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Expression of the arylhydrocarbon receptor in the peri-implantation period of the mouse uterus and the impact of dioxin on mouse implantation*

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Summary. The arylhydrocarbon receptor (AhR) is a nuclear transcription factor mediating toxic effects of chemicals such as dioxins. The 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a member of polyhalogenated aromatic hydrocarbons family, exerts a wide-variety of toxic effects in a tissue- and species-specific manner including the reproduction process. Recently, AhR-mediated direct effects of TCDD on a cell-specific interaction with ovarian steroids have been shown. However, information regarding the effects of TCDD on the mouse implantation is limited. We therefore examined the expression and localization of AhR in the pregnant mouse uterus from 4 to 10 days of gestation (day 4 to day 10) using immunohistochemistry to investigate the effect of TCDD on uterine tissue during the peri-implantation period. Intense AhR expression was detected in the uterine vasculature throughout the periods examined. We also found that implanted blastocysts and their surrounding luminal epithelia and decidualized stroma expressed AhR on day 5. On days 6 and 7, persistent AhR expression was found in the transitional zone between the invading embryonic tissue and decidual tissue. On days 9 to 10, placental vasculature and spongiotrophoblasts displayed AhR immunoreactivity. The administration of TCDD on day 4 decreased the number of surviving implanted embryos on day 7 in a dose-dependent manner. This effect of TCDD was inhibited by the simultaneous administration of an AhR antagonist, alpha-naphthoflavone (α-NF). The spatio-temporal expression of AhR during the peri-implantation phase of the mouse uterus may indicate functional roles of this orphan receptor in feto-maternal interactions as well as substantiate the risk of exposure to chemicals such as dioxins during the reproductive period.

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Introduction

The arylhydrocarbon receptor (AhR) is a ligand-activated transcription factor that belongs to the basic helix-loop-helix/Per-AhR-Arnt-Sim (bHLH/PAS) superfamily of proteins (Wilson and Safe, 1998). Although the physiological ligand of AhR is still unknown, a variety of toxic environmental chemicals such as polyhalogenated aromatic hydrocarbons—including polychlorinated dibenzo-*p*-dioxins, dibenzofrans, polychlorinated biphenyls, and other structurally related molecules—have been shown as possible ligands. These toxic molecules exert carcinogenic, teratogenic and reproductive toxicity *via* their interaction with AhR, and activate its target genes (Poland and Kunutson, 1982; Peterson *et al.*, 1993; Birnbaum, 1995; Rowlands and Gustafsson, 1997).

The 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a member of the polyhalogenated aromatic hydrocarbons family and one of the most toxic environmental congeners, is mainly produced by industrial combustion and chemical manufacturing processes (Birnbaum, 1994, 1995). A wide variety of TCDD mediated toxic effects on humans and other mammals have been described previously (Kitajima *et al.*, 2004). TCDD has been studied as a prototype chemical of the AhR mediated toxicity in laboratory experiments.

In the rodent, reproductive organs such as the uterus and ovary as well as the liver, thymus, and skin have been shown to be the major target tissues of dioxin toxicity (Birnbaum, 1995; Mann, 1997). Exposure of mice to TCDD in the implantation period initiated by estrogen results in reduced implantation rates (Johnson *et al.*, 1992). However, the mechanisms of TCDD toxicity on the implantation process in relation to the expression of AhR in target tissues have not been clearly defined.

Recent studies in mammals including humans have shown that AhR is present in utero-placental tissue (Manchester *et al.*, 1987; Peters and Wiley, 1995; Küchenhoff *et al.*, 1999; Tscheudschilsuren *et al.*, 1999a,b; Buchanan *et al.*, 2000; Hasan and Fischer, 2001; Kitajima *et al.*, 2004). In rabbits, the cellular localization of AhR in preimplantations or sex steroid hormone-treated uterine epithelia showed distinct alterations from supranuclear localization to diffuse cytoplasmic and nuclear localizations on day 6 of gestation (Hasan and Fischer, 2001). This report may indicate the functional role of AhR in relation to maternal steroid hormones during the implantation process (Hasan and Fischer, 2001). AhR is also expressed in the preimplantation embryo of several animals, including the mouse (Peters and Wiley, 1995; Matthews *et al.*, 2001). *An in vitro* study on the exposure of a mouse embryo to TCDD noted a growth promoting effect, though adverse effects of TCDD on mouse embryo have also been reported (Blankenship *et al.*, 1993; Tsutsumi *et al.*, 1998; Matthews *et al.*, 2001).

