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Immune-Specific Up-Regulation of Adseverin Gene Expression by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

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ABSTRACT

To identify genes that are regulated by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and possibly involved in TCDD-induced immunotoxicity, we used the differential display technique to screen for differentially expressed genes in the mouse thymus. Here we show that TCDD increased the expression of adseverin, a Ca^{2+}-dependent, actin-severing protein. The induction of adseverin is dose- and time-dependent in parallel with the induction of CYP1A1, which is currently the most frequently used marker for TCDD exposure. A comparison between mouse strains with different TCDD responsiveness indicated that the induction of adseverin is dependent on the aryl hydrocarbon receptor, a transcription factor known to mediate most of TCDD's biological effects. Examination of additional organs revealed that the up-regulation of the adseverin gene expression is immune-specific. Using an anti-adseverin antibody, we confirmed the induction of adseverin by TCDD at the protein level and it was confined to the thymic cortex, which harbors immature thymocytes that are known target cells of TCDD. Considering adseverin's role in actin cytoskeletal reorganization, our observations reveal new mechanistic aspects of how TCDD might exert some of its immunotoxic effects.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and other structurally related halogenated aromatic hydrocarbons are persistent environmental pollutants that induce a wide variety of biological and toxic effects in mammals (reviewed in Birnbaum and Tuomisto, 2000). The immune system is one of the most sensitive targets for TCDD; signs of TCDD exposure include thymus atrophy and suppression of cell-mediated and humoral immune responses (reviewed in Kerkvliet, 1995).

In fetal and adult mouse thymus, TCDD causes a rapid but transient decrease in cell proliferation followed by a reduction in thymocyte number (Lundberg et al., 1990). In addition, TCDD seems to affect thymocyte differentiation, resulting in a relatively higher number of $CD8^+$ thymocytes (Esser and Welzel, 1993). In contrast, the cell number and CD4/CD8 ratio in peripheral lymphoid organs is largely unaffected by TCDD unless the mice have been challenged with an antigen (Lundberg et al., 1991). Thus, within the immune system, TCDD seems to preferentially affect proliferating and/or differentiating cells.

Different mechanisms have been proposed to explain TCDD-induced thymus atrophy and both the thymic stroma (Greenlee et al., 1985; Kremer et al., 1994) and the thymocytes (McConkey et al., 1988; Staples et al., 1998b) have been suggested to be direct targets. One of the more recently proposed mechanisms comes from an observation in fetal thymus organ cultures where TCDD induced the cell-cycle inhibitor $p27^{Kip1}$ (Kolluri et al., 1999). This up-regulation of $p27^{Kip1}$ could explain the initial decrease in thymocyte proliferation and number observed after TCDD exposure. However, although the proliferation returns to normal within a few days, the cell number in the thymus remains low for several weeks (Lundberg et al., 1990; Staples et al., 1998a). Thus, to explain atrophy duration other or additional mechanisms must be considered. One such mechanism is the reduced ability of early progenitor cells in the bone marrow and fetal liver to seed the thymus after TCDD-exposure (Fine et al., 1990). Another hypothesis is that TCDD initiates apoptosis in immature thymocytes (McConkey et al., 1988), although that theory has been questioned (Staples et al., 1998a). Thus, to get a clear picture of TCDD-induced thymic atrophy more information is needed.

Most effects of TCDD are believed to be mediated through its high-affinity binding to the aryl hydrocarbon receptor (AhR), a ubiquitously expressed transcription factor, and the AhR-nuclear translocator protein. This complex binds to dioxin-responsive elements in the promoter region of the target genes and regulates their transcription (Hankinson, 1995).

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ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, aryl hydrocarbon receptor; PBS, phosphate-buffered saline; FCS, fetal calf serum; IL, interleukin; RT, reverse transcription; PCR, polymerase chain reaction; HPRT, hypoxanthine phosphoribosyl nucleotide transferase; ab, antibody; differential display, differential display RT-PCR; bp, base pair(s); PIP_2, phosphatidylinositol 4,5-bisphosphate; PLCγ, phospholipase Cγ.

Despite much information on the AhR, its physiological function remains unclear and no natural ligand has been identified.

The list of TCDD-regulated genes is continuously growing and includes genes coding for xenobiotic metabolizing enzymes (CYP1A1, CYP1B1, glutathione-*S*-transferase), cytokines (IL-1β, transforming growth factor-β, plasminogen activator inhibitor-2), and others (major histocompatibility complex Q1b, Ecto-ATPase, p27^{Kip1}) (Whitlock, 1993; Okey et al., 1994; Dong et al., 1997; Gao et al., 1998; Kolluri et al., 1999). CYP1A1 is the most characterized of these AhR-regulated genes and has become a useful marker for TCDD exposure. However, none of the above-mentioned genes, except perhaps Kip1 (Kolluri et al., 1999), has shown a direct causal relationship with the adverse effects of TCDD.

To identify genes that might be involved in TCDD-induced immunotoxicity, we used the differential display reverse transcription-polymerase chain reaction (differential display) technique (Liang and Pardee, 1992) to screen for differentially expressed genes in mouse thymus. The thymus is a critical target organ because of its central role in T cell development, which has activation steps similar to those in mature T cells (Zuniga-Pflucker et al., 1993). Therefore, studies of TCDD-induced gene regulation in the thymus may give mechanistic explanations of immunotoxicity in both the thymus and in peripheral lymphoid organs.

Here we show that TCDD induces the expression of adseverin, an actin-binding protein important for normal cell functions. We also examine the induction of adseverin in other organs and in two different mouse strains and conclude that the up-regulation of adseverin gene expression is immune-specific and dependent on the AhR.

