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Characterization of glomerular cell phenotypes following repeated cycles of benzo[*a*]pyrene injury *in vitro*

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Abstract

Exposure of cultured glomeruli to benzo[*a*]pyrene (BaP), a carcinogenic hydrocarbon, modulates mesangial and visceral epithelial cell proliferation *in vivo* and *in vitro*. The present studies were conducted to characterize mitogenic signaling profiles of cultured glomeruli following repeated cycles of BaP challenge. Enhanced rates of DNA synthesis were observed by the third passage in randomly cycling cultures after single or repeated carcinogen exposure. This response was characterized by upregulation of mitogenic sensitivity during early cell cycle transit, and increased cell numbers under restrictive growth conditions. The mitogenic response to platelet-derived growth factor (0.5 to 25 ng/mL), acidic fibroblast growth factor (2.5 to 10 ng/mL), basic fibroblast growth factor (0.05 to 5 ng/mL), epidermal growth factor (0.5 to 5 ng/mL), or conditioned medium was not enhanced by hydrocarbon challenge. BaP-treated cultures exhibited anchorage-independent growth and increased expression of hepatocyte growth factor mRNA and E-cadherin protein. Binding of activator protein-1 to DNA was enhanced in BaP-treated cells, but this change did not involve truncation or mutation of the c-*jun* delta region. Collectively, the data demonstrate that repeated cycles of BaP injury alter mitogenic signaling profiles in cultured glomerular cells. These alterations may contribute to deregulation of proliferative control following carcinogen exposure *in vivo*.

Keywords: AP-1; Benzo[*a*]pyrene; Glomerular cell phenotypes; c-*jun*; Mitogenic signaling

1. Introduction

Chronic human exposure to aromatic hydrocarbons is associated with nephropathy [1], renal cancer [2], and glomerulonephritis [3]. Recent *in vivo* studies have identified the glomerulus as a preferential site of nephrotoxic injury by BaP, a carcinogenic aromatic hydrocarbon [4]. BaP is a widespread contaminant in food, water, and air that is formed during incomplete combustion of fuels [5]. The prevalence of BaP in the environment and its high chemical reactivity following cellular metabolism have led to extensive investigations of the role of BaP in carcinogenesis. BaP is metabolized to electrophilic intermediates via the cytochrome P450 monooxygenase system, a bioactivation process linked to skin and bladder tumorigenesis [6] and vascular atherosclerotic lesions [7]. In cultured glomeruli, CYP1B1 metabolizes BaP to intermediates that bind covalently to DNA [8]. The adduction process is similar to that seen in response to benzo[*a*]pyrene 7,8-diol-9,10-epoxide, a proximate carcinogenic metabolite of BaP [9]. Acute challenge of primary glomerular cultures with BaP inhibits DNA synthesis, depletes antioxidant defenses, and induces mitochondrial injury [10,11]. In contrast, the adaptive response to injury involves activation of glomerular cells and the appearance of proliferative phenotypes with epithelial characteristics [12,13].

Modulation of glomerular cell proliferation by BaP may involve disruption of mitogenic signaling during the course of injury. The AP-1 transcription factor is a potentially significant molecular target since altered c-*jun* expression is observed in cultured glomeruli treated with BaP [12]. AP-1 dimers formed by Jun, Fos, and ATF family members regulate gene expression by binding to consensus TREs in the promoter region of target genes. AP-1 plays an impor-

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Abbreviations: AP-1, activator protein-1; BaP, benzo[*a*]pyrene; DTT, dithiothreitol; FBS, fetal bovine serum; RT–PCR, reverse transcription–polymerase chain reaction; TPA, 12-*O*-tetradecanoyl-13-phorbol acetate; TREs, TPA responsive elements.

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tant role in glomerular pathology [14,15] and, importantly, overexpression of AP-1 proteins is observed during the early stages of tumor development in the Eker rat model [16]. In certain cells, AP-1 is a requisite for neoplastic transformation induced by TPA or epidermal growth factor [17]. In these studies, expression of a dominant-negative Jun protein was shown to reduce growth in soft agar, suggesting that AP-1 is important for conversion to anchorage-independent phenotypes. Using Jun mutants selective for Fos or ATF2 proteins, van Dam *et al.* [18] have shown that Jun is critical for anchorage-independence (Jun:Fos) and growth factor independence (Jun:ATF2). Taken together, these data implicate AP-1 as a critical mediator of several aspects of cellular transformation.

Rigorous characterization of cellular targets of aromatic hydrocarbons is paramount to evaluate the link between environmental aromatic hydrocarbon exposures and nephropathy and renal cancer. The present studies were conducted to evaluate mitogenic signaling profiles of cultured glomeruli following repeated cycles of carcinogen challenge *in vitro*. Evidence is presented that repeated cycles of BaP injury induce glomerular cell phenotypes characterized by upregulation of mitogenic sensitivity, anchorage-independent growth, and enhanced AP-1 DNA binding activity. In addition, the overexpression of E-cadherin and hepatocyte growth factor in BaP cultures is consistent with the predominance of epithelial cell phenotypes following carcinogen challenge.

