Tissue-specific Rescue Suggests That Placental Adenosine Deaminase Is Important for Fetal Development in Mice*

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Adenosine deaminase (ADA, EC 3.5.4.4) is an essential enzyme of purine metabolism that is expressed at very high levels in the murine placenta where it accounts for over 95% of the ADA present at the fetal gestation site. We have recently shown that ADA-deficient fetuses, which also lack ADA in their adjoining placentas, die during late fetal development in association with profound purine metabolic disturbances and hepatocellular impairment. We have now investigated the potential importance of placental ADA by genetically restoring the enzyme to placentas of ADA-deficient fetuses. This genetic engineering strategy corrected most of the purine metabolic disturbances, prevented serious fetal liver damage, and rescued the fetuses from perinatal lethality. Our findings suggest that placental ADA is important for murine fetal development and illustrate a general strategy for the tissue specific correction of phenotypes associated with null mutations in mice.

During mammalian development the first differentiation event gives rise to the trophectoderm, which in turn provides lineages for specialized extraembryonic cells known as trophoblasts. These cells make the physical connection between the embryo and the maternal environment and play important roles in the implantation process and placental function (1). Inadequate placental development is associated with a high incidence of early embryonic mortality (2–4) and serious pregnancy disorders such as preeclampsia (5). Although the formation of a functional placenta is essential for mammalian embryogenesis and fetal development, relatively little is known about the molecular mechanisms that govern trophoblast differentiation and subsequent function. Determining what proteins are produced in trophoblasts and elucidating their physiological roles is an important part of understanding how this cell lineage contributes to the formation of a functional placenta.

One such protein that is highly abundant in the murine placenta is adenosine deaminase (ADA).¹ ADA is an essential enzyme of purine metabolism that is expressed at very high levels in trophoblasts of the murine placenta (6, 7). The physiological importance of ADA in trophoblasts of the placenta is not known. However, recent evidence suggests that ADA is essential during fetal stages of development. ADA-deficient fetuses, which also lacked ADA in trophoblasts of their adjoining placentas, died perinatally in association with profound purine metabolic disturbances and hepatocellular impairment (8, 9). Considering that greater than 95% of ADA enzymatic activity found in the fetal gestation site resides in trophoblasts of the placenta, it is likely that placental ADA plays an essential role during fetal development. Here we show that genetically restoring ADA to placentas of ADA-deficient fetuses rescued them from perinatal lethality, thereby providing compelling evidence for the importance of placental ADA for fetal development.

MATERIALS AND METHODS

ADA Minigene Construction and Transgenic Mouse Generation—The plasmid, −15/+ 21ksmg(6.4), was constructed by cloning a 950-bp EcoRI to HindIII fragment of the murine ADA CDNA from pADA529 (10) into pKSII (Stratagene). A 2.8-kb genomic ADA HindIII fragment containing the endogenous polyadenylation sequences, intron 11, and −2 kb of 3′-untranslated region and 3′ flanking was then inserted at the 3′ end of the cDNA. Next, the endogenous ADA promoter was replaced with an ADA promoter containing a 36-bp deletion in the 5′-untranslated region (11). A 6.2-kb BamHI to EcoRI fragment from the plasmid ADACAT (12) was inserted at the 5′ end of the construct. The construct was digested with NotI and SalI to release the 10-kb ADA minigene construct. This fragment was separated from vector sequences by agarose gel electrophoresis and gel-purified using a Qiaex gel extraction kit (Qiagen, Inc.). DNA was microinjected into FVB/N one-cell embryos at a concentration of 2 ng/μl (13).

Southern Blotting—Genomic DNA was isolated from tails at weaning or from extraembryonic membranes (8, 12). For Southern blotting, 20 μg of genomic DNA was digested with BamHI, separated by agarose gel electrophoresis, transferred to Zeta- Probe membranes (Bio-Rad) and hybridized according to manufacturer’s instructions. A 3.5-kb internal probe (8) was 32P-labeled using a random primer labeling kit (Boehringer Mannheim).

RNase Protection Assays—Fetuses and placentas were isolated on 13.5 days postcoitum (dpc) from a nontransgenic female who was mated with a transgenic male from line 2448, carrying ~20 copies of the ADA minigene. Extraembryonic membranes served as a source of genomic DNA for genotyping. Total cellular RNA was isolated (12) and its integrity analyzed by formaldehyde-agarose gel electrophoresis. RNase protection assays were carried out using ~30 μg of total cellular RNA (12). A 400-bp 32P-labeled ADA antisense probe that protects the first 300 bp of the ADA message was synthesized from linearized ADA plasmid (12) by T7 RNA polymerase.

ADA Enzymatic Assays—Tissue extracts of fetuses and placentas were generated, and ADA enzymatic activity was measured according to established procedures (12).

Analysis of Nucleosides and Nucleotides—25 μl of whole blood was rapidly collected from the thoracic cavity of fetuses on 17.5 dpc for analysis of dATP levels. Remaining fetuses and placentas were quickly frozen in liquid nitrogen for extraction and analysis of nucleosides. Extraembryonic membranes served as a source of genomic DNA for genotyping. Nucleosides and nucleotides were extracted and analyzed by reversed phase HPLC (8, 14, 15).