Materials and Methods

Experimental Animals. Female C57BL/6J (B6) AhR$^{b/b}$ (TCDD high responding) mice were bred in our own animal facilities. Original breeding pairs were purchased from B&K Universal (Solna, Sweden). Four-week-old female DBA/2J AhR$^{d/day}$ (TCDD low responding) mice were obtained from Møllegaard og Bomhultgård, Ry, Denmark, and allowed to acclimatize for a minimum of 1 week before use. All mice were housed and cared for in accordance with the National Research Council guidelines. Briefly, they were housed in a pathogen-free environment on a 12-h light/dark cycle and given a standard pellet diet and tap water ad libitum.

Treatments. TCDD (98.4% pure; Larodan Fine Chemicals, Malmö, Sweden) was dissolved in 1,4-dioxan and subsequently diluted with corn oil. At 5 to 6 weeks of age the mice were weight-matched, randomly allocated into treatment groups with 3–5 mice in each group and then injected i.p. with different concentrations of TCDD in a total volume of 10 μl/mouse. Control mice were administered the vehicle only.

For the differential display experiments, B6 mice were exposed to 50 μg of TCDD/kg for 24 h. In the dose-response experiments, which were performed after 24 h of exposure, B6 mice received 0.5, 2.5, 5, 10, or 50 μg of TCDD/kg and DBA mice received 0.5, 5, 10, or 50 μg of TCDD/kg. In the time-response studies, B6 mice were injected a dose of 10 μg of TCDD/kg and killed 3 h, 6 h, 24 h, 1 week, 2 weeks, or 7 weeks later. For prenatal studies, pregnant B6 mice were administered 10 μg of TCDD/kg at day 12 or 17 of gestation and sacrificed 24 h later by cervical dislocation, after which thymus [gestational day (gd) 18] or liver (gd 13) were dissected from their fetuses.

RNA Preparation. In all experiments, the mice were killed by CO_2 asphyxiation and the thymuses were removed, weighed, and immediately dissolved in guanidinium isothiocyanate. Total RNA was isolated by phenol-chloroform extraction (Chomczynski and Sacchi, 1987) and treated with DNase I. From B6 mice exposed to 10 μg of TCDD/kg for 24 h, RNA samples were also prepared from lymph nodes, spleen, liver, adrenal glands, kidney, thymocytes, and bone marrow cells. Thymocytes were isolated by gently pressing the thymus through a steel mesh in sterile PBS with 3% FCS and passing the cell suspension over cotton wool followed by three rounds of washing in PBS with 3% FCS. Bone marrow cells were collected by cutting of the ends of the femur and flushing the cavity with sterile PBS with 3% FCS using a 25-gauge needle. RNA samples were also prepared from fetal liver and thymus.

Differential Display. Differential display was performed on thymus samples from B6 mice exposed to 50 μg of TCDD/kg for 24 h using the Delta Differential Display Kit (CLONTECH, Palo Alto, CA). Briefly, reverse transcription (RT) of control and TCDD samples were done in a total volume of 10 μl/sample containing 2 μg of denatured total RNA, 0.1 μM oligo dT primer, 2 μl of 5$\times$ RT buffer (Promega, Madison, WI), 1 mM dNTP, and 200 U of Moloney murine leukemia virus reverse transcriptase. The reactions were incubated at 42°C for 60 min and then stopped by heating at 75°C for 10 min. The cDNA was then amplified in the presence of 1 μM arbitrary 5′ primer and 1 μM oligo dT primer according to the manufacturer's recommendations.

Amplified cDNAs were separated on a 5% polyacrylamide gel and visualized by autoradiography. Differentially expressed bands were eluted and reamplified using the same primers and reaction conditions as in the differential display, except that no isotope was added. After reamplification, the length and concentration of the PCR product was checked on a 1% agarose gel containing ethidium bromide.

The reamplified cDNA was cloned into a T/A cloning vector using the AdvanTAge PCR cloning Kit (CLONTECH) according to the manufacturer's instructions. Plasmids with the PCR product were purified with QIAGEN plasmid mini kit (QIAGEN GmbH, Hilden, Germany) and the cDNA was sequenced from both ends using the Cy5AutoRead sequence kit and the ALF express DNA sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden). For sequence analysis we used the BLAST program available at http://www.ncbi.nlm.nih.gov/BLAST/.

RT-PCR. RT of RNA was done as described above for the differential display. The cDNAs were amplified in a total volume of 50 μl containing 0.25 μM 5′ and 3′ primers, 1$\times$ PCR buffer [40 mM Tricine-KOH, pH 9.2 at 25°C,, 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, and 75 μg of bovine serum albumin/ml], 0.2 mM dNTPs containing 2 μCi of [α-^{33}P]dATP and 1 μl of Advantage 2 polymerase mix (CLONTECH). The samples were incubated at 94°C for 4 min and then amplified with 25 cycles: 94°C for 1 min, 60°C for 1.5 min, and 68°C for 1 min with a final extension at 68°C for 7 min. PCR-primers shown in Table 1 were used to detect transcripts from these genes: adseverin, CYP1A1, gelsolin, interleukin-9 (IL-9), and hypoxanthine phosphoribosyl nucleotide transferase (HPRT).

Amplified cDNAs were separated on a 1.5% agarose gel for 1 h. The gel was rinsed in a solution of 10% acetic acid and 1% glycerol and dried. The gel was then exposed overnight to BioMax MR film (Eastman Kodak, Rochester, NY) and the autoradiogram was analyzed using the public domain NIH Image program (developed at the United States National Institutes of Health and available on internet at http://rsb.info.nih.gov/nih-image). The integrated band intensities were normalized to the mRNA expression of the HPRT gene, which was used as an internal standard, and the mRNA levels were calculated and compared: [(target gene$_{treated}$ $\times$ HPRT$_{control}$) / (target gene$_{control}$ $\times$ HPRT$_{treated}$)].