2. Materials and methods

2.1. Chemicals

BaP (> 98% purity) was purchased from the Aldrich Chemical Co., and FBS from Intergen. Insulin, antibiotic/antimycotic solution, BSA, DMSO, and trypsin/EDTA were obtained from the Sigma Chemical Co. RPMI 1640 was purchased from Gibco/BRL. Collagenase and holotransferrin were obtained from Boehringer Mannheim. Hepatocyte growth factor cDNA was purchased from ATCC, and a monoclonal anti-E-cadherin antibody was obtained from Transduction Laboratories. The consensus sequence for the AP-1 transcription factor and T4 polynucleotide kinase were purchased from Promega. [^{3}H]Thymidine (55 Ci/mmol) was purchased from ICN Biomedicals, and [^{32}P]dATP was obtained from NEN. All other chemicals were purchased from Sigma.

2.2. Glomerular cultures

Glomeruli were isolated from female Sprague–Dawley rats and established in serial culture as described in [10]. Subcultures consisted primarily of mesangial cells along with a minor population of visceral epithelial cells [13]. Cultures were maintained in RPMI 1640 medium supplemented with 10% FBS, 1 mg/mL of 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). Cultures were used between passages 6 and 9 for carcinogen treatment, and processed for biochemical measurements between passages 9 and 17.

2.3. Treatments

Randomly cycling cultures at passage 6 were treated with DMSO (0.1%) or BaP (3 μM) for 24 hr, rinsed with PBS, and allowed to recover for 24 hr in fresh medium containing 10% FBS. Cells were maintained under conventional culture conditions for passages 7 and 8 after one round of chemical treatment, or challenged with BaP for two additional rounds of BaP treatment. Estimates of BaP exposure in non-smoking adults range from 0.171 to 1.64 μg/day [19]. Thus, the BaP concentration used (3.0 μM or 2.3 μg/dish) approximates the range of estimated human daily burden, and is within the expected limits in moderately exposed individuals. For simplicity, glomerular cells were designated as BaP-treated or control cells (DMSO treatment). For measurements of growth factor sensitivity, growth-arrested cultures were incubated under serum-free conditions with platelet-derived growth factor (0.5 to 25 ng/mL), acidic fibroblast growth factor (2.5 to 10 ng/mL), basic fibroblast growth factor (0.05 to 5 ng/mL), or epidermal growth factor (0.5 to 5 ng/mL). Stock solutions were prepared in acidified water and protected from light.

2.4. Thymidine incorporation

Cultures were labeled with 0.5 to 1 μCi of [^{3}H]thymidine for 24 hr, except in cell cycle experiments, and were harvested with 5% trichloroacetic acid to precipitate cellular proteins. Following three washes with ethanol, the pellet was dissolved in 1 N NaOH, and neutralized with HCl. Counts per minute were normalized to cellular protein.

2.5. Cell proliferation

Cells were seeded at 200 cells/mm^2 and allowed to attach for 3 hr. Plates were rinsed with PBS before the addition of FBS (0–10%). Cells were grown for 5 days prior to determination of cell numbers [20]. Dishes were rinsed twice with PBS, and cells were fixed with 1 mL of ethanol for 90 sec. Ethanol was aspirated, and the dishes were allowed to air-dry. One milliliter of Janus green dye was added for 1 min, followed by rinsing with PBS twice. The dye was extracted with 2 mL of ethanol and measured spectrophotometrically at 654 nm. Cell number was determined by regression analysis using a standard curve generated by plating known numbers of cells, and measuring absorbance after a 6-hr attachment period.

2.6. Conditioned medium

Naïve glomerular cells were seeded in 12-well culture plates at a density of 100 cells/mm^2 and allowed to attach in medium containing 10% FBS for 24 hr. At this time, cultures were washed three times with sterile PBS and growth arrested for 72 hr by serum restriction in 0.1% FBS. Cells were washed twice with sterile PBS, and serum-free conditioned medium harvested from randomly cycling control and BaP-treated cells over a 24-hr period.

2.7. Cell cycle transit

Control and BaP-treated cells were seeded on 12-well plates at a density of 100 cells/mm^2 and allowed to attach for 24 hr in medium containing 10% FBS. Cells were growth arrested by serum restriction for 72 hr in 0.1% FBS prior to reintroduction of 10% FBS to allow synchronized entry into the cell cycle. Cells were pulse-labeled with 1 μCi/mL of [^{3}H]thymidine for 4 hr. Data for each cell type are presented as fold-induction over cultures maintained in 0.1% FBS for the same period.

2.8. Anchorage-independent growth

Control and BaP-treated cells were suspended in a 0.35% agar solution and overlaid onto 0.7% agar. Cells were seeded at a density of 200 cells/mm^2 in six-well plates, and colony formation was assessed 12 days post-plating by evaluation of select fields using light microscopy. Only colonies greater than 1.0 mm were counted. H4IIE hepatoma cells were used as a positive control.

2.9. Western blotting

One hundred microliters of lysis buffer (4% sodium dodecyl sulfate) was added, and cells were harvested by scrapping. Proteins were quantified using the Bradford assay [21]; 30 μg sample protein was loaded onto an 8% polyacrylamide gel, separated, and transferred to nitrocellulose membrane (Hybond C, Amersham). The membrane was then incubated with a monoclonal anti-E-cadherin (1:1000), and following several washes, was incubated with secondary antibody coupled to alkaline phosphatase. Proteins were visualized using BCIP-NBT tablets (Sigma).

