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Antiproliferative and antiangiogenic effects of 3-methylcholanthrene, an aryl-hydrocarbon receptor agonist, in human umbilical vascular endothelial cells

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Abstract

There is increasing interest in the effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) and polycyclic aromatic hydrocarbons on cardiovascular diseases. Their chemical structures are similar, although polycyclic aromatic hydrocarbons contain no chlorine as does TCDD. The biochemical mechanism of their action is mainly mediated by the aryl hydrocarbon receptor. In addition, oxidative stress also plays a role in the biological and toxic effects of these chemicals. In this study, we used an aryl hydrocarbon receptor agonist, 3-methylcholanthrene (3-MC), to investigate its effect on the proliferation and angiogenesis of human umbilical vascular endothelial cells. 3-MC suppressed DNA synthesis of human umbilical vascular endothelial cells as determined by [^{3}H]thymidine incorporation in a concentration-dependent fashion and arrested cells at the G0/G1 phase of the cell cycle. Interestingly, the inhibition of DNA synthesis by 3-MC was eliminated to a greater extent by aryl hydrocarbon receptor antagonists, alpha-NF (0.5 and 1 μM) and resveratrol (5 and 10 μM), than by the antioxidant, *N*-acetylcysteine (5 and 10 mM). Cell permeability, adhesion, and tube formation in human umbilical vascular endothelial cells exposed to 3-MC decreased in concentration-dependent manners. We also demonstrated that cell adhesion signaling (phosphorylated focal adhesion kinase (FAK)) decreased upon 3-MC treatment, suggesting that cell adhesion inhibited by 3-MC might be due to inhibition of cell adhesion signaling. Additionally, alpha-naphthoflavon (alpha-NF) ameliorated the effects of 3-MC on cell permeability, adhesion and tube formation, indicating the involvement of the aryl hydrocarbon receptor in angiogenesis. The results suggest that the adverse effects of 3-MC are mainly mediated by the aryl hydrocarbon receptor and not via increased oxidative stress.

Keywords: Aryl-hydrocarbon receptor; 3-Methylcholanthrene; Dioxin; Polycyclic aromatic hydrocarbons; Angiogenesis

1. Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) and polycyclic aromatic hydrocarbons are ubiquitous environmental pollutants, of which the former was discovered to be a contaminant in the herbicide Agent Orange and the latter are formed as by-products of the combustion of diverse organic substances (Massaad et al., 2002). These chemicals accumulate in adipose tissue and can produce a variety of effects, such as hepato-, immuno-, neuro-, dermal, and reproductive toxicities as well as cancer in both humans and animals (Brouwer et al., 1995).

The effects of TCDD and polycyclic aromatic hydrocarbons on cardiovascular diseases have elicited much attention lately, including inhibition of coronary vasculogenesis and increases in the number and size of atherosclerotic lesions (Paigen et al., 1985; Ivnitski-Steele and Walker, 2003). Several experimental studies have demonstrated that repeated exposure of avian and rodent species to polycyclic aromatic hydrocarbons such as benzo[a]pyrene,7,12-dimethylbenz(a)anthracene, and 3-

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methylcholanthrene (3-MC) is associated with the development of vascular lesions of atherosclerotic etiology (Revis et al., 1984; Paigen et al., 1985). It has been reported that TCDD induces cardiac structural malformations and disrupts cardiovascular morphology and function in the chicken embryo (Canga et al., 1993; Walker and Catron, 2000; Ivnitski-Steele and Walker, 2003). Furthermore, TCDD-reduced coronary vascular development in chick embryos in vivo has been shown to be ameliorated by vascular endothelial growth factor (Ivnitski-Steele and Walker, 2003).

Biochemical and genetic evidence has indicated that the biochemical mechanism of the actions of TCDD and polycyclic aromatic hydrocarbons is mediated by the aryl hydrocarbon receptor. The aryl hydrocarbon receptor, a ligand-activated transcription factor, is the receptor for polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and TCDD. Mechanistically, TCDD diffuses across the plasma membrane and binds to the aryl hydrocarbon receptor complex present in the cytoplasmic compartment (Ma and Whitlock, 1997). Ligand aryl hydrocarbon receptor complexes are subsequently translocated into the nucleus, and following their association with the nuclear Arnt (aryl hydrocarbon receptor nuclear translocator) protein, the ligand-aryl hydrocarbon receptor-Arnt complex is able to bind to the dioxin responsive element (DRE) on DNA and initiate transcription of genes including cytochrome P-450 (CYP) (Sogawa et al., 1986; Whitlock, 1987; Denison and Whitlock, 1995; Hankinson, 1995).

