POSSIBLE INVOLVEMENT OF DIOXIN-ALTERED SIGNALING PATHWAY IN ANGIOGENESIS IN THE RAT PLACENTA

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Abstract

Vascular development is a sensitive target of dioxins in the fetal period, and is regulated by other conditions such as hypoxia. Arylhydrocarbon receptor (AhR) and hypoxia inducible factor-1α have a direct link to dioxin and hypoxia, respectively, and ARNT is a key mediator for the both transcription factors. We studied histopathology and gene expression on the vascular development in the control and TCDD-exposed placentas of Holtzman rats during the late gestation period. We administered pregnant rats TCDD (1,600 ng/kg. b.w.) on GD15 and found that the morphological development in blood sinusoids and trophoblasts in the labyrinth zone was hampered by TCDD treatment, and that the expression of Tie2 mRNA, that plays a role for both vascular remodeling and apoptosis was suppressed in TCDD-exposed placenta on GD20. The suppressed angiogenesis was also supported by an elevated expression of IP-10 mRNA, a potent angiogenesis inhibitor. The placenta from TCDD-exposed rats was under a hypoxic condition, characterized by an increased expression of GAPDH and glucose transporter-3. Abnormal morphology showed that TCDD-exposed placenta was occupied with glycogen cells on GD20. All these data suggest that TCDD suppressed mainly vascular remodeling through Ang/Tie2 system.

Introduction

The fetus is one of the most sensitive targets in life of mammals, and *in utero* and lactational exposure to dioxins has been reported to exhibit a wide spectrum of biological and toxicological responses, including reproductive, neurobehavioral and immune disturbances in the offspring, by which dams are not affected as much as their fetuses. The use of arylhydrocarbon receptor (AhR)-null mice provided experimental evidence to show that the essentiality of this transcription factor for the manifestation of dioxin toxicities. Earlier studies reported the occurrence of intrauterine fetal death in various animal species, but the precise mechanism underlying fetal death was largely unknown.

The placental vasculature develops at the early phase of gestation but still alters its structure even at late phase of gestation in order to increase the vascular bed size and supports the significant growth of fetus during this period. The labyrinth zone, in which nutrients and oxygen are exchanged between maternal and fetal blood circulations, is mainly composed of endothelial cells of fetal capillaries and trophoblasts that are derived from the fetus. The vascular development in the embryo consists of mainly two stages, vasculogenesis and angiogenesis. In vasculogenesis, blood vessels form through *in situ* differentiation of undifferentiated precursor cells (angioblasts) to endothelial cells that assemble into a vascular network, in which the adhesion of endothelial cells and periendothelial support cells is at the immature stage. The term angiogenesis was used to generally denote the growth and remodeling process of the primitive network into a complex network.[1] During this remodeling, periendothelial support cells are recruited to encase endothelial tubes, providing maintenance and modulatory functions to blood vessels. In each of these stages, growth factors and transcription factors have been identified to act as modulators. Vascular endothelial growth factor (VEGF) and its receptors (VEGFRs), such as fetal liver kinase-1 (Flk1) and fms-like tyrosine kinase-1 (Flt1), named VEGF/VEGFR system, are mainly associated with vasculogenesis. Angiopoietin-1 (Ang1) and Ang2 and their receptor Tie2, named Ang/Tie2 system, are involved mainly in vascular remodeling.[2]

Recently, other workers[3-6] reported that TCDD, 3-methylcolanthrene and cigarette smoke affect the development of vascular development in zebrafish, chicken and HUVEC cells by affecting mainly VEGF/VEGFR system, perhaps triggered by HIF-α signal transduction pathway. In the present paper, we review and introduce the preceding works and new findings on how dioxin exposure induces fetal death in terms of the

alterations in the placental morphology and vascular remodeling via Ang/Tie2 signal transduction pathway.

Materials and Methods

Animals and TCDD administration: Holtzman rats (Harlan Sprague-Dawley Inc.) were used for the experiments according to the guideline of animal experiments at NIES. Pregnant rats (9-week old) were administered TCDD (Cambridge Isotope Laboratory) dissolved in corn oil by gastric intubation at a single dose of 1,600 ng/kg bw. The placentas were collected from the uterus on GD16, 18 and 20, immediately frozen in liquid nitrogen, and kept at -80 °C until used.