2.10. Northern blotting

Following the instructions of the manufacturer, cells were lysed by directly adding 1 mL of a monophasic solution of phenol and guanidine isothiocyanante (TRIzol Reagent). RNA was precipitated with chloroform and isopropanol, washed with 75% ethanol, and dissolved in RNase-free 50% formamide. Total amounts of cellular RNA were determined by UV absorbance at 260 nm, and samples were stored at −70° until used. Total RNA (20 μg/lane) was loaded on a 1.5% agarose gel, electrophoresed, separated, and transferred to a Bright Star-Plus nylon membrane (Ambion) by capillary blotting. The blots were immobilized further by ultraviolet irradiation at energy mode 1800 in an ultraviolet cross-linker. Membranes were preincubated with prehybridization solution (Ambion Inc.) at 42° (overnight) followed by hybridization in the same buffer with a cDNA probe labeled with [α-^{32}P]dCTP (NEN) by random oligonucleotide priming (2×10^6 cpm/mL of a ^{32}P-labeled cDNA probe for hepatocyte growth factor or β-tubulin) and incubation at 55° for 2 hr. Membranes were washed in low stringency wash solution No. 1 (Ambion Inc.) three times at room temperature for 15 min followed by two washes in high stringency wash solution No. 2 (Ambion Inc.) for 20 min at 42°, and were subsequently exposed to x-ray film at −70° for 72 hr.

2.11. Gel shift analysis

Cells were washed in buffer [10 mM Tris–HCl (pH 7.9), 10 mM KCl, 1 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 0.75 mM spermidine, 0.15 mM spermine, 0.5 mM phenylmethylsulfonyl fluoride] and scraped into the same buffer. The cell suspensions were spun at 1000 *g* at 4° for 10 min. The supernatants were removed, and 30 μL of extraction buffer [50 mM Tris–HCl (pH 7.9), 1 mM EDTA, 1 mM DTT, 12.5 mM $MgCl_2$, 0.42 M KCl, 20% glycerol] was added to each pellet for 1 hr (4°). Supernatants were collected after centrifugation (1800 *g* at 4°, 15 min), and nuclear protein concentration was determined by the method of Bradford [21]. The nuclear extracts (5 μg) were incubated for 30 min with 10 fmol of double-stranded, ^{32}P-labeled oligonucleotide containing the consensus AP-1 binding motif [12]. Reactions were separated on 5% non-denaturing polyacrylamide gels, and the gels were dried and exposed to film for 12 hr at room temperature.

2.12. RT–PCR/Sequencing

Two hundred nanograms of RNA was reverse transcribed using 2.5 U/μL of murine leukemia virus reverse transcriptase in a reaction mixture containing 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 5 mM $MgCl_2$, 1 mM dNTPs, 2.5 μM oligo d(T), and 1 U/μL of RNase inhibitor in a total volume of 20 μL. c-*jun* cDNA was amplified with primers designed using OLIGO 5.0 (National Biosciences Institute) based on published sequences (GenBank Accession No. X17163). The annealing temperature was set at 62°. The upstream primer was 5′-TGTCGCTGGGGCTGGTC-3′ and the downstream primer 5′-AGTGGGGGTCGGTG-TAGTGGT-3′, respectively. The PCR product (607 bp) was excised from a low-melting agarose gel and purified (Wizard PCR Prep, Promega). Two hundred fifty femtomoles of cDNA was used for sequencing in buffer containing 10 U/μL of ampliTaq DNA polymerase, 50 mM Tris–

HCl (pH 8.3), 10 mM KCl, 2.5 mM $MgCl_2$, and 0.025% Tween-20 in four individual reactions [2.5 μM dNTPs, 20 μM ddNTPs (one per reaction), 5.625 μM c7dGTP]. The downstream primer (1 pmol) for c-*jun* was used in the reaction. The reactions were cycle sequenced at 95° for 30 sec, 65° for 30 sec, and 72° for 60 sec, 35 cycles. Two U/μL of 1 × terminal deoxynucleotide transferase mix [1 mM dNTPs, 4 mM Tris–HCl (pH 7.5) 2 mM DTT, 0.2 mg/mL of BSA] was added for 20 min followed by 4 μL of stop solution [95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.02% xylene cyanol]. Three microliters of each reaction was loaded onto a 6% polyacrylamide, 8 M urea gel and separated for 2 hr at 75 W. The gel was dried and exposed to x-ray film for analysis.

2.13. Statistics

Analysis of variance in conjunction with Fisher's post-hoc test was used to assess the statistical significance of differences between control and BaP-treated cells. Values are means ± SEM (N = 4–6). Data was considered significant at $P < 0.05$.

