Transformation of Mouse Liver Cells by Methylcholanthrene Leads to Phenotypic Changes Associated with Epithelial-mesenchymal Transition

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Environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs) have been implicated in cancer development and progression. However, the effects of PAHs on carcinogenesis are still poorly understood. Here, we characterized a mouse cancer cell line BNL 1MEA.7R.1 (1MEA) derived by transformation of non-tumorigenic liver cell line BNL CL.2 (BNL) using 3-methylcholanthrene (3MC), a carcinogenic PAH. RT-PCR and immunoblot analysis were used to determine the expression level of mRNA and proteins, respectively. To determine functionality, cell motility was assessed in vitro using a transwell migration assay. Both mRNA and protein levels of E-cadherin were significantly decreased in 1MEA cells in comparison with BNL cells. While the expression levels of mesenchymal markers and related transcription factors were enhanced in 1MEA cells, which could lead to increase in cell motility. Indeed, we found that 7-day exposure of BNL cells to 3-MC reduced the level of the adhesion molecule and epithelial marker E-cadherin and increased reciprocally the level of the mesenchymal marker vimentin in a dose-dependent manner. Taken together, these results indicate that the process of epithelial-mesenchymal transition (EMT) may be activated during premalignant transformation induced by 3-MC. A mechanism study to elucidate the relation between 3-MC exposure and EMT is underway in our laboratory.

Key words: Environmental pollutant, Polycyclic aromatic hydrocarbon, 3-Methylcholanthrene, Carcinogenesis, Epithelial-mesenchymal transition

INTRODUCTION

Environmental pollutants such as dioxins and polycyclic aromatic hydrocarbons (PAHs) exert a variety of long term toxic effects in animals and humans, including alterations in development, abnormal immune response, and carcinogenesis (1-4). A large number of these pollutants are potent ligands and activators of aryl hydrocarbon receptor (AhR), which is a ligand-dependent transcription factor (5). Ligand binding leads to AhR dimerization with aryl hydrocarbon receptor nuclear translocator (ARNT) followed by transcriptional activation of several phase I and phase II xenobiotic-metabolizing enzymes, such as cytochrome P4501A and glutathione-S-transferase, respectively (6,7). Because phase I enzymes metabolize inert carcinogens to active genotoxins, AhR plays a pivotal role in tumor initiation (7). In addition to this typical route, AhR mediates tumor promotion and recent evidence suggests that the AhR could play a role in tumor progression (8-10). However, so far, there was no clear mechanistic evidence of a connection between the xenobiotic metabolism-mediated classical pathway and AhR-dependent tumor progression.

A hallmark of tumor promotion is unbalanced proliferation, whereas tumor progression is characterized by dedifferentiation, increased motility and invasiveness of tumor cells. Tumor promotion and progression are triggered by loss of cell–cell contact (11). Disruption of E-cadherin- and
desmosomes-mediated cell-cell adhesion permits the epithelial cells not only to undergo uncontrolled proliferation but also to dedifferentiate to a mesenchymal phenotype which could induce cell migration, a process referred to as epithelial–mesenchymal transition (EMT) (12-16). Increasing evidence is provided that the AhR can stimulate EMT and migration in several cell lines (17-19). Exposure of the human breast cancer epithelial cell line MCF-7 to 2,3,7,8-tetrachlorodibenzodioxin (TCDD) or 3-methylcholanthrene (3-MC) leads to down-regulation of E-cadherin, loss of cell-cell adhesion and increased mobility of the cells (17). In search for an AhR target gene in MCF-7 cells, the authors further identified Nedd9/Hef1/Cas-L as a gene most consistently induced in response to 3-MC. Using the human liver hepatoma cell line HepG2, they also showed that Nedd9/Hef1/Cas-L is an AhR target gene, which contains two xenobiotic-responsive elements (XRE) in its promoter and mediates the effects of TCDD and 3-MC on JNK activation and E-cadherin down-regulation (18). Although some important signaling pathways and key players have been identified, it is clear that we are still at the beginning of understanding the role of the AhR in these processes.

3-Methylcholanthrene (3-MC) is one of the most potent carcinogenic PAHs (20). Metabolism of 3-MC by cytochrome P450 enzymes and epoxide hydrolase leads to the formation of chemically reactive intermediates that can bind covalently to DNA, a critical step in the initiation of carcinogenesis (21). Even though several mechanisms mediating its carcinogenic potential are suggested, the effects of 3-MC on carcinogenesis are still poorly understood.

In the present study, in order to investigate the effect of 3-MC on carcinogenesis, we characterized a tumorigenic liver cell line BNL 1ME A. 7R.1 (1MEA) transformed by 3-MC from a non-tumorigenic liver cells BNL CL.2 (BNL). In addition, we examined the regulation of E-cadherin and vimentin following BNL cell exposure to 3-MC for 7 days.

