Renal Glomerular Mesangial Cell Proliferation Is Enhanced by Sequential Challenge With Benzo(a)pyrene and 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin

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1. INTRODUCTION

The contribution of aberrant glomerular mesangial cell (GMC) proliferation to the onset and progression of renal glomerular disease has been the subject of intense research in recent years [1]. GMCs are mesenchymal cells which provide structural support for the glomerular tuft [2] as well as regulate glomerular hemodynamics. Several glomerular disorders involve glomerular hypercellularity as a result of enhanced GMC proliferation [3] and recent studies have suggested that xenobiotics may initiate GMC injury and contribute to the onset of renal disease [4-6]. Of particular significance are reports that human environmental and occupational exposures to aromatic hydrocarbons result in glomerulonephritis [7].

Previous studies in this laboratory have demonstrated that acute exposure of cultured GMCs to benzo(a)pyrene (BaP) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), two distinct aromatic hydrocarbons, differentially modulates the DNA synthetic rates [8]. Challenge of GMCs with these hydrocarbons was associated with induction of aryl hydrocarbon hydroxylase activity and enhanced formation of BaP/DNA adducts [9]. This is consistent with reports showing that treatment of mice with BaP in vivo results in kidney DNA adducts [10] and benzo(a)pyrene 7,8-diol-9,10-epoxide, the carcinogenic intermediate of BaP, (BPDE)-induces DNA adducts which persist for up to 14-20 days [11]. In contrast, the responses of the kidney to TCDD involves alterations in glomerular hemodynamics and renal tubular function [12]. These findings, along with evidence linking aromatic hydrocarbon exposure to increased incidence of renal cancer [13] and glomerular pathologies [7], suggest that injury to the kidney is part of the toxicologic spectrum of these chemicals.

We have recently reported that GMCs exposed to repeated cycles of chemical treatment with BaP and methoxamine, a growth promoting α_1-adrenergic agonist, exhibit a highly proliferative phenotype characterized by enhanced protooncogene expression [14]. The present studies were conducted to determine if the combinatorial responses elicited by BaP and methoxamine were promoter specific or instead sustained by chemical promoters which operate via different mechanisms. For this purpose, we chose to examine the influence of TCDD, a recognized growth promoter in other systems [15,16], and a known stimulator of DNA synthesis in GMCs [8].

2. MATERIAL AND METHODS

Animals. Female Sprague-Dawley rats between 175-200 g (12-21 wk of age) were purchased from Sasco (Houston, TX). Female rats are more susceptible to the toxicologic/carcinogenic effects of PAHs and PCDDs than male counterparts [17-19].

Chemicals. BaP (>98% purity) was purchased from Aldrich Chemical Co. (Milwaukee, WI). TCDD was a kind gift from Dr. Stephen H. Safe (Texas A&M University, College Station, TX).

Cell Culture of Glomerular Mesangial Cells. GMCs were isolated and established in culture as described previously [8]. Subcultures of GMCs between passage 6 and 14 were used for all experiments.

DNA Synthesis and Cell Proliferation in GMCs Exposed to Sequential Treatments with BaP and TCDD. Subcultured GMCs were treated with DMSO or BaP (3 mM) for 24 hr, rinsed with PBS, and allowed to recover for 24 hr in fresh media containing 10% serum. Control and BaP-treated cultures were then incubated with TCDD (1 nM) for 24 hr period. This cycle of sequential chemical treatment was repeated two additional times. Cycling cultures of GMCs were incubated in the presence of 0.5 mCi [^{3}H]thymidine/dish for 24 hr to assess DNA synthetic rates. Measurements of [^{3}H]thymidine incorporation were conducted as described previously [21]. For measurements of cell proliferation, GMCs were seeded at 30 cells/mm^2 and incubated for 24 hr in media containing 10% FBS. Attachment rates were determined 24 hr after seeding and relative proliferation rates monitored 1 or 7 days after mitogenic stimulation in media containing 10% FBS. Cell numbers were counted using a hemacytometer or the Janus Green assay [20].

Data Analysis. Analysis of variance (ANOVA) in conjunction with Fisher's post-hoc test was used to assess the statistical significance of differences between control and treated cultures ($P > 0.05$). Values always represent mean $\pm$ the standard error of the mean (n=4). Experiments were always performed in duplicate or triplicate.

3. RESULTS

Treatment of GMCs with BaP alone or in combination with TCDD resulted in a 80% inhibition of [^{3}H]thymidine incorporation relative to controls, while TCDD alone did not modulate DNA synthetic rates (Fig. 1). After 3 cycles of chemical treatments, BaP/TCDD cells exhibited increased DNA synthetic rates relative to controls, while cells exposed to BaP or TCDD alone exhibited [^{3}H]thymidine incorporation profiles comparable to controls (Fig. 2). To further evaluate the proliferative effects of these compounds, cell numbers were examined 1 or 7 days after mitogenic stimulation of quiescent cultures with 10% FBS. Only BaP/TCDD cells exhibited a significant enhancement in cell numbers from days 1 to 7 (Fig. 3). These data indicate that the combined treatment of GMCs with BaP and TCDD cells induces a highly proliferative phenotype. The reproducibility of these responses was confirmed in a separate dosing regimen using a different cell strain where the same profiles were observed (data not shown).

4. DISCUSSION

Compensatory proliferation of GMCs following renal injury is considered a key early step in the pathogenesis of glomerular disorders [23]. In fact, renal pathologic lesions

involving proliferation and/or expansion of GMCs comprise a significant percentage of glomerulonephritides 2). In this study, we present evidence GMCs exposed to one cycle of sequential chemical treatments with BaP and TCDD exhibit depressed DNA synthetic rates, while three rounds of treatment promote the acquisition of a highly proliferative phenotype.