TCDD was reported to produce oxidative stress through CYP1A induction in vascular endothelium, resulting in local circulatory failure and apoptosis in the dorsal midbrain of zebrafish embryos (Dong et al., 2002; Chen et al., 2004). CYP1A1- and CYP1B1-catalyzed catechol estrogen formation in TCDD-induced oxidative damage was eliminated by resveratrol, an aryl hydrocarbon receptor antagonist and antioxidant, by blocking the metabolic formation of catechol estrogens and scavenging the reactive oxygen species generated during their redox cycling. It was also documented that TCDD inhibits cell proliferation through aryl hydrocarbon receptor-mediated G1 arrest in SK-N-SH human neural cells, which is significantly prevented by pretreatment with alpha-naphthoflavone (alpha-NF), a partial aryl hydrocarbon receptor antagonist.

Angiogenesis, the formation of new blood vessels from preexisting vessels, occurs extensively during embryonic development and in wound healing. TCDD has been shown to inhibit coronary vasculogenesis (Ivnitski-Steele and Walker, 2003). We attempted to investigate whether in addition to TCDD, other similar chemicals acting as aryl hydrocarbon receptor agonists have corresponding effects on vasculogenesis. In this study we investigated the potential mechanism and the adverse effects of the aryl hydrocarbon receptor agonist, 3-MC, on cell proliferation and angiogenesis in human umbilical vascular endothelial cells and their dependence on the aryl hydrocarbon receptor. Furthermore, the effects of the aryl hydrocarbon receptor antagonists such as alpha-NF and resverarol, and the antioxidant, *N*-acetylcysteine, on the antiproliferation and antiangiogenesis of 3-MC were examined.

2. Materials and methods

2.1. Materials

3-MC, resveratrol, alpha-naphthoflavon (alpha-NF), *N*-acetylcysteine, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), HEPES, and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical (St. Louis, MO, USA). Culture medium 199 (M199), fetal bovine serum (FBS), and tissue culture reagents were obtained from Invitrogen (Carlsbad, CA, USA). Protein assay agents were purchased from Bio-Rad (Hercules, CA, USA).

2.2. Cell culture and media

Human umbilical vein endothelial cells were isolated from an umbilical cord vein by collagenase treatment as described previously (Jaffe et al., 1973) and grown in M199 supplemented with 10% FBS, an endothelial cell growth supplement (0.03 mg/ml), kanamycin (50 U/ml), and 50 units/ml heparin (Sigma) in a humidified 37 °C incubator. After the cells had grown to confluence, they were dispersed in a trypsin solution, washed with phosphate-buffered saline (PBS), and mixed with M199 containing 10% FBS, then centrifuged at 125 ×*g* for 5 min, resuspended, and subcultured according to standard protocols. Cells from passages 5~9 were used.

2.3. [^{3}H]Thymidine incorporation

As previously described (Jain et al., 1996; Lin et al., 2002; Shih et al., 2004), human umbilical vascular endothelial cells at a density of 2×10^4 cells/cm^3 were applied to 24-well plates in growth medium (M199 plus 10% FBS). After the cells had grown to 70~80% confluence, they were rendered quiescent by incubation for 24 h in M199 containing 2% FBS. The concentration of 3-MC as indicated or dimethyl sulfoxide (DMSO) in 10% FBS was added to the cells, and the mixture was allowed to incubate for 24 h. During the last 4 h of the incubation with or without 3-MC, [^{3}H]thymidine was added at 1 μCi^{-1} (1 μCi=37 kBq). The incorporated [^{3}H]thymidine was extracted in 0.2 N NaOH and measured in a liquid scintillation counter.

2.4. Flow cytometry

Cells were centrifuged at 1500 ×*g* for 10 min and washed twice with ice-cold PBS. Cells were fixed at 1 ml alcohol (70%) and left at −20 °C for at least 1 h. Cell numbers with tryptan blue staining were counted using a microscope. One milliliter of PBS-based reaction mixture containing propidium iodide (5 μg/ml), RNase (50 μg/ml), and 0.1% Triton X-100 (Sigma) was added to the cell samples at a density of 1×10^6 cells/ml and incubated in the dark for 30 min prior to the analysis of DNA content by flow cytometry.

2.5. Adhesion assay

The adhesion assay was performed as previously described (Aguzzi et al., 2004). human umbilical vascular endothelial cells (2.5×10^4 cells/well) were allowed to adhere onto 24-well tissue culture plates coated with 1% gelatin or 0.5 mg/ml mouse collagen-IV (Becton-Dickinson, Bedford, MA, USA). Adhesion was allowed to progress for 1 h at 37 °C in the presence or absence of 3-MC. Plates were washed with PBS and incubated for another 20 h. The amounts of cells adhering to the plates were determined by an MTT assay and measurement at a wavelength of 570 nm.