On the other hand, the implantation process may differ from species to species. In mice, a fertilized oocyte may develop into a blastocyst by the 4th day post ovulation—when the implantation process begins in the antimesometrial part of the uterus (Theiler, 1989; Kaufman, 1992; Dey, 1996). Along with embryonic development, the decidual reaction, i.e., the morphological and functional differentiation of endometrial tissue, which involves the endometrial epithelium, connective tissue, and vessel systems, may take place in the uterus (Theiler, 1989; Kaufman, 1992). In these processes, sex steroids and a related series of growth factors, chemokines, and prostaglandins participate in concert (Paria *et al.*, 2002). Endocrine disruptors such as TCDD may exert critical effects on the mouse uterus at this period *via* interaction with AhR.

Previously, we found a strong AhR expression in the mouse endometrial vasculature as well as endometrial epithelial cells, and the cell-specific expression of AhR was increased by TCDD administration (Kitajima *et al.*, 2004). Studies of the AhR KO mouse have shown a development of vascular hypertrophy and mineralization in the uterus and an impaired reproductive outcome (Fernandez-Salguero *et al.*, 1997; Abbott *et al.*, 1999;). AhR may have possible functions in the implantation process, and its expression in these tissues may increase the sensitivity to chemicals such as TCDD. Although these studies suggest that the uterine AhR may play important roles in mice implantation processes, the spatio-temporal expression pattern of AhR in the implantation period of the mouse uterus has not been demonstrated.

In this study, we examined spatial and temporal patterns of AhR expression in the peri-implantation period of the mouse uterus by immunohistochemistry. In addition, we studied the effect of the exposure to TCDD in the mouse uterus in this period by a subcutaneous dosing of TCDD and/or AhR antagonist, alpha-naphtoflavone (α-NF) on day 4 of gestation.

Materials and Methods

Animals

Female B6C3F$_1$ mice at seven weeks of age were purchased from Charles River Japan Inc. and allowed to acclimate for one week. The mice were caged in groups of three or four under controlled environmental conditions and received food (PicoLab Rodent Diet 20, Japan SLC, Hamamatsu) and water ad libitum. After confirmation of the presence of estrus cyclicity by vaginal smear, the mice were injected (i.p.) with 5 IU of pregnant mere serum gonadotropin (PMSG, G4527, Sigma, St. Louis, MO, USA) and 48 h later with 5 IU of human chorionic gonadotropin (hCG, Mochida Pharmaceutical Co Ltd., Tokyo). Then they were housed individually with male mice of the same strain with proven fertility. Mating was confirmed the following morning by the presence of a vaginal copulatory plug and was designated as day 1 of gestation. From day 4 to day 10 of gestation, three animals were sacrificed and laparotomized each morning. Experimental procedures applied in these studies were approved by the Institutional Animal Care of Use Committee, Nagasaki University, according to the Guiding Principles in the Use of Animals in Toxicology.

TCDD treatment

In another group (n=5), 0.01, 0.1, 1, 10 μg/kg body weight (BW) of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, D-404S, Lot. B1040436, AccuStandard, New Haven, CT) were administered subcutaneously on day 4 of gestation. TCDD were dissolved and administered in 0.1mL of corn oil (C8267, Sigma) as a vehicle. We set 10 μg/kg BW of TCDD as the highest dosage in this experiment because previous studies showed significant effects on the mouse endometrium or hormonal environment at this concentra-

