Kidney International, Vol. 57 (2000), pp. 1571–1580

Phenotypic profiles of cultured glomerular cells following repeated cycles of hydrocarbon injury

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Phenotypic profiles of cultured glomerular cells following repeated cycles of hydrocarbon injury.

Background. The glomerulus has been implicated as a target of hydrocarbon injury in vitro and in vivo. In the present studies, the phenotypic profiles of cultured rat glomerular cells (GCs) following repeated cycles of hydrocarbon injury were evaluated. Cultured GCs were incubated for 24 hours with benzo[a]pyrene (BaP; 3 μmol/L), a prototypical polycyclic aromatic hydrocarbon, and were allowed to recover overnight before two additional cycles of chemical challenge during serial propagation in vitro. At the end of this regimen, control cultures were characterized by predominance of fusiform cells that grew in "hills and valleys," while GCs subjected to hydrocarbon injury displayed an epithelial morphology characterized by a rounded, polygonal shape clearly distinct from that normally exhibited by glomerular mesangial cells (GMCs) in culture.

Methods. Indirect immunofluorescent detection of cell markers was conducted to identify cells of mesenchymal or epithelial origin. Measurements of DNA synthesis and cell number were performed to determine proliferative capacities of the different cell types in response to hydrocarbon challenge.

Results. Immunofluorescence studies revealed that control GC cultures contained mostly α-smooth muscle (SM) actin-positive cells, with a few (5.1% ± 2.6) E-cadherin–positive cells occasionally identified. In contrast, BaP-treated cultures exhibited a mixed cell population in which E-cadherin–positive cells were predominant (66.6% ± 4.1). Single-cell cloning of naive cultures of GCs yielded four clones, three of which exhibited a fusiform morphology and were α-SM actin positive (SCC 1 through SCC 3) and one (SCC 4E) that exhibited epithelial characteristics similar to those found in hydrocarbon-treated cultures. Immunofluorescence studies showed that epithelial cells in hydrocarbon-treated cultures, as well as SCC 4E-derived clones, were vimentin positive and cytokeratin negative, characteristics similar to glomerular visceral epithelial cells (GVECs). DNA synthesis and cell proliferation in clone SCC 1 were decreased following acute BaP challenge, while growth rates in SCC 4E-derived clones were unaffected by hydrocarbon injury. Repeated cycles of hydrocarbon challenge in clonal populations yielded different profiles of DNA synthesis, with significant decreases in SCC 1 and no changes in SCC 4E.

Conclusions. These observations suggest that hydrocarbon injury induces differential responses in cells of the glomerulus, resulting in inhibition of GMCs and selective growth advantage of GVECs. These alterations are reminiscent of critical events described in the pathogenesis of focal segmental glomerulosclerosis and raise important questions about the pathogenesis of hydrocarbon-induced nephropathies.

The glomerulus is a complex structure that requires cooperation of several cell types, namely endothelial cells, glomerular mesangial cells (GMCs), glomerular visceral epithelial cells (GVECs), and glomerular parietal epithelial cells (GPECs), for the preservation of its structural and functional integrity. Chemically induced injury to one or more of these cells may contribute to the pathogenesis of glomerular disease. Glomerular injury in human and laboratory animals has been observed following exposure to several drugs and environmental contaminants. Toxic glomerulopathies may involve changes in the intact nephron, hyperfiltration, and/or complex deposition [1]. For example, puromycin, an aminonucleoside antibiotic, directly injures GVECs, while N,N′ diacetylbenzidine and hexadimethrine induce dysfunctional filtration [2]. Inorganic mercury and aromatic hydrocarbons also induce glomerular injury [3, 4], but the mechanisms of toxicity remain undefined. These findings emphasize the significance of epidemiologic reports linking toxic environmental exposures and glomerulonephritis [4–6].

Benzo[a]pyrene (BaP), a prototypical polycyclic aromatic hydrocarbon (PAH), is formed during the incomplete combustion of organic materials [7]. Because of its prevalence in the environment and high chemical reactivity following activation by cytochrome P-450s (CYP450s), the role of BaP in the onset and progression of carcinogenesis has been extensively examined. In the kidney, the treatment of mice with BaP induces a spectrum of

Key words: glomerular mesangial cells, visceral epithelial cells, endothelial cells, hydrocarbon induced nephropathies, focal segmental glomerulosclerosis, nephrotoxicity.

Received for publication May 20, 1999
and in revised form September 17, 1999
Accepted for publication November 2, 1999

DNA adducts comparable to those seen in liver [8], with BaP 7,8-diol-9,10-epoxide (BPDE)-DNA adducts present for lengthy periods [9]. Studies in our laboratory have confirmed that formation of BaP-DNA adducts in glomerular cells (GCs) requires CYP450 metabolism of the parent compound [10]. These findings are in agreement with epidemiological studies suggesting a correlation between occupational exposure to PAHs and the occurrence of chronic nephropathies [6] and renal cancers [11].

The present studies demonstrate the effects of BaP on the phenotypic profiles of GCs following repeated cycles of chemical injury in vitro. Evidence is presented that inhibition of GMC growth by BaP and selective growth advantage to GVECs leads to significant changes in the phenotypic spectrum of hydrocarbon-treated GC cultures.

METHODS

Animals

Female Sprague-Dawley rats (175 to 200 g) were purchased from Harlan (Houston, TX, USA). Female rats were used in these studies because they have been shown to be more susceptible to the toxicological/carcinogenic effects of PAHs than their male counterparts [12–14].