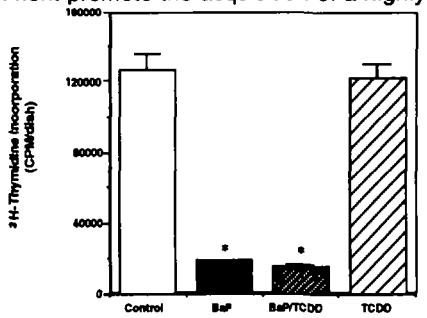

Figure 1. [^{3}H]Thymidine Incorporation into DNA of Subcultured Glomerular Mesangial Cells After One Cycle of Sequential Chemical Treatments With Benzo(a)pyrene (3 mM) and 2,3,7,8-TCDD (1 nM). Randomly cycling GMCs were challenged with test chemicals and processed for measurements of [^{3}H]thymidine incorporation as described in methods. Experiments were conducted in duplicate using three or four replicate dishes/group. * Significantly different from respective control (P<0.05).

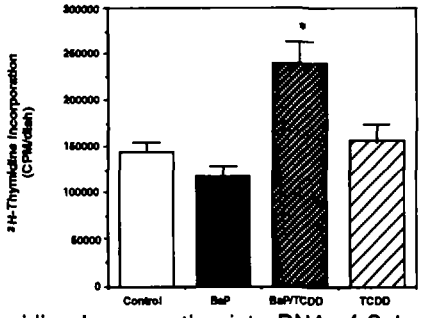

Figure 2. [^{3}H]Thymidine Incorporation into DNA of Subcultured Glomerular Mesangial Cells After Three Cycles of Sequential Chemical Treatments With Benzo(a)pyrene and 2,3,7,8-TCDD. Randomly cycling GMCs were challenged with test chemicals and processed for measurements of [^{3}H]thymidine incorporation as described in methods. Experiments were conducted in duplicate using three or four replicate dishes/group. * Significantly different from respective control (P<0.05).

The initial inhibition of DNA synthesis elicited by BaP alone or in combination with TCDD is consistent with previous studies which showed that BaP interferes with early G_1 cell cycle progression [14]. This inhibitory response is comparable to that observed in other cells of mesenchymal origin [24], and likely involves oxidative metabolism of the parent compound. BaP undergoes bioactivation by the sequential actions of cytochrome P-450 and epoxide hydrolase to form BPDE, a reactive epoxide which preferentially binds to guanine nucleotides with DNA to initiate genotoxid effects [25,26]. BPDE binds to, and inhibits DNA polymerase-a activity in human lymphocytes [27] and blocks chain elongation

transiently by binding within DNA replication forks [28]. GMCs possess inducible aryl hydrocarbon hydroxylase activity which metabolizes BaP to reactive intermediates that bind to DNA [9]. These cytochrome P450-derived reactive intermediates could induce permanent genomic mutations leading to growth deregulation as reported in other cell systemes.

The expression of a proliferative phenotype in GMCs following repeated cycles of chemical treatments with BaP in combination with TCDD is reminiscent of the growth deregulation observed using an initiation/promotion strategy in a skin model of carcinogenesis [29]. In this model, DNA damage by a genotoxic agent is followed by clonal expansions of initiated cells leading to the appearance of a population of cells with altered phenotypes. PAHs have been identified as initiating agents in mesenchymal cells [30] and TCDD promotes the development of tumors or tumor-like lesions of mesenchymal origin [31-33]. Previous studies in this laboratory have shown that methoxamine also enhances the acquisition of a proliferative phenotype in GMCs exposed to BaP [14]. Since methoxamine modulates gene expressin by a different mechanism than that of TCDD, these studies suggest that irrespective of the mechanism of promotion, BaP can alter genetic programs to induce a highly proliferative state in GMCs.

GMCs challenged with BaP in combination with TCDD exhibited the most pronounced alterations in proliferative behavior relative to the other groups tested. The mechanism of promotion by TCDD presumably involves ligand interaction with the aryl hydrocarbon receptor (AhR). The ligand activated AhR complex binds to consensus sequences known as dioxin responsive elements (DREs) found upstream of a number of growth-related genes. Puga et al. [15] have shown that TCDD binding to the AhR enhances the expression of c-*jun* and c-*fos* in Hep1 cells. We have demonstrated that binding of the AhR complex to a DRE is associated with induction of c-Ha-*ras* and c-*myc* in vascular smooth muscle cells [16].

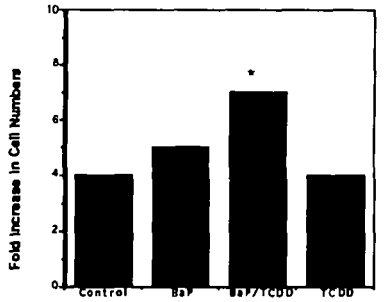

Figure 3. Relative Cell Numbers of Subcultured Glomerular Mesangial Cells After Three Cycles of Sequential Chemical Treatments With Benzo(a)pyrene and 2,3,7,8-TCDD. Randomly cycling GMCs were challenged with test chemicals and processed for measurements of cell growth as described in methods. Experiments were conducted in duplicate using three or four replicate dishes/group. * Significantly different from control counterpart ($P<0.05$).

In summary, our study demonstrates that injury of cultured GMCs by BaP followed by treatment with TCDD induces a highly proliferative phenotype. The ability of environmental chemicals to modulate GMC growth *in vitro*, coupled with recent reports implicating aromatic hydrocarbons as etiologic agents in glomerular disease, raise provocative questions regarding the impact of environmental agents, and specifically BaP and TCDD, in

the onset and progression of glomerular diseases involving aberrant GMC proliferation. (Supported by NIEHS Superfund Program Project Grant 04917).

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