Activation of arylhydrocarbon receptor (AhR) in T lineage cells inhibits cellular growth

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Introduction

Dioxins, including the most toxic congener, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), exert their toxic effects by binding and activating the arylhydrocarbon receptor (AhR), a ligand-dependent transcription factor [1, 2]. Upon binding dioxins, the AhR in the cytoplasm is activated and translocated to the nucleus, where it heterodimerizes with another transcription factor, ARNT. The AhR/ARNT heterodimer modulates expressions of various genes by binding xenobiotic responsive elements (XREs) in their enhancer regions or modifies cellular functions through protein-protein interactions. The AhR activation by TCDD exposure induces various immunotoxic reactions including thymus involution and suppression of T cell-dependent antibody production [3]. We have investigated the roles of AhR activation in T lineage cells and their underlying mechanisms by generating transgenic (Tg) mice expressing a constitutively active AhR (CA-AhR) mutant specifically in T cells and by transiently expressing the CA-AhR mutant in Jurkat T cells.

Materials and Methods

Generation of T cell-specific CA-AhR Tg mice: Transgene expression construct was generated by inserting a CA-AhR mutant or GFP into T cell-specific expression vector VA hCD2 [4]. Transgenic founder mice were obtained by microinjecting the transgene expression constructs into C57BL/6J x DBA/2 eggs [5]. For some lines, VA hCD2-GFP was coinjected with VA hCD2-CA-AhR. One line carrying both CA-AhR and GFP constructs and two lines with only CA-AhR construct were chosen for further studies and subsequently backcrossed into C57BL/6J mice. Founders and subsequent littermates were genotyped by PCR of tail DNA.

Flow cytometry: Splenocytes and thymocytes were prepared and their cellular populations were examined using a FACSCalibur (BD Biosciences, San Diego, CA) as described previously [6].

Transient transfection study [7]: Jurkat T cells were transiently transfected with pEB6CAGFP for green fluorescent protein (GFP) expression or with pEB6CAG-CA-AhR-GFP for CA-AhR-GFP fusion protein using DMRIE-C reagent (Invitrogen, Carlsbad, CA). After 2 days, GFP-positive cells were sorted using a FACSVantage SE (BD Biosciences). The efficiency of the sorting was confirmed using a FACSCalibur and 98-99% of the sorted cells were GFP-positive. The sorted

cells were cultured at 1×10^5 cells/ml to examine growth rate, apoptosis, and cell cycle distribution, as described below.

Detection of Apoptosis [7]**:** Apoptotic cells were detected using an Annexin V-biotin apoptosis detection kit (BioVision, Palo Alto, CA). On days 0, 2, and 4 after sorting, the cells were stained with biotin-labeled annexin V plus streptavidin-labeled allophycocyanin, followed by propidium iodide (PI), and were analyzed using a FACSCalibur.

The induction of apoptosis was also confirmed by apoptotic morphological changes. The sorted cells were cultured for 2 days, then stained with bis-benzimide (Hoechst 33342, ICN Biomedicals Inc., Aurora, OH), and were examined by the changes in their nuclear morphology under a UV-visible fluorescence microscope.

Cell Cycle Analysis [7]**:** On days 0, 2, and 4 after sorting, the cells were stained with PI using a CycleTEST PLUS DNA Reagent Kit (BD Biosciences), and their DNA content was measured using a FACSCalibur. The percentages of cells in the G_1, S, and G_2/M phases were analyzed using ModFit software (BD Biosciences).

Affymetrix GeneChip Analysis [7]**:** After sorting, total RNA was isolated using an RNeasy Mini Kit (Qiagen, Chatsworth, CA). Affymetrix GeneChip analysis was performed using a Human Genome U133A array (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions with a slight modification. The data were analyzed using Affymetrix Microarray Suite 5.0 software. A comparison analysis was performed to obtain genes with at least two-fold changes in Jurkat T cells expressing CA-AhR-GFP as compared with cells expressing GFP-alone.

RT-PCR: Total RNA was isolated from cells using an RNeasy Mini Kit (Qiagen). RT-PCR was conducted as described elsewhere [7,8].

Results and Discussion

T cell-specific CA-AhR Tg mice shows thymus involuiton: To generate T cell-specific CA-AhR Tg mice, we used a CA-AhR mutant having a deletion in the PAS B domain, which is constitutively translocated into nucleus and activates AhR/ARNT-dependent transcription, independently of ligands (Fig. 1A). We obtained one line (line A) carrying both CA-AhR and GFP constructs and two lines (line J and N) with only CA-AhR construct working under the control of the T cell-specific expression vector VA hCD2. Fig. 1B shows CA-AhR mRNA expressions in various organs in line A mice. As expected, CA-AhR mRNA was detected in the thymus and spleen. The expression of CYP1A1, one of the genes activated by the AhR/ARNT heterodimer, was also detected in the thymus and spleen without exposure to ligands (Fig. 1C). In the line J and N mice, CA-AhR and CYP1A1 mRNA were confirmed to be expressed in the thymus and spleen (data not shown). Expressions of the transgene in immune cells were detected by flow cytometry of the GFP expression in line A mice. Thymocytes, and CD4 and CD8 T cells in the spleen, were confirmed to be GFP-positive, and B cells did not express GFP (data not shown). In these Tg mice of all lines, the thymus weight and total thymocyte number were decreased. In addition, the ratio of CD8 single positive/CD4 single positive cells in the thymus was elevated when compared with wild-type mice (data not shown). All these phenotype changes in the thymus are characteristic