Western Analysis. For detection of adseverin protein levels, total protein extracts were made from thymocytes by disrupting 50 $\times$ 10^6 cells in 50 μl of lysis buffer [PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Pefabloc (Roche Molecular Biochemicals, Mannheim, Germany), 10 μg/ml aprotinin, and 1 mM sodium orthovanadate]. After centrifugation at 10,000*g* for 10 min,

TABLE 1
Oligonucleotide primers used for gene expression analyses by RT-PCR

Gene	5′ Primer sequence	3′ Primer sequence	Product size (ab)	Cycles[a]
Adseverin/Ads D5	5′-TGGCTTTGGGAAGGTGTACATCA	5′-TGAGATGGGAAACAGCAAGGCAT	1595/1296	25
Adseverin	5′-TGGCTTTGGGAAGGTGTACATCA	5′-GCCATTGTTTCGTGGCAGTTTTA	591	25
CYP1A1	5′-GGCATTCATCCTTCGTCCCCTT	5′-TCACAGCGGGCGTGTTTTAAAGT	384	30
Gelsolin	5′-AAGGCAAGCAGGCCAACATGGA	5′-CAAAGAGTCGAGGAGGATGGGCAT	977	25
IL-9	5′-CATCCTTGCCTCTGTTTTGCTCTTCA	5′-TCTTCATGGTCGGCTTTTCTGCC	427	30
HPRT[b]	5′-CACAGGACTAGAACACCTGC	5′-GCTGGTGAAAAGGACTCT	249	25

[a] The number of cycles used in the PCR reactions was optimized to ensure that analyzes of gene expression levels were done in the exponential phase.
[b] To normalize the amount of cDNA used in the PCR reactions, the housekeeping gene HPRT was amplified in the same reaction as the target gene as an internal control. For CYP1A1 and IL-9, the HPRT primers were included during the last 25 PCR cycles.

the protein concentration in each supernatant was determined according to standard methods (bicinchoninic acid protein assay; Pierce, Rockford, IL). The protein extracts (30 μg/lane) were resolved by SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970), followed by transfer to a polyvinylidene difluoride membrane (Hybond P; Amersham Pharmacia Biotech). The membrane was blocked in 10% nonfat dry milk in 0.1% Tween-20 in PBS for 1.5 h at room temperature and incubated overnight at 4°C with a rabbit anti-mouse adseverin antibody (ab) (Lueck et al., 1998), a kind gift from Dr. David J. Kwiatkowski. The membrane was washed in 0.1% Tween-20 in PBS, and immunoreactive bands were then detected with peroxidase-conjugated donkey anti-rabbit IgG ab and the ECL enhanced chemiluminescence system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Immunohistochemistry on Whole Thymus Sections. Dissected thymuses were fixed in PBS containing 4% formaldehyde, dehydrated in a series of ethanol/water, and embedded in paraffin. Sections (4.5 μm thick), were dewaxed in xylene overnight and rehydrated before quenching endogenous peroxidases with 1% H_2O_2 in 0.3% Triton-X in PBS for 30 min. After washing (three times in 0.3% Triton-X/PBS and twice in PBS), the sections were blocked with 4% bovine serum albumin in PBS for 1 h at room temperature. Anti-adseverin ab, diluted in PBS was applied overnight at 4°C and washed as above. Staining was done with biotinylated goat anti-rabbit IgG ab, ABComplex (DAKO A/S, Glostrup, Denmark) and 3-amino-9-ethylcarbazol according to the manufacturer's instructions. The stained sections were mounted in glycerol-gelatin and photographed with a Coolpix 990 digital camera (Nikon, Tokyo, Japan) mounted on a Olympus BH2 microscope.

Statistics. Two-tailed Student's t test for paired variables was used to evaluate differences between treated and control groups, all of which contained a minimum of three animals. Differences were considered significant at $p < 0.05$.

Results

TCDD Increases the mRNA Level of the Actin-Severing Protein Adseverin in Mouse Thymus. Using the differential display technique, we found three TCDD-inducible PCR-products when analyzing thymus RNA from B6 mice exposed to 50 μg of TCDD/kg for 24 h (Fig. 1). These products were sequenced and a GenBank search revealed that one of them was 100% identical to the 3′ end of the mRNA coding for the actin-binding protein adseverin (GenBank accession number U04354) and a proposed splicing variant, adseverin D5 (GenBank accession number Y13971). Neither of these proteins has been associated with TCDD-induced toxicity. Hence, they might represent new mechanistic pathways for TCDD immunotoxicity. We identified the other two products as the mRNAs for CYP1A1 and CYP1B1. These genes are well known TCDD targets, and their induction confirms the reliability of the technique.

Confirmation and Characterization of the TCDD Up-Regulated Adseverin mRNA Level. To confirm the up-regulation of adseverin found in the differential display and to investigate whether TCDD induces both adseverin and adseverin D5, RT-PCR was carried out. PCR primers were used that bind outside the sequence that is missing in adseverin D5—base pair (bp) 1657–1956 of adseverin (Robbens et al., 1998); two PCR products were amplified: one band of 1595 bp corresponding to adseverin and a shorter band of 1296 bp corresponding to adseverin D5. Interestingly, under the present conditions, only full-length adseverin was induced upon TCDD exposure (data not shown). Hence, for subsequent experiments, PCR-primers were designed to amplify adseverin only (Fig. 2).

