EXOSURE TO THE LIGANDED AH-RECEPTOR RESULTS IN THE EXPRESSION OF VARIANT CD44 ISOFORMS ON EMIGRATING THYMOCYTES IN THE MOUSE

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Introduction

The CD44 transmembrane glycoproteins exist as a standard form (CD44s) and as numerous variant isoforms (CD44v), generated by differential splicing. CD44 has been implicated in cell-cell and cell-matrix interactions, in particular in lymphocyte homing, cell migration, and tumor metastasis[1,2]. Also, CD44 has been described as one of the pivotal mediators for both homing to the thymus and between pre-T cells and the thymic microenvironment[3,4]. Recent studies on T cell development with CD44 variant-specific antibodies revealed the importance of CD44 variant isoforms containing exons v6 and v7[5].

Very little is known about thymocyte emigration. Emigration could be either stochastic, or, alternatively, a signal-dependent event[6,7].We had observed an Ah-receptor (AhR) dependent CD44 upregulation on thymocyte subpopulations in mouse fetal thymus organ cultures (FTOC) exposed to dioxins and chlorinated biphenyls. Also, we observed differing frequencies of certain thymocyte subpopulations found outside the thymus lobe after exposure. Based on the observation that the frequency of CD44-expressing cells is upregulated on very immature $CD4^-CD8^-$ (DN) thymocytes by TCDD and TCB treatment[8], we have analyzed the differential expression of CD44v isoforms of TCDD/TCB-treated fetal thymocytes and thymic emigrants.

Methods and Materials

Thymi from dioxin-sensitive C57BL/6 fetuses of day 15 of gestation were cultivated in the presence or absence of 10mM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) under conditions which mimick in vivo development. CD4/CD8 surface marker bearing cells can be detected outside of organ-cultured fetal thymus lobes after several days. These cells were collected in PBS. They are referred to here as thymus emigrants. Afterwards, the lobes were lifted off the filter single cell suspensions were prepared. Both cell populations (emigrants and thymocytes) were counted and stained for FACScan analysis. Also, RNA from sorted subpopulations was prepared and RT-PCRs performed. Calibrated cDNA samples were tested for CD44 isoforms by southern-blotting with probes specific for the ten isoforms.

Results and Discussion

Fetal thymus lobes of gestation day 15 were organ-cultured for five or six days with or without 10 nM TCDD. Thymocytes and thymus emigrants were collected, stained with anti-CD4 and anti-CD8 antibodies, and analyzed flow-cytometrically. After six days of organ culture with TCDD the normal distribution pattern for thymocyte subsets had changed: only 40 % of CD4+CD8+ and 20 % of CD4-CD8- were found (no TCDD: 60% and 15%). At 40%, CD4-CD8+ were significantly more abundant after TCDD treatment ($p<0.001$). Moreover, when TCDD was added to the cultures, the otherwise equal subset pattern changed between inner thymocytes and emigrants. Now more CD4-CD8- cells (about 60 %) were found as emigrants, at the same time the frequency of emigrant CD4+CD8+ cells was decreased to 20 % ($p<0.001$) Overall TCDD decreased the number of emigrants. Whereas in unexposed thymi about 10% of cells were found as emigrants, in TCDD-treated lobes this number dropped to 3%, i.e. either emigration itself or the survival of emigrated cells is impaired.

CD44 is known as one of the markers of hematopoietic precursor cells, populating the thymus[13]. After six days of FTOC plus TCDD, more than 50 % of all emigrants expressed pan-CD44 with high density ($CD44^{high}$) compared to only 8 % of control emigrants. In addition, 20 % of CD44 intermediate-density-expressing cells ($CD44^{int}$) were found in emigrants from TCDD-treated cultures, compared to 12 % in the controls. Most of these cells derived from TCDD-treated FTOCs expressing CD44 belong to the CD4-CD8-

subset. Interestingly, CD4+ and CD8+ single positive and double positive emigrant subpopulations which are mainly $CD44^{-/low}$ *in vivo* continued to express CD44 upon TCDD treatment, but to a lesser extent than CD4-CD8- emigrants. In absolute cell numbers this result was even more striking. Only CD4-CD8-$CD44^{high}$ cell numbers were found outside of the thymus lobes in equally high numbers as inside, all other subsets decreased drastically.

Ten variant exons of the complete CD44 gene may be used in different combinations in the final RNA product flanked by the standard exons. Different biological functions have been ascribed to different isoforms. We used anti-CD44v6, anti-CD44v7 and anti-CD44v10-antibodies to differentiate between pan-CD44 and specific variant isoforms on the cell surface of fetal thymus emigrants exposed to TCDD. Taken together, the data shows that the TCDD-induced increase of expression of CD44 on CD4-CD8- emigrants described above is due to the presence of CD44v10 containing isoforms. After six days of FTOC, almost 80 % of CD4-CD8- thymic emigrants expressed CD44v10 on their cell surface (see Table 1). $CD4^+$ and CD4+CD8+ emigrants expressing CD44v10 were also increased from about 2 % to 38 % and 21 %, respectively, in TCDD-treated cultures. In contrast, no change in CD44v10 expression was found in CD8+ emigrants. Because the bulk of thymic emigrants from TCDD-treated FTOC were CD4-CD8- and most of them expressed CD44v10 but not CD44v7, TCDD seems to induce in particular all the isoforms containing this particular exon product.