3. Results

Mitogenic stimulation of growth-arrested, or randomly cycling, cultures with 10% FBS significantly increased DNA synthesis in BaP-treated cultures over controls (Fig. 1). This increase was seen in cells challenged with BaP once or three times, indicating that upregulation of mitogenesis as part of the adaptive response did not require multiple carcinogenic challenges. Significant elevations in [^{3}H]thymidine incorporation were observed at 8 and 12 hr in pulse-labeled BaP-treated cells compared with controls (Fig. 2). Peak DNA synthesis was observed in BaP-treated cells at 12 hr, while control cultures exhibited progressive increases for up to 24 hr. The effects of BaP were specific for cells of the glomerulus since challenge of cultured tubular epithelial cells with BaP did not modulate mitogenic sensitivity or cellular proliferation (not shown). BaP-treated cultures proliferated at faster rates than controls irrespective of FBS concentrations (Fig. 3). Significant increases in cellular proliferation were observed in the absence of exogenous mitogenic stimulation and under serum-restrictive conditions. BaP-treated cultures reached higher saturation densities than control counterparts.

Because the appearance of proliferative phenotypes may involve modulation of autocrine/paracrine control, conditioned medium was harvested from randomly cycling control or BaP-treated cultures. Conditioned medium from control or BaP-treated cells did not modulate [^{3}H]thymidine incorporation in synchronized naïve glomerular cultures (data not shown). Challenge of control and BaP-treated cultures with platelet-derived growth factor (0.5 to 25 ng/mL), acidic fibroblast growth factor (2.5 to

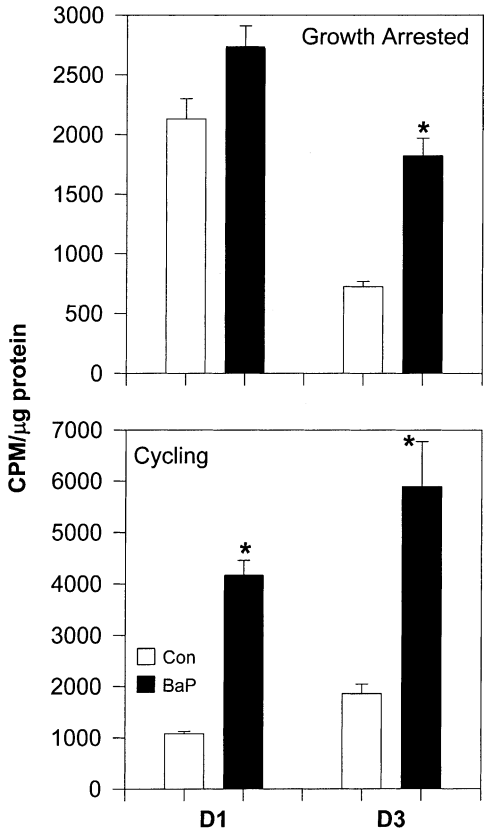

Fig. 1. DNA synthesis in growth-arrested and randomly cycling cultures of glomerular cells following one (D1) or three (D3) rounds of 3 μM BaP treatment. Cells were seeded at 150 cells/mm^2 and allowed to attach for 24 hr. Cultures were growth arrested by serum restriction in 0.1% FBS for 72 hr or allowed to cycle randomly for the same period. Fresh medium containing 10% FBS and 1 μCi/mL of [^{3}H]thymidine was then added for 24 hr, and label incorporation was measured as described in Section 2. Each data point represents the mean ± SEM of six replicate dishes. Key: (*) significantly different from respective controls ($P < 0.05$).

10 ng/mL), basic fibroblast growth factor (0.05 to 5 ng/mL), or epidermal growth factor (0.5 to 5 ng/mL) increased DNA synthesis in control and BaP-treated cells (Fig. 4). Control cultures were most responsive to basic fibroblast growth factor, the major growth factor for glomerular mesangial cells [22]. While basal rates of DNA synthesis were consistently higher in BaP-treated cells under mitogen-restricted conditions, their relative sensitivity to all growth factors examined was reduced compared with respective controls. This reduction was most pronounced for basic fibroblast growth factor, and likely reflects down-regulation of growth factor sensitivity in proliferative phenotypes.

BaP-exposed cells proliferated in an anchorage-independent manner (Fig. 5), demonstrating that the BaP phenotype was characterized by loss of contact inhibition and anchorage-independent growth. At least 50 colonies of 1.0 mm diameter were identified per dish in BaP-treated cultures (control < 5; H4IIE > 80). Acquisition of anchorage-independent growth may involve the expansion of a unique epithelial cell phenotype since SCC-4, a clonal

