conjunction with Fisher’s Protected LSD or Scheffé’s post hoc tests were used to assess differences between control and treated groups (P < 0.05). Experiments which contained only two treatment groups were analyzed with a two-way paired Student’s t-test. Experiments were performed in duplicate or triplicate as indicated in the legend to respective figures.

**RESULTS**

Kidney function and glomerular morphology. Compromised renal function was observed in female rats challenged with 10 mg/kg BaP for up to 16 wk. While no changes were detected within the first 8 wk of treatment, significant increases in total urinary protein were observed by 16 wk (Fig. 1) in the absence of a detectable increase in glomerular filtration rate.
Fig. 5. Immunohistochemical expression of Thy-1, fibronectin, and α-smooth muscle actin in glomeruli from BaP-treated rats. Sprague-Dawley rats were challenged with BaP (10 mg/kg ip) once a week for 8 or 16 wk. A: Thy-1. B: morphometric analysis of Thy-1 immunoreactivity. C: morphometric analysis of fibronectin immunoreactivity. Images shown are representative of 6 different animals (∗×200). *Significant at the $P < 0.05$ level.
of changes in blood urea nitrogen (BUN) or creatinine levels (not shown). Average BUN and creatinine values in control animals were 24 and 0.3 mg/dl, respectively. Urinary albumin levels increased above control levels in two of five rats by 8 wk (Fig. 2A), with loss of selective permeability becoming progressive in five of seven rats by 16 wk (Fig. 2, B and C). Urinary excretion of other high-molecular-weight proteins was increased, but excretion of low-molecular-weight proteins remained unchanged. Computer-assisted morphometric analysis revealed significant increases in glomerular cellularity after eight and 16 wk of hydrocarbon treatment (Fig. 3, A and B). Other endpoints of glomerular morphology (glomerular tuft, Bowman’s capsule, and urinary space) remained unchanged. Alterations of podocyte structure and regions of podocyte foot fusion were observed by 8 wk of hydrocarbon challenge (Fig. 4A) and sustained over the course of treatment (Fig. 4B). Changes in podocyte morphology correlated with thickening of the basement membrane (not shown). No alterations in renal parenchyma were noted at any time, indicating that tubular structure was not affected by hydrocarbon treatment. Loss of renal function in hydrocarbon-treated animals was not seen in female rats treated with 1 mg/kg BaP for up to 16 wk (not shown).

**Immunohistochemical analyses.** To identify specific cell type(s) contributing to changes in glomerular cellularity, the expression of Thy-1, a GPI-anchored membrane glycoprotein of the Ig superfamily in mesangial cells, was examined in glomeruli of control and BaP-treated rats (22). Changes in Thy-1 immunoreactivity were observed after both 8 and 16 wk of treatment (Fig. 5A), with modest reductions by 8 wk and rebound increases by 16 wk of treatment (Fig. 5B). The pattern of Thy-1 closely matched that of fibronectin immunoreactivity in the kidneys of hydrocarbon-challenged rats (Fig. 5C). α-Smooth muscle actin expression, a marker of mesangial activation, was unchanged at 8 wk and increased by 16 wk in treated rats relative to controls (Table 2). Consistent with previously published work (36, 46, 52), immunohistochemical analysis localized α-smooth muscle actin-immunoreactive signal within injured glomeruli.

Next, WT-1 immunoreactivity was evaluated to quantify podocye cell numbers. WT-1 is a nuclear transcription factor associated with urogenital development and expressed exclusively in podocyte nuclei within the adult glomerulus (30). Transient increases in WT-1 immunoreactivity were observed after 8 wk of hydrocarbon treatment (Fig. 6, A and B), indicating that podocyte proliferation contributes to early changes in glomerular cellularity. No changes in factor 8 antigen, L1/macrophage/calprotectin Ab-1, or CD3 staining were observed at any time in kidneys of hydrocarbon-treated animals (data not shown). Factor 8-related antigen (von Willebrand Factor) is an endothelial-specific plasma glycoprotein that assembles into disulfide-linked multimers (12), L1/macrophage/calprotectin Ab-1 is a cytoplasmic protein commonly used as a macrophage marker (13), and CD3 is membrane protein marker of T cells.

BaP exposure did not modulate the lipid peroxidation marker, 4-HNE (not shown), but altered expression of Cu/Zn superoxide dismutase (Fig. 7, A and B). Decreased enzyme expression was observed at 8 wk followed by marked induction at 16 wk, suggesting that oxidative injury contributes to mesangial cell injury. This change occurred in the absence of local peritubular or systemic inflammation in BaP-treated animals.

