Aryl hydrocarbon receptor controls adhesion and migration of colon cancer cells
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Received: July 24, 2018; Accepted: August 07, 2018; Published: August , 2018

Abstract
The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor mediating various cellular responses to xenobiotics as well as to physiological stimuli. We previously showed that AhR-deficient mice spontaneously develop cecal tumors with severe inflammation. To further elucidate the molecular mechanism of the tumor suppressor function of AhR, we investigated cell migration in colon cancer cells transfected with siRNA against AhR. Cell motility examined in an in vitro wound healing assay was up-regulated in the AhR-knockdown (AhR-KD) cells. Migration was further studied using a real-time cell analyzer system and showed that cell migration was remarkable on substrates coated with either fibronectin or Matrigel. The GTPase Rac was activated in these cells as assessed by GST pull-down assay. The AhR knockdown phenotype also included enhanced cell spreading, increased amounts of focal adhesion kinase phosphorylation at Y397 and decreased integrin β5 as detected by immunoblotting using anti-cytoplasmic domain antibodies. Collectively, these data suggest that AhR function contributes to tumor suppression by regulating cell adhesion and migration signaling.

Keywords: aryl hydrocarbon receptor, colon cancer, migration, adhesion, focal adhesion kinase

Introduction
The aryl hydrocarbon receptor (AhR) mediates various biological responses to environmental pollutants such as dioxins, PCBs, and benzo[a]pyrene (BP), as well as to natural chemical substances. When AhR is activated by ligand binding, it translocates to the nucleus to form a heterodimer with AhR nuclear translocator (ARNT). In the nucleus, AhR/ARNT binds to the xenobiotic responsive elements located in the 5′-flanking regions of target genes such as CYP1A1 and various other factors involved in cellular responses [1, 2]. Because the tumorigenic activity of BP requires metabolic activation of CYP1A1 or CYP1B1 enzymes into their ultimate DNA-damaging forms [3, 4], AhR has been investigated in the context of tumorigenesis.

Apart from toxicological studies, AhR has been shown to have critical endogenous function involved in embryonic development [5, 6], cell cycle regulation [7], and immune responses [8-10]. We have reported on the tumor suppressor function of AhR using AhR-deficient mice that spontaneously develop cecal tumors [11]. In addition, treatment of ApcMin/+ mice with an AhR ligand, which is a model mouse for the study of carcinogenesis, suppresses intestinal tumorigenesis. Our study also found an up-regulation of inflammatory cytokines in the colorectal tissues of AhR-deficient mice and that suppression of inflammatory responses repressed tumor formation in these animals [12]. Immunohistochemical analysis using human colon cancer tissues has revealed that AhR expression is increased in both tumor and stromal cells [13]. Up-regulation of AhR expression has also been reported in other tissues such as lung [14], breast [15], and pancreatic cancer [16], indicating the possibility of therapeutic treatment with AhR ligands. We have also previously observed that cell growth is suppressed by treatment with methylcholanthrene or indole-3-carbinol (I3C), an AhR ligand, in human colon cancer cell lines [13]. These results indicate a tumor suppressor function of AhR.

In this study, we investigated whether changes in AhR expression alter cell migration in cultured human colon cancer cells. Cell migration is a critical step during tissue formation during fetal development and wound healing, as well as in tumor formation. Migration requires
orchestration of various responses, including actin polymerization regulated by small GTPases and cell adhesion dynamics involving molecules such as integrins, focal adhesion kinase (FAK), and other related signaling proteins. Gonzalez JM et al. [17] have reported that AhR transcriptionally regulates vav3, a Rho/Rac GTPase, resulting in modulation of cell shape and adhesion of mouse embryonic fibroblast. AhR also regulates SRC and FAK activities [18, 19] as well as integrin expression [20], with subsequent alterations to cell migration. Although many studies suggest a regulatory function of AhR in cell motility [21-23], its effects appear to be cell-context dependent. The focus of this study was to examine cell migration of colon cancer cells in an in vitro wound healing analysis using a real-time cell analyzer system combined with AhR-KD techniques.

