Aryl hydrocarbon receptor exogenous ligand 3-methylcholoranthrene inhibited endometrial cancer cells proliferation

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Abstract. – OBJECTIVE: Endometrial cancer (EC) is the most prevalent gynecologic malignancy among women worldwide. Increasing evidence has disclosed the potential role of aryl hydrocarbon receptor (AhR) in the cancer development; however, little is known about its roles in the EC development. In the present study, we evaluated AhR expression in EC tissues as well as cell lines, and investigated the effects of AhR knockdown and exogenous ligand 3-methylcholoranthrene (3-MC) on EC cells proliferation and invasion using Ishikawa and ECC-1 cells lines.

MATERIALS AND METHODS: In this study, tissue microarray and immunohistochemistry were used to investigate the expression and localization of AhR in EC tissues. RT-PCR and Western blot were performed to detect the AhR expression. AhR specific siRNA was used to knockdown the AhR expression. MTT and transwell assay were carried out to study the EC cells proliferation and invasion, respectively.

RESULTS: Our results showed that AhR was highly expressed in the EC tissues and cell lines when compared with its expression in the normal endometrial tissues. AhR siRNA significantly decreased (p < 0.05) AhR protein expression in both Ishikawa and ECC-1 cells. Knockdown of AhR did not alter EC cells proliferation and invasion. However, 3-MC dose-dependently inhibited (p < 0.05) EC cells proliferation via AhR-mediated pathway.

CONCLUSIONS: The results from the current application will provide critical information on roles of 3-MC/AhR pathway in mediating EC growth, which could be useful for future therapeutic intervention in this lethal human disease.

Key Words: AhR, Endometrial cancer, 3-MC, Proliferation, Invasion.

Abbreviations

EC = endometrial cancer; AhR = aryl hydrocarbon receptor; 3-MC = 3-methylcholoranthrene; PAS = [Per (period circadian protein), ARNT (aryl hydrocarbon receptor nuclear translocator protein), Si (single-minded protein)]; BNF = β-naphthoflavone; TCDD = 2,3,7,8-tetra-chlorodibenzo-p-dioxin; PAHs = polycyclic aromatic hydrocarbons; DAB = diaminobenzidine; PBS = phosphate buffered saline; DMEM = Dulbecco’ modified eagle medium; GAPDH = Glyceraldehyde-3-phosphate dehydrogenase; FBS = fetal bovine serum.

Introduction

Endometrial cancer (EC) is the most prevalent gynecologic malignancy among women worldwide1. EC has been traditionally divided into two types based on the histopathology and clinical behavior: type I and type II. Women with these cancers often have risk factors such as long-term unopposed estrogen therapy, obesity, tamoxifen therapy, polycystic ovarian syndrome (PCOS), obesity, diabetes mellitus, and hypertension2,3. Over the past decades, great advances in our understanding of endometrial cancer biology, the patients still have an extremely poor prognosis4. Undoubtedly, a better understanding of molecular pathogenesis of EC will assist the development of new therapeutic methods for this deadly disease.

The aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor of the Per-Arnt-Sim (PAS) family, mediates multiple biological processes including immune system...
homeostasis, cell growth and differentiation, vascular remodeling. Unliganded AHR exists in the cytoplasm as part of a multimeric, upon ligand binding, AHR translocates to the nucleus where it associates with ARNT to form a functional transcription factor complex, binds to specific enhancer sequences adjacent to target promoters termed dioxin responsive elements and subsequently regulates the expression of Cytochrome P4501A1 (CYP1A1) and other xenobiotic target genes to combat the effects of environmental contaminants such as 3-methylcholanthrene (3MC), β-naphthoflavone (BNF), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).

For the last decades, AhR has been widely studied as a receptor for environmental toxicants. Recently, increasing evidence has disclosed the potential role of AhR in the tumor development. The important roles of AhR in carcinogenic pathways is evidenced in AhR knockout mice, which are refractory to polycyclic aromatic hydrocarbons (PAHs)-induced carcinogenesis. Increased expression of AhR has been observed in breast, skin, and lung cancers in humans. Meanwhile, several studies have clearly demonstrated that AhR-mediated responses are closely correlated with proliferation in different types of cancer cells, suggesting that AhR might be as a potential target for cancer treatment. However, the precise role of AhR in EC tumorigenesis is still lacking.