**CYP gene expression.** The profile of cellular changes seen during the course of BaP nephropathy in vivo is comparable to changes seen in vitro where BaP preferentially injures mesangial cells in culture and allows podocyte expansion (1). To examine mechanistic relationships contributing to the loss of renal function in vivo, the expression of CYPs involved in the bioactivation of BaP to oxidative intermediates was evaluated in clones of mesangial cells (SCC 1) and podocytes (SCC 4E). These clones preserve the differentiated features characteristic of cell populations within the intact glomerulus and allow for rigorous analysis of the respective contributions of individual glomerular cell populations to the nephropathic response. CYP1A1 mRNA was detected under constitutive conditions in mesangial cells, but not podocytes (Fig. 8, A and B). Challenge with 3 μM BaP for 24 h was without effect in mesangial cells and markedly induced CYP1A1 in podocytes. CYP1B1 expression was undetectable under constitutive conditions but highly inducible by hydrocarbon challenge in mesangial cells and podocytes (Fig. 8C). Ethoxyresorufin-O-deethylase was expressed at low levels in both cell types, and refractory to hydrocarbon challenge (not shown), while AHH was not detectable under constitutive conditions but inducible by BaP in both cell types (Fig. 9). Induction was most significant in mesangial cells compared with podocytes, indicating that expression of CYP-associated enzymatic activity closely correlates with patterns of hydrocarbon sensitivity.

**Cell-cell and cell-matrix interactions.** Because cell-cell interactions are critical for the regulation of glomerular cell functions, preferential injury to mesangial cells may disrupt critical cellular interactions that afford podocytes a proliferative advantage. To test this hypothesis, proliferation of glomerular mesangial cells and podocytes was evaluated under in vitro coculture conditions. In the first set of experiments, mesangial cells were seeded in the presence of serum at increasing densities (75, 150, and 300 cells/mm²) in direct contact with podocytes (150 cells/mm²) to yield ratios ranging from 1:0.5 to 1:2. Mesangial cells significantly inhibited epithelial cell proliferation at all ratios, as evidenced by significant reductions in E/P-cadherin positive cells (Fig. 10A). E/P-cadherin is a marker of epithelial identity within the rodent glomerulus (41). In the second set of experiments, mesangial

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<th>α-Smooth Muscle Actin expression in glomeruli from BaP-challenged rats</th>
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<td>8 wk</td>
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Rats were given 10 mg/kg benzo(a)pyrene (BaP) intraperitoneally once a week for 8 or 16 wk. Immunoreactivity scores were 0–4, depending on the intensity and number of positive cells within the glomerulus. Immunoreactivity scores 0 = no signal, 1 = weak signal, 2 = moderate signal, 3 = strong signal in <50% of glomeruli, 4 = strong signal in >50% of glomeruli. All analyses were conducted blindly to eliminate bias. A Wilcoxon rank sum test, a nonparametric one-way analysis method, was used to determine statistical significance. *Significantly different from respective control (n = 6 rats).
cells were seeded at varying densities on permeable inserts placed inside culture wells containing 150 cells/mm² visceral epithelial cells to determine whether cell-cell contact was required for trophic interactions. Mesangial cells inhibited DNA synthesis in cultured epithelial cells, as evidenced by marked reductions in [3H]thymidine incorporation into TCA-insoluble material at all ratios examined (Fig. 10B). DNA synthesis of mesangial cells was unaffected, suggesting that trophic factor(s) derived from mesangial cells negatively regulate epithelial cell proliferation, and that this effect is not reciprocal.

Given that trophic factors secreted by mesangial cells may be associated with the extracellular matrix, or themselves represent matrix components, the last set of experiments was conducted to evaluate the influence of the seeding extracellular matrix on mesangial and podocyte proliferation. In these experiments, cell populations were cross-seeded on a matrix deposited by the opposite cell type and processed for measurements of cellular proliferation. Figure 11 shows that podocyte proliferation was markedly inhibited when cells were seeded on a matrix deposited by mesangial SCC 1 cells, while mesangial cell growth was unaffected by a podocyte-derived matrix. These results indicate that the negative influence of mesangial cells on podocytes involves deposition of a growth-inhibitory matrix that regulates epithelial cell growth.