2.6. Immunoprecipitation and Western blot analysis

To prepare whole-cell lysates, cells were washed twice with ice-cold PBS, resuspended in ice-cold extraction buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% Na deoxycholate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, and 1 μg/ml aprotinin). Focal adhesion kinase (FAK) was immunoprecipitated from 200 μg protein using an anti-FAK antibody (2 μg) (BD Biosciences) and Protin G PLUS-Agarose beads (20 μl) (Santa Cruz, CA, USA) for overnight incubation at 4 °C on a rotation wheel. The precipitates were washed five times with washing buffer (20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40). The pellet was then resuspended in sample buffer (50 mM Tris (pH 6.8), 100 mM bromophenol blue, and 10% glycerol) and incubated at 90 °C for 10 min before electrophoresis to release the proteins from the beads. The samples were electrophoresed on a 10% SDS-polyacrylamide gel and then transblotted onto a Hybond-P membrane (Pharmacia, Hong Kong). Membranes were blocked in PBS containing 0.1% Tween-20 and 5% skim milk at room temperature for 30 min. Western blot analysis was carried out using the following antibodies: aryl hydrocarbon receptor from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA), Cyp1A1 from Oxford Biomedical Research (Oxford, Michigan, USA), phosphotyrosine from Upstate Biotechnology (Lake Placid, NY, USA) and FAK. Blots were incubated with the indicated antibodies (with dilutions used according to the manufacturer's instructions), in blocking buffer for 1 h at room temperature. After 3 washes with PBS containing 0.1% Tween-20, blots were incubated with peroxidase-conjugated goat anti-mouse immunoglobulin G (1 : 2000) for 1 h at room temperature, followed by another washing. Expression of protein was detected by an enhanced chemiluminescence system.

2.7. Cell migration assay

Endothelial-cell migration assays were performed using a 24-well microchemotaxis chamber (Costar, Cambridge, MA, USA) (Falk et al., 1980). Polyvinylpyrrolidone-free polycarbonate filters with a pore size of 8 μm (Nuclepore, North Point, Hong Kong) were coated with 1 mg/ml collagen for 30 min in a 37 °C incubator. 3-MC at 1 mM was diluted to appropriate concentrations in M199 supplemented with 10% FBS, and 600 μl of the final dilution was placed in the lower chamber of a microchemotaxis chamber. Confluent cell cultures were washed and trypsinized for the minimum time required to achieve cell detachment. After placing the filter between the lower and upper chambers, 2.5×10^4 cells suspended in 100 μl of M199 containing 2% FBS and indicated concentrations of 3-MC were seeded in the upper compartment. The apparatus was then incubated for 20 h at 37 °C in a humidified chamber with 5% CO_2 to allow cell migration. After the incubation period, the filter was removed, and nonmigrated cells on the upper side of the filter were scraped away with a cotton applicator. The filter was fixed for 30 min at room temperature with 4% paraformaldehyde, washed 2 times with PBS and stained with crystal violet. The number of cells that had migrated through to the lower surface of each membrane was counted. Each experimental point was repeated 6 times.

2.8. In vitro tube formation assay

Wells of a 96-well plate were coated with 50 μl Matrigel (BD Biosciences, San Jose, CA, USA) and allowed to gel by incubating at 37 °C for 30 min. Human umbilical vascular endothelial cells were then seeded onto the Matrigel-coated wells at a density of 1×10^4 cells/well. 3-MC was added to each well at a concentration of 0, 5, 50, or 500 nM and incubated for 4 h at 37 °C. Cells that had formed differentiated tubes were photographed under a microscope equipped with a camera. The experiment was repeated 5 times.