Chemicals

Benzo[a]pyrene (>98% purity) was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA, USA). Insulin, antibiotic/antimycotic solution, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), trypsin/ethylenediaminetetraacetic acid (EDTA), and transferrin were obtained from the Sigma-Aldrich Corporation. RPMI 1640 was purchased from GIBCO/BRL (Grand Island, NY, USA). Collagenase was obtained from Boehringer Mannheim (Indianapolis, IN, USA). α-Smooth muscle (α-SM) actin (clone: 1A4) and cytokeratin (clone: K8.13) antibodies were purchased from the Sigma-Aldrich Corporation. E-cadherin (clone: 36) antibody was purchased from Transduction Laboratories (Lexington, KY, USA). Vimentin (clone: V9) antibody was purchased from Zymed Laboratories (San Francisco, CA, USA). The FITC-labeled antimouse IgG and normal goat serum were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Prolong antifade was purchased from Molecular Probes (Eugene, OR, USA). Tritiated thymidine was purchased from ICN Biomedicals (Costa Mesa, CA, USA). Scintillation fluid was purchased from Packard Instrument Company (Meriden, CT, USA). All other chemicals were purchased from Sigma-Aldrich Corporation.

Culture of glomerular cells

Glomerular cells were isolated and cultured as described previously [15]. Subcultured GCs were obtained by trypsinization of confluent primary cultures and were serially propagated. Cells were maintained in RPMI 1640 culture medium supplemented with 10% FBS, 1 mg/mL BSA, insulin (0.66 U/mL), transferrin (10 μg/mL), L-methionine (30 μg/mL), penicillin (100 U/mL), streptomycin (100 mg/mL), and amphotericin B (25 mg/mL). Single-cell clones were isolated from female Sprague-Dawley rats derived-GC subcultures by the limited-dilution technique [16]. Final cell suspensions were of 0.5, 1, and 5 cells per well plated on 96-well culture dishes and were grown in RPMI media supplemented with 20% FBS. After two weeks in culture, clones were replated at a ratio of 1:2. Following three passages, cells were then maintained in RPMI media supplemented with 10% FBS.

Chemical treatments

Randomly cycling GCs (between passages 6 through 9) and single cell clones (between passages 11 through 15) were treated with DMSO (0.1%) or BaP (3 μmol/L) for 24 hours, rinsed with phosphate-buffered saline (PBS), and then allowed to recover for 24 hours in fresh media containing 10% serum. This treatment was repeated twice for a total of three BaP exposures. Estimates of BaP exposure in nonsmoking adults range from 0.171 to 1.64 μg/day. The BaP concentration used in our experiments was 3.0 μmol/L (2.3 μg/dish), a concentration that approximates the range of the estimated human daily burden [17]. The dosing regimen used was designed to replicate an initiation/promotion protocol for the study of nephrocarcinogenic effects of the hydrocarbon.

DNA synthesis

Cultures were incubated with 0.5 to 1 μCi [^{3}H]-thymidine for 24 hours and harvested into 5% trichloroacetic acid (TCA). Following a standard washing protocol, the acid-precipitable material was dissolved in 1 N NaOH, neutralized with HCl, and counted as previously described [18]. Protein concentrations were determined by the method of Bradford [19].

Immunofluorescence studies

Subconfluent monolayers of GCs growing on Lab-Tek chamber slides were rinsed in 20 mmol/L PBS (pH 7.2), fixed with 100% methanol at −20°C for 10 minutes, and air dried. Slides were subsequently washed three times with 0.3% Tween/PBS and incubated with blocking solution (1:50 goat serum in 1.0% BSA/Tween/PBS) for 10 minutes. Cells were incubated with the desired primary antibody, α-SM actin (1:100 dilution in 1.0% BSA/Tween/PBS), cytokeratin (1:100), E-cadherin (1:50), or vimentin (1:50) for one hour at 25°C, followed by three five-minute washes in Tween/PBS. Cells were then incubated with a FITC-conjugated secondary antibody (goat antimouse IgG, 1:200 dilution) for one hour in the dark. Slides were washed once with PBS and mounted with

prolong antifade. Slides were stored at 4°C until analysis using an Olympus Vanox AHBs3 and NIH Image Analysis Software (version 1.61). α-SM actin and E-cadherin–positive cells were quantitated based on fluorescence intensity and staining patterns using NIH Image [20].

Statistics

Analysis of variance (ANOVA) in conjunction with Fisher's Protected LSD and Scheffe's post hoc tests were used to assess the statistical significance of differences between control and treated cultures ($P < 0.05$). Values always represent the mean ± SEM. Experiments were performed in duplicate or triplicate, as indicated.

RESULTS

Repeated cycles of hydrocarbon injury are associated with the appearance of proliferative phenotypes in cultured GCs [18]. To define phenotypic profiles in these cultures, experiments were designed to examine markers of cellular identity following three consecutive cycles of BaP injury. Control cultures were fusiform shaped and exhibited mesenchymal cell characteristics (Fig. 1A). In contrast, BaP-treated cultures displayed an epithelial-like morphology (Fig. 1B). Serial cultures of cells challenged only once with BaP yielded comparable phenotypes, showing that a single round of chemical injury is sufficient to alter phenotypic profiles [18].

To determine the identity of cell populations and the basis for epithelialization of BaP-treated cells, various immunofluorescence markers were examined. The majority of cells (95.4% ± 2.7) in control GC cultures were of mesenchymal character and exhibited positive staining for α-SM actin (Fig. 2A). A minor (5.1% ± 2.6) population of E-cadherin–positive cells was identified in control cultures (Fig. 3A). In contrast, a mixed population containing α-SM actin-positive cells (22.2% ± 3.8; Fig. 2B), but dominated by E-cadherin–positive cells (66.6% ± 4.1) was identified in hydrocarbon-treated cultures (Fig. 3B). To characterize the origin of these cells, clonal populations of GCs were isolated by limited serial dilution. Four single cell clones were generated, three of which exhibited mesenchymal characteristics (SCC 1 through SCC 3) and one (SCC 4E) that displayed epithelial features similar to those seen in BaP-treated cultures. α-SM actin and E-cadherin immunofluorescence of SCC 1 and SCC 4E before hydrocarbon challenge are shown in Figure 4. Cells in SCC 1 cultures were α-SM actin positive and E-cadherin negative, while cells in SCC 4E were α-SM actin negative and E-cadherin positive. Changes in immunofluorescence staining for either protein were not observed after chemical challenge in SCC 1 or SCC 4E, and E-cadherin expression was not detectable at any time in mesenchymal SCCs before or after BaP injury.

