Expression of indoleamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase in early concepti

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Indoleamine 2,3-dioxygenase (IDO)-initiated tryptophan degradation in the placenta has been implicated in the prevention of the allogeneic fetus rejection [Munn, Zhou, Attwood, Bondarev, Conway, Marshall, Brown, and Mellor (1998) *Science* **281**, 1191–1193]. To determine how IDO is associated with the development of the fetus and placenta, the time course of IDO expression (tryptophan-degrading activity, IDO protein and IDO mRNA) in the embryonic and extra-embryonic tissues as well as maternal tissues of mice was examined. A high tryptophan-degrading activity was detected in early concepti on days 6.5 and 7.5, whereas IDO protein and its mRNA were not expressed during early gestation, but appeared 2–3 days later, lasted for

about 3 days and declined rapidly thereafter. The expression of IDO basically coincided with the formation of the placenta. On the contrary, the early tryptophan-degrading activity was due to gene expression of tryptophan 2,3-dioxygenase (TDO), as shown by Northern and Western analysis. These findings indicate that IDO is transiently expressed in the placenta but that the expression does not last until birth, and that the IDO expression is preceded by expression of another tryptophan-degrading enzyme, TDO, in the maternal and/or embryonic tissues in early concepti.

Key words: gene expression, IDO, placenta, TDO, tolerance.

INTRODUCTION

Indoleamine 2,3-dioxygenase (IDO; EC 1.13.11.42) is a monomeric haemoprotein with a molecular mass of about 45 kDa. It catalyses the initial and rate-limiting step in the metabolism of tryptophan along the kynurenine pathway in mammals [1–4]. IDO is widely induced in many tissues under various pathological conditions associated with immune activation, and it has been suggested that the induced IDO may inhibit proliferation of some pathogens, including tumours [5–7] and intracellular parasites [8–10], by deprivation of tryptophan.

It is also known that IDO is ubiquitous in healthy normal tissues, and its expression level is higher in the placenta, intestine and lung [11,12]. However, the role of IDO in these organs has not been elucidated fully. Munn et al. [13] recently proposed that the placental IDO might play an important role in the prevention of allogeneic fetus rejection by maternal T-cells. They administered 1-methyl-tryptophan, an inhibitor of IDO, to pregnant mice, and found that the inhibitor induced rapid abortion of allogeneic concepti due to T-cells. They also reported that IDO, which was induced in monocytes stimulated by macrophage colony-stimulating factor, suppressed T-cell proliferation *in vitro* by the rapid and selective degradation of tryptophan [14]. These data suggest the presence of a mechanism by which antigen-presenting cells regulate T-cell activation via tryptophan catabolism.

Assuming that IDO is involved in fetal immune tolerance during pregnancy, the expression of IDO should be associated closely with murine development of both the fetus and placenta. To address this question, a detailed analysis of IDO expression

in terms of enzyme activity, protein and mRNA was performed. Here we will show that IDO is transiently expressed in the placenta, and that IDO expression is preceded by expression of another tryptophan-degrading enzyme, tryptophan 2,3-dioxygenase (TDO; EC 1.13.11.11) in the early concepti.

EXPERIMENTAL PROCEDURES

Mice

Mature outbred ICR mice (SLC, Shizuoka, Japan) were mated naturally (a total of 23 pairs). Noon of the day after copulation was considered as 0.5 day post-coitus (0.5 dpc). For biochemical measurements, tissues were obtained from the concepti on days 5.5–9.5, and from the placenta and fetus on days 10.5–18.5, because separation of the extra-embryonic and embryonic tissues is difficult before day 10.5. The current study was approved by the Animal Research Committee of Kawasaki Medical School, Okayama, Japan, and conducted according to the Guide for the Care and Use of Laboratory Animals of Kawasaki Medical School.