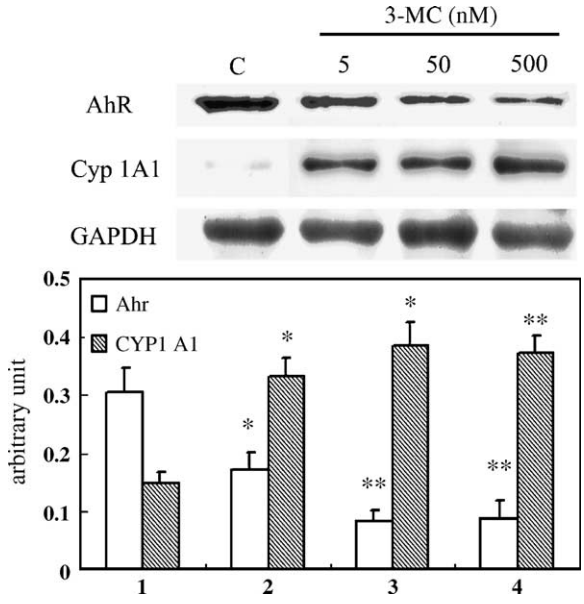
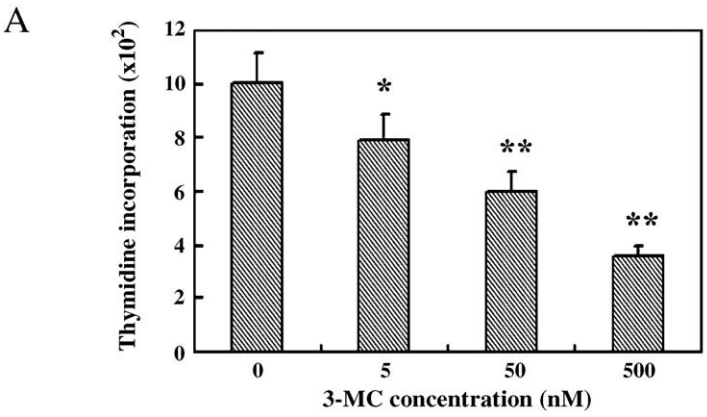
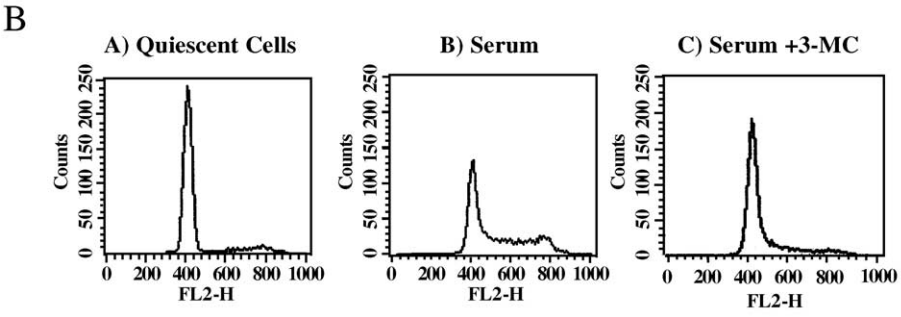


Fig. 1. Down-regulation of aryl hydrocarbon receptor and induction of Cyp1A1 by 3-MC. Cells were exposed to the indicated concentrations of 3-MC in M199 supplemented with 10% fetal bovine serum for 3 h. Crude cell lysates were prepared, and Western blot analysis of aryl hydrocarbon receptor and Cyp1A1 in cells exposed to increasing concentrations of 3-MC was examined. GAPDH was used as an internal control. A representative experiment is shown. Bar charts in the lower panel show the band intensity of aryl hydrocarbon receptor and Cyp1A1 proteins by densitometry. Data were derived from three independent experiments and are presented as the mean±S.D. $*P<0.05$ and $**P<0.01$ indicate significant differences from the DMSO-treated group.





	G0/G1	S	G2/M
Quiescence	92.25%	2.20%	5.55%
Serum	50.38%	25.23%	24.39%
3-MC	82.20%	10.60%	7.20%

Fig. 2. Effects of 3-MC on [^{3}H]thymidine incorporation and cell cycle arrest in human umbilical vascular endothelial cells. (A) 3-MC inhibited DNA synthesis in a concentration-dependent manner. To study the effect of 3-MC on cell proliferation, [^{3}H]thymidine incorporation was investigated by incubation in culture media supplemented with 10% fetal bovine serum, DMSO, or indicated concentrations of 3-MC in DMSO. Data shown are the mean ± S.D. of 5 independent experiments. *$P<0.05$ and **$P<0.01$ indicate significant differences from the DMSO-treated group. (B) 3-MC abrogated serum-induced cell cycle progression of human umbilical vascular endothelial cells. Cells were incubated in 2% serum M199 for 24 h to induce quiescence (A), followed by the addition of serum for 20 h (B) or serum plus 50 nM 3-MC for 20 h (C). The cell cycle was analyzed. The cell number is represented on the *y*-axis and DNA content on the *x*-axis. Results of a representative experiment are shown.

2.9. Statistical analysis

Values are expressed as the mean ± S.D. The significance of the difference between the control and each experimental test condition was analyzed by Student's *t*-test. A value of $P<0.05$ was considered statistically significant. $^{\#}P<0.05$ and $^{\#\#}P<0.01$ demonstrated a significant difference between indicated groups, as analyzed by paired Student's *t*-test.