To identify cell types involved in the glomerulotoxic response to BaP in vitro, cells were analyzed for expression of additional cell-specific immunofluorescence markers. Control (DMSO-treated) and BaP-challenged GCs showed a diffuse and punctate staining pattern for cytokeratin, a molecular marker for GPEC (Fig. 5 A, B). A similar pattern was seen in SCC 4E cells (Fig. 5C). This pattern was different from that of cytokeratin-positive LLC-PK1 cells (Fig. 5D) in which a characteristic filamentous staining pattern was observed. In contrast, a filamentous expression pattern was observed for vimentin, a molecular marker for GVECs, in the epithelial population of BaP-treated GCs (Fig. 6B) as well as SCC 4E cells (Fig. 6C). The characteristics of BaP-treated GCs and SCC 4E relative to those ascribed to GVECs and GPECs suggest that the epithelial population present in hydrocarbon-treated cultures is of GVEC origin (Table 1).

Experiments were then conducted to determine whether BaP treatment of GC cultures affords a selective growth advantage to GVECs relative to GMCs. The rate of DNA synthesis was determined by [^{3}H]-thymidine incorporation into TCA-precipitable material. BaP treatment inhibited DNA synthesis in mesenchymal clones (SCC 1; Fig. 7A), but had no effect on DNA synthesis in epithelial cells (SCC 4E; Fig. 7B). Cell numbers were reduced in BaP-treated SCC 1 cultures (Fig. 8A), but unaffected in SCC 4E cell populations (Fig. 8B). These results indicate that selective growth inhibition of GMCs allows epithelial cells to proliferate in culture after hydrocarbon injury.

DISCUSSION

Thorough characterization of cellular targets of renal toxicity is paramount in evaluating the link between aromatic hydrocarbon (AH) exposure and renal nephropathy [6], or carcinogenesis [11]. The kidney has often been considered resistant to AH injury, a notion based on the lack of cytotoxicity in AH-treated corticotubular epithelial cells. This laboratory has confirmed the relative insensitivity of tubular cells to cytotoxic insult by AHs [15, 21] and identified GCs as critical targets for this class of chemicals.

Despite improvements in preparation of primary cultures, a major set back often encountered is the heterogeneity of cell populations established in culture. Primary glomerular cultures isolated by enzymatic methods yield predominantly mesangial cells (>95%) [15, 22], along with a minor GVEC population. As such, these cultures have been advocated as useful in vitro models for the study of GMC biology. The evidence presented here, however, indicates that caution must be exercised when using serial cultures of GCs, since differential patterns of cellular sensitivity to environmental stressors may be