Materials and Methods

Cell culture and siRNA transfection

The human colon cancer cell lines HCT116 and HT29, kindly donated by Dr. Kawajiri K. [11], were incubated in RPMI-1640 supplemented with 10% fetal calf serum. The cells were maintained in a humidified incubator at 37 °C in the presence of 5% CO₂. For AhR-KD experiments, HCT116 cells or HT29 cells were seeded in 24 well plates. After overnight incubation, cells were transfected with siRNA at 10 nM in culture medium using Lipofectamine RNAiMAX reagent (Invitrogen, Waltham, MA, USA). The siRNAs used were as follows: human AhR siRNA (part number 4390824, Ambion, Austin, TX, USA), human ARNT siRNA (part number 439240, Ambion), and control siRNA (Ambion).

Cell migration analysis

HCT116 cells were seeded to a density of 5 × 10³/well in a 96-well plate (Oris cell migration assay kit, Platypus Technologies, WI, USA). After incubation to allow cells to adhere, stoppers were removed to create a central cell-free zone in the center of each well. Cells were further incubated to promote migration and were photographed to determine cell index at the indicated time points. Migration was monitored every 15 min for 24–48 h. For quantification, the cell index at the indicated time points was averaged from four independent experiments. We used fibronectin-coated membranes for cell migration assays and Matrigel matrix-coated membranes for cell invasion assays in accordance with the manufacturer's instructions.

RT-PCR and immunoblotting

RNA was extracted from cultured cells using Isogen (NipponGene, Tokyo, Japan), and reverse transcription was performed using a PrimeScript RT reagent kit (Takara Bio, Shiga, Japan), according to the manufacturer's instructions. PCR analyses were performed using ExTaq DNA polymerase (Takara Bio) and a GeneAmp PCR system 9700 (Applied Biosystems, Waltham, MA, USA). PCR products were visualized by electrophoresis on 2% agarose gels. For immunoblot analysis, tissue samples were prepared by homogenization in SDS lysis buffer followed by boiling. The protein samples were mixed with 100 mM DTT and bromophenol blue before loading on 8% polyacrylamide gels, electrophoresed and then transferred to nitrocellulose membranes. The membranes were incubated in blocking solution containing 5% skimmed milk dissolved in Tris-buffered saline. The antibody probes used were anti-actin (Sigma, A2066), anti-AhR (BIOMOL, Plymouth Meeting, PA, USA, catalog no. SA-210), anti-Arnt (Santa Cruz, sc-5580), anti-AhR (Bioworld Technology, BS60270), anti-ARNT (Santa Cruz, sc-5580), anti-RhoA (NB-100-91273), anti-Rac (Sigma, R2650), anti-Cdc42 (Bioworld Technology, BS60270), anti-phosphoFAK (Y397, CST 8556), anti-FAK (CST 13009), anti-integrin β1 (CST 9699), and anti-integrin β5 (cytoplasmic, CST 3629 and extracellular, ABGENT, AP14000). The membrane was incubated with secondary antibodies conjugated with HRP and detected using the Immobilon Western Chemiluminescence HRP substrate (Millipore, Billerica, MA, USA) followed by visualization with a LAS-4000 (Fujifilm, Tokyo, Japan).

GST pull-down assay

Colon cancer cells were lysed on ice in RIPA buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na₃P₂O₅, 1 mM EDTA, 2 mM Na₂VO₅, 1% NP40, and 1% Triton X-100. GST-hPAK (67-150) fused protein bound to glutathione sepharose beads was added to cell lysate and incubated for 2 h at 4 °C. Beads were washed four times with RIPA buffer, and bound proteins were eluted in Laemli sample buffer. Samples were analyzed by immunoblotting using antibodies against Rac.

Results

AhR-knockdown increases cell migration

In order to study AhR function in cell migration, wound healing analysis was performed using AhR-KD cells. AhR expression was efficiently inhibited by transfection of siRNA against AhR (Figure 1A), and wound
area recovered faster in these cells compared with those transfected with control siRNA (Figure 1B). The time course of wound healing revealed that cell migration increased significantly in AhR-KD cells. Interestingly, AhR-KD promoted cell mobility even more on substrates coated with fibronectin (Figure 1C).

These results led us to study cell migration using a real-time system. In this case, higher migration signals were detected in AhR-KD cells on fibronectin-coated plates (Figure 2A). A cell invasion assay using Matrigel, which mimics basement membrane, showed that AhR-KD cells exhibited higher invasion signals at the lower (2.5%) Matrigel densities but not at higher (5%) densities (Figure 2B).