3. Results

3.1. Down-regulation of aryl hydrocarbon receptor and induction of Cyp1A1 by 3-MC

To confirm whether the human umbilical cord endothelial cells express the aryl hydrocarbon receptor and that after exposure to 3-MC it is down-regulated by the 3-MC ligand binding. After 3 h of incubation with the concentrations of 3-MC from 5 to 500 nM, cell lysates were prepared, and protein level of aryl hydrocarbon receptor in treated cells were assessed by Western blot analysis. In Fig. 1, a 95 kDa protein band corresponding to aryl hydrocarbon receptor was concentration-dependently degraded in cells exposed to 3-MC, which agrees with the results demonstrated by Ma and Baldwin (2000). Additionally, aryl hydrocarbon receptor target gene, Cyp1A1, was up-regulated by the increasing concentrations of 3-MC (Fig. 1). Likewise, these results confirm that 3-MC is a ligand for aryl hydrocarbon receptor in endothelial cells and consequently results in the degradation of the aryl hydrocarbon receptor similar to the finding by using TCDD (Ma and Baldwin, 2000).

3.2. Inhibition of [^{3}H]thymidine incorporation into human umbilical vascular endothelial cells and arrest of the cell cycle at G0/G1 by 3-MC

To study the effect of 3-MC on cell proliferation, [^{3}H] thymidine incorporation (a measurement of DNA synthesis) was investigated by incubation in culture media supplemented with 10% fetal bovine serum, DMSO, or indicated concentrations of 3-MC in DMSO after cells were quiescent as described in Materials and methods. As illustrated in Fig. 2A, 3-MC

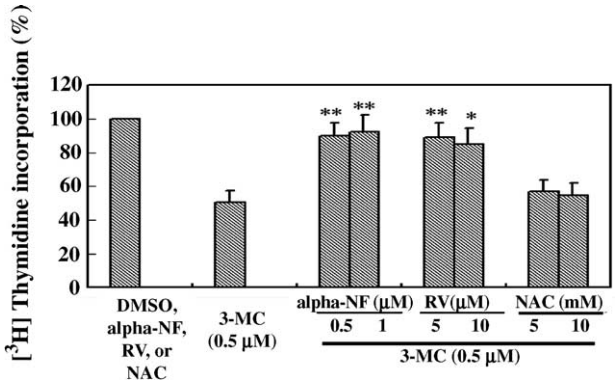


Fig. 3. Effects of the aryl hydrocarbon receptor antagonists, alpha-NF and resveratrol, and the antioxidant, *N*-acetylcysteine, on DNA synthesis in human umbilical vascular endothelial cells exposed to 3-MC. Cells plated at 2×10^4 cells/well in 24-well plates were pretreated with alpha-NF, resveratrol (RV), and *N*-acetylcysteine (NAC) for 30 min prior to adding DMSO or 3-MC, as described in the text. The method for the [^{3}H]thymidine incorporation assay is described in Materials and methods. The value of the [^{3}H]thymidine incorporation assay in the presence of a respective aryl hydrocarbon receptor antagonist or *N*-acetylcysteine without additional 3-MC was used as a standard (100%). Bars are the percentages of the amount of [^{3}H]thymidine incorporation with additional 50 nM 3-MC treatment with respect to that of each individual pretreatment. Data were derived from 5 independent experiments and are presented as the mean±S.D. *$P<0.05$ and **$P<0.01$ indicate significant differences from the 3-MC-treated group.

inhibited [^{3}H]thymidine incorporation into human umbilical vascular endothelial cells. This inhibition was dose-dependent, and additionally it occurred at a dose of 3-MC of as low as 500 pM (data not shown).

In order to further examine the actions of 3-MC on the cell cycle, cells were cultured in media with 2% FBS for 24 h to synchronize their cell cycle activities. Fig. 1B shows the fluorescence-activated cell sorter (FACS) analyses of DNA contents at various times by incubation in culture media supplemented with 10% FBS and 0.1% DMSO or 50 nM 3-MC in 0.1% DMSO. The data revealed that 3-MC induced a significant accumulation of cells at the G0/G1 phase of the cell cycle, suggesting the observed growth inhibition effect of 3-MC was due to arrest of DNA replication, which thereby inhibited further progression of the cell cycle.