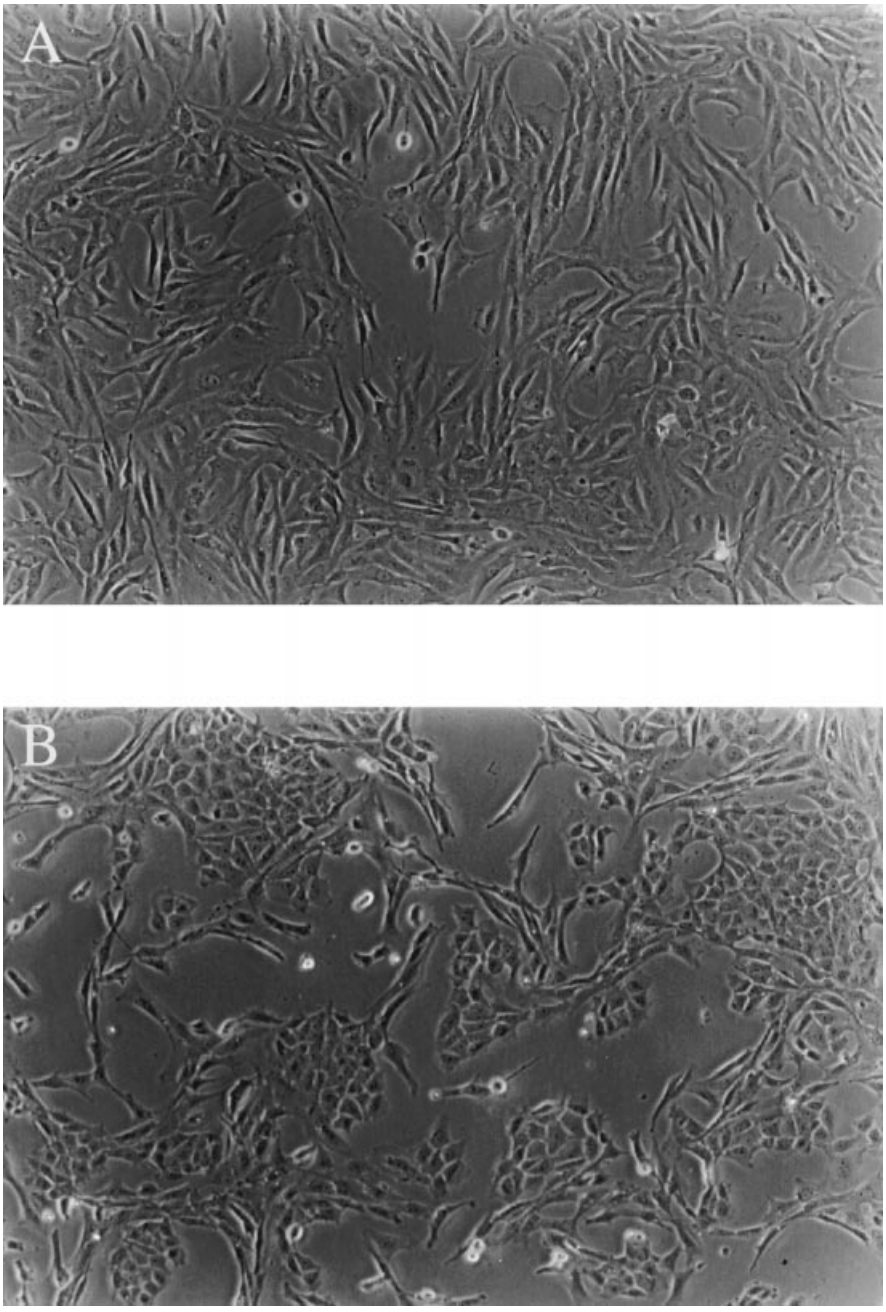

Fig. 1. Cell morphology of glomerular cultures following three cycles of repeated benzo-[a]pyrene (BaP) injury. Exponentially growing cultures were examined by light microscopy (×25). Control cultures exhibited hill and valley patterns of growth and fusiform cells characteristic of mesangial cells. In contrast, a mixed population of cells that mostly displayed an epithelial morphology was found in BaP-treated cultures. (*A*) Control. (*B*) BaP-treated cultures.

encountered during experimental manipulations. In support of this conclusion, we have shown that BaP treatment provides a selective growth advantage to a minor population of epithelial cells present in GC cultures. This cell type-specific difference in proliferation leads to distinct patterns of phenotypic expression in vitro, with GVECs becoming the predominant cell type following repeated cycles of chemical injury.

To identify cell populations involved in the glomerulotoxic response to BaP, SCCs were prepared to study pure cell populations. Our results indicate that mesenchymal and epithelial cells exhibit differential sensitivity to BaP injury. Immunofluorescence studies showed that SCC 4E and BaP-treated epithelial cells expressed vimentin, but not cytokeratin. This pattern is consistent with that described for GVECs in culture [23]. To date, little research has been done to characterize GVECs because of inherent difficulties in establishing long-term viable cultures [24]. The SCC 4E clone described here may overcome some of these limitations. However, more extensive characterization of this clone is warranted, since glomerular epithelial cells have been most exten-

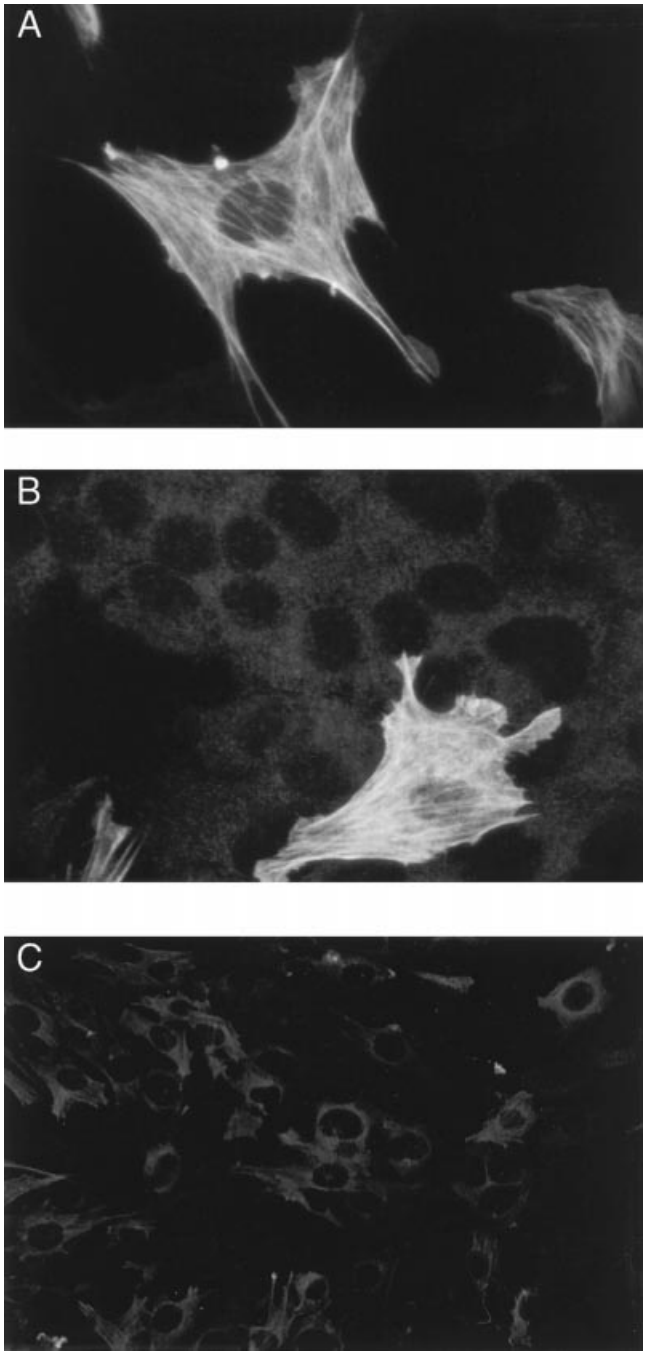

Fig. 2. Expression of α-smooth muscle (α-SM) actin in glomerular cell cultures following BaP injury. Subconfluent cultures of glomerular cells were analyzed for α-SM actin expression by immunofluorescence, as described in the **Methods** section. Diffuse staining patterns were seen in the epithelial population of BaP-treated cells, as compared with the characteristic filamentous staining pattern seen in α-SM actin positive cells (×1030). (*A*) Control. (*B*) BaP. (*C*) Negative control (aortic smooth muscle cells incubated with mouse IgG; ×500). A lower magnification was used for panel C to illustrate the lack of α-SM actin immunoreactivity in multiple cells.