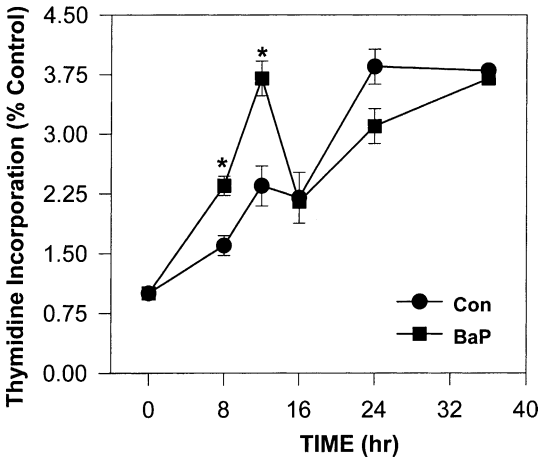

Fig. 2. Serum-stimulated kinetics of [^{3}H]thymidine incorporation in growth-arrested glomerular cultures following three rounds of 3 μM BaP treatment. Cells were seeded at 150 cells/mm^2 and allowed to attach for 24 hr. Cultures were growth arrested by serum restriction in 0.1% FBS for 72 hr before stimulation with medium containing 10% FBS, and 1 μCi/mL of [^{3}H]thymidine was added for various times as indicated. Label incorporation was measured as described in Section 2. Data are presented as percent of unstimulated controls. Each data point represents the mean ± SEM of six replicate dishes. Key: (∗) significant difference from control ($P < 0.05$). Cellular protein (cpm/μg) in serum-restricted control cultures was 1272.2 ± 115 versus 1548 ± 67 in BaP cultures.

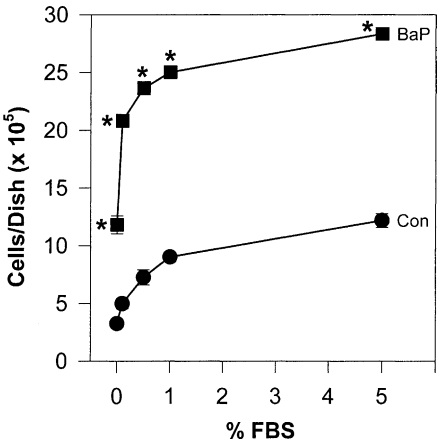

Fig. 3. Serum responsiveness of glomerular cultures following three rounds of 3 μM BaP treatment. Cells were seeded at 200 cells/mm^2 and were allowed to attach for 3 hr. Cultures were grown for 5 days in various concentrations of FBS (0, 0.1, 0.5, 1, 5%) before cell numbers were counted as described in Section 2. Each data point represents the mean ± SEM of four replicate dishes. Key: (∗) significantly different from control ($P < 0.05$).

population of glomerular epithelial cells, also grew in soft agar (not shown). BaP-treated cultures expressed hepatocyte growth factor mRNA and E-cadherin protein, markers of epithelial identity (Fig. 6).

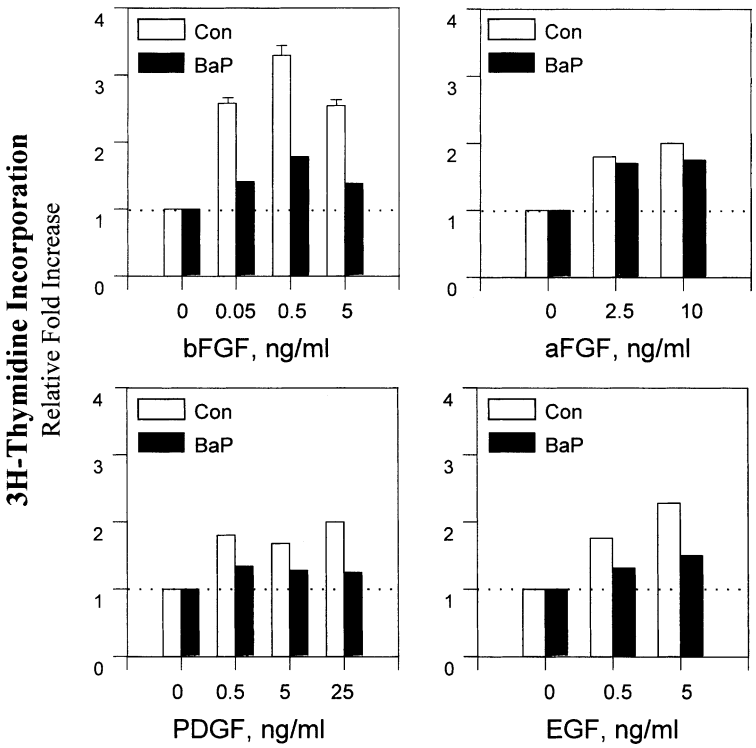

Fig. 4. Growth factor responsiveness of glomerular cultures following three rounds of 3 μM BaP treatment. Cells were seeded at 200 cells/mm^2 and allowed to attach for 3 hr. Cultures were grown for 3 days in various concentrations of 0.1% FBS before stimulation of [^{3}H]thymidine incorporation in the presence of basic fibroblast growth factor (0.05 to 5 ng/mL), acidic fibroblast growth factor (2.5 to 10 ng/mL), platelet-derived growth factor (0.5 to 25 ng/mL), or epidermal growth factor (0.5 to 5 ng/mL). Thymidine incorporation was measured as described in Section 2. Each data point represents the mean ± SEM of six replicate dishes; data are presented as the relative fold-increase in thymidine incorporation. These results are representative of four different experiments.