Measurement of IDO activity

IDO activities in tissue homogenates were assayed as described elsewhere [2]. Briefly, the homogenate (50 μ l) was added to the standard reaction mixture (50 μ l) containing 100 mM potassium phosphate buffer (pH 6.5), 40 mM ascorbate, 20 μ M Methylene Blue, 200 μ g/ml catalase and 800 μ M L-tryptophan. The tissue homogenates and the reaction mixture were incubated at 37 °C for 30 min and the reaction was terminated by adding 0.2 ml of

Abbreviations used: IDO, indoleamine 2,3-dioxygenase; TDO, tryptophan 2,3-dioxygenase; RT-PCR, reverse transcriptase PCR; dpc, days post-coitus; GADPH, glyceraldehyde-3-phosphate dehydrogenase.

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30% (w/v) trichloroacetic acid. They were boiled for a further 5 min to hydrolyse the *N*-formylkynurenine produced by IDO into kynurenine. After centrifugation (21880 g, 10 min), the supernatant was analysed by HPLC (Waters 600 E) using a reversed-phase column (S-5 300A ODS, YMC Co., Kyoto, Japan).

Polyclonal antibodies

Serum containing anti-(mouse IDO) polyclonal antibody was obtained from rabbits that were immunized with a mixture of three peptides corresponding to amino acids 1–18, 156–175 and 359–377 of mouse IDO protein.

Anti-mouse TDO polyclonal antibody was produced in the same way as that for IDO, but rabbits were immunized with a peptide corresponding to amino acids 391–406 of mouse TDO protein.

Western-blot analysis

The sample (30 µg of protein/lane) was subjected to electrophoresis on 10% (w/v) polyacrylamide gel in the presence of 0.1% SDS, and then transferred on to nitrocellulose membrane according to the method of Towbin et al. [15]. After blocking of the nitrocellulose membrane with PBS containing 5% skimmed milk for 1 h at room temperature, it was soaked in PBS containing anti-(mouse IDO) polyclonal antibody. The immunized rabbit serum was diluted to 2000-fold with PBS plus 0.05% (w/v) Tween 20 (PBST). The membrane was then washed extensively with PBST and incubated with horseradish peroxidase-conjugated anti-rabbit IgG for 1 h at room temperature. Finally, the membrane was washed extensively with PBST and developed with ECL Western blotting detection reagent (Amersham, Little Chalfont, Bucks., U.K.).

Reverse transcriptase PCR (RT-PCR) analysis of IDO mRNA

Total RNA samples were extracted from the tissues using RNA-isolation kits (Trizol reagent, Gibco BRL, Gaithersburg, MD, U.S.A.). Total RNA (5 µg) was reverse transcribed into cDNA using a transcription kit (Ready-to-Go You-Prime First-Strand Beads, Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). The resultant cDNAs were used for PCR: 95 °C for 3 min followed by 35 cycles of 95 °C for 30 s, 62.8 °C for 30 s, 72 °C for 1 min, and finally 72 °C for 5 min. PCR primers for IDO were the same as those reported by Munn et al. [13]. The primers and PCR conditions for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were the same as those reported previously [16].

Northern-blot analysis of IDO or TDO

Total RNA (5 µg per lane) was electrophoresed in a formaldehyde gel (1.2% agarose), blotted on to nylon membranes (Hybond N, Amersham) and probed with radiolabelled IDO cDNA fragments (*Eco*RI fragments) [17] as described previously [18]. In the case of TDO Northern-blot analysis, the probe was made by RT-PCR (5' primer, 5'-TGGGAAGTAGATTCTGTTTCG-3'; 3' primer, 5'-TCGCTGCTGAAGTAAGAGCT-3', obtained from GenBank, accession no. U24493). The PCR fragment was confirmed to be TDO by automatic nucleotide sequencing. For the standardization of data, radiolabelled rRNA probes were used. The signals of IDO and rRNA were quantified using an image analyser (BAS 2000, Fuji Film, Tokyo, Japan).

RESULTS

IDO activity during gestation

To clarify how the IDO-initiated tryptophan degradation is related to embryonic development, the time course of changes in IDO activity was examined throughout the developmental period from day 5.5 to 18.5 after copulation. Because the placenta and fetus become separable only after day 10.5, the concepti, consisting of extra-embryonic tissues and embryos, were collected from day 5.5 to 9.5 and assayed. IDO activity was very low on day 5.5, but 1 day later (day 6.5) the activity increased dramatically, reached the peak level, and then decreased gradually between days 7.5 and 14.5 in the concepti or placenta (Figure 1). On day 16.5, no activity was detected in the placenta or fetus. None of the embryos/fetuses between days 10.5 and 18.5 showed any tryptophan-degrading activity.