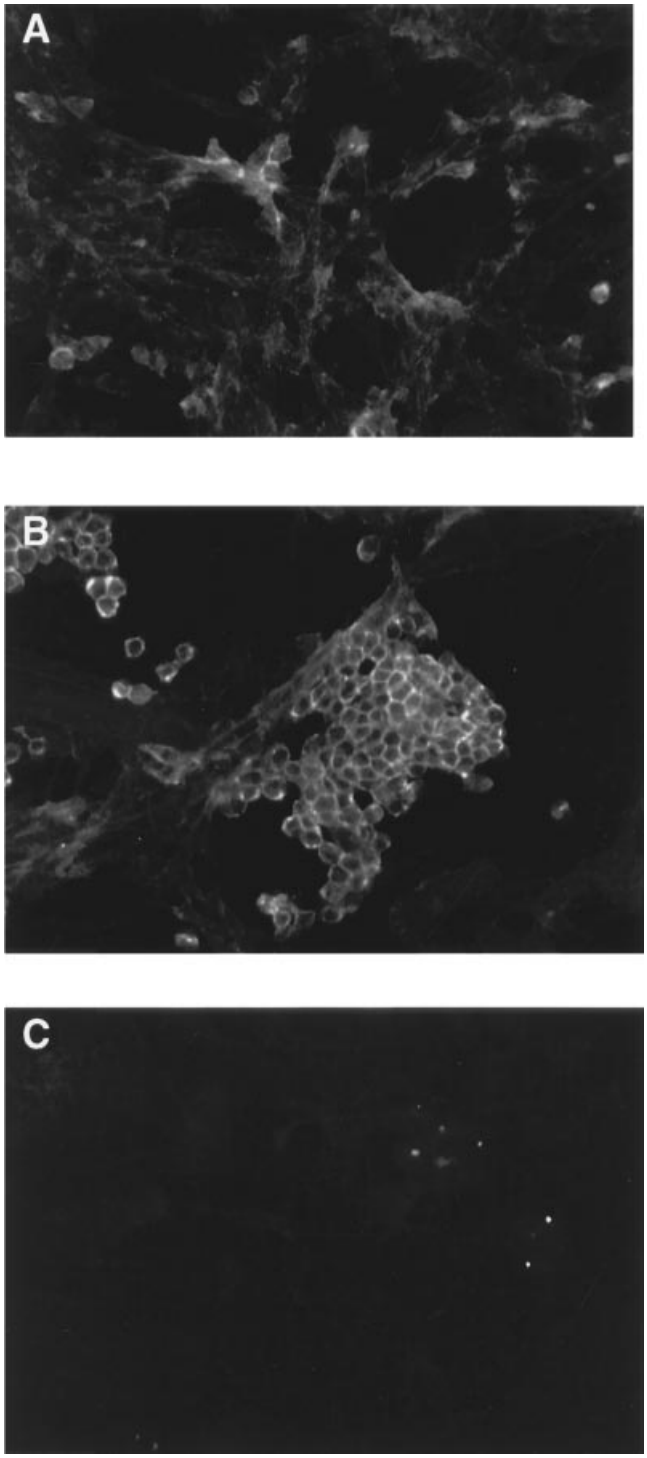

Fig. 3. Expression of E-cadherin in glomerular cell cultures following BaP injury. Subconfluent cultures of glomerular cells were analyzed for E-cadherin by immunofluorescence as described in the **Methods** section. Intense staining was localized at cell-to-cell adhesion regions in epithelial cells, consistent with E-cadherin staining (×500). (*A*) Control. (*B*) BaP. (*C*) Negative control (aortic smooth muscle cells).