3.3. Attenuation of 3-MC-induced inhibition of DNA synthesis by aryl hydrocarbon receptor antagonists, but not by the antioxidant, N-acetylcysteine

Given that the toxic effects of 3-MC might occur through the aryl hydrocarbon receptor and oxidative stress, we utilized the aryl hydrocarbon receptor antagonists, alpha-NF and resveratrol, and the antioxidant, *N*-acetylcysteine, to examine 3-MC-induced inhibition of DNA synthesis to determine which mechanism plays a major role. Cells plated at 2×10^4 cells/well in 24-well plates were treated with alpha-NF (0.5 or 1 μM), resveratrol (5 or 10 μM), or *N*-acetylcysteine (5 and 10 mM) for 30 min prior to incubation with 50 nM 3-MC for 24 h. As shown in Fig. 3, *N*-acetylcysteine had almost no effect, whereas alpha-NF and resveratrol significantly eliminated the 3-MC-induced inhibition of DNA synthesis.

3.4. Inhibition of cell adhesion and migration in human umbilical vascular endothelial cells exposed to 3-MC and reverse of these effects by alpha-NF

When human umbilical vascular endothelial cells were plated on a collagen or gelatin-coated plate, the cells spread out well evenly. However, with treatment using 3-MC, the cells were rounded and their spreading out on the plates was inhibited. Fig. 4A shows that 3-MC inhibited human umbilical

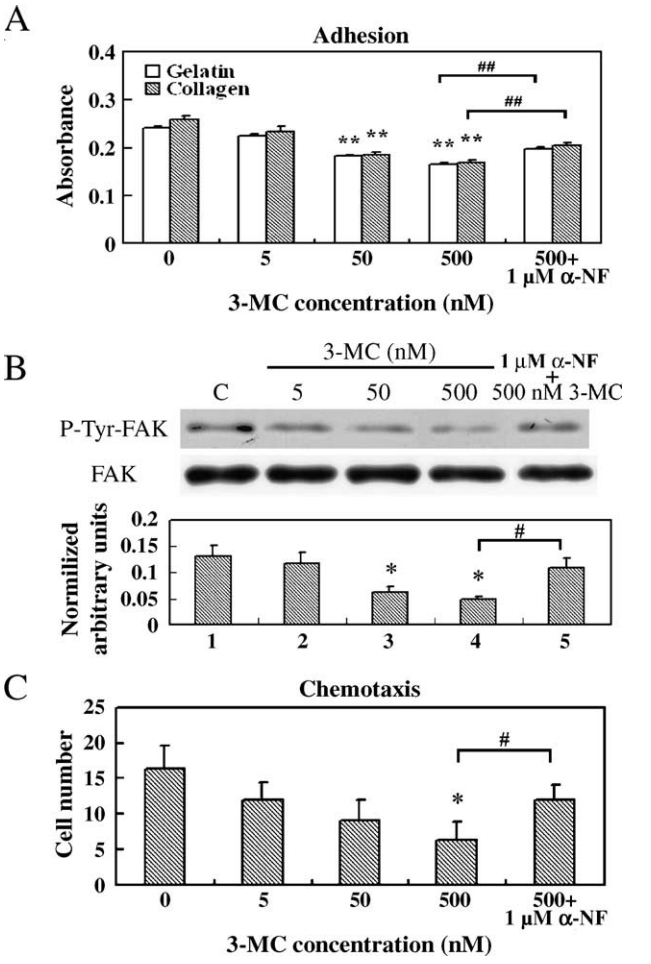


Fig. 4. Effects of 3-MC on cell adhesion (A), cell adhesion signaling (B) and cell migration in human umbilical vascular endothelial cells (C). (A) human umbilical vascular endothelial cells adhesion to gelatin or collagen was evaluated. Cells were seeded in the presence of 10% FBS and treated for 1 h at 37 °C with serial dilutions of 3-MC. Cell adhesion was then quantified using the MTT assay and measured at a wavelength of 570 nm. 3-MC reduced cell adhesion to 1% gelatin or 0.5 mg/ml collagen in a concentration-dependent manner. (B) To assay cell adhesion signaling upon 3-MC treatment in human umbilical vascular endothelial cells, cell lysates were immunoprecipitated with an anti-FAK antibody and immunoblotted with anti-phosphotyrosin and anti-FAK antibodies. Bar charts in the lower panel show the band intensity of normalized P-Tyr-FAK by densitometry. Data were derived from 3 independent experiments and are presented as the mean±S.D. (C) The chemotactic migration of human umbilical vascular endothelial cells was carried out in microchemotaxis chambers. Human umbilical vascular endothelial cells were exposed to increasing concentrations of 3-MC as described in Materials and methods. Six samples were analyzed in each group, and values represent the mean±SD; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. 500 nM 3-MC, *$P<0.05$ and **$P<0.01$ vs. DMSO-treated group.

vascular endothelial cells adhesion to collagen or gelatin in a dose-dependent manner, which were partially reversed by alpha-NF. We also set out to determine the effect of 3-MC on the cell adhesion signaling pathway. As demonstrated in Fig. 4B, 3-MC dose-dependently inhibited the phosphorylation of FAK and alpha-NF can block the inhibition, These results presented in this study suggest that the antiadhesion effect of 3-MC is through aryl hydrocarbon receptor and the adhesion signaling pathway is involved.