The Increase in Adseverin mRNA Level Is Dependent on the Dose and Exposure Time of TCDD. RT-PCR analyses of adseverin mRNA from thymuses of B6 mice exposed for 24 h to different doses ranging from 0.5 to 50 μg of TCDD/kg revealed that TCDD up-regulates adseverin mRNA in a dose-dependent manner (Fig. 3, A and B). At a dose of 0.5 μg of TCDD/kg, already a 2-fold increase of the adseverin mRNA level was observed. At 10 μg of TCDD/kg, the ad-

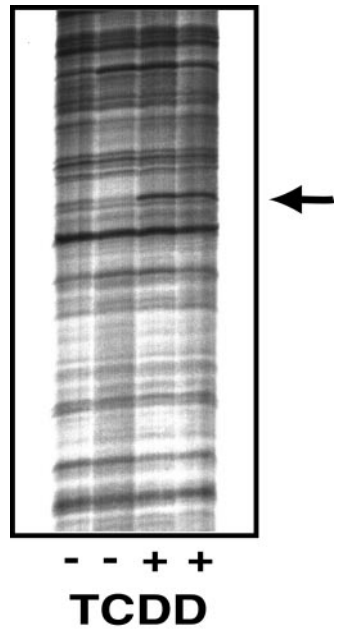

Fig. 1. Identification of TCDD-regulated genes by differential display. RNA was isolated from thymus of B6 mice treated with 50 μg of TCDD/kg (+) or vehicle (−) for 24 h and analyzed by differential display PCR. The lanes represent one mouse each and the arrow indicates the TCDD inducible cDNA product, later identified as adseverin.

severin mRNA level was increased about 7-fold, which was also the maximal induction observed at 24 h. In addition, the induction of adseverin was time-dependent (Fig. 4). After a dose of 10 μg of TCDD/kg there was already a 1.5-fold induction of the adseverin mRNA level at 3 h after exposure (earliest time point studied). Maximal induction of about 9-fold was reached 1 week after exposure. Thereafter, the mRNA level of adseverin decreased. However, at 7 weeks after exposure, the mRNA level of adseverin was still significantly higher in the TCDD-exposed mouse thymus compared with the control thymuses. CYP1A1 was induced in parallel with the adseverin gene in both the dose- and time-response studies, but to a higher level of expression (Fig. 3C and 4).

The Induction of Adseverin Gene Expression by TCDD Shows AhR Dependence. The AhR/AhR nuclear translocator heterodimer mediates most of TCDD's effects (Fernandez-Salguero et al., 1996). To test whether the induction of adseverin gene expression is mediated via the AhR complex, a dose-response experiment was performed with DBA mice. These mice have an AhR that is less responsive to TCDD and thus requires higher doses of TCDD to elicit the same degree of AhR-mediated responses compared with B6 mice. We found that the DBA mice required a dose more than 10-fold higher than that of the B6 mice to induce comparable levels of adseverin as well as CYP1A1 mRNA (Fig. 3, B and C). This difference corresponds to earlier differences found in TCDD sensitivity between the two strains (Abel et al., 1996).

The Induction of Adseverin Gene Expression Is Tissue Specific. Adseverin was first described in bovine adrenal glands as a factor important for exocytosis (Rodriguez Del Castillo et al., 1990). Since then, adseverin has been detected in other species, in several other tissues and cell types, and at different developmental stages (Lueck et al., 1998; Robbens et al., 1998; Arai and Kwiatkowski, 1999). We performed RT-PCR focusing mainly on tissues in the immune system (i.e., adult and fetal thymus, spleen, lymph nodes, bone marrow, and fetal liver). We also included adult kidney, adrenal glands, and liver in the study. All tested organs showed a constitutive level of adseverin mRNA, except for adult liver (Fig. 5A). Even using the same adseverin primers used by Robbens et al. (1998), we were unable to detect adseverin in adult liver (data not shown). Interestingly, we observed TCDD-induced levels of adseverin mRNA only in adult and fetal thymus, adult spleen, and fetal liver (Fig. 5A). In contrast, TCDD induced CYP1A1 gene expression in all organs tested (Fig. 5B).

Adseverin Is Induced in Thymocytes. Considering that one of the most prominent effects of TCDD is thymus atrophy, it could possibly be argued that the increase in adseverin mRNA level observed in whole thymus after TCDD exposure is not a direct effect on gene regulation but merely a consequence of a decrease in thymocyte number. To sort this out, RT-PCR was performed on mRNA from thymocytes

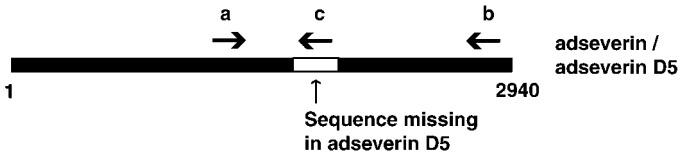

Fig. 2. The binding sites of the PCR-primers used for amplifying adseverin and its proposed splicing variant adseverin D5. Primers a and b were used in initial experiments to amplify both adseverin and adseverin D5, giving two products of 1595 and 1296 bp, respectively. Primers a and c, which amplify only adseverin, yield a 589-bp product. See also Table 1.