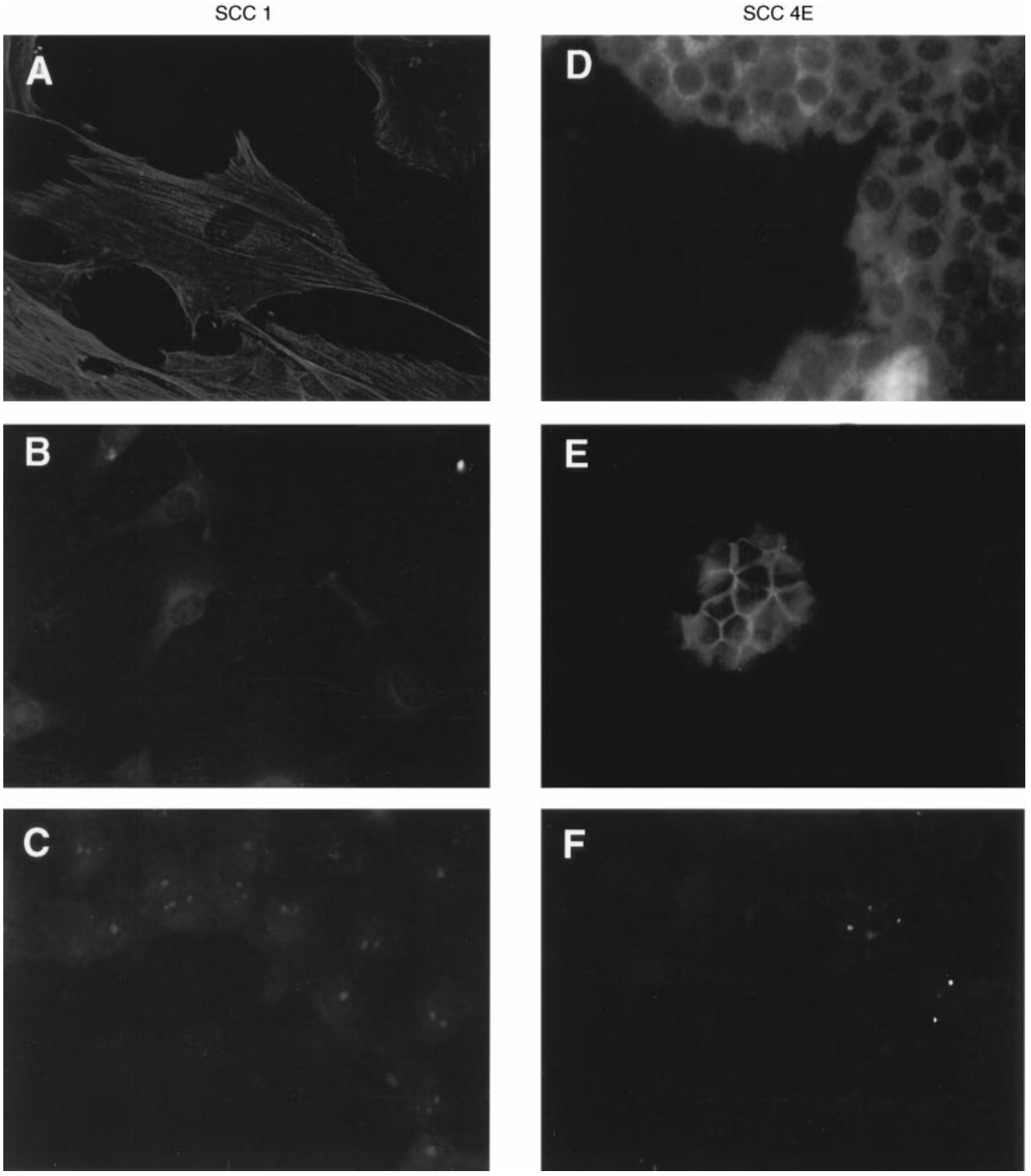

Fig. 4. Expression of α-SM actin and E-cadherin in SCC 1 and SCC 4E. Subconfluent cultures of SCC 1 and SCC 4E were analyzed for α-SM actin and E-cadherin expression by immunofluorescence, as described in the **Methods** section (×1030). (*A* and *D*) α-SM actin. (*B* and *E*) E-cadherin. (*C*) α-SM actin negative control (LLC-PK1). (*F*) E-cadherin negative control (aortic smooth muscle cells).

sively characterized in vivo. For instance, cultured renal cells show altered characteristics as a function of serial passage, as demonstrated by the loss of WT1 expression in GVECs in vitro [25]. WT1, a tumor suppressor gene intricately involved in the differentiation of podocytes, is expressed at high levels in GVECs in vivo [26].

Support for the hypothesis that BaP affords epithelial cells a proliferative advantage over mesangial cells was obtained from DNA synthesis, cell number, and E-cadherin expression experiments. While repeated challenges of mixed GC cultures with BaP were associated with the occurrence of highly proliferative phenotypes [18], this

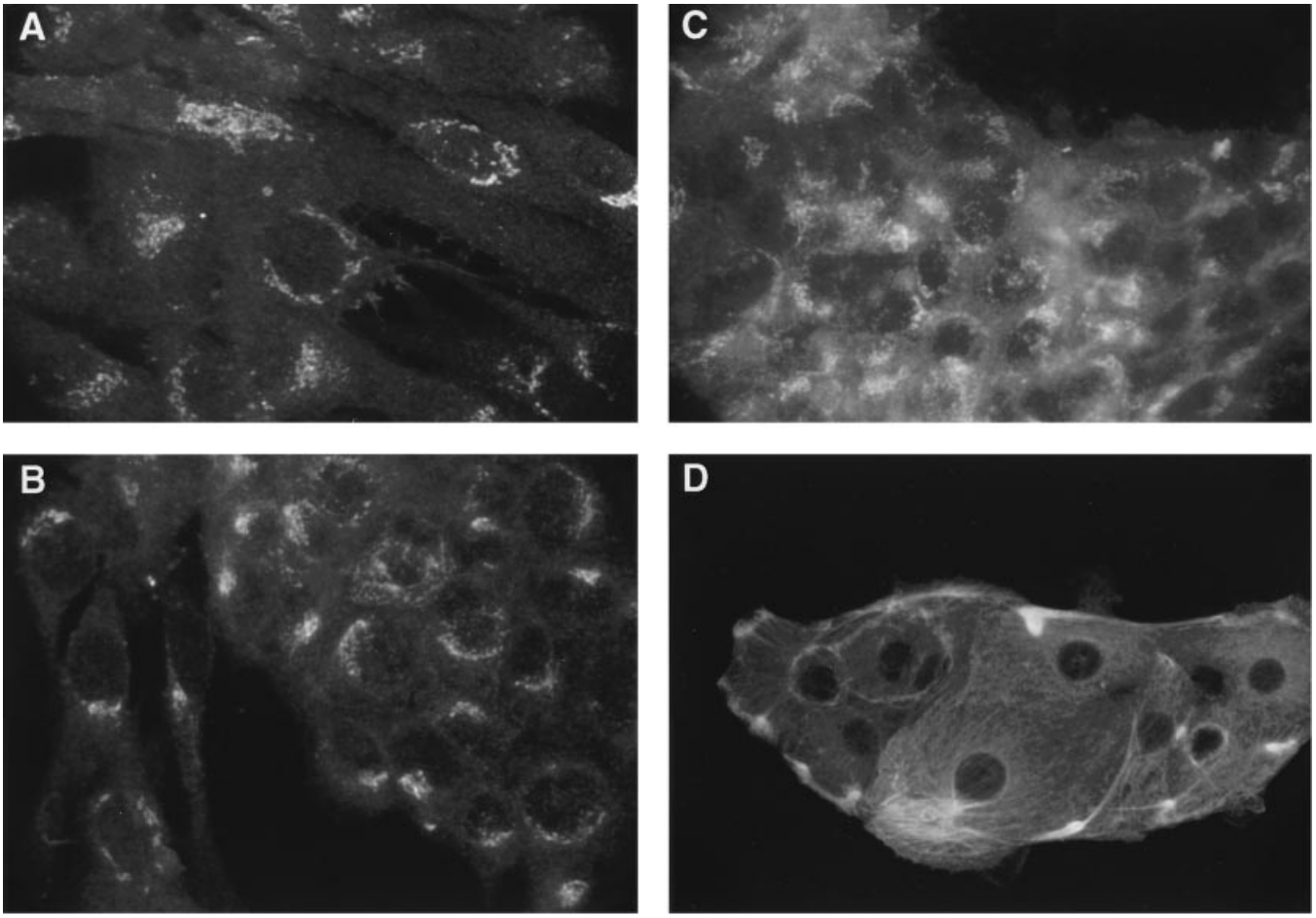

Fig. 5. Expression of cytokeratin in glomerular cells following BaP injury. Subconfluent cultures of GC and SCC 4E were analyzed for cytokeratin expression by immunofluorescence, as described in the **Methods** section. Perinuclear staining of cytokeratin was seen in epithelial cells of BaP cultures and SCC 4E, as compared with the filamentous pattern seen in the LLC-PK1 cells (×1030). (*A*) Control. (*B*) BaP. (*C*) SCC 4E. (*D*) Positive control (LLC-PK1).