The effect of 3-MC on human umbilical vascular endothelial cells migration was evaluated using a microchemotaxis chamber. The data presented in Fig. 4C demonstrate that there was a dose-dependent inhibition of the chemotactic migration of human umbilical vascular endothelial cells. Inhibition of the chemotactic migration was significant at a dose of 5 nM and maximal at 500 nM for 3-MC.

3.5. Attenuation of antiangiogenic effect of 3-MC by alpha-NF

To confirm the antiangiogenic effect of 3-MC, we conducted a capillary-like tube formation assay. As illustrated in Fig. 5, 3-MC dose-dependently inhibited capillary-like tube formation. To our knowledge, this is the first demonstration of the antiangiogenic effect of 3-MC in human umbilical vascular endothelial cells.

It is known that 3-MC exerts both biological and toxic effects via the aryl hydrocarbon receptor; nevertheless, oxidative stress derived from 3-MC might also be attributable to its effects on cellular damage (Dong et al., 2002). Therefore, an aryl hydrocarbon receptor antagonist, alpha-NF and an antioxidant, *N*-acetylcysteine, were included to investigate their effects on the anti-tube formation property of 3-MC. Interestingly, alpha-NF at the concentrations of 0.5 and 1 μM partially reversed the antiangiogenic effect of 3-MC to the extent of cells treated with alpha-NF alone, as shown in Fig. 5e∼h. However, the anti-tube formation property of 3-MC was not affected by treatment with 5 or 10 mM *N*-acetylcysteine (data not shown).

4. Discussion

In the present study, we examined the adverse effects of an aryl hydrocarbon receptor agonist, 3-MC, on cell proliferation and angiogenesis, and the dependence on the aryl hydrocarbon receptor using a human endothelial cell system. We have demonstrated that cell adhesion as well as the chemotactic migration of endothelial cells was inhibited by 3-MC in a concentration-dependent manner. Interestingly, the inhibition of cell adhesion by 3-MC occurs through inhibition of the cell adhesion signaling pathway, as presented in Fig. 4B which shows that 3-MC inhibited the phosphorylation of FAK. These adverse effects of 3-MC on the properties of angiogenesis could be partially reversed by an aryl hydrocarbon receptor, alpha-NF.

Ivnitski-Steele and Walker (2003) demonstrated that TCDD inhibits coronary vasculogenesis using coronary blood vessels derived from the epicardium of a chick embryo, which could be rescued by vascular endothelial growth factor. Furthermore, Ivnitski-Steele et al. (2005) showed that TCDD inhibition of coronary vasculogenesis is associated, in part, with the reduced VEGF-A secretion in response to endogenous angiogenic stimuli. In agreement with previous findings using a similar chemical to exert its actions through the aryl hydrocarbon receptor, 3-MC also inhibited the angiogenic effect of endothelial cells, with evidence of the inhibition of capillary-like tube formation in human umbilical vascular endothelial cells (Fig. 5). Furthermore, the antiangiogenesis of 3-MC could be partially inhibited by the aryl hydrocarbon receptor antagonist, alpha-NF, but not by the antioxidant, *N*-acetylcysteine (data not

