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Note

The regulation mechanism of AhR activated by benzo[*a*]pyrene for CYP expression are different between 2D and 3D culture of human lung cancer cells

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ABSTRACT

Most of cytochrome P450 (CYP) expressions are regulated by nuclear receptors. The regulation pathways of transcription are activated by binding of the ligand to the receptor. Many combination of CYPs and nuclear receptors in transcriptional regulation have been reported. However, we have reported that the combination changes depending on culture condition on the same type of cells. The regulation pathway of *CYP1A* expression is different between 2D monolayer cultured cells and 3D spheroids of human liver cancer cells. Aryl hydrocarbon receptor (AhR) is one of the transcription factors for *CYP1A* and *CYP1B1* expression, and this pathway is important for inducing human lung cancer. *CYP1B1* expression in human lung cancer cells are regulated by AhR in 2D and 3D cells. But *CYP1A* expression are not induced by AhR in 3D cells. As with liver cancer cells, the function of AhR in lung cancer cells is different between 2D cells and 3D spheroids. These results important for understanding relationship between AhR and *CYP* expression before and after cell neoplastic formation in human lung.

1. Introduction

Aryl hydrocarbon receptor (AhR) is a transcriptional factor that belongs to the family of basic-loop-helix/Per-ARNT-Sim (bHLH-PAS) transcriptional factors [1,2]. When exogenous ligands bind to and activate AhR, the AhR translocates into the nuclear after dimerizing with aryl hydrocarbon receptor nuclear translocator (ARNT) to form a complex to bind to xenobiotic response elements (XREs) of AhR-response gene [3–5]. Benzo[*a*]pyrene (B[*a*]P) is well known as a ligand for AhR [6]. B[*a*]P has toxic effects towards human health, especially smoking-related diseases including lung cancer effects [7], as an AhR ligand, B[*a*]P induces expression of CYP1A and CYP1B1. The CYP1A1 induction associates with lung cancer initiation [8] and CYP1B1 is known enzyme responsible for the metabolism and synthesis of estrogen and procarcinogenic derivatives [9]. The relationship between AhR and carcinogenesis has been extensively analyzed. But documented of the role of AhR after carcinogenesis has not been investigated at molecular levels. In meta-analysis studied on subsequent smokers and non-smokers who have been diagnosed with early stage lung cancer, it has also been reported that patients who were current smokers have a significantly poorer prognosis than ex-smokers [10].

We suppose that the difference in prognosis was due to the influence of carcinogenic polycyclic aromatic hydrocarbons such as B[*a*]P intake by smoking on cancer progression and being more malignant. Analyzing the influence of B[*a*]P and the function of AhR in lung cancer cells are important to know whether smoking is involved in poor a prognosis. In order to proceed this research, analyzing the target genes of AhR will greatly contribute, and there are huge amount of knowledge for the target genes in the cultured cancer cells. We have reported that target genes of AhR in liver cancer cells are different between 2D cells and 3D spheroids [11]. As cluster of cancer cells *in vivo* are generally three-dimensional spheroids (3D spheroids), the target genes of AhR in lung cancer cells also are different from the results obtained in 2D cultured cells.

List of abbreviations: 3D, Three dimension; AhR, Aryl hydrocarbon receptor; CYP, Cytochrome P450.

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In this investigation, we examined whether the function of transcription of downstream gene of AhR under B[*a*]P stimulation in human lung cancer cells, A549 (adenocarcinoma cell line), H520, H1703 and H2170 (squamous cell lung cell line) are different between 2D cells and 3D spheroids, using *CYP1A1*, *1A2* and *1B1* genes as indexes.

2. Materials and methods

2.1. Cell culture

Human lung cancer cells, A549 (ATCC; CCL-185), H520 (ATCC; HTB-182), H1703 (ATCC; CRL-5885) and H2170 (ATCC; CRL-5928) were cultured in RPMI 1640 or Dulbecco's modified Eagles medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% FBS (Hyclone, South Logan, VT USA) and 1% antibiotic-antimycotic (Life Technologies). 2D and 3D culture protocol are described in our previous report [11]. 100 μL of lung cancer cell suspension (1.0×10^4 cells/mL) was applied to a Prime Surface 96 V Plate (Sumitomo Bakelite, Tokyo, Japan) for 3D culture. The 2D cultured cell concentration were 1.0×10^5 cells/mL cell suspension. The suspension was applied 3 ml per culture dish (60 mm). Benzo[*a*]pyrene (Tokyo Chemical Industry, Tokyo, Japan) was dissolved in dimethyl sulfoxide (DMSO) as 10 mM stock and add to the mediums in starting culture. The cells were cultured at 37 °C in a CO_2 incubator for 48 h.

2.2. RNA extraction and quantitative RT-PCR

Total RNA was extracted from the cells using an RNeasy Mini Kit (Qiagen, Dusseldorf, Germany), following the manufacturer's instructions. Total RNA was used for cDNA synthesis with a cDNA synthesis kit (Roche, Basel, Switzerland). Using first-strand cDNA, real-time PCR was performed using a 7500 Real Time PCR system (Applied Biosystems, Tokyo, Japan). Primer sequences for the genes are described in Terashima et al. [12]. The data from three independently cultured samples were averaged, and standard deviations were calculated. A statistical analysis was performed using the Student's t-test. Statistical significance was determined at $p < 0.05$.