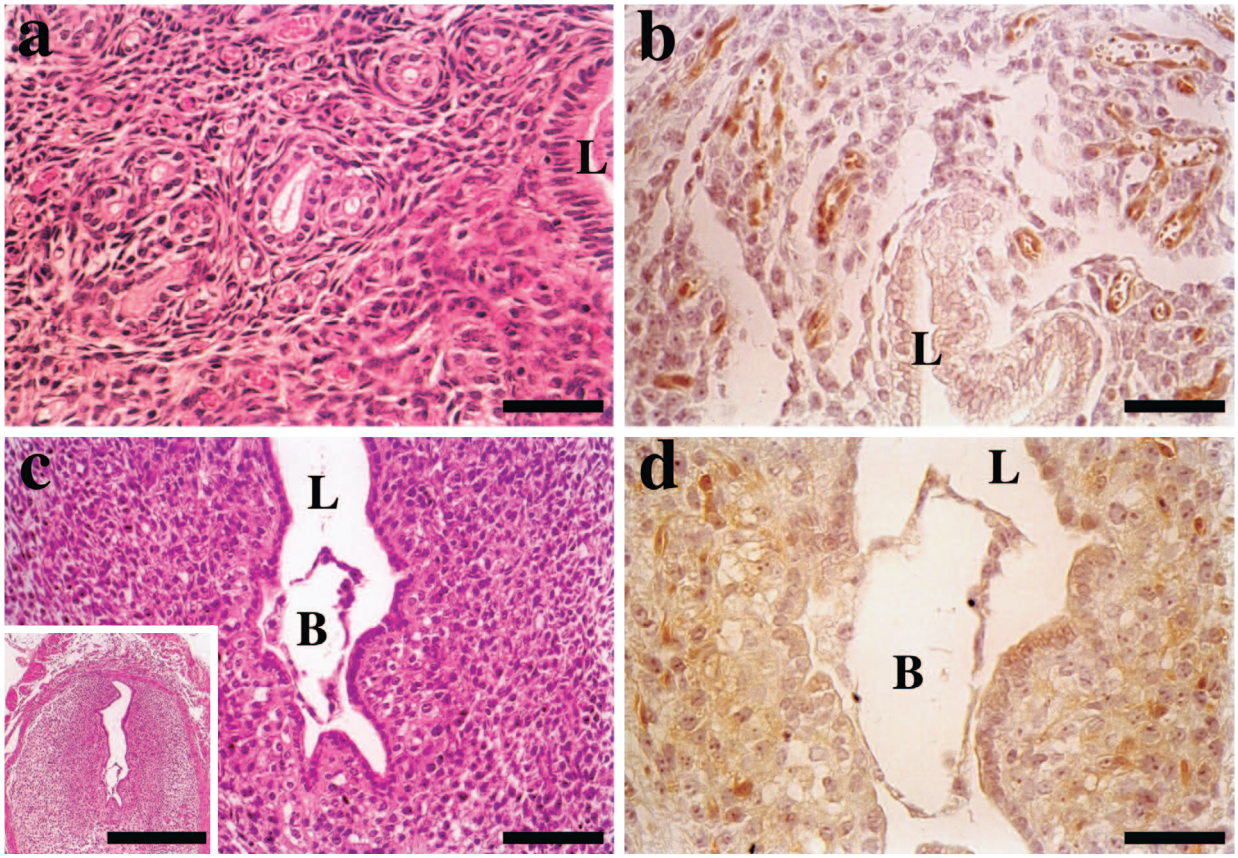

Fig. 1. Expression and localization of the arylhydrocarbon receptor (AhR) in the mouse uterus on days 4 and 5 of gestation. **a:** Photomicrograph of hematoxylin and eosin (H-E) staining of the mouse uterus on day 4. Bar=50 μm. **b:** Photomicrograph of AhR immunostaining of the mouse uterus on day 4. AhR immunoreactivity was dominantly found in blood vessels in the stroma. Immunostaining in the uterine luminal epithelium or glandular epithelium are faint. bar=50 μm. **c:** Photomicrograph of H-E staining of the mouse uterus on day 5. A blastocyst (B) is floating in the uterine lumen (L), ultimately to attach to the uterine luminal epithelium. Bar=100 μm; 0.5 mm (small inlet). **d:** Photomicrograph of AhR immunostaining of the mouse uterus on day 5. AhR immunoreactivity can be found in the cytoplasm of the cells surrounding the implanted blastocyst, including the uterine luminal epithelium. Some of the cells in these regions have positively stained nuclei. The implanted blastocyst also presents immunoreactivity for AhR. Blood vessels in the outer zone of the decidual reaction show a strong immunopositivity for AhR. Bar=50 μm

tion (De Vito *et al.*, 1992; Li *et al.*, 1995; Kitajima *et al.*, 2004). In another five animals in each group, 10 μg/kg BW alpha-naphthoflavone (α-NF, N5757, Sigma), an AhR antagonist (Merchant *et al.*, 1993), was administered subcutaneously singly or simultaneously with 10 μg/kg BW TCDD on day 4 of gestation. On day 7, the mice were sacrificed and laparotomized. The uterus was removed and weighed, and the numbers of implantation sites were counted.

Tissue preparation

The uteri were removed and fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS), processed through graded alcohols, and embedded in paraffin wax. Five-μm sections were attached to glass slides coated with aminopropyltriethoxysilane (APS coated slide, Matsunami Glass, Osaka) for morphological analyses including that with hematoxylin and eosin staining and immunohistochemical staining.