growth advantage is likely attributable to the presence of an expanded GVEC population with high proliferative potential. The specificity for the growth inhibitory effects of BaP was shown by the inability of BaP to alter proliferation rates in SCC 4E under conditions in which significant GMC growth inhibition occurred. In addition, the number of SCC 1 was significantly reduced after BaP treatment, while SCC 4E was unaffected. E-cadherin immunofluorescence studies verified the presence of epithelial cells in GC cultures prior to BaP challenge and identified this population as a minor component of primary GC cultures. The staining profiles of α-SM actin along with E-cadherin showed that the relative numbers of GMCs decreased as a function of chemical challenge and serial passage in hydrocarbon-treated cultures. On the basis of these findings, we conclude that inhibition of GMC growth following damage by BaP affords a small resident GVEC population a proliferative advantage in vitro. The mesenchymal/epithelial interaction observed in our culture model may be analogous to other organ systems, such as prostate, where stimulation of smooth muscle cells by androgens influences the proliferative capacity of the epithelium [27]. As such, podocytes may not be able to proliferate in the presence of GMCs under co-culture conditions [26]. It is important to recognize, however, that some epithelial cells in BaP-treated cultures were immunoreactive for α-SM actin, thus raising the possibility that mesenchymal-to-epithelial or epithelial-to-mesenchymal transition may contribute to the appearance of altered phenotypes.

The modulation of mesangial cell growth and viability by BaP reflects the overall injury response of these cells to hydrocarbon challenge. Because GMCs participate in the regulation of matrix disposition and filtration function of the glomerulus, injury to mesangial cells may be of pathophysiological significance in the context of mesangial–podocytic interactions. In this manner, injury to mesangial cells by BaP may result in unregulated

