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Induction of Tryptophan 2,3-Dioxygenase in the Mouse Endometrium during Implantation

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Indoleamine 2,3-dioxygenase (IDO) is expressed in trophoblasts and defends the conceptus against rejection by reducing the tryptophan level and suppressing the T cell activity. We isolated a cDNA for tryptophan 2,3-dioxygenase (TDO), another key catabolizing enzyme of tryptophan, from a mouse uterus cDNA library enriched with pregnancy-induced genes. Northern blot and *in situ* hybridization analyses demonstrated that the *TDO* mRNA was induced in the decidualized stromal cells around the implanted embryo at the time of implantation. The expression was then upregulated and primarily localized at the mesometrial decidua. *TDO* mRNA was induced by decidуoma formation as well as embryo transfer but not by ovarian steroid hormones. These findings demonstrated that TDO is induced in the endometrial stromal cells concomitant with decidualization and suggested its involvement in the implantation process by regulating the tryptophan level at the implantation site. © 2000 Academic Press

Key Words: **tryptophan 2,3-dioxygenase; mouse; endometrial stromal cell; implantation; *in situ* hybridization.**

Successful implantation and development of embryo require complex interactions between the embryo and the uterus. In the mouse, when a blastocyst reaches to the uterus 4 days after fertilization, the uterine endometrium becomes receptive to implant by the embryo (1, 2). After the attachment of embryo, endometrial stromal cells proliferate and differentiate to decidua cells, which is called decidualization. Though these processes are regulated by a timely interplay of the maternal hormones, estrogen and progesterone, some endometrial changes, such as increased permeability of the subepithelial capillaries surrounding the blastocyst, seem to occur in response to the blastocyst (1, 2). Although signals and molecular pathways to regulate these phenomena have been gradually recognized, among these are cell surface adhesion molecules, cytokines and growth factors and their receptors (3–5), the mechanism of the complex series of interactions required for implantation is still largely unknown.

We previously used the complementary DNA (cDNA) subtractive hybridization technique and demonstrated that calcium binding protein D-9k mRNA is induced in the endometrial epithelia under the control of progesterone and that its level is reduced by the presence of embryo in the glandular epithelia (6). In the present study, we performed another set of subtractive hybridization and identified one of the pregnancy-induced genes as the *tryptophan 2,3-dioxygenase* (*TDO*) gene. TDO, a tetrameric hemoprotein consisting of four equivalent subunits and two protoheme IX, is the key regulatory enzyme that degrades tryptophan into n-formylkynurenine by catalyzing the insertion of molecular oxygen into the 2,3-bond of the indole moiety of L-tryptophan (7). It is reported to be expressed in the liver, brain (8), and skin (9), and induced by glucocorticoid (10). Recently, another tryptophan catabolizing enzyme, indoleamine 2,3-dioxygenase (IDO), has been reported to be expressed by trophoblasts and defend the conceptus against rejection by suppressing the T cell activity (11). In this study, we examined the expression profile of *TDO* mRNA in the uteri of nonpregnant and early pregnant mice, especially during implantation period. We also investigated the effect of steroid hormones on the induction of *TDO* mRNA.

MATERIALS AND METHODS

Animals. Male and female CD-1 mice were obtained from Charles River Japan Inc. (Kanagawa, Japan). Six-week-old females were mated with adult males, and the presence of vaginal plug after mating was designated day 1 of pregnancy. Pseudopregnant mice were prepared by mating with vasectomized males. Estrus stage was ascertained by examining vaginal smears. Experiments were con-

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ducted in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by the Society for the Study of Reproduction.

Construction of a subtracted cDNA library. The cDNA subtraction was performed as described previously (6, 12) with some modifications. Total RNA was extracted from the uteri of mice at pseudopregnancy day 6 or pregnancy day 6 by acid-guanidinium-phenol-chloroform method using Trizol Reagent (Life Technologies, Rockville, MD) according to the manufacturer's protocol. Polyadenylated (poly(A)$^+$) RNA was prepared using a mRNA Purification kit (Pharmacia, Piscataway, NJ). After the synthesis of a double stranded cDNA from each poly(A)$^+$ RNA using a Time Saver cDNA Synthesis kit (Pharmacia), suppression subtractive hybridization was performed using a PCR Select Subtraction kit (Clontech, Palo Alto, CA) according to the manufacturer's protocol. The subtracted cDNAs were cloned into pBluescript SK(−) plasmid (Stratagene, La Jolla, CA), and the cDNA sequence of each clone was determined using autosequencer DSQ-1000 (Shimadzu, Kyoto, Japan).