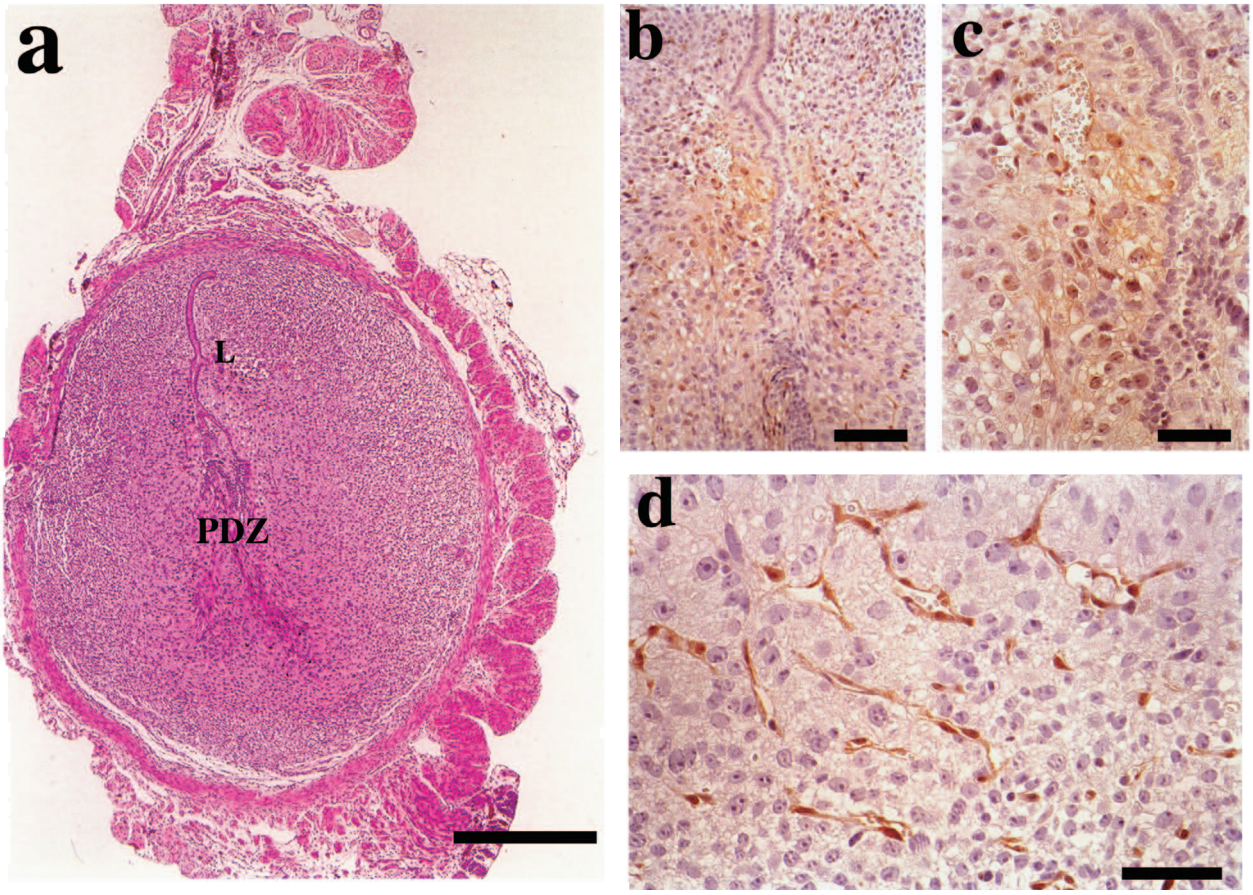

Fig. 2. Expression and localization of the arylhydrocarbon receptor (AhR) in the mouse uterus on day 6 of gestation. **a:** Photomicrograph of H-E staining of the mouse uterus on day 6. Decidualization initiated at the anti-mesometrial pole of the uterus, primary decidualizing zone (PDZ). L: uterine lumen. Bar=0.5 mm. **b–d:** Photomicrographs of AhR immunostaining of the mouse uterus on day 6. AhR immunoreactivity can be found in the boundary zone of the uterine lumen and decidualizing reaction (**b**). Walls of dilated blood vessels are immunopositive for AhR in these regions. Some of the cells surrounding the vessels have positively stained nuclei (**c**). Blood vessel wall in the outer zone of PDZ show strong immunopositivity for AhR (**d**). Bars=100 μm (b); 50 μm (c,d) .