2.3. RNAi assay

siRNA sequences were constructed to target human *AhR* mRNA (HSS100336; Invitrogen, Carlsbad, CA, USA), and negative control siRNAs were designed by scrambling these nucleotide sequences. Negative control siRNAs were not homologous to any other gene. 2D and 3D cultures of lung cancer cells were individually transfected with 50 nM siRNA; transfections were performed with Lipofectamine RNAiMAX Reagent (Invitrogen), and added these to the cells on starting culture. The suppression efficiency of *AhR* are shown as supplemental data (Supplemental Fig. 1).

3. Results and discussion

3.1. CYP1A and 1B1 expression and the regulation in 3D spheroids

The expression levels of some CYPs in 2D culture cells are lower than those *in vivo* [13]. When the cells are cultured in 3D culture system, the CYP expressions are higher than those expression of 2D culture cells [11,14,15]. *CYP1A1* and *CYP1B1* expression levels in all kind of the 3D spheroid cells were higher than the expression levels in 2D culture cells and *CYP1A2* expression levels in 3D spheroids except for H1703 also were higher than the expression levels in 2D culture cells (Fig. 1A). We have reported that the regulation pathway of *CYP1A* expression in 3D spheroids of human liver cancer cells are different from the pathway in 2D culture cells [11]. Also in human lung cancer cells, the regulation pathways of *CYP1A* expression were different between 2D cells and 3D spheroids, on the other hand *CYP1B1* expression were regulated by AhR in both of 2D cells and 3D spheroids (Fig. 1B). *CYP1A1* expression in the lung cancer cells were suppressed by AhR knock down in 2D cells, but not suppressed in H1703 and H2170 3D spheroids and *CYP1A1* expression in A549 showed decreasing trend in 3D spheroid. There

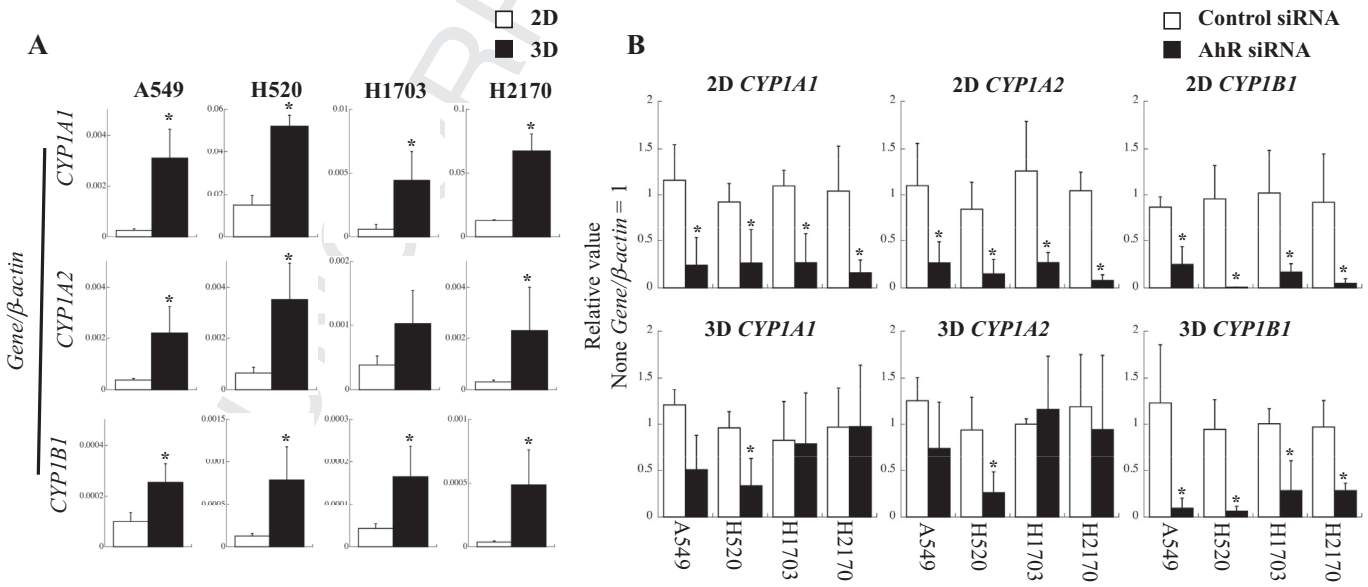

Fig. 1. Comparison of *CYP1A1, 1A2* and *1B1* expression between 2D cells and 3D spheroids. A: *CYP1A1*, *CYP1A2* and *CYP1B1* expression levels. Closed column and open column indicate 3D and 2D cultures, respectively. Bars indicate the standard deviation of independent triplicate experiments. Statistical analysis was carried out between 2D and 3D, $^*p < 0.05$. B: *CYP* expression after AhR knockdown in 2D cultured cells and 3D spheroids. The open and closed columns indicate *CYP* expression following transfection with control siRNA and 50 nM AhR siRNA, respectively. The graphs are indicated as relative value based on *gene/β-actin* in the control of none (w/o siRNA). The bars indicate the standard deviation of independent triplicate experiments. The expression levels between the 2 aforementioned conditions were statistically analyzed $^*p < 0.05$.

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