The small GTPase protein Rac1 is activated by AhR-KD. Because the Rho GTPase family is a key regulatory factor in cell migration, we examined the expression of Rho A, Rac1, and CDC42. Immunoblot analysis revealed that Rac was most abundant in HCT116 cells (Figure 3A). To measure Rac1 GTPase activity, active Rac1 was pulled down using GST-PAK-PBD beads (Figure 3B, C). In the absence of fibronectin coating, Rac1 activity was elevated in AhR-KD cells compared with control, in the presence of 1% FCS.

Enhanced cell adhesion by AhR-KD

To further study the underlying mechanism of migration, we examined cell adhesion and shape. siRNA-transfected cells were trypsinized and maintained in cell suspension during incubation on ice for 60 min. Cell shape was examined after plating on fibronectin-coated dishes. Cell adhesion increased in AhR-KD cells (Figure 4A), and 3 h after plating, AhR-KD cells spread more efficiently on fibronectin-coated plates than control cells. Moreover, we also examined autophosphorylation of FAK and found that FAK activity was increased in AhR-KD cells compared with controls (1.4- and 1.5-fold at time 0 and 10 min, respectively). These results suggest that cell spreading associated with FAK activation contributes to the promotion of cell motility observed in AhR-KD cells.

AhR-KD reduces the cytoplasmic region of integrin β5

We next investigated the expression of integrin isoforms, which play central roles in cell adhesion and migration. Among them, immunoblotting analysis revealed that integrin β5 was reduced in AhR-KD cells (Figure 5A). In contrast, expression of integrin β1 was unaffected. Reduction of integrin β5 was not observed by transfection of siRNA targeted against ARNT, a dimerization partner with AhR to activate transcription of target genes (Figure 5A). RT-PCR analysis showed that the expression level of integrin β5 mRNA did not change in AhR-KD cells (Figure 5B). The anti-integrin β5 antibody used in the immunoblot shown in Figure 5A detects the cytoplasmic region. Detection of integrin β5 was further compared using two kinds of antibodies, against the cytoplasmic and extracellular domains of integrin β5 (Figure 5C, D). A decrease in integrin β5 was observed when using the anti-cytoplasmic region antibody, but not with the anti-extracellular region probe, in the two cell lines, HCT116 and HT29.

Figure 1. AhR depletion promotes cell migration. (A) siRNA against AhR was transfected into HCT116 cells. AhR expression was detected by RT-PCR. (B) Wound healing analysis using siRNA-transfected cells. Wounded area is indicated by the yellow line. Representative photographs at day 3 are shown. (C) Wounded areas were analyzed with ImageJ software. Values are given as the mean ± SD (n = 4). *P < 0.001.
Figure 2. Cell migration detected in real time. siRNA-transfected HCT116 cells were plated on either fibronectin (A) or Matrigel (B) coated membranes. Values are indicated as the mean ± SE. MG, Matrigel.

Figure 3. Rac-GTP expression increases in AhR-depleted cells. (A) Different amounts of HCT116 cell lysates were subjected to SDS-PAGE, and Rho family GTPases were probed by immunoblot analysis using anti-RhoA, anti-Rac, and anti-CDC42 antibodies. (B) Rac pull-down activation assay. Lysates from either control or AhR siRNA-transfected cells were incubated with GST-PAK-PBD, and GTP-bound Rac was analyzed by pull-down assay. Cells were cultured on dishes with or without fibronectin coating in the presence of 1% FCS. GTP-bound Rac was quantitated using ImageJ software (C).
Figure 4. Knockdown of AhR promotes cell adhesion. (A) siRNA-transfected HCT116 cells were harvested at 2 days post-transfection. Cell suspension was incubated on ice for 60 min and re-plated on dishes to observe changes in cell shape. (B) Immunoblot analysis of phosphorylated and total FAK. Re-plated cells were collected after incubation for the indicated durations, and cell lysates were subjected to SDS-PAGE. The amount of phosphorylated FAK was quantified by ImageJ and normalized with total FAK (see text).