Northern blotting. Total RNA (10 μg/lane) from uteri of mice was electrophoresed on a 1.0% agarose-formaldehyde gel and transferred to a nylon membrane (Hybond-N$^+$, Amersham, Arlington, IL). The membrane was incubated with prehybridization solution (Rapid Hybridization Buffer, Amersham) for 30 min at 65°C, and then hybridized with the ^{32}P-labeled probes for 2 h at 65°C in the same solution. After hybridization, the filter was washed in 2 × standard saline citrate (SSC) at room temperature for 5 min, followed by 0.2 × SSC and 0.1% sodium dodecyl sulfate at 65°C for 30 min, and then subjected to autoradiography. The membrane was washed and rehybridized with mouse β-actin probe or stained with methyleneblue to correct for the amount of loaded RNA.

In situ hybridization. In situ hybridization was performed as described previously (6). Briefly, a plasmid containing the murine *TDO* cDNA was linearized with the appropriate enzymes to provide sense and antisense templates. Digoxigenin-labeled single strand RNA probes were transcribed with T3 or T7 RNA polymerase, using a DIG RNA Labeling kit (Boehringer Mannheim Biochemica, Mannheim, Germany). Tissues were fixed with freshly prepared 4% paraformaldehyde in 0.1 M PBS for 24 h, and then embedded in polyester wax after dehydration. The tissues were cut into 4 μm thick sections. Before hybridization, the sections were dewaxed and hydrated with a graded ethanol series, fixed with 4% paraformaldehyde for 15 min, and pretreated successively with proteinase K, 0.2 N HCl, and 0.25% acetic anhydride in 0.1 M triethanolamine. The sections were dehydrated and air-dried. Hybridization was then carried out for 16 h at 50°C with approximately 0.5 μg/ml digoxigenin-labeled RNA probes in 50% formamide, 10% dextran sulfate, 10 × Denhardt's solution, 600 mM NaCl, and 250 μg/ml *E. coli* transfer RNA. After hybridization, the sections were treated with ribonuclease A (1 μg/ml) at 37°C for 30 min and washed twice in 2 × SSC and 0.2 × SSC for 20 min each at 50°C. The hybridized digoxigenin-labeled probe was visualized with a Nucleic Acid Detection kit (Boehringer Mannheim). Hybridization with the sense probe was carried out at the same time under identical conditions and served as a negative control.

Treatment of mice with sex steroid hormones. Oophorectomy was performed under anaesthesia with nembutal (Dainabot, Osaka, Japan) as described (13). Progesterone and 17β-estradiol (Sigma, St. Louis, MO) were dissolved initially in 100% ethanol and then diluted in sesame oil. Two weeks after the oophorectomy, the mice were given an injection subcutaneously with progesterone (2 mg/mouse) and/or estradiol (1 μg/mouse). The animals were killed by cervical dislocation 24 h after the injection, and their uteri were collected for RNA isolation. Oophorectomized mice injected with vehicle (sesame oil) alone served as a control. Two animals were used for each treatment group in these experiments.

Embryo transfer and the induction of deciduoma. Embryo transfer experiment was performed as described previously (6). Donor mice were injected intraperitoneally with pregnant mare serum gonadotropin (Teikoku Zoki, Tokyo, Japan) (5 IU/mouse) followed by human chorionic gonadotropin (Teikoku Zoki) (5 IU/mouse) 48 h later to induce ovulation, and mated with male mice. Blastocysts were collected by flushing the uteri of donor mice on pregnancy day 4. The recipient pseudopregnant mice were prepared by natural mating. On pseudopregnancy day 4, 10 blastocysts were transferred to the left uterine horn of the recipient mice. The medium without blastocysts was injected to the right horn of the same animals as a control. To induce the deciduoma, sesame oil was transferred to recipient mice in place of blastocysts. Uteri were collected 36 h later, fixed in 4% paraformaldehyde and used for *in situ* hybridization. Three recipients were prepared for embryo transfer experiment and two animals were used for deciduoma.