**MATERIALS AND METHODS**

**Materials.** 3-Methylcholanthrene (3-MC) and crystal violet were purchased from Sigma Aldrich (St. Louis, MO, USA). Anti-E-cadherin antibody was obtained from BD Transduction Laboratories (San Jose, CA, USA). Anti-vimentin antibody was purchased from Cell signaling (Danver, MA, USA) and anti-β-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Peroxidase conjugated antibodies against mouse or rabbit IgG were obtained from Thermo scientific (Waltham, MA, USA).

**Cell culture.** BNL CL.2 (BNL) and BNL 1ME A. 7R.1 (1MEA) cell lines were obtained from Korea Research Institute of Bioscience and Biotechnology (KRIIBB, Cheongwon, Chungbuk, Korea). Both cell lines were grown in Dulbecco’s Modified Eagle’s Medium containing 10% Fetal Bovine Serum (FBS) and 100 U/ml penicillin and 100 μg/ml streptomycin (GenDEPOT, Barker, TX, USA) at 37°C in a humidified incubator with 5% CO2. One day before treatment with indicated concentrations of 3-MC, BNL cells were cultured in DMEM without phenol red supplemented with 5% charcoal-stripped FBS and maintained in the same media during all treatments.

**RNA extraction and semi-quantitative RT-PCR.** Total RNA was purified from cells using the RNeasy Mini kit (QIAGEN, Valencia, CA, USA). cDNA synthesis was performed with iScript cDNA Synthesis system (Bio-Rad, Hercules, CA, USA), followed by PCR using GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA). PCR products were analyzed by gel electrophoresis on a 1% agarose (w/v) gel. The sequences of murine specific primers for E-cadherin, N-cadherin, fibronectin, vimentin, Snail, Slug, Twist1, Zeb1, and Zeb2 are provided in Table 1.

**Immunoblot analysis.** Cells were lysed with ice-cold PRO-PREP protein extract solution (iNtRON, Sungnam, Gyeonggi, Korea) and protein concentration was quantified using the BCA procedure (Thermo scientific, Waltham, MA, USA). Equal amounts of protein samples were separated by SDS-PAGE using 10% polyacrylamide gel and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The mem-

| Table 1. List of murine primers used for RT-PCR |
|---|---|---|
| Primer | Forward | Reverse |
| E-Cadherin | 5'-CAAGTGACGTGAGTCCA-3' | 5'-TGATGACCGCTAGTGAAT-3' |
| N-cadherin | 5'-TTTCAAGGGTGCAGGGAGC-3' | 5'-CTTCTCAATGTGAACCCGAGGT-3' |
| Fibronectin | 5'-AGAGGTGGAGGACCTTAC-3' | 5'-GGCTTGAAGAGACTCTG-3' |
| Vimentin | 5'-ATGACGGCTTTGCAAACACT-3' | 5'-GTGCCAGAGAAGCATTGTA-3' |
| Snail | 5'-CACCCTCCTGAGGACCTTC-3' | 5'-CTTCACTCCAGGTTGCT-3' |
| Slug | 5'-GCGAATCTGGACACAAGAAGTTT-3' | 5'-CCCGATGTGAGTTCTAAATTGTGCC-3' |
| Twist1 | 5'-GGGACCGTACATCGACTTC-3' | 5'-TAAAAAGTGTGCGCCACCAGC-3' |
| Zeb1 | 5'-GGGTCAGCTCGATGTTA-3' | 5'-GGGGCGCTCAGGATAATG-3' |
| Zeb2 | 5'-GGGTCAGCTCGATGTTA-3' | 5'-GGGGCGCTCAGGATAATG-3' |
brane was blocked with 5% skim milk in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.2% Tween-20 (TBST) for 1 hr at room temperature. The membranes were incubated with TBST containing 5% milk and the primary antibodies. After three washes with TBST, the blot was incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. The antigen was detected using an Western Bright ECL HRP substrate kit (Advansta, Menlo Park, CA, USA).

Migration assay. Cell migration assay was assessed using 24-transwell migration chamber (Corning Life Sciences, Corning, NY, USA). A total of $3 \times 10^5$ cells were placed in the upper compartment of the migration chamber and incubated for 16 hr at 37°C. The migrating cells at the bottom side of the membranes stained with crystal violet.

RESULTS

BNL and 1MEA cells are morphologically different. As shown in Fig. 1A, BNL cells reserved the morphological characteristics of clustered cobblestone-shaped epithelial cells, whereas 1MEA cells exhibited a fibroblast-like spindle shape (Fig. 1A). To determine whether these morphological changes are associated with EMT, we examined the protein expression of epithelial and mesenchymal markers. Consistent with morphological change, 1MEA cells showed down-regulation of the epithelial marker E-cadherin and up-regulation of the mesenchymal marker vimentin (Fig. 1B). These results suggest that EMT process might be involved in the transformation of BNL cells by 3-MC treatment.

1MEA cells exhibit an EMT-like phenotype compared with BNL cells. To quantify the EMT-like process, we performed RT-PCR analysis for EMT marker genes as well as

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Fig. 1. BNL and 1MEA cells show morphological changes. (A) BNL cells show typical epithelial characteristic, while 1MEA cells show fibroblastic morphology (200X magnification). (B) Immunoblot analysis of E-cadherin and vimentin in BNL and 1MEA cells.