Morphology: The placental tissue sections were prepared horizontally as described earlier.[7] Images of maternal blood sinusoids and fetal capillaries in stained endothelial sections were obtained by scanning sections under an Olympus microscope connected to a digital camera. Areas of the sinusoids and capillaries were subjected to morphometry. Apoptotic cells were detected by a kit (ApopTag S7101; Serologcical Co.) . All the placental tissues were prepared from live fetuses.

Semiquantitative RT-PCR: The expression of VEGF, Flk1, Flt1, Ang1, Ang2 and Tie2 mRNA was analyzed by the semiquantitative RT-PCR method.[8]

Statistical analysis: Student's *t*-test was used for morphometric measurements, band intensity of the semiquantitative RT-PCR analysis, and the number of apoptotic cells. To minimize dam-dependent effects, six placentas from a different dam used.

Results and Discussion

Blood vessel formation and development of placenta: The placenta plays a very important role in the transport of oxygen and nutrients from the maternal circulation to the fetal blood circulation, and at the same time, it acts as a barrier to xenobiotics, known as feto-placental barrier. The rodent placenta consists of two morphologically distinct zones, the junctional zone and labyrinth zone. Although an adequate orchestration of cell proliferation, differentiation and apoptosis is considered to be essential for the normal development of vasculature development in the labyrinth zone, how vasculature in the labyrinth zone develops even in the normal rat was largely unknown. In our study, we found a significant change in the morphology of the maternal sinusoids in the placenta from control rats. On GD16, both maternal blood sinusoids and fetal capillaries were constricted, and the trophoblasts were large and the trophoblastic layer of the interhemal membrane was thick. During a late gestation period (GD16-GD20), both maternal blood sinusoids and fetal capillaries became dilated, and the trophoblasts became smaller. These morphological changes are thought to be congruent with the physiological requirement of the growing fetus to take up oxygen and nutrients effectively. And during this period, we observed an increase in both VEGF/VEGFR system and Ang/Tie2 system at the same time in the control placenta.

Suppressive effects of TCDD on vascular remodeling in the placenta: When we administered pregnant Holtzman rats TCDD (1600 ng/kg b.w., p.o.) on GD15, we found a suppression of vascular dilation in both maternal sinusoids and fetal capillaries of the labyrinth zone on GD20, the morphology of which was very similar to that of GD16 of the control placenta. The proteomics analysis using 2-D gel electrophoresis and protein sequencing on the TCDD-exposed placental tissue revealed that amounts of heat shock protein 27 and beta-tropomyosin, an indicator for oxidative stress, were increased between GD16 and GD20, whereas that of glyceraldehydes 3-phosphate dehydrogenase (GAPDH), an indicator for hypoxia, was significantly elevated on GD20. We also found that in TCDD-exposed rat placenta, glycogen cells occupy the junctional zone at the time of which they disappear in the control placenta, and accordingly, glycogen contents were found increased in a TCDD dose-dependent manner. The up-regulation of glucose transporter-3 (GLUT-3) mRNA level, another indicator for hypoxia, supports not only the presence of hypoxia but also the accumulation of glycogen in the TCDD-exposed placenta. Taken together, these data suggest that TCDD exposure retarded the placental growth and development.

In addition, in the late gestation period, TCDD-exposure decreased a number of apoptotic cells in trophoblasts

less than half of that of the control placenta. In the human placenta, apoptosis in trophoblasts observed in pregnancies is triggered by hypoxia and is thought to be responsible, in part, for the vascular development.[9] Studying the expression of genes associated with blood vessel formation on GD20, we found that Tie2 mRNA, but not other genes in the VEGF/VEGFR system, was significantly suppressed. This observation is consistent with the activity of Tie2 that has a suppressing role in apoptosis in the endothelial cells as well as vascular remodeling. In a collaborative study, Mizutani and coworkers[10] used a separate set of the TCDD-exposed placental tissue under the same experimental protocol and performed a global gene expression analysis by microarray and quantitative RT-PCR analysis. A remarkable profile of the gene expression was that many (15 out of 18) interferon-inducible genes were up-regulated by the TCDD treatment. In particular, interferon-γ-inducible protein-10 (IP-10), an inhibitor for angiogenesis, was significantly up-regulated on GD20.