Immunohistochemistry

For Immunohistochemistry, five-μm sections were deparaffinized and dehydrated. The sections were pretreated in a 10 mM citrate buffer solution (pH 6.0) at 121℃ for 15 min. Endogenous peroxidase activity was quenched by immersion in 0.3% H_2O_2 in methanol for 15 min. The sections were incubated with goat IgG (500μg/m*l*, I9140, Sigma) and 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature to block any nonspecific binding of the antibodies. Then the sections were reacted with a rabbit polyclonal anti-AhR antibody (SA-210, BIOMOL, Plymouth Meeting, PA, USA) diluted with 1% BSA in PBS (1:500) overnight at room temperature in a moist chamber. After three 15 min washings in PBS with 0.075% Brij 35 (Sigma), the sections were incubated with horse radish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulins (1:200, P0448, DAKO, Glostrup, Denmark) diluted with 1% BSA in PBS for 1 h at room temperature, and washed as described above. The specific immunoreactivity was visualized by 3,3'-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2. The sections were counter-stained with Mayer's hematoxylin, dehydrated, and coverslipped for analysis. As a control, some of the sections were reacted

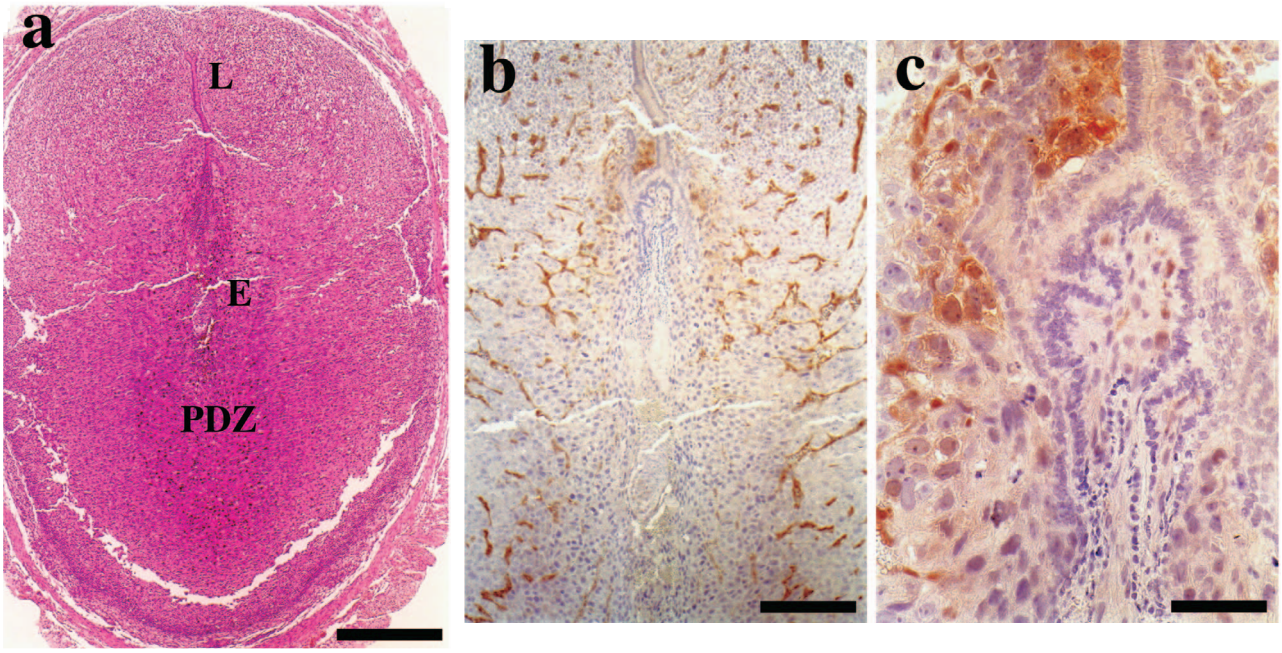

Fig. 3. Expression and localization of the arylhydrocarbon receptor (AhR) in the mouse uterus on day 7 of gestation. **a:** Photomicrograph of H-E staining of the mouse uterus on day 7. Decidualization spreads through the uterus. PDZ: primary decidualizing zone, L: uterine lumen, E: embryo. Bar=0.5 mm. **b** and **c:** Photomicrographs of AhR immunostaining of the mouse uterus on day 7. Strong AhR immunoreactivity is found in the wall of blood vessels in the outer zone of the PDZ. Decidual cells in the PDZ are devoid of staining for AhR (**b**). Strong AhR immunoreactivity is also found in the boundary zone of the uterine lumen and decidualizing reaction. Some of the cells in the central part of the PDZ have positively stained nuclei (**c**). Bars=200 μm (b); 50 μm (c)

with rabbit IgG instead of the specific antibodies at the same dilution.

Statistical analysis

Results were expressed as mean ± SE, and the statistical differences among treatment groups were determined by one-way analysis of variance (ANOVA) with Fisher's PLSD test using StatView version 5.0 (SAS institute Inc., Cary, NC, USA). $P<0.05$ was defined as significant.