Fig. 6. Immunolocalization of WT-1 in glomeruli from BaP-treated rats. Female Sprague-Dawley rats were challenged with BaP (10 mg/kg ip) once a week for 8 or 16 wk. Images shown are representative of 6 different animals. Control (×240); BaP (×310). A: WT-1 immunoreactivity signals. B: morphometric analysis of WT-1 immunoreactivity. *Significant at the \( P < 0.05 \) level.
DISCUSSION

Chronic treatment of female rats with 10 mg/kg BaP, a dose considerably lower than that required to elicit a carcinogenic response in rodents (10), was associated with loss of selective permeability, glomerular injury, and albuminuria by 16 wk of exposure. Albuminuria is a hallmark of the nephrotic syndrome and often linked to poor prognosis in the management of glomerular diseases, including diabetic nephropathy, focal and segmental glomerulosclerosis, and mesangiocapillary and membranous glomerulonephritis (11, 25, 38, 56). Mizuno and co-workers (41) reported that albuminuria in nephrotic mice does not involve abnormal BUN and creatinine levels as long as the tubular architecture is intact. This parallels the profiles seen in BaP-treated rats where renal parenchymal structure remained unchanged and proteinuria did not involve significant leakage of low-molecular-weight proteins.

Hydrocarbon nephropathy was characterized by significant changes in glomerular cellularity. A central role for mesangial cells in the nephropathic response was evidenced by drastic reductions in cell numbers at 8 wk, followed by rebound increases at 16 wk and increases in \( \alpha \)-smooth muscle actin expression. These changes are similar to those seen in minimal change glomerular disease (3) and may involve phenotypic modulation of mesangial cell phenotypes and increased deposition of extracellular matrix components (26). Changes in mesangial and podocyte cell densities preceded the occurrence of significant increases in Cu/Zn superoxide dismutase expression.

Fig. 7. Immunolocalization of Cu/Zn superoxide dismutase in glomeruli from BaP-treated rats. Female Sprague-Dawley rats were challenged with BaP (10 mg/kg ip) once a week for 8 or 16 wk. Images shown are representative of 6 different animals (×200). A: Immunocytochemical signals for Cu/Zn superoxide dismutase expression. B: morphometric analysis of Cu/Zn immunoreactivity. Statistical differences were evaluated using ANOVA and Fisher’s protected LSD. *Significance at the \( P < 0.05 \) level.
Fig. 8. CYP1A1 and CYP1B1 gene expression in SCC 1 and SCC 4E clones challenged with BaP. SCCs were seeded on culture dishes for 24 h at a density of 150 cells/mm² and treated with DMSO (vehicle control) or BaP (3 μM) for an additional 24 h. A: RT-PCR of CYP1A1 in SCCs clones challenged with BaP. β-Actin gene expression was used as a loading control. Control for RT-PCR included a minus template (−temp), minus RT (−RT), cells expressing CYP1A1 (CYP1A1), and cells expressing β-actin (β-actin). B: densitometric quantification of hybridization signals. C: Northern analysis of CYP1B1 in SCCs challenged with BaP. α-Tubulin gene expression was used as a control for loading and transfer. Total RNA from mesangial cells treated with anthracene (30 μM) for 24 h was used as a positive control for 1B1. Statistical differences were evaluated using ANOVA and Fisher’s protected LSD. *Significance at the P < 0.05 level.
Fig. 9. AHH activities in SCC 1 and SCC 4E clones under constitutive conditions or following challenge with BaP. SCCs were seeded at 150 cells/mm² for 24 h and then challenged with BaP (3 μM) for an additional 24 h. AHH assays were conducted as described in METHODS. *Statistically significant differences between treatment and controls (P < 0.05). **Statistically significant differences between SCC 1 and SCC 4E BaP-challenged cultures (P < 0.05). No changes in ethoxyresorufin-O-deethylase activity were observed at any time.

Fig. 10. Proliferation profiles of SCC 4E using direct and indirect coculture systems. Using lab-tek chamber well slides for direct cocultures (A), SCC 1 were seeded at different densities, whereas SCC 4E were seeded at 150 cell/mm². E/P-cadherin-positive cells were counted as described in METHODS. Using a transwell coculture system for indirect cocultures (B), SCC 1 were placed on the inserts at different densities. SCC 4E were seeded at 150 cell/mm² on the bottom wells. Cultures were maintained in 10% fetal bovine serum at all times. DNA synthesis was conducted as described in METHODS. Experiments were conducted using 3 replicate wells/group. Statistical differences were evaluated using ANOVA and Scheffe’s post hoc test. *Significantly different from respective control (P < 0.05). The x-axis represents the seeding matrix deposited by individual cells before cross-plating was completed.