Western-blot and RT-PCR analysis of IDO

To examine the changes of IDO protein levels after copulation, Western-blot analysis using an anti-IDO antibody was performed (Figure 2). The 45 kDa band was clearly detected between days 9.5 and 12.5. This 45 kDa band was not detected between days 5.5 and 7.5, indicating that IDO protein is not expressed in the early embryonic stages. None of the embryos/fetuses had any IDO proteins (results not shown). The 60 kDa band, which appeared from day 5.5 to 10.5, was not related to IDO protein, because the expression pattern was not consistent with that of IDO mRNA. This was most likely due to accidental non-specific binding of anti-IDO antibody to maternal and/or fetus protein.

The expression pattern of IDO gene was examined by RT-PCR analysis (Figure 3). Total RNAs were obtained from the tissues of placenta (or extra-embryonic tissues) or fetus between days 5.5 and 18.5, and they were analysed by RT-PCR. A very faint band (about 500 bp in size) was detected on day 7.5, but there was no band on day 5.5 and 6.5. From day 8.5 to 12.5, a very distinct band was observed, and this indicated that the IDO gene was actively expressed in this development stage (days 7.5–12.5). After day 12.5, IDO gene expression became very weak.

Northern-blot analysis of IDO and TDO mRNA

Total RNA of the tissues obtained at each stage of gestation was examined by Northern-blot analysis to confirm the expression pattern of the IDO gene observed by RT-PCR (Figure 4A). As found by RT-PCR, the signal was very faint on day 7.5 and became strong on day 8.5 to 10.5, after which it fell to the level of day 7.5 until day 18.5. Figure 4(B) shows quantification by Northern-blot analysis. The figures were obtained by normalizing IDO signals with the amount of 18 S rRNA. IDO expression was very low before day 8.5, and peaked on day 9.5.

In order to examine whether the early high tryptophan-degrading activity (peak on days 6.5–7.5) originated from another tryptophan-degrading enzyme, TDO, the same filter was used for hybridization with TDO probe. Figure 5 shows that TDO expression could be detected from day 5.5 to 10.5, and that the peak was obtained on days 6.5–8.5, which roughly coincided with the pattern of tryptophan-degrading activity.

RT-PCR and Western-blot analysis of TDO

The expression pattern of the TDO gene was examined by RT-PCR. Figure 5(B) shows that strong TDO expression again could be detected from day 5.5 to 10.5.

Figure 1 Changes of IDO activity/g wet weight during murine gestation

IDO activity was determined in concepti (5.5–9.5 dpc) or placenta (10.5–18.5 dpc). Each point represents the mean $\pm$ S.D. from triplicate experiments. Kyn, kynurenine.

Figure 2 Immunoblot analysis of IDO protein during the gestation period

Arrows indicate 45 kDa bands. Samples were subjected to SDS/PAGE (10% gels), transferred on to nitrocellulose membrane and analysed with anti-IDO antibody. Size markers are shown on the left. Results are representative of three separate experiments.

Using an anti-TDO antibody, the changes in TDO protein levels were examined. Figure 5(C) shows that the 41 kDa band was detected clearly on days 6.5–14.5, and that weak bands were seen until day 18.5.

Effect of 1-methyl-tryptophan on tryptophan-degrading activity

Tryptophan-degradation activities of extracts from concepti on 6.5, 9.5 and 12.5 dpc, in addition to those of adult liver extracts and recombinant IDO, were measured in the presence or absence of an IDO-specific inhibitor, 1-methyltryptophan (Table 1), in order to determine the contribution of IDO and TDO to tryptophan-degradation activity. Whereas the activity of recombinant IDO was almost completely inhibited, that of liver extracts

Figure 3 RT-PCR analysis of IDO transcription during the gestation period

PCR products (about 500 bp in size) were generated by RT-PCR amplification of RNA samples from concepti (5.5–9.5 dpc) or placenta (10.5–18.5 dpc) at the gestation times indicated. M, molecular-size marker. Results are representative of three separate experiments.