Results

Immunohistochemical localization of AhR in mouse uterus during peri-implantation period

The expression and localization of AhR were immunohistochemically evaluated in the pregnant mouse uterus from 4 to 10 days of gestation. On day 4, when the floating blastocysts entered the uterus, AhR was localized in blood vessels in the stroma and smooth muscle cells. Immnunopositive AhR in the uterine luminal or glandular epithelium was the lowest, and no nuclear staining was found (Fig. 1a, b). On day 5, the immunoreactivity was also found in cells within the inner zone of decidual tissue around the blastocyst, and a positive signal was also detected in implanting blastocysts. Some of the cells in the decidual tissue had positively stained nuclei (Fig. 1c, d). On day 6, as decidualization spread in the uterus (Fig. 2a), positive staining for AhR was found in the transitional part of the uterus from the luminal epithelial gland to the decidua (Fig. 2b, c). Intensely immunopositive cells were detected in blood vessels in the outer zone of the decidual tissue (Fig. 2d). On days 7 and 8, when decidualization is prominent around the embryo (Fig. 3a), the positive signal was intense in the wall

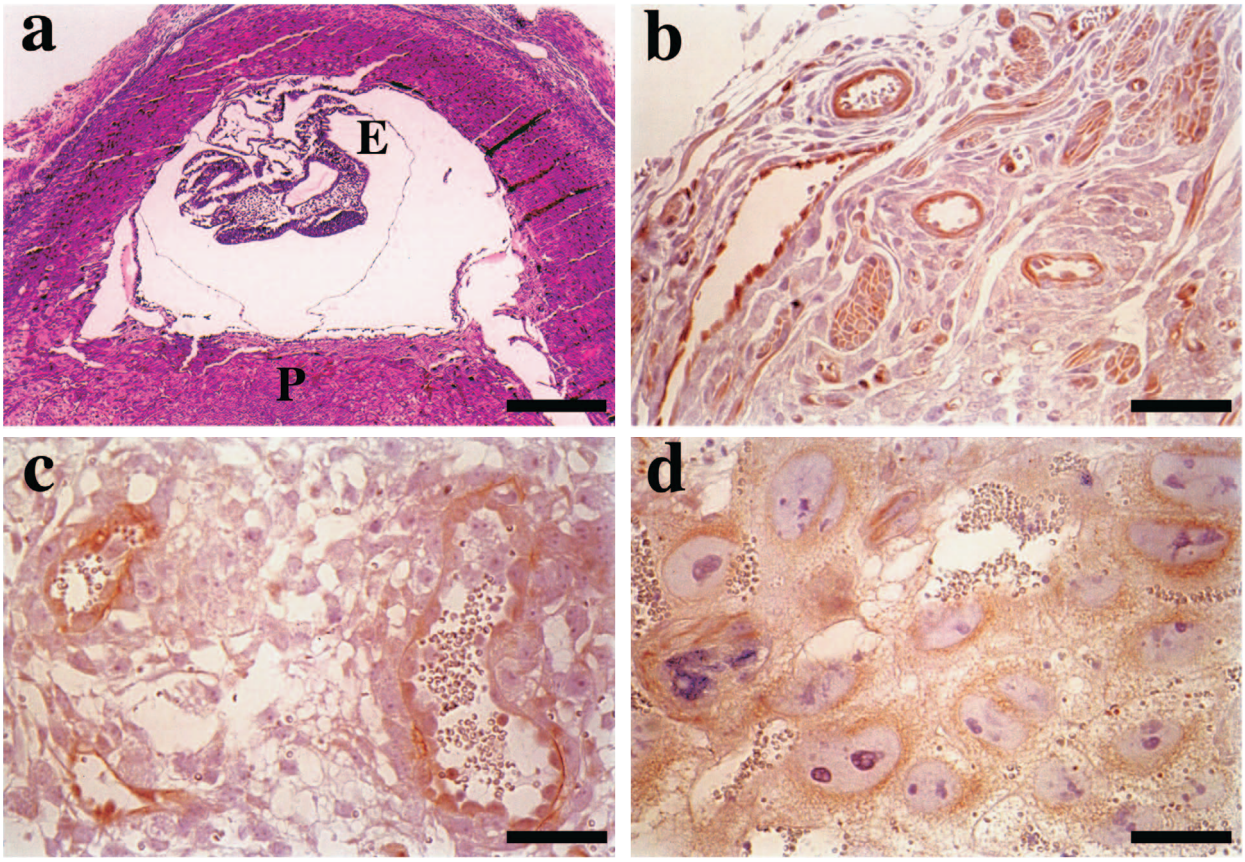

Fig. 4. Expression and localization of the arylhydrocarbon receptor (AhR) in the mouse uterus on days 9 and 10 of gestation. **a:** Photomicrograph of H-E staining of the mouse uterus on day 9. The embryo (E) is now under development in the amniotic cavity. The placenta (P) has also formed and is under differentiation. Bar=0.5 mm. **b:** Strong AhR immunoreactivity is found in dilated maternal blood vessels at the periphery of the placenta in part of the outer longitudinal layer of myometrial smooth muscles on day 9. **c:** Positive staining for AhR is also found in the wall of dilated blood vessels in the labyrinthine part of placenta on day 10. **d:** Immunopositivity for AhR is found in the spongiotrophoblast layer at periphery of placenta on day 10. Bars=50 μm (b–d)