Fig. 11. Proliferation profiles of cross-plated SCC 1 and SCC 4E. SCC1 and SCC4E were grown to confluence for 5 days to allow for matrix deposition. Cultures were trypsinized and immediately cross-plated onto the culture matrix laid by the opposite cell type at a density of 150 cells/mm² for 24 h. Results represent means ± SE of tritiated thymidine incorporation in 3 separate cultures. *Significance at P < 0.05. The x-axis represents the seeding matrix deposited by individual cells before cross-plating was completed.
variety of glomerular nephropathies including IgA nephropathy, diabetic nephropathy, and idiopathic focal glomerulosclerosis. Of importance is that increased formation of reactive oxygen species on exposure of mesangial cells to high glucose results in antioxidant-sensitive increases in fibronectin expression (19). Thus parallel changes in mesangial cell numbers and fibronectin expression in BaP-treated rats may involve oxidative mechanisms. Increased production of oxygen free radicals may contribute to loss of mesangial cell function as Cu/Zn superoxide dismutase protein expression was significantly altered at both 8 and 16 wk.

Early changes in podocyte density at 8 wk may involve loss of paracrine control secondary to mesangial cell injury. This interpretation is in keeping with the results of in vitro studies showing that coculture of SCC4E with SCC1 cell clones decreases epithelial cell numbers and protein expression. Podocyte proliferation has been reported in focal segmental glomerulosclerosis (17) as well as in experimental models of membranous nephropathy (51). The fusion of podocyte foot processes may be secondary to disruption of cell-cell and cell-matrix interactions and directly contribute to deficits in permeability (40, 55). Because foot process effacement may be reversible, it is unknown if BaP nephropathy subsides with time or continues to progress. The loss of podocyte functional integrity in hydrocarbon-treated rats may also involve dedifferentiation of podocytes secondary to changes in matrix composition, an interpretation consistent with the observation that reduced WT-1 immunoreactivity occurs in the absence of TUNEL staining (data not shown). The specificity of the injury response for glomerular mesangial and epithelial cells was evidenced by the finding that BaP exposure in vivo did not change endothelial cell numbers or trigger recruitment of inflammatory cells to sites of glomerular injury. In vitro challenge of SCC1 or SCC4E cells with BaP inhibits SCC1 growth and affords SCC4E cells a selective growth advantage (2), a pattern that is consistent with the changes seen at 8 wk of treatment.

Glomerular mesangial cells are selectively injured by aromatic hydrocarbons (7), suggesting that preferential injury of mesangial cells may involve differential expression of constitutive and hydrocarbon-inducible CYPs. As noted, cytochrome P-450s participate in the biotransformation of polycyclic aromatic hydrocarbons to reactive intermediates that bind DNA, induce oxidative stress, and damage cellular macromolecules. BaP-induced renal injury may disrupt the expression and function of mesangial cell-derived factor(s) that regulate growth via autocrine and paracrine mechanisms. In this manner, mesangial injury may interfere with deposition of extracellular matrix components and disrupt mesenchymo-epithelial interactions that afford a proliferative advantage to visceral epithelial cells. Coculture experiments suggested that the proliferative advantage afforded to podocytes involves loss of negative regulatory factors produced by mesangial cells. The inhibitory influence of mesangial cells on epithelial cell growth has been observed in other organs such as trachea (14) or prostate (21), where smooth muscle cells support the expression of quiescent, differentiated epithelial cell phenotypes. Dysfunctional mesenchymal-epithelial interactions have also been described in the mammary gland (15). Interestingly, p20 has been identified as a mesenchymal-derived paracrine inhibitor of epithelial cell proliferation (50). While the identity of paracrine regulators of mesangial cell function is not known, IP-10 and its receptor CXCR3 are chiefly expressed on podocytes, where inhibition of IP-10 function disturbs podocyte function and increases the severity of Thy-1.1 glomerulonephritis (20).

The cellular and molecular mechanisms of hydrocarbon nephropathy are complex and likely involve disruption of cell:cell and cell:matrix interactions that compromise glomerular function. Oxidative stress may mediate the activation of signaling cascades involved in regulation of mesangial cell expansion (19, 32) and basement membrane deposition giving rise to a microenvironment that fails to support podocyte differentiation. In our study, loss of renal function was seen after early changes in mesangial and podocyte cell densities had been corrected and possibly overcompensated. This suggests that mesangial cell hyperplasia and excess fibronectin after 16 wk of exposure may change glomerular basement density and composition in a manner that compromises podocyte function. It is important to note that mesangial cell proliferation alone can be associated with glomerular disease (31).

Because many of the etiological agents and mechanisms of glomerular injury are not yet known, it is possible that disruption of mesangial/epithelial interactions by aromatic hydrocarbons contributes to the development of glomerular disease in humans. Hydrocarbon-induced deficits of renal function are similar to those observed in minimal change glomerulonephritis and early focal segmental glomerulosclerosis in humans (18), suggesting that common etiological links may exist in the onset of renal disease.

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