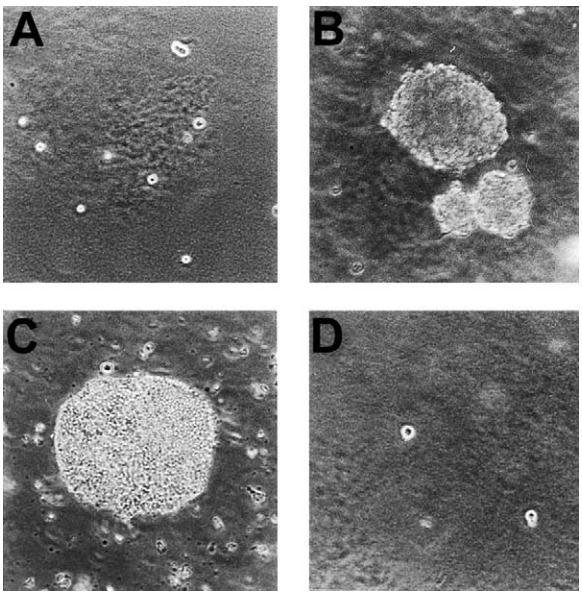

Fig. 5. Anchorage-independent growth of glomerular cells following three rounds of 3 μM BaP treatment. Glomerular cells were suspended in 0.35% agar solution and plated at a density of 200 cells/mm^2 on 0.7% agar plates. Colony formation was assessed 12 days following plating as described in Section 2. A = control; B = BaP; C = H4IIE; D = naïve cells. Similar results were seen in three independent experiments.

Unregulated expression of the c-*jun* proto-oncogene has been observed in glomerular cells following BaP treatment [12]. Thus, experiments were conducted to assess the impact of BaP challenge on AP-1 DNA binding activity

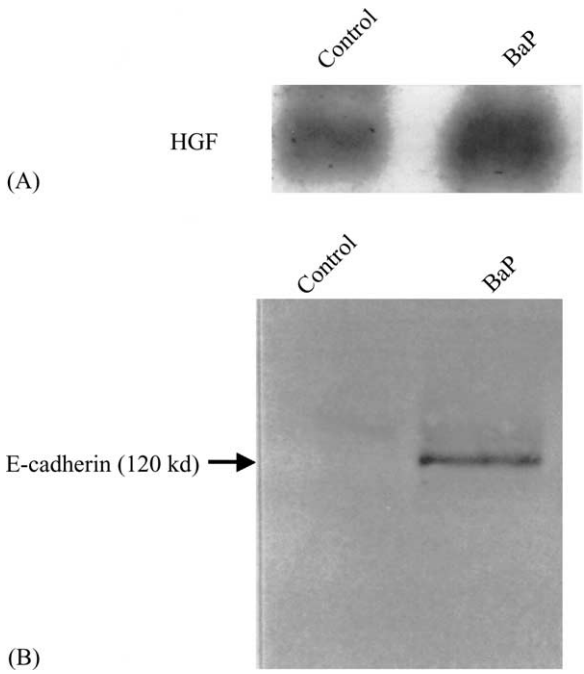

Fig. 6. Expression of hepatocyte growth factor mRNA (A) and E-cadherin (B) in glomerular cultures following three rounds of 3 μM BaP treatment. Randomly cycling cultures were harvested for total cellular RNA or protein, and the expression of hepatocyte growth factor and E-cadherin was measured by northern and western blotting, respectively, as described in Section 2. Similar results were seen in two separate experiments.

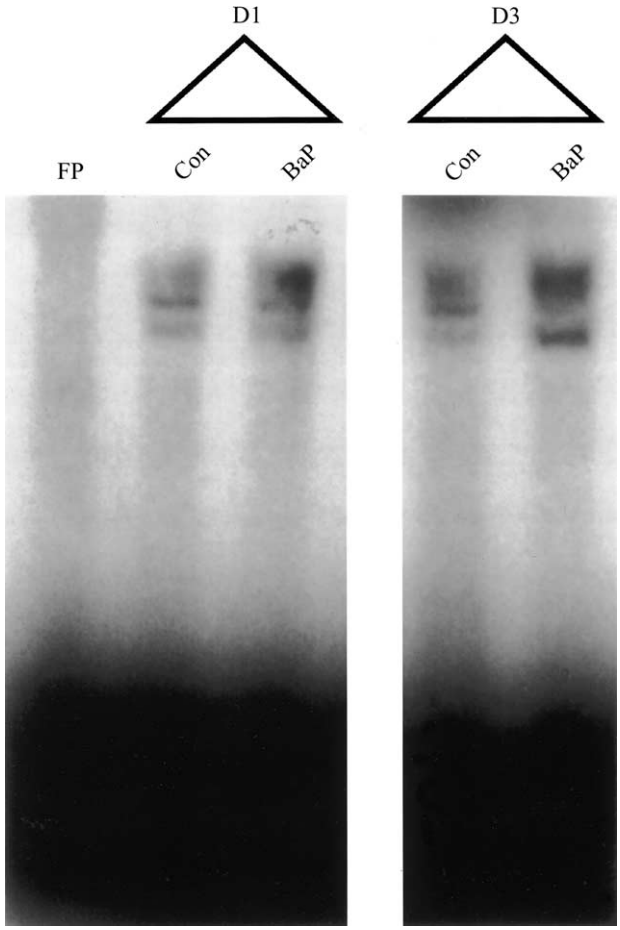

Fig. 7. Binding of nuclear proteins to the AP-1 consensus sequence in glomerular cultures following one (D1) or three (D3) rounds of 3 μM BaP challenge. Cells were seeded at 150 cells/mm^2 and allowed to cycle randomly for 48 hr. Nuclear proteins were isolated and gel shift analysis was performed as described in Section 2. FP = free probe; Con = control; BaP = benzo[*a*]pyrene. Similar results were seen in two separate experiments.