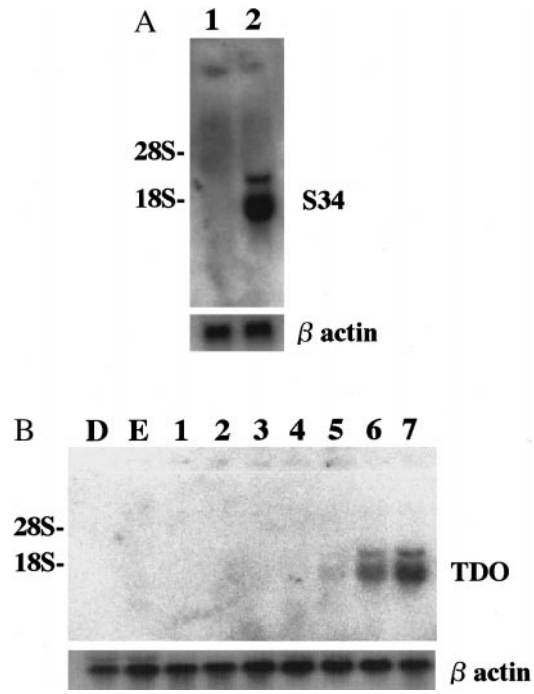

FIG. 1. Northern blot analysis of expression of *TDO* mRNA in the mouse uterus. Ten μg of total RNA from uteri of mice were analyzed using a ^{32}P-labeled clone S-34 (*TDO* cDNA) as a probe (upper panels). β-actin cDNA was also used as a probe to correct for the amount of RNA loaded (lower panels). (A) Expression in the uteri of mice on pseudopregnancy day 6 (lane 1) and pregnancy day 6 (lane 2). (B) Expression in the uteri of mice at diestrus phase (lane D), estrus phase (lane E), and pregnancy days 1 to 7 (lanes 1 to 7, respectively). Results are representative of two separate experiments.

RESULTS

Isolation of TDO Partial cDNA Clone (S-34)

After analyzing the subtracted cDNA library of mouse uterus enriched with pregnancy-induced genes, we isolated one clone (clone S-34). Northern blot analysis using the clone S-34 as a probe revealed two distinct transcripts in the pregnancy day 6 uterus whereas no band was detected in the pseudopregnancy day 6 uterus (Fig. 1A). The nucleotide sequence of the 682-bp insert of this clone was determined, which

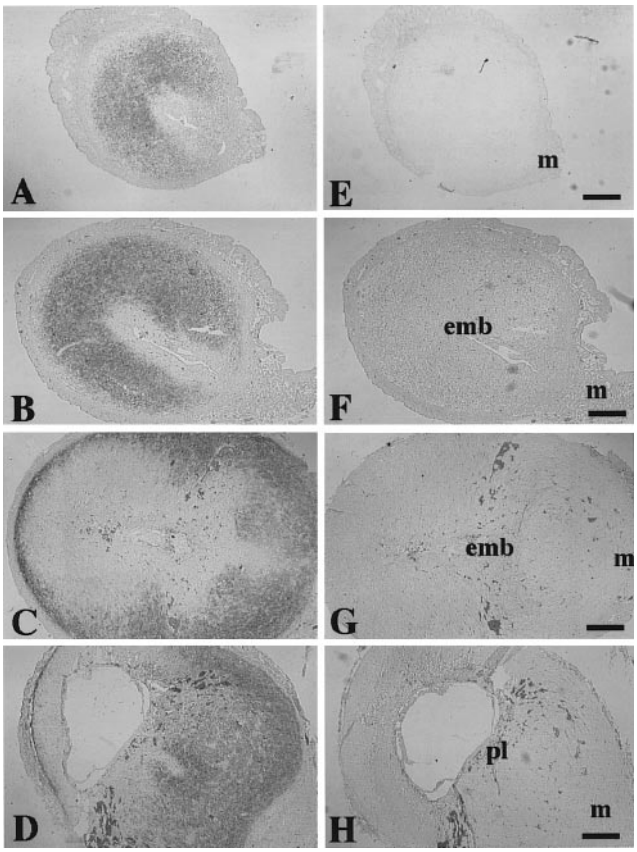

FIG. 2. *In situ* hybridization analysis of *TDO* mRNA expression in mouse uterus. Sections of the uterus made from mice on pregnancy day 6, 7, 8, or 9 were hybridized with antisense probe (A, B, C, and D, respectively) or sense probe (E, F, G, and H, respectively) for mouse *TDO*. Expression was observed in the endometrial stroma. Hybridization with sense probe did not give significant signals. emb: embryo, m: mesometrium, pl: placenta. Bars: 100 μm.