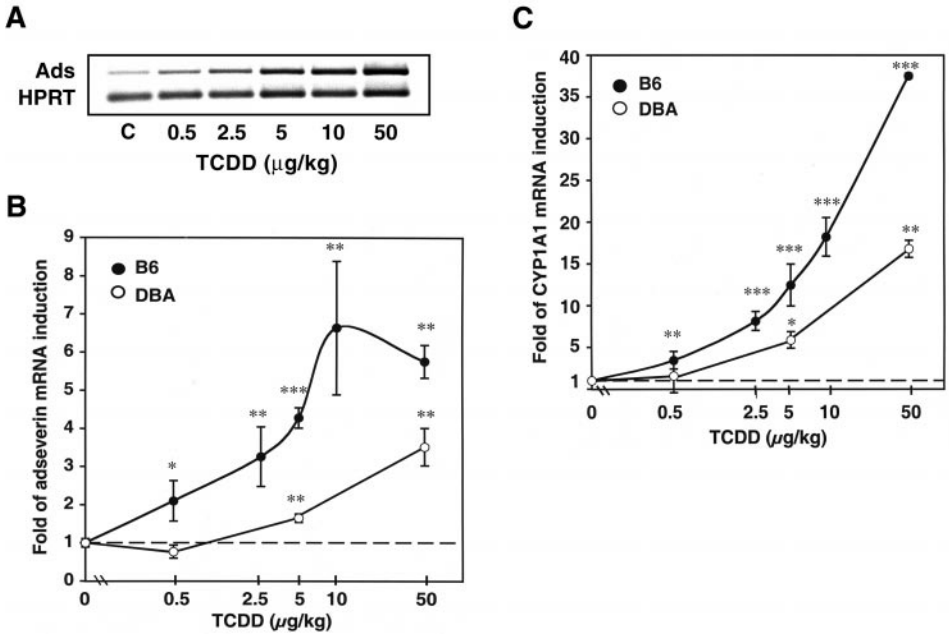

Fig. 3. Dose- and strain-dependent increase in thymic adseverin and CYP1A1 mRNA levels by TCDD. B6 mice (TCDD high responding, ●) and DBA mice (TCDD low responding, ○) were exposed to the indicated TCDD doses or the vehicle for 24 h. RNA was isolated from thymus and subjected to RT-PCR and the amplified cDNA species were separated by electrophoresis and visualized by autoradiography. A, autoradiogram showing the dose-dependent increase of the adseverin mRNA level (upper band) in B6 mice in relation to the internal standard HPRT (lower band), amplified in the same reaction as adseverin. B and C, TCDD-induced increase of mRNA levels of adseverin and CYP1A1, respectively, calculated by densitometric analysis. Each data point is the mean of three to five independent experiments, with the ratio of target gene/HPRT in the matched controls (vehicle treated mice of the same strain) set to 1. Error bars indicate S.D. *$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$, with respect to vehicle-treated, matched controls (broken line).

and Fig. 6 shows that the induction of adseverin mRNA, at 24 h after exposure to 10 μg of TCDD/kg, was even greater in thymocytes than in whole thymus. In addition, thymuses were always weighed before RNA isolation; Fig. 7 shows that a dose of 10 μg of TCDD/kg had no effect on thymus weight during the first 24 h. Neither was the thymocyte number affected within the first 24 h (data not shown). However, at 1 week after exposure, the thymus weight was reduced by roughly 50% indicating that 10 μg of TCDD/kg was indeed causing thymus atrophy. At 7 weeks after exposure, when the mice had reached an age of 12 weeks, we observed a reduction in thymus weight in the control mice, which was interpreted as age-related thymic involution. Interestingly, the thymus from TCDD-treated mice had instead regained weight, which was comparable with the thymus weight of 5-week old control mice (Fig. 7).

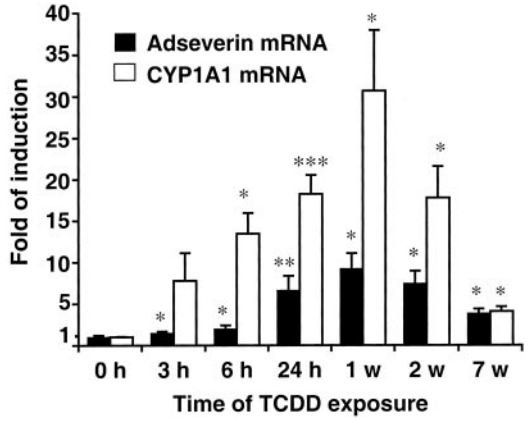

Fig. 4. Time-dependent increase in thymic adseverin and CYP 1A1 mRNA levels by TCDD. The time course of adseverin (■) and CYP1A1 (□) induction was compared by RT-PCR of RNA from thymus of B6 mice exposed to 10 μg of TCDD/kg for the indicated times. The autoradiograms were quantified by densitometric analyses and the data shown are the mean of three to five independent experiments with the ratio of target gene/HPRT in vehicle treated controls defined as 1. Error bars indicate S.D. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$, with respect to vehicle treated, matched controls.

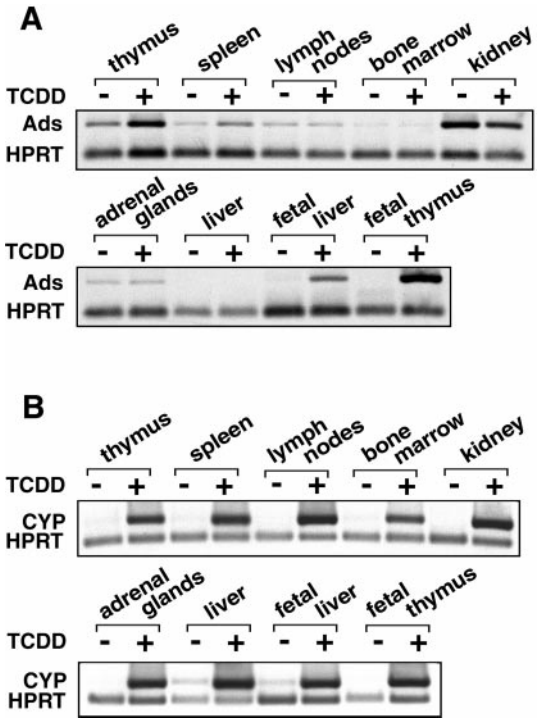

Fig. 5. Normal and TCDD-induced adseverin and CYP1A1 expression in different tissues. RNA isolated from the indicated organs of B6 mice exposed to 10 μg of TCDD/kg (+) or vehicle (−) for 24 h were subjected to RT-PCR analysis of adseverin and CYP1A1 expression using HPRT as an internal standard. The PCR products were separated by electrophoresis and visualized by autoradiography. A, a constitutive level of adseverin mRNA was detected in all tested organs except the adult liver. Note that there was a low but significant constitutive level of adseverin in the control fetal thymus and liver although it is invisible on this autoradiogram. TCDD treatment only induced adseverin mRNA levels in adult and fetal thymus, adult spleen, and fetal liver. B, a low constitutive level of CYP1A1 was observed only in adult and fetal liver. However, it was induced after TCDD treatment in all tested organs. Each band represents three individual experiments, all showing the same results. Ads, adseverin; CYP, CYP1A1.