(TDO activity) was not significantly suppressed by 1-methyl-tryptophan. The activity of early concepti (day 6.5) was not significantly inhibited, but on day 12.5 the activity was suppressed to nearly half by the inhibitor. This suggested that the contribution of IDO in the early activities was very low and that later in development the IDO contribution increased.

DISCUSSION

In this study, we examined the detailed time course of IDO expression during the development of embryo/fetus and placenta. Our results showed that IDO protein is strongly expressed from 9.5 to 12.5 dpc, but that the expression does not last until birth. The period of peak expression of IDO basically coincides with the

Figure 4 Northern-blot analysis of IDO expression during the gestation period

(A) Expression pattern of IDO and 18 S rRNA. Each lane contains 5 μ g of total RNA from concepti (5.5–9.5 dpc) or placenta (10.5–18.5 dpc) at the gestation times indicated. IDO mRNA level was estimated by Northern-blot analysis. Fractionated 18 S rRNA stained with ethidium bromide is shown at the top of the panel. (B) Quantitative analysis of IDO transcripts in terms of the ratio of IDO transcript to 18 S rRNA.

Figure 5 Changes of TDO expression during the gestation period

(A) Northern-blot analysis. See the legend of Figure 4(A) for details. (B) RT-PCR analysis of TDO. (C) Western-blot analysis of TDO protein. See the legend of Figure 2 for details.

formation of the placenta [19]. If IDO plays a role in suppression of proliferation of T-cells cytotoxic to the fetus by tryptophan consumption, as proposed by Munn et al. [13], this

Table 1 Effect of 1-methyl-tryptophan on tryptophan-degrading activity

Tryptophan-degrading activities in concepti 6.5, 9.5 and 12.5 dpc, in adult liver extracts and of recombinant IDO in the presence (1MT +, 3.75 mM) or absence (1MT –) of 1-methyl-tryptophan were determined as the amount of kynurenine produced (nmol/g wet weight of tissue per h). Inhibition was calculated as [(activity of 1MT – minus activity of 1MT +)/activity of 1MT –] $\times$ 100%. Means $\pm$ S.D. of triplicate measurements are shown.

	Activity (nmol/h per g)		Inhibition (%)
	1MT –	1MT +	
Day 6.5	2233 $\pm$ 678	2198 $\pm$ 675	2.25 $\pm$ 1.26
Day 9.5	642 $\pm$ 272	637 $\pm$ 240	4.00 $\pm$ 3.61
Day 12.5	502 $\pm$ 465	274 $\pm$ 236	44.67 $\pm$ 5.13*
Liver	694 $\pm$ 252	657 $\pm$ 241	6.00 $\pm$ 4.90
Recombinant IDO	3075	540	83

* Significantly different ($P < 0.001$) from the values of 6.5 and 9.5 dpc or liver.

transient expression of IDO would be sufficient to induce immune tolerance. In other words, once tolerance is established in this period, it may last until the birth without tryptophan deprivation.

The unexpected finding in this study was the high tryptophan-degrading activity in early gestation, i.e. on 6.5 dpc. Surprisingly, very abundant expression of another tryptophan-degrading enzyme, TDO, was detected in RNA from early concepti of days 5.5–10.5, indicating that early tryptophan-degrading activity is due to TDO activity. In addition, the activity was not inhibited by the IDO inhibitor 1-methyltryptophan [13] (see Table 1). Taken together, these facts suggest that the early activity is not due to IDO, but to TDO. This conclusion is consistent with the finding that there was no expression of IDO protein and mRNA at such an early stage.

So far, TDO has been known to be localized exclusively in the adult liver and is not expressed in embryonic hepatocytes [20], and there are no hepatocytes in such early concepti. Therefore, for the first time, we can demonstrate clearly the extra-hepatic expression of TDO. It is reasonable to speculate that, in addition to IDO, TDO activity contributes mainly to tryptophan catabolism during the development of the fetus/embryo and placenta. Studies on the function of IDO and TDO will deepen our understanding of tryptophan catabolism in mother–fetus interactions, including fetal tolerance.

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