In this study, we evaluated AhR expression in EC tissues as well as cell lines, and investigated the effects of AhR protein levels and 3-MC on endometrial cancer cell growth using Ishikawa and ECC-1 as cell models. Our data clearly showed that knockdown of AhR did not alter EC cells proliferation and invasion. However, 3-MC dose-dependently inhibited EC cells proliferation via AhR-mediated pathway.

Materials and Methods

Tissue Microarray and Immunohistochemistry

Human endometrial cancer tissue microarray (US Biomax, Rockville, MD, USA) was used for immunolocalization of AhR in endometrial cancer tissues. This microarray contained 12 cases of endometrial cancer, 12 cases of adjacent normal endometrial tissues. Two microarrays were run in parallel: one was probed with AhR antibody AhR (1:500; Enzo Life Sciences Inc., Farmingdale, NY, USA) and another was probed with IgG control. Sections were microwaved in citrate buffer to extract antigens. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide/methanol for 10 min. Sections were incubated with blocking solution and the primary antibody AhR, then incubated with biotin-labeled secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 10 min. A diaminobenzidine (DAB) kit (Sigma Diagnostics, St Louis, MO, USA) was used to detect chromogens. The primary antibodies were replaced with PBS for a negative control.

To semi-quantitatively analyze the AhR levels, two observers blindly and independently assessed the immunohistochemical expression of AhR. We defined a score that corresponded with the sum of the percentage of positive cells (0, 0-24% immunopositive cells; 1, 25-50% positive cells; 2, 51-74% positive cells; 3, ≥75% positive cells) and staining intensity (0, negative; 1, weak; 2, moderate; 3, strong).

Tissue Samples

Fresh tissues of endometrial cancer compared with their adjacent normal ones were obtained from 5 patients who underwent surgery at Dalian Gynaecology and Obstetrics Hospital (Dalian, China). Written informed consent to participate in the study was obtained from each patient before surgery, according to the ethical guidelines of Dalian Gynaecology and Obstetrics Hospital. None of the patients has received any preoperative treatment.

Cell Lines and Cell Culture

Human endometrial cancer cell lines Ishikawa and ECC-1 cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). All these cells were grown in DMEM/F12 (Gibco, Grand Island, NY, USA) supplemented with with 2 mM glutamine (Gibco), 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin), within a humidified atmosphere containing 5% CO2 at 37°C.

RNA Isolation and RT-PCR

Total RNA was prepared from human endometrial tissues and endometrial cancer cell lines using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was generated from 3 μg of total RNA with oligo dT primers and Super-
script II reverse transcriptase (Invitrogen), all according to the manufacturer’s instructions. The cDNA product was used as a template for subsequent PCR amplifications for AhR. PCR was then performed with the following sets of primers: AhR subunit, Forward 5’-CATGCTTTTGCTTTTATGC-3’, Reverse 5’-TTCCTTTCTTTCTGTCC-3’; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Forward 5’-GCACCGTCAAGGCTGAGAAC-3’; Anti-sense: 5’-TGGTGAAGACGCCAGTGGA-3’.

Amplification condition was performed as follows: 94°C for 2 min, followed by 30 cycles (denature for 2 min at 95°C, anneal for 30 s, and extend for 30 s at 72°C), and then 72°C for 5 min. GAPDH acted as an internal control. The reaction products were separated on 1% agarose gel and analyzed by an imaging system.

**Transient Transfection and Small Interfering RNA**

A pool of four small interfering RNAs (siRNA) that target AhR was purchased from DHarmaco (Chicago, IL, USA). Cells were cultured in 35 mm dishes in DMEM/F12 medium containing 10% fetal bovine serum (FBS). After reaching 70% of confluence, cells were washed with DMEM/F12. The AhR siRNA duplexes and non-targeting control (Scrambled siRNA) were mixed with Lipofectamine 2000 Reagent (Life Technologies Inc., Carlsbad, CA, USA) respectively, then incubated for 30 min at room temperature. Cell transfections were performed using Lipofectamine 2000 according to the manufacturer’s instructions. After 4 hrs of transfection, the medium was supplemented with serum and antibiotics. Cells were harvested 48 hrs after the transfection. Western blot was used to examine transfection efficiency.