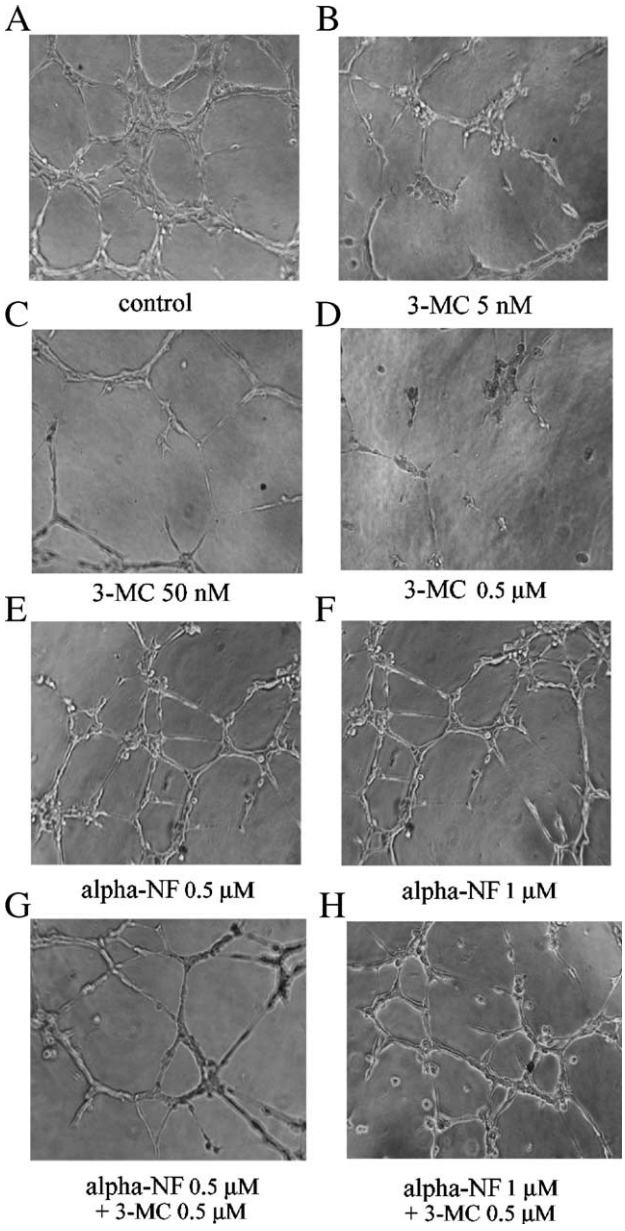


Fig. 5. Inhibition of capillary-like tube formation in human umbilical vascular endothelial cells by 3-MC in Matrigel. Human umbilical vascular endothelial cells were suspended in M200 media supplemented with 10% FBS and an endothelial cell growth supplement and were plated onto a layer of Matrigel at a density of 1×10^4 cells/well without (A) or with 3-MC at a concentration of 5 (B), 50 (C) or 500 nM (D). Additionally, cells were pretreated with alpha-NF at a concentration of 0.5 (E) or 1 μM (F) for 30 min, before cells were challenged with 500 nM of 3-MC (G and H). The method is described in Materials and methods. (magnification: a∼h ×100).

shown). The results suggest that aryl hydrocarbon receptor is involved in the antiangiogenesis of 3-MC in human umbilical vascular endothelial cells.

Jin et al. (2004) showed that TCDD suppresses DNA synthesis of SK-N-SH human neuronal cells. Similarly, the results of our study using the human endothelial system also showed that 3-MC inhibited [^{3}H]thymidine incorporation into human umbilical vascular endothelial cells and arrested cells at the G0/G1 phase of the cell cycle, as shown in Fig. 2. Therefore, the use of similar chemicals, such as TCDD and 3-MC, to exert their actions through the aryl hydrocarbon receptor, revealed that they exerted similar effects on human neuronal and endothelial cells, although benzo[a]pyrene has been shown to stimulate cell proliferation by modulation of proto-oncogene expression in rat aortic smooth muscle cells (Sadhu et al., 1993).

TCDD-suppressed DNA synthesis in neuronal cells is significantly prevented by pretreatment with either alpha-NF, a partial aryl hydrocarbon receptor antagonist, or 8-methoxypsoralen (MOP), a binding inhibitor of activated aryl hydrocarbon receptor to dioxin responsive element (Jin et al., 2004). Given that TCDD-initiated toxicity can be mediated by the aryl hydrocarbon receptor pathway and/or via increased oxidative stress (Dong et al., 2002), an antioxidant such as *N*-acetylcysteine was included to examine this possibility. In our study, administration of the antioxidant, *N*-acetylcysteine, had almost no effect against 3-MC-induced growth inhibition in human umbilical vascular endothelial cells. Nevertheless, alpha-NF or resveratrol exposure resulted in a significant decrease in 3-MC-induced inhibition of DNA synthesis, as shown in Fig. 3. These results suggest that 3-MC-initiated inhibition of DNA synthesis in human umbilical vascular endothelial cells is mediated by the aryl hydrocarbon receptor and does not occur via increased oxidative stress. Consistent with the present findings, a recent study by Chan et al. (2004) also demonstrated that TCDD-induced homologous recombination in CHO 3–6 cells is not mediated by oxidative DNA damage, but does involve the AhR pathway.

In conclusion, results of our present study demonstrate that 3-MC is a potent antiadhesive and antichemotactic molecule in human umbilical vascular endothelial cells. The antiproliferative and antiangiogenic effects of 3-MC occur mainly through the aryl hydrocarbon receptor, not through oxidative stress. These findings may contribute to the understanding of the potency of an aryl hydrocarbon receptor agonist in inducing vasculotoxicity and its potential mechanism.

Acknowledgments

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