TCDD-Induced Adseverin Gene Expression Is Evident at the Protein Level and Confined to the Thymic Cortex. We examined the expression of adseverin in isolated thymocytes by Western blot and by immunohistochemical analysis of thymic tissue sections. The Western blot shows that the TCDD-induced gene expression can be detected also at the protein level (Fig. 8), and Fig. 9 shows that the induction is confined to cells in the thymic cortex, which normally don't express high levels of adseverin. In control mice, adseverin is primarily expressed in the medulla, possibly by dendritic cells (Fig. 9).

No General Effect on Actin-Severing Proteins. Adseverin belongs to the gelsolin family of actin-binding proteins, which have similar structures, functions, and to some extent colocalize in the cell (Arai and Kwiatkowski, 1999). Gelsolin, the family member with highest structural homology and functional similarity to adseverin, showed a constitutive mRNA expression in mouse thymus but no change in mRNA levels 1 week after TCDD treatment when the induction of adseverin reached its maximum (Fig. 10).

The Up-Regulation Is Not a Secondary Effect to Changes in IL-9 Level. In mouse, adseverin was first described as one of several genes induced in a number of T-helper cell clones in response to interleukin-9 (IL-9) (Robbens et al., 1998). To test if the induction of adseverin was a secondary response to a TCDD-mediated increase in IL-9 gene expression, RT-PCR was performed on RNA isolated from whole thymus with primers specific for IL-9. A constitutive level of IL-9 was observed but no IL-9 gene induction by TCDD was seen at the time points tested (3 and 24 h) (Fig. 11).

Discussion

Despite years of research the mechanisms behind TCDD-induced immunotoxicity are still largely obscure, indicating

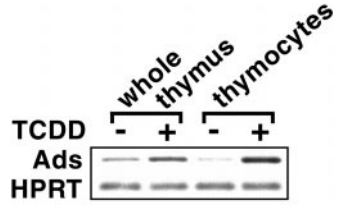

Fig. 6. Induction of adseverin mRNA levels in whole thymus and isolated thymocytes. Two micrograms of RNA isolated from either whole thymus or isolated thymocytes of B6 mice exposed to 10 μg of TCDD/kg (+) or vehicle (−) for 24 h were subjected to RT-PCR analysis of adseverin gene expression using HPRT as an internal standard. The autoradiogram is typical of three individual experiments. Ads, adseverin.

that the effects might be caused by induction or suppression of yet unidentified genes via AhR activation.

Using differential display and RT-PCR, we have identified a new TCDD-inducible gene coding for the actin-severing protein adseverin, also known as scinderin. The induction was evident at both the mRNA and protein level. In contrast, the mRNA level of adseverin D5, a proposed splicing variant of the same gene, was not induced, suggesting that the transcription of the two variants is differently regulated.

The increase of adseverin mRNA was observed already at 3 h after TCDD exposure, and at least 24 h before any signs of thymus atrophy was apparent. This indicates that the up-regulated adseverin mRNA level is a direct effect of TCDD on gene regulation, and not a consequence of a decrease in thymocyte number. This is further supported by the fact that the adseverin mRNA level was also up-regulated in thymocytes exposed in vivo and that the up-regulation was confined to the thymic cortex, where adseverin expression is normally low. Additionally, the constitutive level of gelsolin mRNA was unaffected by TCDD, suggesting that the induction of adseverin was not the result of toxicity affecting the expression of actin-binding proteins in general. Rather, adseverin is specifically up-regulated in an AhR-dependent manner, as indicated by the different TCDD-induced adseverin gene expression levels in B6 and DBA mice.

The increase of adseverin mRNA in the thymus after TCDD exposure was dose- and time- dependent and expressed similar kinetics to that of CYP1A1 induction. With a dose of 10 μg of TCDD/kg, maximum induction of adseverin (9-fold) was reached 1 week after exposure, and the effect remained for several weeks, possibly as a result of the long TCDD half-life. Even 7 weeks after TCDD exposure, the levels of adseverin mRNA were still significantly higher than

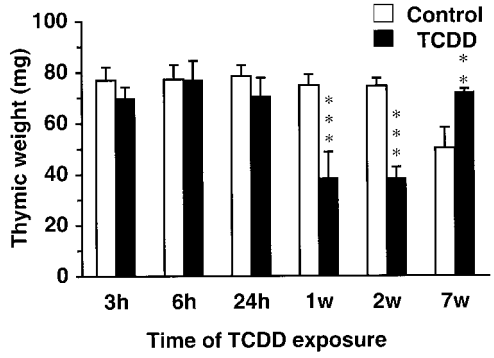

Fig. 7. Time course of thymic atrophy and recovery after TCDD exposure. Thymic weight was measured for individual B6 mice at the indicated time-points after exposure to 10 μg of TCDD/kg (■) or vehicle (□). Each column represents a mean of three mice per treatment group. Error bars indicate the S.D. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, compared with the vehicle-treated, matched controls.