and Jun function. A 2-fold increase in DNA binding to a consensus AP-1 element was observed in randomly cycling cultures following either one or three rounds of BaP treatment compared with controls (Fig. 7). The size of the c-*jun* transcript was unchanged in glomerular cultures following three rounds of BaP challenge (Fig. 8A). No mutations of the delta region (bp 453–550) of c-*jun*, a region known to negatively regulate c-*jun* expression, were observed following BaP treatment (Fig. 8B). Thus, carcinogen treatment did not induce truncations or mutations in this region of the c-*jun* proto-oncogene.

4. Discussion

The data presented here demonstrate that the adaptive response of glomerular cells to BaP treatment involves alterations in mitogenic sensitivity, anchorage-dependent growth, expression of hepatocyte growth factor mRNA and E-cadherin protein, and AP-1 DNA binding activity.

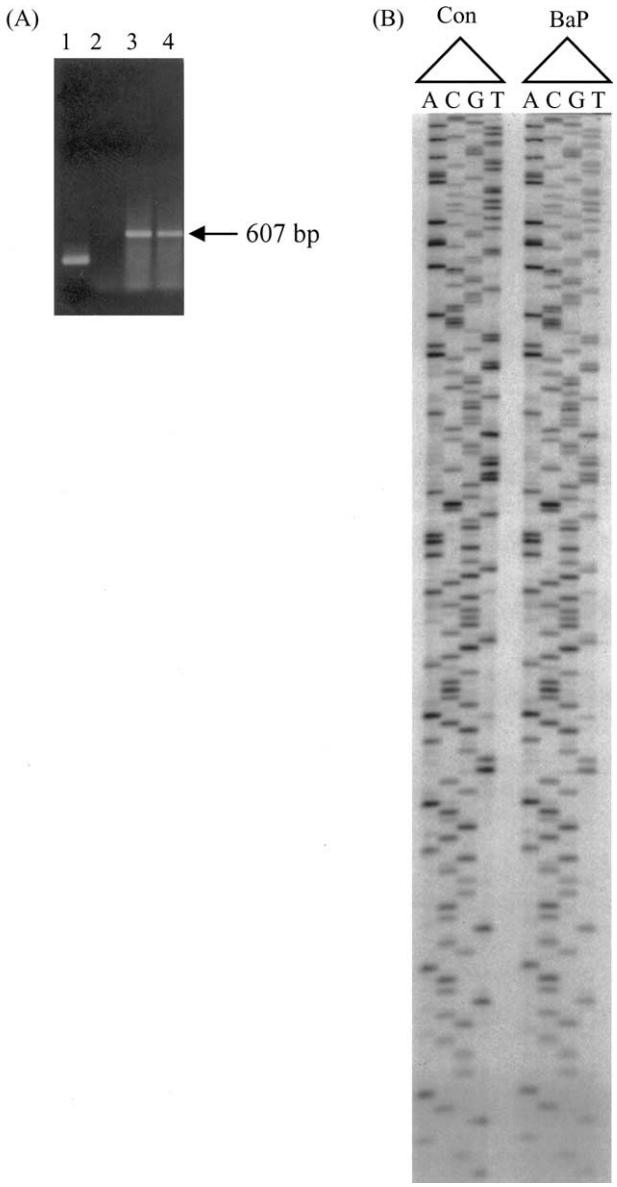

Fig. 8. (A) RT–PCR products of c-*jun* mRNA in glomerular cultures following three rounds of 3 μM BaP challenge. c-*jun* cDNA was amplified as described in Section 2. Lane 1 = control amplicon interleukin-1α in a pAW109 plasmid. The fragment was 308 bp. The primers used for the control amplicon were (DM151) upstream 5′–GTCTCTGAATCAGAA-ATCCTTCTATC and (DM152) downstream 5′–CATGTCAAATTTCAC-TGCTTCATCC; lane 2 = no template control; lane 3 = 607 bp c-*jun* DNA control; lane 4 = 607 bp c-*jun* DNA from BaP-treated cells. (B) DNA sequencing of the c-*jun* delta region in glomerular cultures following three rounds of 3 μM BaP challenge. The cDNA sequence reads 3′ to 5′ (top to bottom) and corresponds to the antisense strand. The sequence of the sense strand (bp 433–591) was: 5′-CTA CAG TAA CCC TAA GAT TCT GAA GCA GAG CAT GAC CTT GAA CCT GGC CGA CCC GGT GGG CAA TCT GAA GCC GCA CCT CCG AGC CAA GAA CTC GGA CCT TCT CAC GTC GCC CGA CGG TCG GGC TGC TCA AGC TGG CGT CGC CGG AAG CTG GAG CGC CTG AT-3′.