**Western Blot Analysis**

Western blot analysis was carried out as following described. Tissues were pulverized in liquid nitrogen using a mortar and pestle, followed by homogenized, and further lysed by sonication. Cells were washed twice with cold PBS, and then harvested and lysed by sonication in buffer (4 mM sodium pyrophosphate, 50 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM EDTA, 2 mM sodium orthovanadate [Na3VO4], 1 mM PMSF, 1% Triton X-100, 5 mg/ml leupeptin, 5 mg/ml aprotinin). The protein concentrations in supernatants of the lyses were determined. The protein samples were separated on 10% SDS-PAGE gels, and electrically transferred to polyvinylidene fluoride (PVDF) membranes. The blot for tissue samples was first probed with the AhR antibody (1:2000; Enzo Life Sciences Inc., Farming, NY, USA) followed by reprobing with a rabbit GAPDH (1:4000; Research Diagnostics, Concord, MA, USA) as a loading control. Proteins on the membrane were visualized using electrochemiluminescence (ECL) reagents. The immunoreactive signals were analyzed by densitometry. Data on AhR were normalized to GAPDH.

**Cell Invasion**

The invasion of cells was performed using 24-well transwell chamber with 8.0 µm pore polycarbonate filter inserts (Costar, Cambridge, MA, USA). Cells (8 x 10⁴) suspended in DMEM/F12 media were seeded into the upper compartment of invasion chamber coated with matrigel. In each lower chamber, 600 µl of DMEM/F12 containing 10% FBS was added. Then, the inserts were incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 16 hrs. The invaded cells attached to the bottom side were were stained, and counted under a microscope. The value of invasion was expressed as the average number of invaded cells per microscopic field over the 4 fields in each assay from three independent experiments.

**MTT Assay**

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) viability assay was performed as following steps. Briefly, cells were seeded into 96-well plate with 3000 cells/well and incubated for 48 h. Then the cells were washed with PBS and 200 µl MTT (0.5 mg/ml) was added to each well and further incubated for 4 hrs. The MTT solution was carefully removed by aspirating, and the formazan product was dissolved in 150 ml dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm on a microplate reader (Bio-TEK Instrument, Winooski, VT, USA).

**Statistical Analysis**

All data were expressed as means ± SEM of at least three independent experiments. The statistically significant differences compared with untreated group were analyzed using one-way ANOVA or Student’s t test. p < 0.05 were considered to be statistically significant.
Results

AhR Expression in the Tissue Microarray

In the endometrial cancer tissue microarray, the AhR immunoreactivity was mostly present in cancer tissue. The AhR staining was localized primarily in epithelial cells, but not other cell types (Figure 1A). No positive staining was observed in the preimmune rabbit IgG (data not shown). The semi-quantification analysis revealed that the AhR staining score in the endometrial cancer tissue was much higher than that in the adjacent normal tissue (Figure 1B).

AhR is Expressed in Human Endometrial Cancer Tissues and Cell Lines

Both mRNA and protein levels of AhR in human endometrial cancer tissues and cell lines were determined by RT-PCR and Western blot analysis (Figure 2). The AhR antibody detected a band approximately at 90 kDa (Figure 2B). After normalized to GAPDH, AhR protein levels in cancer tissues were 2.0 fold higher ($p < 0.05$) than those in normal tissue. Also, we detected high protein levels of AhR in both Ishikawa and ECC-1 cells (Figure 2B).

Figure 1. Immunohistochemical analysis for AhR in human endometrial cancer tissues and normal endometrial tissues. Brown color indicates positive AhR staining. A, Representative images from CANCER (a) and NORM (b) tissue are shown. B, Semi-quantitative analysis for AhR staining score. Semi-quantitative data are expressed as Means ± SEM fold of the NORM, *differ from NORM ($p < 0.05$).

Figure 2. AhR mRNA and protein expression in human endometrial cancer (C) and their corresponding adjacent normal tissues (N). AhR expression was detected by RT-PCR (A) and Western blot and band intensities of AhR were normalized with corresponding band intensities of GAPDH (B). *Differ from the NORM ($p < 0.05$).
**Effects of AhR siRNA on AhR Protein Expression**

Given AhR is highly expressed in the human endometrial cancer tissues and cell lines, we investigate whether knockdown of AhR would alter the malignant features of EC cells. As shown in Western blot results (Figure 3), the transfection of AhR siRNA reduced the AhR protein levels in both Ishikawa and ECC-1 cells in comparison with the cells treated with the scrambled siRNA. And this inhibitory effects maintained at least 72 hrs.