Table 1

CD44v7 and CD44v10 expression on thymic emigrants in FTOC exposed to 10 nM TCDD[a]

	CD44v7				CD44v10			
	$CD4^+$	DP	DN	$CD8^+$	$CD4^+$	DP	DN	$CD8^+$
control	2%[b]	3%	3%	4%	2%	2%	37%	20%
TCDD	9%	18%	3%	8%	38%	21%	77%	19%

[a]Thymus lobes were organ cultured for six days with either 10 nM TCDD or 1,4-dioxane alone. Thymic emigrants were stained with anti-CD4, anti-CD8, and anti-CD44v7 or anti-CD44v10, and analyzed flow-cytometrically. Results are from one represantative experiment [b]Numbers represent percentage of CD44v positive cells

In order to analyze the composition of CD44v isoforms in thymocytes and emigrants, we amplified CD44 cDNAs from sorted thymocyte subpopulations by PCR. Subsequently the amplified fragments were detected with radiolabeled variant exon-specific probes. We found that CD4-CD8- emigrant populations express at a higher level and more numerous CD44 variant exons, either singly or in combination with neighbouring exons after TCDD treatment (see Table 2). Expression level is higher than in controls. The expression of isoforms containing the variant exon v10 is conspicuously elevated in exposed cells. Like cell surface expression of CD44v10, i.e. the CD44 isoform which contains only the variant exon v10, appearance of CD44v mRNA with additional variant exons is enhanced by TCDD (Fig) Interestingly, also more CD44s RNA was detected in CD4-CD8- emigrants in TCDD-treated FTOCs compared to controls. The increase of CD44v7 in DP cells seen by flow-cytometry (Table II) could not be detected in this experiment due to the low amount of cDNA available for the less abundant DP cells in TCDD-treated cultures.

Table 2

	Experiment A				Experiment B			
	Thymocytes		Emigrants		Thymocytes		Emigrants	
Exon	Control	TCDD	Control	TCDD	Control	TCB	Control	TCB
v1								+
v2				+		+		
v3				++		++	+	++
v4				+				+
v5								++
v6						+		+
v7			+	++		++	+	+
v8						+	+	
v9				-/+		+		-/+
v10	+	+++	++	+++	+	+++	++	++
v7-v10			+			++	+	++
v8-v10		+		+		+		+
v9-v10				++		++		++

Thymocytes were organ-cultured for six days with 10 nM TCDD, 3.3 µM TCB or 0.1 % dioxane, respectively. Emigrants and thymocytes were collected, stained with anti-$CD4^{PE}$, anti-$CD8^{BIO}$ and TRI-COLOR®, and DN cells were isolated. After preparation of the cDNA of the DN cells, RT-PCRs with CD44-specific primers were performed Southern blots were hybridized with CD44v1 to CD44v10-specific probes. -/+ very weak expression; + weak expression; ++ strong expression; +++ very strong expression.

Conclusions

Thymus atrophy is a hallmark of TCDD exposure. We and others have previously ascribed the thymus atrophy caused by TCDD, TCB, and other Ah-receptor ligands to changes in thymocyte proliferation and differentiation[8,10,11,12]. Another possibility is a changed/enhanced emigration of thymocytes. We analyze here whether TCDD induces distinct isoforms of CD44 on thymocyte subsets and whether, in correlation with this, also the emigration of thymocytes or thymocyte subsets might be affected by xenobiotic overstimulation of the Ah-receptor.

CD44 is found on lymphoid, myeloid, epithelial and endothelial cells. CD44 is a homing receptor for the thymus[3]. Moreover, CD44-deficient mice appear to have an impaired progenitor egress from the bone marrow[13], pointing to a role of CD44 in cell migration. Functionally, CD44 can mediate both cell attachment and motility, often by virtue of its hyaluronan binding capacity[14,15]. The variant isoforms appear to differentially sequester these general properties of CD44, for instance, a number of metastatic responses with variant-correlated migration patterns have been observed[16].

We used FTOCs as a model to explore the effects of TCDD on thymocyte emigration. In the control cultures, we found about one tenth of the number of the inside thymocytes on the outside. In TCDD-exposed thymi, emigrant numbers dropped to only 3% of cell number inside. At present, we cannot distinguish whether the observed effects are due to (a) inhibited emigration, (b) a changed survival rate of emigrants, or (c) a changed proliferation capacity of emigrated cells by TCDD. Further studies, e.g. BrdU labeling experiments, are needed to clarify these issues. Strikingly though, CD4-CD8- cells were found not to be affected as much as the other subsets. Thus the most immature thymocytes from which all the other thymocytes are eventually generated, appear as the major population of emigrants.

In our model system of FTOC plus TCDD a higher percentage and also number of CD4-CD8- emigrants expressed $CD44^{high}$ on their surface. Gene modulation by the TCDD/Ah-receptor complex has been

demonstrated for a variety of genes involved in cell proliferation and differentiation. Some cytokines, e.g. IL-1ß, IL-2, TNFα, and TGFß3 mRNA are inducible by TCDD. TGFß inhibits proliferation and differentiation of CD4-CD8- thymocytes, which then might accumulate[17]. TNFα has been shown to activate the transcriptional activity of CD44 variant isoforms[18]. It is currently unknown how the different CD44 isoforms might be regulated.

CD44 variant isoforms were differentially upregulated on emigrants in the presence of TCDD. CD44v are markers of immature cells and are most likely involved in cell-cell and cell-matrix interactions. Thus, it will be interesting to further analyze the consequences of the upregulation of specific isoforms of a gene (CD44) that assumes different roles in T-cell developmental stages and their respective functions.

CD44v10, which was highly abundant on TCDD treated thymocytes, has been suggested to be involved in cell migration, for instance by acting as T cell target structure for chemokines provided by the contacted cell partner[19]. It is tempting to speculate that TCDD/TCB-induced expression or - respectively - the lack of downregulation of CD44v10-containing isoforms could be the reason for an enhanced emigration of CD44-positive DN thymocytes. Likewise, the role of CD44 isoforms in tumor metastasis and its possible connection to the proposed effect of TCDD as a tumor promotor might prove an interesting line of experimentation.

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