Upregulation of mitogenic sensitivity was consistent with leftward shifts in the serum concentration–response curve and cell cycle kinetics in BaP-treated cultures, respectively. Because a similar regimen did not elicit a proliferative phenotype in proximal tubular epithelial cells, disruption of renal cell growth by BaP appears to be selective for cells of the glomerulus. The effects of carcinogen treatment on mitogenic sensitivity did not involve altered secretion of mitogenic factors since conditioned medium harvested from proliferative cells was without effect on basal or serum-stimulated proliferation of naïve glomerular cells. Likewise, modulation of mitogenic activity was independent of platelet-derived growth factor, acidic or basic fibroblast growth factor, or epidermal growth factor signaling. This was an interesting finding since autocrine regulation of glomerular mesangial cell growth is mediated primarily by basic fibroblast growth factor [22]. The possibility remains that the growth advantage afforded by carcinogen challenge involves changes in the synthesis and deposition of extracellular matrix proteins, a suggestion consistent with the growth-restrictive properties of the extracellular matrix deposited by glomeruli in culture (unpublished observation).

BaP treatment selectively injures mesangial cells and affords visceral epithelial cells a growth advantage [13]. Thus, upregulation of proliferative activity in BaP-treated cultures may reflect differences in mitogenic sensitivity between these two cell populations. Since only transformed cells grow on soft agar *in vitro*, the loss of contact inhibition and acquisition of anchorage-independent growth of glomerular cells may represent expansion of a unique undifferentiated epithelial-like phenotype. This interpretation is consistent with the epithelioid morphology of cultured glomerular cells following carcinogen challenge [13], and the expression of E-cadherin protein, an epithelial specific cell-cell adhesion marker. A role for mutational activation of critical target genes is unlikely since carcinogen challenge of clonal epithelial cells does not modulate proliferative activity (unpublished observation). The expression of hepatocyte growth factor mRNA is of interest since this gene is expressed by cells of mesenchymal origin, and has been proposed to function as part of a paracrine loop mediating stromal-epithelial signaling [23]. Previous studies have shown that hepatocyte growth factor is expressed by glomerular mesangial cells, and induces tubulogenesis of kidney epithelial cells and anchorage-independent growth of SV-40 transformed tubular epithelial cells [24–27]. Although the relationship between hepatocyte growth factor expression and anchorage-independent growth of BaP-treated glomerular cultures is unclear, recent studies demonstrate that hepatocyte growth factor-induced invasive growth of mouse embryo liver cells is mediated by osteopontin [28]. Of note, BaP-treated glomerular cells expressed high levels of osteopontin that may contribute to invasive capacity (unpublished observation). Since elevated osteopontin levels have been associated with tumor invasiveness [29–31], BaP treatment may be responsible for initiating a cascade of deregulated growth factor/matrix protein expression that ultimately leads to altered proliferative phenotypes and disruption of normal mesenchymal–epithelial interactions.

BaP is known to influence the abundance of c-*jun* transcripts in cultured glomerular cells [12]. This finding is consistent with altered expression of c-*jun* in renal tumors of mesenchymal origin [32–34]. The mechanism of aberrant c-*jun* expression is unknown, but may involve activation of c-*jun* itself since a consensus TRE sequence is located upstream of the c-*jun* promoter [35]. Studies by Oehler *et al*. [36] have demonstrated that mutation of serine 226 of Jun enhances DNA binding and transactivation of Jun. Thus, increases in AP-1 DNA binding activity in BaP-treated cells may involve mutational activation of c-*jun*. Several positive and negative regulatory domains have been identified in c-*jun*, most notably a 27 amino acid sequence located near the amino-terminus termed the delta region [37]. The oncogenic counterpart of c-*jun* lacks this sequence and is refractory to protein phosphatase 2 [38]. However, no truncations or mutations within the delta region of c-*jun* were observed following three rounds of BaP treatment. The possibility remains that mutations in the carboxy-terminus and/or the promoter region of c-*jun* contribute to increased Jun function. In this regard, Dolan *et al*. [39] have shown that the promoter region of c-*jun* in Clara cells is unresponsive to a genotoxic BaP metabolite. As noted previously, overexpression of AP-1 proteins has been demonstrated in the early stages of tumor development in the Eker rat model [16]. In certain cells, AP-1 is requisite for neoplastic transformation induced by TPA or epidermal growth factor and for conversion to the anchorage-independent phenotype [17]. Using Jun mutants selective for binding to Fos or ATF2 proteins, van Dam *et al.* [18] have shown that Jun is critical for both anchorage-independent growth and growth factor independence.

The kidney has often been considered resistant to aromatic hydrocarbon injury, a notion based on the lack of toxicity to cortico-tubular epithelial cells. This laboratory has confirmed the relative insensitivity of tubular cells to BaP and related hydrocarbons [10,11], and identified glomerular cells as critical targets for this class of chemicals. The modulation of mitogenic sensitivity, anchorage-independent growth, hepatocyte growth factor mRNA and E-cadherin protein, and AP-1 DNA binding activity indicates that chemical treatment influences growth regulatory programs of cultured glomerular cells. Because BaP is a nephrocarcinogen *in vivo*, the results presented here suggest that cells of the glomerulus may be critical targets during the carcinogenic response elicited by BaP and related aromatic hydrocarbons.

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