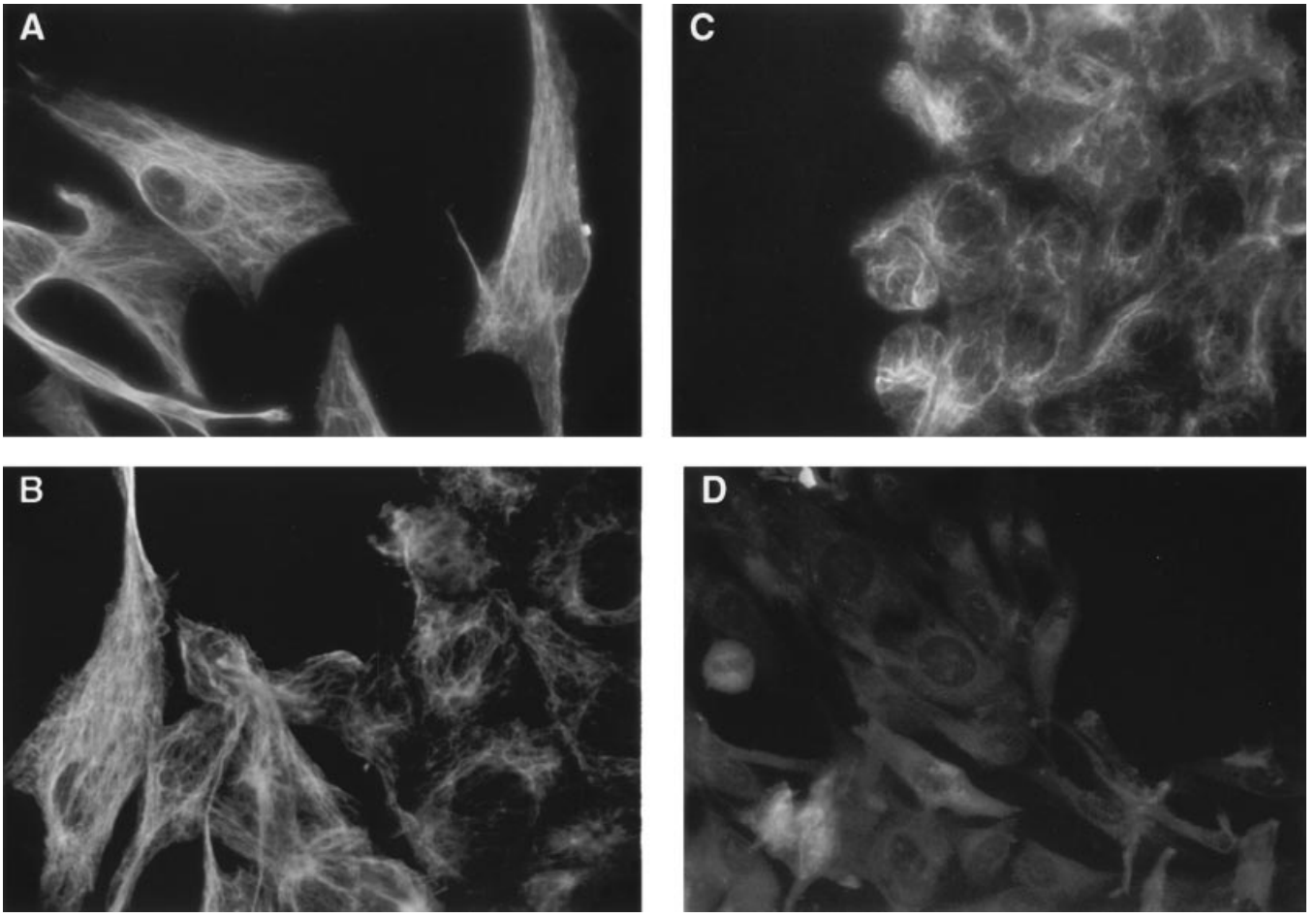

Fig. 6. Expression of vimentin in glomerular cells following BaP injury. Subconfluent cultures of glomerular cells and SCC 4E were analyzed for vimentin expression by immunofluorescence as described in the **Methods** section. A filamentous staining pattern for vimentin was seen in all three groups (×1030). (*A*) Control. (*B*) BaP. (*C*) SCC 4E. (*D*) Negative control (LLC-PK1).

Table 1. Expression of selected cytoskeletal markers in GVECs, GPECs, and SCC 4E

	Cell type		
Marker	GVEC	GPEC	SCC 4E
Cytokeratin	−	+	−
Vimentin	+	−	+
α-Smooth muscle actin	−	−	−

Detection of cytoskeletal markers was conducted by immunofluorescence. Characteristics of glomerular visceral epithelial cells (GVECs) and glomerular parietal epithelial cells (GPECs) have been described by Ardaillou et al [23]. SCC 4E is single cell clone 4E.

paracrine control of podocytic function, a hypothesis that is currently under investigation. Our findings are particularly intriguing in light of reports that deregulation of GMC function, coupled to increased GVEC proliferation, is a critical event in the pathogenesis of focal segmental glomerulosclerosis (FSGS). FSGS is associated with detachment of podocyte foot processes from the glomerular basement membrane, leading to proliferation of GVECs, as well as GPECs [28]. This step may dictate whether the glomerulus undergoes resolution or chronic injury. Since many of the etiological agents and mechanisms of glomerular injury are not yet well understood, it is possible that interactions between GMCs and GVECs, such as those reported here, contribute to the pathogenesis of FSGS. Future studies focusing on critical molecular targets of hydrocarbon injury in GCs may help define pathogenetic mechanisms.

ACKNOWLEDGMENTS

This work was supported in part by NIEHS Superfund Program Project Grant 04917 to K.S.R. A.R.P. and R.C.B. III were predoctoral fellows in NIEHS Toxicology Training Grant ES 07273-02. N.F.A. is the recipient of a minority graduate fellowship from Texas A&M University. The authors thank Dr. Rick Metz for helpful comments during manuscript preparation.

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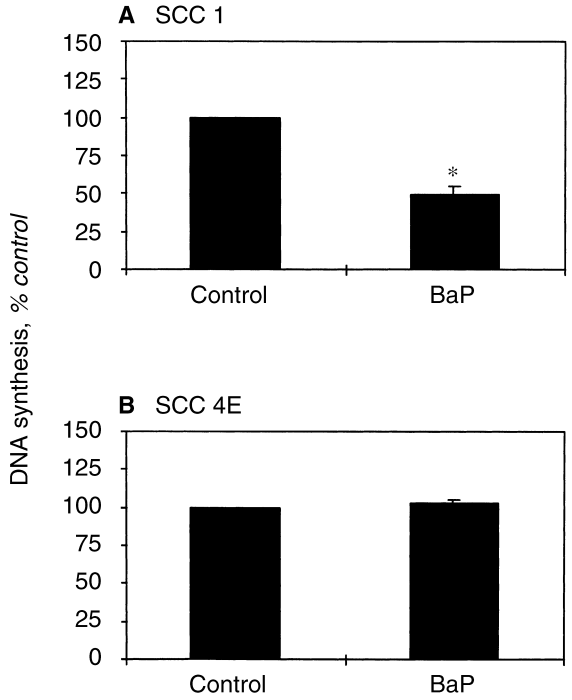

Fig. 7. [^{3}H]-Thymidine incorporation into DNA of SCC 1 and SCC 4E following BaP exposure for three consecutive passages. Randomly cycling SCC 1 (*A*) and SCC 4E (*B*) were incubated with [^{3}H]-thymidine (0.5 to 1.0 μCi/mL) and analyzed as described in the **Methods** section. Experiments were done in triplicate with three replicate wells/group. *Significantly different from the corresponding control value ($P < 0.05$).

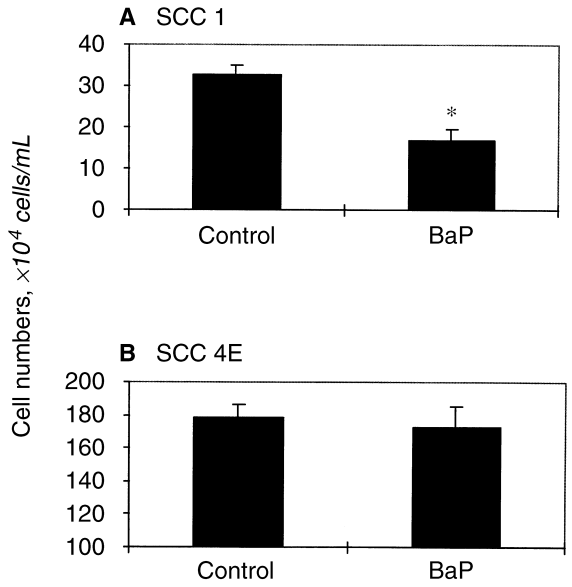

Fig. 8. Cell numbers in SCC 1 and SCC 4E following BaP exposure for three consecutive passages. Cell counts were conducted using a hemacytometer in randomly cycling SCC 1 (*A*) or SCC 4E (*B*) clones challenged with 3 μmol/L BaP. Experiments were done in triplicate. *Significantly different from the corresponding control ($P < 0.05$).

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