AhR, ARNT and HIF-1α in Ischemia-induced Angiogenesis: The current paradigm of AhR dependent toxicity is outlined as follows. First, activated AhR by ligands translocates to the nucleus and dimerizes with AhR nuclear translocator (ARNT; also called as HIF-1β); second, this AhR/ARNT complex binds xenobiotic responsive element (XRE; also known as dioxin responsive element) in the promoter region of genes; third, the genes transcribe the corresponding mRNAs, followed by production of proteins, such as CYP1A1 and UDP-glucuronosyl transferase, and finally, biological and/or toxicological responses are triggered. Besides AhR/ARNT-dependent toxicities, HIF-1α is presumably another transcriptional factor that modulates dioxin toxicities as well as a hypoxic signal transduction pathway because it is heterodimerized with ARNT to bind hypoxia response element (HRE) in the promoter region of genes whose expression may induce functionally active genes such as vascular endothelial growth factor (VEGF). For these two distinct signal transduction pathways, ARNT is a common transcription factor that shares its role with AhR and HIF-1α to modulate XRE- and HRE-dependent pathways, respectively. Thus, it has been speculated that AhR/ARNT pathway and HIF-1α/ARNT pathway is on a balance between which pathway is activated under particular physiological conditions, although there is another supposition that amounts of ARNT are so sufficient enough not to affect these pathways. And yet, the presence of dioxin and other arylhydrocarbons and/or the occurrence of hypoxia are thought to modulate these signal transduction pathways which is thought to be related vasculogenesis and angiogenesis.

Ivnitski-Steele and Walker[4] reported that TCDD inhibits early events in coronary endothelial tube formation and outgrowth and blocks vasculogenesis in chick embryos, and that its inhibition was rescued by the treatment of VEGF. Using human umbilical vein endothelial cells (HUVEC), Michaud and coworkers[11] found that cigarette smoke extract inhibits tube formation by HUVEC by inhibiting the expression of VEGF. Transfection of adenoviral vector carrying HIF-1α in these cells rescued this inhibition. They also found the cigarette smoke extract suppressed the increased level of VEGF and HIF-1α mRNAs induced by the ischemic-induced increase in these genes. An AhR agonist, 3-methylcholanthrene, has been shown to inhibit DNA synthesis as determined by ^{3}H-thymidine incorporation, but its inhibition was compromised by an AhR antagonist, α-naphthoflavone. Recently, Ichihara and coworkers[12] found that hypoxia produced by ligation of femoral artery markedly induced angiogenesis in AhR-null mice and wild-type mice, with a significant increase of the former to the latter. In these ischemic AhR-null mice, HIF-1α and ARNT as well as target genes for these transcription factors, such as VEGF, were found up-regulated for not only mRNA but also protein, compared to those in wild-type animals. In addition, the DNA-binding activity of the HIF-1α-ARNT complex as well as the association of these transcription factors with the promoter region of VEGF gene was increased by ischemia by a greater extent in AhR-null mice than in wild-type animals. Thus, the authors suggest that an increased quantity and activity of the HIF-1α-ARNT heterodimer in ischemia-induced AhR-null mice may explain at least in part the enhancement of ischemia-induced VEGF and angiogenesis.

The data obtained so far demonstrated that dioxin and other AhR ligands affect the vasculogenesis and angiogenesis via HIF-1α-HRE and AhR-XRE signal transduction pathways, depending upon the tissue and cell specific, and developmental stage specific manners. During the late gestation period, the placenta becomes hypoxic because of an increase in oxygen consumption by fetal growth and development, and the remodeling of the placental vasculature is perhaps needed for fetal tissues to cope with this hypoxic state. It is conceivable that hypoxic fetal tissues enhance the apoptosis of trophoblasts so that the remodeled labyrinth can manage more

oxygen transport. Because of the hypoxic condition, this remodeling might be regulated by HIF-1α-ARNT-dependent signal transduction pathway. And, TCDD administration *in utero* disrupts this pathway in the case of Holtzman rats. On the other hand, we recently found that pregnant Sprague-Dawley rats that are not prone to vascular remodeling in the labyrinth zone in contrast to the Holtzman rats harbor the same AhR gene as Holtzman rats do. And the Sprague-Dawley rats were resistant to a high TCDD dose (10 μg/kg b.w.) and that such a high dose failed to cause any pathological alterations in morphology of the placenta.[13] These observations suggest a difference in the development of placental vasculature, not the AhR sequence may explain the strain difference in the TCDD toxicity in the placenta and fetuses. Future investigations on the morphological and molecular basis for such a difference in susceptibility between the two rat strains rats will provide at least a part of the intriguing questions about how HIF-1α-ARNT as well as AhR-XRE-dependent signal transduction pathways are involved not only in the manifestation of the toxicity of dioxins in the placenta and fetuses, but also the normal development of the vascular development in the placenta.

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