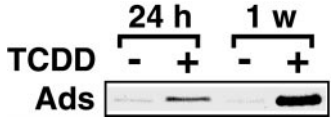

Fig. 8. Effect of TCDD on adseverin protein level. Proteins were extracted from isolated thymocytes of B6 mice exposed to 10 μg of TCDD/kg (+) or vehicle (−) for 24 h or 1 week and analyzed by Western blot. Equivalent amounts of cellular proteins (30 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis, conjugated with an anti-adseverin ab and visualized as described under *Materials and Methods*. The western blot shown is typical for three individual experiments.

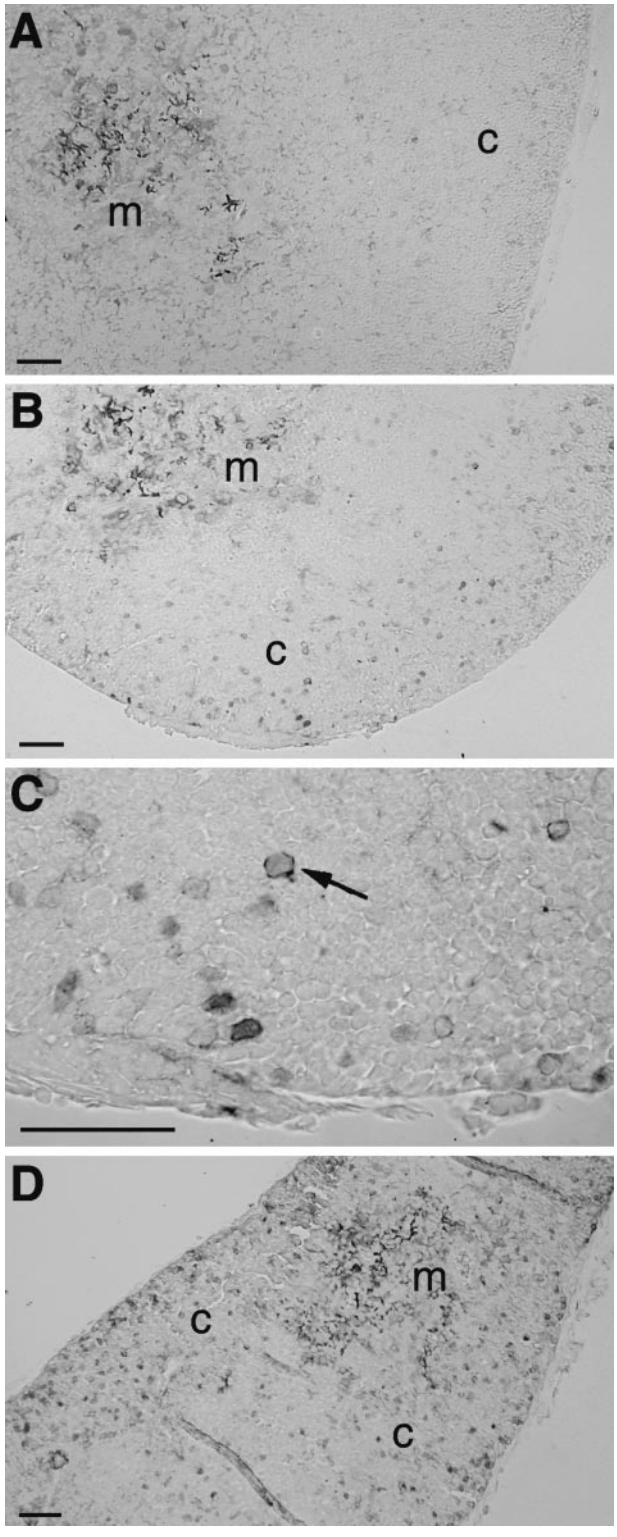

Fig. 9. Localization of TCDD-induced adseverin protein levels in the thymus: immunohistochemical localization of adseverin on paraffin sections of thymus from B6 mice. A, thymus of a vehicle-treated mouse showing a constitutive expression of adseverin in the medulla. B, thymus treated with 10 μg of TCDD/kg for 24 h showing adseverin staining in the medulla and in the cortex. C, high magnification of the cortical region of panel B (10 μg of TCDD/kg, 24 h exposure time). The arrow point to one of several cells with characteristic cellular staining of adseverin, with most adseverin localized beneath the cell membrane. D, thymus treated with 10 μg of TCDD/kg for 1 week showing high levels of adseverin staining in the cortex. m, medulla; c, cortex. Bars represent 50 μm.

in control animals. At this point, there were no longer any signs of thymus atrophy in the TCDD-treated animals that had regained the thymus weight of 5-week old control mice, whereas control animals showed signs of age-related thymic involution. This observation also discredits the possibility that the adseverin induction is a consequence of thymus atrophy. Moreover, it indicates that TCDD exposure can delay age-related thymic involution.

We also show that adseverin is constitutively expressed in a number of tissues such as the adult thymus, spleen, lymph nodes, bone marrow, kidney, adrenal glands, fetal thymus, and liver but not in adult liver. This agrees with earlier published results on adseverin expression in the mouse by Lueck et al (1998) and Arai and Kwiatkowski (1999), except that by using the more sensitive RT-PCR method, we also detected a low expression of adseverin in adult spleen. The adseverin expression in lymph nodes, bone marrow, and fetal thymus that we report here had not been examined previously.