features that have been observed in TCDD-exposed mice. These results demonstrate that AhR activation only in thymocytes (immature T cells) can induce the thymus involution.

CA-AhR inhibits growth of Jurkat T cells: In order to investigate the underlying mechanism of effects caused by activated AhR in T cells, Jurkat T cells, which expressed ARNT but not AhR, were transiently transfected with an expression vector for either CA-AhR-GFP or GFP-alone. Two days after the transfection, GFP-positive cells were sorted. RT-PCR analysis showed that CA-AhR-GFP, but not GFP-alone, markedly induced CYP1A1 mRNA expression, indicating that the CA-AhR mutant is functional in Jurkat T cells. In addition, the green fluorescence emitted from CA-AhR-GFP was mainly found in the nuclear compartment (data not shown).

To examine the effect of CA-AhR on the growth rate of Jurkat T cells, the sorted cells were cultured for up to 4 days and the cell numbers were counted. As shown in Fig. 2, the cells expressing GFP-alone increased ten-fold, 4 days after sorting. In contrast, the expression of CA-AhR-GFP completely inhibited the increase in the cell number, indicating that the activation of AhR greatly inhibits the growth of Jurkat T cells.

CA-AhR induces both apoptosis and G1 arrest in Jurkat T Cells: Since CA-AhR was shown to induce growth inhibition, we examined whether the expression of CA-AhR induces apoptosis in Jurkat T cells. As shown in Fig. 3, cells transfected GFP-alone did not show remarkable changes in the ratio of dead cells. On the other hand, CA-AhR-GFP increased the percentage of apoptotic cells, especially 2 and 4 days after sorting. The apoptosis induction by CA-AhR-GFP was also confirmed by nuclear morphological changes under a microscope (data not shown).

We also investigated the possibility that CA-AhR induces cell cycle arrest. Immediately after sorting (0 day), no difference was observed in the DNA profile among non-transfected cells, cells expressing GFP-alone and those expressing CA-AhR-GFP (Fig. 4). Two and four days after sorting, the percentage of cells in the G_1 phase was increased in the CA-AhR-GFP-expressing cells (Fig.4). These results suggest that CA-AhR affects cell cycle progression, especially in the G_1 phase.

CA-AhR induces expression changes of genes related to apoptosis and cell cycle arrest: In order to examine whether CA-AhR changes the expression of genes related to apoptosis and cell cycle arrest, total RNA was prepared from GFP- or CA-AhR-GFP transfected cells two days after transfection, and the gene expression was analyzed using Affymetrix GeneChips. Genes related to apoptosis and cell cycle arrest were selected from the genes that showed at least two-fold expression changes in the CA-AhR-GFP-expressing cells, as compared with GFP-alone-expressing cells. Their expression changes were confirmed by semiquantitative RT-PCR. We found that CA-AhR upregulates genes related to apoptosis (caspase 8, c-jun, and Fas) and cell cycle arrest (cyclin G2, growth arrest and DNA-damage-inducible, alpha (GADD45A), $p21^{waf1}$, cell division autoantigen-1 (CDA1), IL-9 receptor). CA-AhR also upregulated the genes involved in both apoptosis and cell cycle arrest (dual specificity phosphatase 6 (DUSP6), GADD34, and TGF-β receptor II). On the other hand, protooncogene c-myc, which plays an important role in the G_1/S transition, was downregulated in cells expressing CA-AhR-GFP.

In the present study, we demonstrated that the CA-AhR expressed only in the thymocytes causes reduction in the cell number in addition to the population change. TCDD exposure to mice is known to reduce the number of the thymocytes, but not the splenocytes or spleen T cells. The

same feature was observed in the T cell-specific CA-AhR Tg mice in the present study, although the CA-AhR was fully expressed in the spleen T cells as well as in the thymocytes. On the other hand, TCDD suppresses the increase in the splenocytes including the T cells induced by immunization in mice [9]. These results may indicate that AhR/ARNT heterodimer inhibits cell growth of activated and proliferating T lineage cells by interacting with transcription factors or proteins which function in cellular activation. In consistence with this speculation, the present study showed that transfection of CA-AhR in Jurkat T cells, which are in an activated and proliferating state, induces inhibition of cellular growth. Furthermore, we deonstrated that the growth inhibition of Jurkat T cells by CA-AhR accompanies an increase in apoptosis and the accumulation of the cells in the G_1 phase, and expression changes of several genes related to apoptosis and cell cycle arrest. Further study is needed to clarify whether the genes identified in the present study are responsible for the growth suppression of mature T cells and thymocytes. The growth inhibition of activated T cells by AhR/ARNT may also be involved in the inhibition of T cell-dependent immune reactions by TCDD.