of dilated blood vessels in the outer part of the primary decidualizing zone (PDZ). Cells in the center of PDZ were negative for AhR (Fig. 3b, c). On days 9 and 10, an intense signal was observed in the wall of dilated blood vessels in the labyrinthine part and in the periphery of the developing placenta (Fig. 4b, c). The immunoreactivity was also strongly positive in spongiotrophoblasts at the periphery of the placenta (Fig. 4d). No staining was discovered with normal rabbit IgG in place of the anti-AhR antibody (data not shown).

Effects of TCDD administration on the expression of AhR in mouse uterus during peri-implantation period

As AhR, a specific dioxin receptor, was immunohistochemically expressed in the peri-implantation phase of the mouse uterus, TCDD was administered to pregnant mice on day 4 of gestation to examine any effects on the implantation process. When the mice were exposed to 0.01 or 0.1 μg/kg BW of TCDD, we did not find significant differences in the number of implantation sites compared with the mice, which received the vehicle alone on day 7. On the other hand, when mice were exposed to 1 or 10 μg/kg BW,

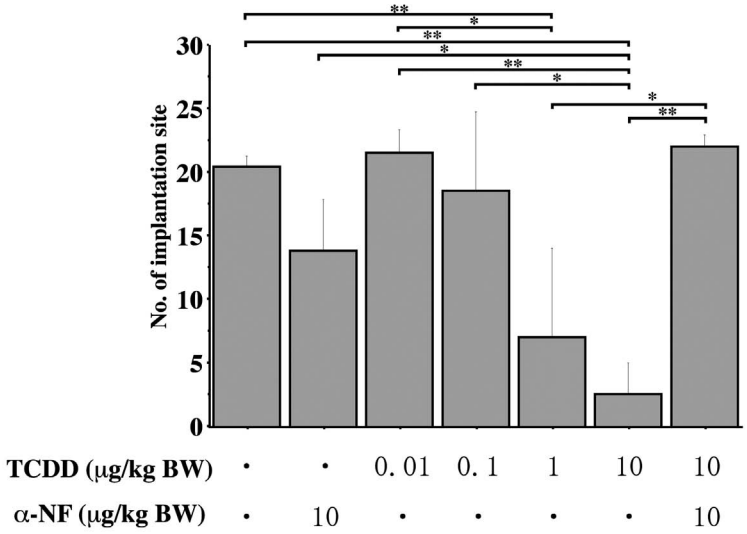

Fig. 5. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and alpha-naphthoflavone (α-NF) on mouse implantation. TCDD (0.01, 0.1, 1, 10 μg/kg BW) and/or αNF (10 μg/kg BW) were administered subcutaneously on day 4 of gestation. On day 7, implantation sites were counted by laparotomy. TCDD had affected mice implantation in a dose dependent fashion, which was reversed by the administration of the competitive AhR antagonist, α-NF, simultaneously with TCDD. Data are presented as the means ± SE. Statistical analysis was performed by one-way ANOVA with Fischer's PLSD test. * P<.05, **P<.01.

the numbers of implantation sites significantly decreased in a dose-dependent manner (Fig. 5). To delineate that these effects of TCDD were mediated through AhR, TCDD and α-NF were simultaneously administered to pregnant mice on day 4. The number of implantation sites after combined treatment with α-NF and TCDD increased to the values of the mice that had been treated with the vehicle only on day 7. The number of implantation sites in the animals with α-NF alone decreased slightly compared with those treated with the vehicle only, although the numbers between these two groups did not significantly differ (Fig. 5).

Discussion

Although the expression of the AhR mRNA and protein was identified in utero-placental tissue in several species (Dolwick *et al.*, 1993; Igarashi *et al.*, 1999; Küchenhoff *et al.*, 1999; Tscheudschilsuren *et al.*, 1999a, 1999b; Buchanan *et al.*, 2000; Pitt *et al.*, 2001; Kitajima *et al.*, 2004), this is the first report on the spatio-temporal expression of the AhR protein in the pregnant mouse uterus with implanted embryos during the peri-implantation period. In the present study, intense AhR expression was detected in the mouse uterine vasculature throughout this dynamic reproductive process. The implantation process in mice requires dramatic histo-morphological alterations in the uterus, which undergoes decidualization. While decidualization spreads out in the uterine endometrium, the re-establishment of uterine vasculature occurs concomitantly with normal implantation. In fact, although adult male and female mice deficient in AhR are fertile, the female AhR −/− suffers from vascular hypertrophy and mineralization in the uterus, resulting in abortion and poor survival during pregnancy and lactation (Fernandez-Salguero *et al.*, 1997; Abbott *et al.*, 1999). These together with our present findings suggest that AhR may be involved in the maintenance of the vascular system in the pregnant uterus.