showed more than 98% identity with that of the mouse *TDO* cDNA (GenBank Accession No. U24493). Thus, we concluded that the clone S-34 was a *TDO* partial cDNA clone.

Northern Blot Analysis of TDO Expression in the Uteri during Early Pregnancy

To examine uterine *TDO* mRNA expression during early pregnancy and pseudopregnancy, Northern blot analysis was carried out. In the uteri of non-pregnant and pregnancy day 1, 2, 3, or 4 mice, the *TDO* mRNA expression was not detected. On pregnancy day 5, the expression of *TDO* mRNA was observed, which increased until day 7 (Fig. 1B). In pseudopregnant mice, no *TDO* expression was detected throughout the observation period (data not shown).

In Situ Hybridization Analysis of TDO mRNA in the Uteri during Early Pregnancy

By *in situ* hybridization histochemistry, *TDO* mRNA was detected in the stromal cells around the implanted embryo, but not in the epithelial cells on day 6 of gestation (Fig. 2A). On day 7 of gestation, strong *TDO* mRNA signal was observed throughout the endometrial stroma surrounding the implanted embryo (Fig. 2B). There was no *TDO* mRNA signal in the embryo proper and the differentiated endometrium immediately adjacent to the embryo. On day 8 of gestation, the signal was decreased in the stromal layer of antimesometrial region (Fig. 2C). On day 9 of gestation, the signal was primarily localized at the mesometrial decidua around the placenta (Fig. 2D). No specific signal was observed when sections were hybridized with a sense *TDO* probe (Figs. 2E–2H).

Effects of Progesterone and Estradiol on the Expression of TDO mRNA in the Uteri of Oophorectomized Mice

To determine whether the *TDO* mRNA expression is regulated by sex steroid hormones in the uterus, we administered progesterone and/or estradiol to oophorectomized mice. By Northern blot analysis, no induction of *TDO* mRNA was observed with progesterone nor estradiol (Fig. 3).

Effects of the Presence of Embryo on the Expression of TDO mRNA in the Uterus

To determine whether *TDO* mRNA expression in the decidualized endometrial stromal cells is affected by the presence of embryo, we transferred blastocysts into the uterus of mice on pseudopregnancy day 4. *In situ* hybridization analysis showed that the endometrial stromal cells at the implantation site expressed *TDO* mRNA 36 h after the embryo transfer (Fig. 4A). The induction was not observed in the control horn of the same animal (data not shown). The endometrial stro-

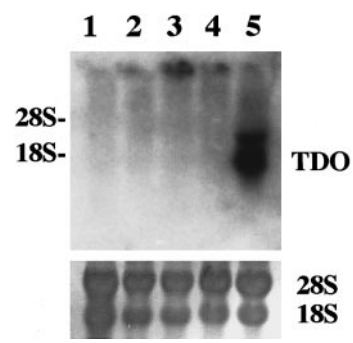

FIG. 3. Effects of progesterone and estradiol on the *TDO* mRNA expression in the uterus of oophorectomized mice. Ten μg of total RNA from the oophorectomized mice injected with vehicle alone (lane 1), progesterone (lane 2), estradiol (lane 3), or progesterone, and estradiol (lane 4) were subjected to Northern blot analysis using a ^{32}P-labeled *TDO* cDNA probe (upper panel). Uterine RNA from pregnancy day 6 mice was also hybridized as a positive control (lane 5). The membrane was stained with methyleneblue to visualize and assess the amount of 18S and 28S ribosomal RNAs (lower panel).