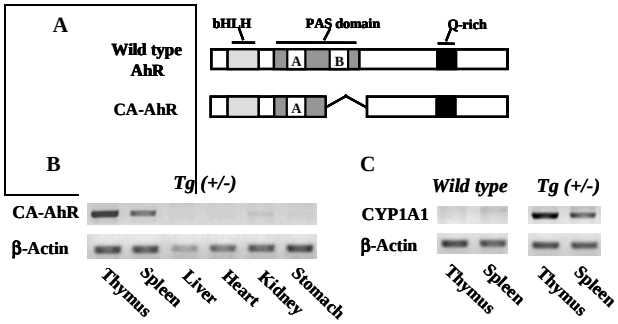

Figure 1. Generation of Tcell-specific CA-AhR transgenic mice. A, Structures of the wild type AhR and the CA-AhR mutant used in the present study. B, RT-PCR analysis of CA-AhR mRNA in the different tissues from heterozygous Tg mice (line A). C, RT-PCR analysis of CYP1A1 mRNA in the thymus and spleen from heterozygous Tg mice (line A).

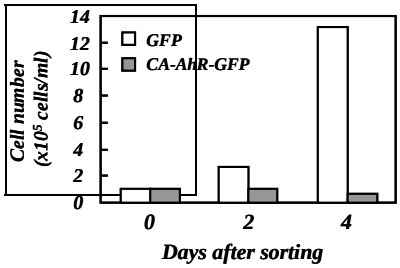

Figure 2. CA-AhR inhibits growth of Jurkat T cells. The expression vector for either CA-AhR-GFP or GFP-alone was transiently transfected into Jurkat T cells. After 2 days, GFP-positive cells were sorted and then cultured at 1×10^5 cells/ml. The cell numbers at the indicated times were determined by trypan blue dye exclusion.

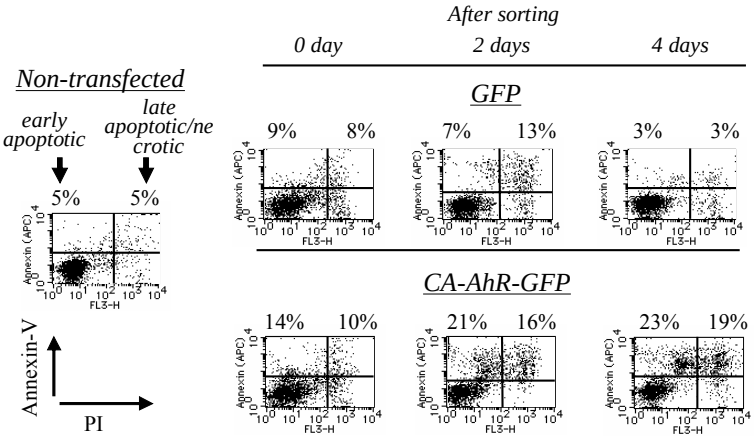

Figure 3. CA-AhR induces apoptosis in Jurkat T cells. Apoptotic cells were measured as described in Materials and Methods. The *upper left quadrant* represents early apoptotic cells, while the *upper right quadrant* represents late apoptotic/necrotic cells.

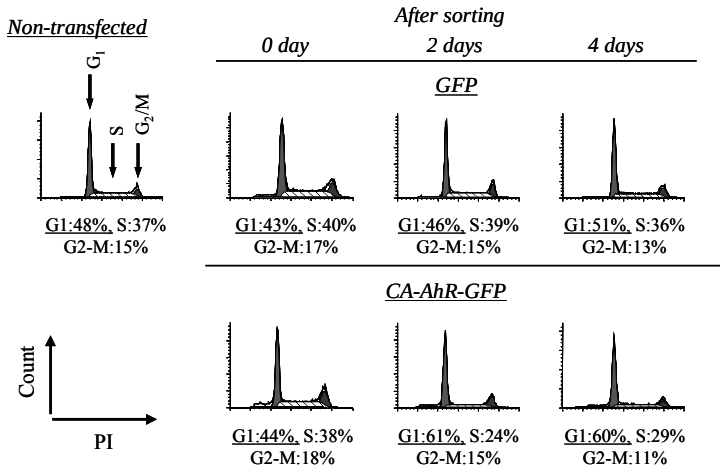

Figure 4. CA-AhR increases the percentage of cells in the G_1 phase. The percentages of cells in the G_1, S, and G_2/M phases were measured as described in Materials and Methods.

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