**Effects of AhR siRNA on the Proliferation and Invasion of EC Cells**

To gain further insight into the potential role of AhR in the proliferation and invasion of EC cells, we performed cell proliferation and invasion assays after transfected with AhR siRNA. As shown in Figure 4 A and B, the transfection of AhR siRNA did not change cell proliferation and invasion in EC cells in comparison with the cells treated with the scrambled siRNA. These results suggested that AhR protein levels may not be involved in the features of malignant in endometrial cancer.

**Effects of 3-MC on the Proliferation and Invasion of EC Cells**

As a widely used exogenous AhR ligands, 3-MC is capable of binding to AhR and activating AhR. In this study, we found that 3-MC dose-dependently inhibited EC cell proliferation (Figure 5A), but not invasion (Figure 6). Importantly, knockdown of AhR using AhR specific siRNA completely blocked 3-MC-inhibited EC cell pro-

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**Figure 3.** Effects of AhR siRNA on AhR protein expression in Ishikawa and ECC-1 cells. Cells cultured in the serum free media were transfected with the scrambled or AhR siRNA. Proteins were subjected to Western blot analysis. Quantified data are expressed as means ± SEM from four independent experiments. *Differ (p < 0.05) from the Scrambled siRNA treatment.
AhR protein levels do not change cell proliferation and invasion in EC cells. However, exogenous AhR ligand 3-MC significantly inhibits EC cells proliferation in vitro, and this inhibitory effect may be partially mediated through AhR pathway. Thus, AhR could potentially be used as therapy target for future intervention in EC development.

In the past decades, AhR was extensively investigated to regulate the expression of drug metabolizing enzymes involved in metabolic activation of some toxicants and carcinogens\textsuperscript{16,17}. Recently, several studies has disclosed the potential role of AhR in the cancer development\textsuperscript{18,19}. In the present study, we found that AhR is highly expressed in human EC tissues in comparison with adjacent normal tissues. Overexpression of AhR may not be confined to endometrial cancers, couple of reports showed that AhR expression was also up-regulated in other cancer types, including breast cancer\textsuperscript{10}, lung cancer\textsuperscript{12}, and pancreatic cancer\textsuperscript{20}. The above findings suggested that AhR may represent a cancer-associated marker in a wide variety of human cancers and high AhR expression inside the endometrial tissues could be used as a tissue marker for endometrial cancers.

Although in the absence of a ligand AhR promotes progression of the cell cycle as shown in HepG2 human hepatoma cells transfected with AhR siRNA\textsuperscript{21}. Similarly, an increase in invasiveness of breast cancer cells was also observed after the AhR knockdown\textsuperscript{22}. In the current study, the AhR knockdown, however, does not alter EC cells proliferation and invasion in the absence of ligands, implying that AhR protein levels is unlikely involved in the regulation of endometrial cancer cell proliferation and invasion. However, treatment with exogenous AhR ligand 3-MC significantly inhibited cell proliferation in the current study. Knockdown of AhR using siRNA duplexes blocked 3-MC-inhibited EC cell proliferation, suggested that 3-MC/AhR signaling pathway is actively involved in the EC cell proliferative action. In agreement with above results, several studies have demonstrated that AhR is a potential drug target for cancer therapy\textsuperscript{20,23}. AhR agonists inhibited the growth of tumor cells in couple of cancer types, including prostate\textsuperscript{20}, ovarian\textsuperscript{24} and breast cancers\textsuperscript{25}. In contrast, other reports showed that AhR activated enhanced human gastric cancer cell\textsuperscript{26} and urothelial cancer invasion\textsuperscript{27}. These findings suggested that the role of AhR and its agonist on cancer is highly tumor-specific.
Conclusions

The present studies demonstrate that the highly expressed AhR may perform important roles during EC development. The AhR ligand 3-MC possesses potent anti-endometrial cancer activity through AhR pathway. Thus, our current findings suggest that interruption of AhR-activated signaling pathways may be a potential target for treatment of endometrial cancers.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

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