Interestingly, under the experimental conditions described in this study, TCDD induced adseverin gene expression only in adult and fetal thymus, fetal liver, and adult spleen. This is in contrast to CYP1A1 gene expression, which was induced by TCDD in all organs tested. The immune-specific induction of adseverin may mirror the presence of a certain cell, or of a cell in a certain state of activation or differentiation that expresses additional regulatory factors needed for induction of the adseverin gene expression above baseline levels. Common to these organs (i.e., the fetal liver, the fetal and adult thymus, and the adult spleen, but not the lymph nodes) is the presence of hematopoietic progenitor cells that have the potential to develop into mature lymphoid cells (Reisner et al., 1978; Shortman and Wu, 1996; Kawamoto et al., 1997). The lack of TCDD-induced adseverin expression in the bone marrow, which also contains hematopoietic progenitor cells (Spangrude et al., 1988), is interesting and in agreement with earlier findings from our group, showing that acute exposure to TCDD had no effect on cell proliferation in the bone marrow while it was inhibited in the thymus (Lundberg et al., 1990). One explanation for this, which may also be applicable to the lymph nodes, could be that the environment of a cell is also important for the outcome of TCDD exposure. A certain cytokine milieu might be necessary for either making the promoter region of the adseverin gene available to binding by the TCDD-AhR-complex or for expression of other transcription factors needed for adseverin regulation. IL-9 has been shown previously to induce adseverin gene expression (Robbens et al., 1998), indicating that induction of adseverin by TCDD could be a downstream event of IL-9 induction. In our study, however, the involvement of IL-9 seems less likely because TCDD-exposed mouse thymus showed no increased IL-9 gene expression at the times tested (3 and 24 h).

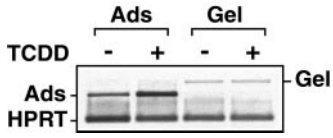

Fig. 10. Comparison of the effects of TCDD on the mRNA levels of adseverin and gelsolin. RNA isolated from whole thymus of B6 mice exposed to 10 μg of TCDD/kg (+) or vehicle (−) for 1 week were subjected to RT-PCR analysis for comparison of the expression of adseverin and gelsolin mRNA with HPRT as the internal standard. The autoradiogram shown is typical for three individual experiments. Ads, adseverin; Gel, gelsolin.

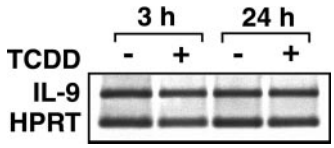

Fig. 11. Effects of TCDD on the mRNA level of IL-9 in the thymus. RNA isolated from whole thymus of B6 mice exposed to 10 μg of TCDD/kg (+) or vehicle (−) for either 3 or 24 h were subjected to RT-PCR analysis of IL-9 gene expression using HPRT as an internal standard. The autoradiogram shown is typical for three individual experiments.

Adseverin belongs to a highly conserved family of actin-binding proteins with multiple functions in the reorganization of actin filaments (Kwiatkowski, 1999). The actin-modulating activity is regulated through interactions with Ca^{2+} and phosphoinositides, mainly phosphatidylinositol 4,5-bisphosphosphate (PIP_2), and changes in pH (Rodriguez Del Castillo et al., 1992).

An increasing number of studies point out the importance of actin reorganization in response to external cell stimuli, and there is a well-established link between lymphocyte activation and the actin cytoskeleton. (reviewed in Bauch et al., 2000). The formation of a contact zone between a T cell and an antigen-presenting cell or—as in the thymus—a thymocyte and an epithelial cell induces early signaling events such as tyrosine phosphorylation, activation of Rho family GTPases, PIP_2 production, and activation of phospholipase Cγ (PLCγ) (Serrador et al., 1999; Dustin and Cooper, 2000). The subsequent increase in intracellular Ca^{2+} concentration activates actin-binding proteins such as adseverin and gelsolin, and these, through severing and capping, reorganize the cortical F-actin cytoskeleton and consequently stabilize the contact zone. Once the contact zone is stabilized, T cell receptor occupancy is prolonged, resulting in sustained Ca^{2+}-mobilization. This prolonged signaling is a prerequisite for activation of T cell effector functions such as proliferation and cytokine production (Valitutti et al., 1995; Delon et al., 1998), as well as thymocyte commitment to the $CD4^+$-lineage (Yasutomo et al., 2000).

The role of adseverin in TCDD-induced immunotoxicity remains to be investigated. We envision that inducibility of the adseverin gene and subsequent severing activity is a natural response and beneficial for cells that need to encounter an increased demand of actin reorganization. Such demands arise when a cell changes from a resting state to high proliferation and differentiation, as in the case of early hematopoietic progenitor cells, thymocytes, and mature T cells upon activation. However, persistently high levels of adseverin, induced by TCDD, might overdo the actin severing and, instead of reorganizing the actin cytoskeleton, sever and depolymerize F-actin. Thereby, formation of an actin scaffold and subsequently the stabilization of a contact zone and T cell activation or thymocyte selection, might be prohibited. This is supported by previous studies showing that depolymerization of the actin cytoskeleton prevents the formation of contact zones and of subsequent Ca^{2+}-responses (Valitutti et al., 1995; Delon et al., 1998). It is also possible that persistently high levels of adseverin, by competing with other PIP_2-binding proteins, such as PLCγ, are blocking the cleavage of PIP_2, an event important for further signaling. In-

creased levels of gelsolin have, through binding to PIP_2, been shown to inhibit the activity of PLCγ (Sun et al., 1997).

With the observed induction of adseverin it is possible to explain TCDD-induced thymus atrophy and the poor activation of the immune system when TCDD-exposed mice are challenged with antigen. It is also possible to explain the skewing toward the production of $CD8^+$ thymocytes because abrogation of T cell receptor-signaling favors commitment to the $CD8^+$ lineage (Yasutomo et al., 2000).

In conclusion, we have found that TCDD induces an immune-specific increase in adseverin mRNA and protein levels. The induction seems to be a primary, AhR-mediated response, not a consequence of general toxic effects or changes in cell number. It is possible that TCDD, by increasing adseverin levels, disturbs cellular signaling and subsequent thymocyte development and T cell activation. Thus, the adseverin gene could be a critical target for TCDD and could be involved in TCDD-induced immunotoxicity.

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