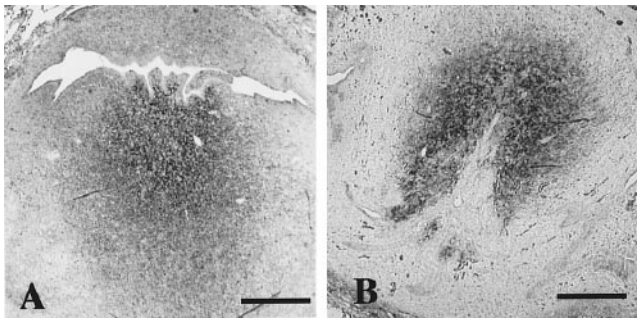

FIG. 4. *In situ* hybridization analysis of *TDO* mRNA expression in the uterus transferred with embryo or deciduoma. *TDO* mRNA was expressed in the decidualized endometrial stromal cells both of embryo-transferred uterus (A) and oil-induced deciduoma (B). Bars: 50 μm.

mal cells in deciduoma also expressed *TDO* mRNA (Fig. 4B). Thus, the expression of *TDO* mRNA in the endometrial stromal cells was correlated with the presence of decidualization and not embryo.

DISCUSSION

Tryptophan is an essential amino acid which is used by vertebrates in protein synthesis and as a precursor to serotonin, melatonin, kynurenine, and quinolinic acid. Tryptophan can be metabolized to n-formylkynurenine by either IDO or TDO (14). Further catabolism of n-formylkynurenine yields kynurenine and quinolinic acid. Although both IDO and TDO degrade tryptophan via the same pathway, they are different proteins (15). Besides tryptophan, IDO is induced by interferon-γ (16), while TDO is induced by glucocorticoid in the liver (17). L-tryptophan is the only substrate of TDO (18), whereas IDO metabolizes D- and L-tryptophan as well as other indoleamines (19). Though IDO is present in many tissues including brain, lung, placenta, epididymis, and intestine (20), TDO has been observed in liver, brain, and skin in the rat. Thus, two pathways of tryptophan catabolism may control the amount of tryptophan in the tissues, and concurrently regulate, especially in the brain, the level of serotonin or melatonin (8). To our knowledge, this is the first report that *TDO* mRNA was induced in the decidualized endometrium around the embryos at the time of implantation. *TDO* mRNA was not induced by sex steroid hormones, although they are the most effective factors for endometrial differentiation. The presence of embryo was demonstrated to induce *TDO* mRNA in the decidualized endometrium. *TDO* mRNA was also induced in artificially-formed deciduoma, which consists of the endometrial stromal cells decidualized functionally and morphologically similar to those at the implantation site. These findings indicated that the induction of *TDO* was due to the decidualization which is physiologically induced by the embryo signals.

Several reports have suggested that tryptophan catabolism contributes to successful pregnancy. IDO is expressed by human syncytiotrophoblast cells (21), and systemic tryptophan concentration falls during normal pregnancy (22). On the other hand, certain macrophages, induced to express IDO in response to interferon-γ or other signals from activated T cells, inhibit T cell proliferation *in vitro* by rapidly consuming tryptophan (23). Munn *et al.* (11) examined the physiological role of IDO in the early pregnancy using a pharmacologic IDO inhibitor, 1-methyl-tryptophan, and demonstrated that *IDO* expression at the maternal-fetal interface is necessary to suppress T cell activity and prevent immunological rejection of fetal allograft. In mice, *IDO* transcripts were detected in concepti from 8 to 10 days post coitus. At later gestation time, *IDO* transcripts were detected in placenta but not in maternal uterus or embryonic tissues. We demonstrated that *TDO* mRNA was expressed in the decidualized endometrium around the implanted embryos. Thus the tryptophan level around the implanted embryo is expected to be regulated by dual factors, TDO as a maternal factor and IDO as an embryonic factor, at the maternal-fetal interface to prevent immunological rejection of embryos.

Tryptophan homeostasis has been suggested to be particularly important during preimplantation period. For example, preimplantation blastocysts have five good systems for transport of tryptophan that contribute to homeostasis of this amino acid (24). High tryptophan level in culture medium has inhibitory effect on the *in vitro* development of 1-cell embryo to blastocyst even at 0.05 mM in protein-free culture medium (25). The present findings suggest that, besides its immunological role, the decidual TDO prevents the toxicity of tryptophan by reducing the tryptophan level in the implantation site. Considering the role played by IDO in human pregnancy, the expression and the role of TDO should be investigated in human endometrium.

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