At implantation sites, AhR immunoreactivity was found in both implanted embryos at the blastocyst stage and the luminal epithelium and adjacent connective tissues surrounding the embryo. In accord, it was reported that murine preimplantation embryos express the AhR mRNA and protein, and cultured embryos in the medium with an AhR

antisense oligodeoxynucleotide result in the mal-development of embryos (Peters and Wiley, 1995). Moreover, the AhR mRNA is shown to be induced in the luminal and glandular epithelium at the site of transient blastocyst attachment to the antimesometrial uterine compartment during early gestation in the rabbit uterus (Tscheudschilsuren *et al.*, 1999). Recently, a cell-specific alteration in AhR expression has been demonstrated in the pre-implantation phase of the rabbit uterus by maternal steroid hormones (Hasan and Fischer, 2001). These lines of evidence may indicate functional roles for AhR in the early embryogenesis and implantation. Our finding on the implantation-site specific expression of AhR may further strengthen the importance of functional roles of this orphan receptor in feto-maternal interaction.

Besides the developing embryos, the expression of AhR is also found in the early phase of the placenta. There have been few reports delineating the expression of AhR in the placenta. In the rabbit uterus at 12 days of gestation, the placental syncytiotrophoblast displays the expression of the AhR mRNA, but not its protein product (Tscheudschilsuren *et al.*, 1999). According to our unpublished data, in the human chorionic villi, strong immunostaining for AhR is observed in syncytiotrphoblasts (our unpublished observation). In this report, AhR expression was demonstrated in the placental vasculature as well as spongiotrophoblasts in the periphery of the placenta, whereas most of the placental cells in the labyrinthine part of the primitive placenta lacked the AhR expression. These discrepancies could be due to species-specific differences in placental structure and development. Therefore, AhR may also be functionally involved in the development of the placenta in mice.

Our results may indicate that stromal vascular endothelial and smooth muscle cells as well as the luminal epithelium and implanted embryos are possible targets of dioxin toxicity during the peri-implantation period of the mouse uterus. The present experiments with TCDD exposure to pregnant mice in this period verified toxic effects of TCDD on the early gestational stage of mice. This specific inhibition of AhR by α-NF further demonstrated that AhR mediates dioxin toxicity to the mouse implantation processes. Therefore, although the endogenous ligand of AhR is still unknown, it is worthy to note the presence of the functional role of AhR in the normal implantation process. Considering the strong AhR expression during the peri-implantation period, it is reasonable to assume that rodent embryos are quite sensitive to chemical exposure such as dioxins.

It has been demonstrated that α-NF antagonizes anti-estrogenic effects of TCDD *via* AhR in human breast cancer cells (Merchant *et al.*, 1993). Moreover, α-NF alone did not show any significant differences in the expression level of AhR binding to dioxin responsive elements and anti-estrogenic effects, as compared with these of the control (Merchant *et al.*, 1993). Our findings concerning the number of implantation sites may also indicate that α-NF serves as an AhR antagonist only in the presence of TCDD. In addition, our present results suggest that an administration of α-NF alone might not disrupt the putative functional roles of AhR in the implantation process. It is possible that the molecular mechanism of the activation of the AhR gene battery by exogenous xenobiotics such as TCDD may differ from that of putative endogenous ligands.

Our present findings may suggest that AhR mediates TCDD toxicity on the implantation processes in mice. TCDD is known to have its anti-estrogenic property (Astroff *et al.*, 1991), though the exact mechanisms of anti-estrogenic effects of TCDD have not been fully determined. On the other hand, estrogen exposure at the time of initiation of implantation is mandatory in mice (Paria *et al.*, 1993). In addition, ovarian steroid hormones as well as related mediators such as growth factors, chemokines, prostaglandins, and a family of adhesion molecules are key substances that may affect implantation processes (Day, 1996; Ghosh and Sengupta, 1998; Paria *et al.*, 2002). Therefore, it is possible that TCDD may exert its anti-estrogenic toxicity via AhR by altering the estrogen-mediated induction of various key substances that are essential in implantation processes.

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