Figure 5. Effect of AhR knockdown on expression of integrin β5. siAhR-transfected HCT116 cells were collected to detect integrin isoforms β1 and β5 by immunoblot (A) and RT-PCR (B). (C) A decrease in integrin β5 was observed using an anti-cytoplasmic region antibody, but not with an anti-extracellular region antibody. Immunoblots of cell lysates from HCT116 and HT29 cells are shown. (D) Quantification of integrin β5 expression using ImageJ software.
Discussion

Tumor cells are characterized by abnormal growth and cell motility. We have previously demonstrated tumor formation at the ileocecal junction in AhR-deficient mice, suggesting a tumor suppressive function for this protein [11]. Furthermore, AhR-KD in colon cancer cell lines results in growth promotion [13]. In this study, we found an up-regulation of cell migration in AhR-KD cells. These data suggest that AhR controls cell growth and migration, resulting in a tumor suppressor function.

Regulation of cell motility requires reorganization of the cytoskeleton and is mainly controlled by Rho family GTPases [24]. Because higher amounts of Rac protein were detected in HCT116 cells compared with RhoA and CDC42, GFP-bound Rac GTPase was examined in a pull-down assay through its specific interaction with PAK1 protein-binding domain. Interestingly, Rac GTPases were activated in AhR-KD cells when compared with controls, suggesting a regulatory role for AhR on guanine nucleotide exchange factors (GEF) or GTPase activating enzymes. Gonzalez JM et al. [25] have reported that vav3, a GEF for Rho/Rac GTPases, is up-regulated by AhR in mouse embryonic fibroblasts, but vav3 mRNA was rarely detected in HCT116 in our experiments. Neither did we observe increased expression of βPIX [26], a GEF for Rac/CDC42, nor Tiam1 [27], a specific GEF for Rac1, in AhR-KD cells. Further analysis is needed to fully understand AhR function in controlling cell motility through Rac1 activation.

Cell migration is coordinated by cell–cell and cell–substrate adhesion. We next examined whether cell adhesion was affected by suppressing AhR expression. We observed efficient cell adhesion to culture dishes and increasing amount of activated FAK in AhR-KD cells. Before plating (at time 0), higher amounts of the autophosphorylated form of FAK (pFAK[Y397]) were detected in AhR-KD cells compared with controls, suggesting a contribution of AhR to the negative regulation of cell–substrate adhesion involving FAK activity. Focal adhesions are composed of adhesion molecules and signaling proteins such as kinases and phosphatases. Among them, it has been reported that Src kinase is associated with AhR [18, 20] and that the tyrosine phosphatase SHP-1 is regulated by AhR/ARNT [28]. AhR function may contribute to cell adhesion and migration through the interaction to these signaling proteins.

To further examine cell–substrate adhesions, expression of integrin isoforms in AhR-KD cells was investigated by immunoblotting. We found reduction of integrin β5 when the cytoplasmic region of integrin β5 was used for detection in immunoblot analysis. Previous studies have indicated an alteration in integrin β1 expression by suppression of AhR [17, 29], but this was not observed in our experiments.

It has been suggested that reduced expression of integrin β5 is controlled post-transcriptionally, because transfection of siAhR does not affect mRNA levels. Integrin binding to ECM induces intracellular signaling by protein complex formation with the cytoplasmic domain of integrin, resulting in activation of cell adhesion as well as migration. In this study, we observed promotion of cell migration as well as alterations in adhesion molecules containing FAK and integrin β5. It is interesting to consider the possibility, therefore, that AhR contributes negatively to cell migration through protein–protein interaction with signaling molecules such as FAK and Src in association with integrin β5. Collectively, these results suggest that AhR negatively regulates cell migration as its contribution to tumor suppression. Further analysis is needed to study regulatory mechanisms of AhR on cell migration.

Acknowledgements

This study was supported by the Ministry of Education, Culture, Sports and Technology in Japan (26430131). The authors would like to thank Ms. K. Ono for her excellent assistance in molecular biology experiments. We would like to thank Enago (www.enago.jp) for the English language review.

Conflict of Interest

No conflicts

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To cite this article: Ikuta T, Okabe-Kado J. Aryl hydrocarbon receptor controls adhesion and migration of colon cancer cells. British Journal of Cancer Research. 2018: 1:3. doi: 10.31488/bjcr.113