Fig. 2. 1MEA cells transformed by 3-MC induce mesenchymal markers accompanied by EMT-activating transcription factors. RNA expression of (A) EMT markers such as E-cadherin, N-cadherin, fibronectin, and vimentin and (B) EMT-activating transcription factors Snail, Slug, Twist1, Zeb1, and Zeb2 was analyzed using RT-PCR. Each value represents the mean ± SD of three independent experiments. *, **Significantly different from the BNL cells at $p < 0.01, 0.001$, respectively (Student’s t-test).
the EMT master transcription factors. As shown in Fig. 2A, the level of epithelial marker E-cadherin was drastically reduced in 1MEA cells. In contrast, the expression levels of mesenchymal markers fibronectin and vimentin were significantly higher in 1MEA cells than in the BNL cells (Fig. 2A). Consistent with the expression of the EMT markers, the expression of the EMT-activating transcription factors Snail, Slug, Twist1, Zeb1, and Zeb2 was also significantly increased in 1MEA cells (Fig. 2B).

**1MEA cells show higher motile activity than BNL cells in transwell migration assay.** Epithelial cells undergoing EMT lose their adhesive phenotype and concomitantly acquire the migratory phenotype, a functional hallmark of EMT (22). 1MEA cells were more motile than BNL cells, as assessed by a transwell migration assay (Fig. 3). These results suggest that 3-MC-mediated carcinogenesis involves EMT-like process, resulting in more motile phenotype, which might be a functional consequence of EMT critical for tumor cell invasion and metastasis (22).

**Exposure to 3-MC for 7 days induces mesenchymal characteristics in BNL cells.** To further confirm the direct role for 3-MC in EMT, we examined whether 3-MC can regulate the mesenchymal and/or epithelial markers. BNL cells were treated with different concentration of 3-MC for 7 days. After 3-MC exposure, there was a significant increase in mesenchymal marker vimentin expression whereas epithelial marker E-cadherin expression was reciprocally reduced in a dose-dependent manner (Fig. 4).

**DISCUSSION**

This study was undertaken to determine whether the exposure to environmental carcinogen 3-MC could lead to alterations in cellular phenotype. We found that 1MEA cells, derived from BNL cells by transformation with 3-MC, showed down-regulation of the epithelial marker E-cadherin and up-regulation of mesenchymal markers vimentin and fibronectin, accompanied by a dramatic increase in EMT activating transcription factors. Moreover, the exposure of the BNL cells to 3-MC for 7 days induced a phenotypic shift from epithelial to mesenchymal characteristics, as demonstrated by a decrease in E-cadherin and increase in vimentin expression in a concentration-dependent manner.

Although the oncogenic potential of 3-MC is well known, only a few studies have investigated the 3-MC-mediated EMT processes. The zinc finger proteins of the SNAIL superfamily such as SNAI1 (also known as Snail) and SNAI2 (Slug), the Twist family of bHLH factors, and zinc finger and E-box binding proteins of the ZEB family such as Zeb1 and Zeb2 are the typical developmental EMT master transcription factors that bind to the promoters of epithelium- and mesenchyme-specific genes and regulate promoter activities (13,14,23,24). Regarding the effect of 3-MC on EMT, a recent study suggested Slug as direct target gene of the AhR (19). AhR activation by 3-MC or calcium depletion in MCF-7 or HaCaT cells induces transcriptional activation of Slug by binding of the AhR-ARNT complex to an identified XRE in the Slug promoter. Although we used different cell lines, Slug could be involved in the cellular effects of 3-MC exposure in this study.

Most interestingly, we found that Slug and other master EMT-activating transcription factors (Snail, Twist1, Zeb1, and Zeb2) were dramatically up-regulated in 1MEA cells. It
is considered that a large number of molecular and cellular changes such as gene expression, cell adhesion and migratory phenotype, which are associated with normal developmental EMT, are recapitulated during human cancer promotion and progression (25). EMT in cancer is manipulated by different members of the EMT master transcription factors depending on the genetic history of the cells and extracellular environments. Recently, it is suggested that the SNAIL and TWIST families are upstream of the ZEB factors for the regulation of EMT (26). This hierarchical relationship supports the strong correlation between the ZEB factors, especially Zeb1, and E-cadherin loss and EMT across cancer cell types (27). However, the exact mechanism of the EMT-inducing effect of 3-MC and the crosstalk between 3-MC and EMT transcription factors still remains to be elucidated.

Although many endogenous chemicals or signaling factors have been suspected to mediate carcinogenesis and tumor progression, little is known about the effects of pollutants on cancer promotion and progression. Thus, further research is warranted to better understand the interactions of between environmental carcinogen, such as 3-MC and TCDD, and EMT process and the subsequent functional significance which may provide molecular insights into the invasive and metastatic tumor cell programs in cancer patients exposed to environmental pollutants.

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