1 The abbreviations used are: ADA, adenosine deaminase; dpc, days postcoitum; HPLC, high performance liquid chromatography; AdoHcy, 5′-adenosylhomocysteine; kb, kilobase pair(s); bp, base pair(s).

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To genetically restore ADA activity to placentas associated with ADA-deficient fetuses, mice heterozygous for a null Ada allele (8) were intercrossed with mice hemizygous for the ADA minigene locus. Among the progeny of such matings were animals hemizygous for the ADA minigene locus and heterozygous for the null Ada allele. When intercrossed, these animals served as a source of fetuses that were homozygous for the null Ada allele (ada<sup>m1</sup>/ada<sup>m1</sup>), some of which carried the ADA minigene locus. To determine if ada<sup>m1</sup>/ada<sup>m1</sup> fetuses were rescued by the presence of the ADA minigene locus, litters from these matings were weaned at 3 weeks of age and genotyped (Fig. 2). As a control, we also examined the progeny of intercrosses between animals heterozygous for the null Ada allele and lacking a minigene. Consistent with our previous results (8), no ada<sup>m1</sup>/ada<sup>m1</sup> mice were present at weaning; however, ada<sup>m1</sup>/ada<sup>m1</sup> mice carrying the ADA minigene locus were detected at a percentage suggesting a 100% rescue of these animals (Table I). These data suggest that expression of the ADA minigene in the placentas of ADA-deficient fetuses is sufficient to rescue them from perinatal lethality.

To verify that this genetic rescue was coupled to the restoration of ADA enzymatic activity in the placenta, we measured ADA activity in fetuses and placenats during late fetal development (Fig. 3). No detectable ADA activity was seen in ada<sup>m1</sup>/ada<sup>m1</sup> fetuses or placentas. In contrast, ada<sup>m1</sup>/ada<sup>m1</sup> placenta containing the ADA minigene locus showed high levels of placental ADA activity, whereas little or no ADA activity was associated with the adjoining fetuses. The small amounts of activity found in some of these fetuses was likely to reside in the forestomach, since activity was not found outside the gastrointestinal tract in adult rescued mice. This pattern of expression is consistent with the regulatory elements used (12) as well as observations that forestomach ADA expression in...
Placental ADA Rescues ADA-deficient Fetuses

Table 1: Genotypes of mice at weaning

| Genotype | Quantity at weaning |
|----------|---------------------|
| +/+      | 77                  |
| m1/+    | 160                 |
| m1/m1   | 0                   |
| Tg, +/+  | 11                  |
| Tg, m1/+ | 44                  |
| Tg, m1/m1| 14                  |

* +/+, wild type; m1/+, heterozygous; m1/m1, homozygous; Tg, presence of ADA minigene locus.

Fig. 3. ADA enzymatic activity in fetuses and placentas on 17.5 dpc. Animals hemizygous for the ADA minigene locus and heterozygous for the null Ada allele were intercrossed. Pregnant females were sacrificed on 17.5 dpc, and individual fetuses and placentas were separated and assayed for ADA enzymatic activity. Measurements were made on fetuses and placentas that were heterozygous for the null Ada allele (m1/+, n = 3) and homozygous for the null Ada allele without (m1/m1, n = 2) or with (Tg, m1/+, n = 4) and samples homozygous for the null Ada allele without (m1/m1, n = 2) or with (Tg, m1/+, n = 4) the ADA minigene locus. Values are given as means specific activities ± S.E., N.D., not detected.

Fig. 4. Levels of ADA substrates and dATP in fetuses and placentas on 17.5 dpc. Fetuses and placentas from matings described in the legend to Fig. 3 were collected on 17.5 dpc, and nucleosides and nucleotides were extracted and analyzed using reversed phase HPLC. A, fetal adenosine levels; B, fetal deoxyadenosine levels; C, dATP levels measured in fetal blood; D, placental adenosine levels; E, placental deoxyadenosine levels. Measurements were made on samples that were heterozygous for the null Ada allele with the ADA minigene locus (Tg, m1/+), and samples homozygous for the null Ada allele without (m1/m1, n = 2) or with (Tg, m1/+, n = 4) the ADA minigene locus. Tg, m1/+ values are essentially the same as wild type values. Values are given as means ± S.E.; N.D., not detected at a lower limit of detection of ≤0.001 nmol/mg protein.

DISCUSSION

In the current study we show that genetically restoring ADA enzymatic activity to placentas of ADA-deficient fetuses prevents most of the purine metabolic disturbances seen in ADA-deficient fetuses. This suggests that disturbances in purine metabolism may be responsible for the liver damage and perinatal lethality seen in ADA-deficient fetuses (8, 9). The highest levels of ADA found during fetal stages of development are in the placenta, suggesting placental ADA is playing an important role during these stages of development. A major function of this enzyme in the placenta may be to prevent the accumulation of substrates that are potentially toxic to the developing embryo.

In attempting to understand the physiological importance of ADA, attention is invariably focused on the metabolic impact of its substrates, adenosine and deoxyadenosine (17). Adenosine is an extracellular signal that elicits a vast array of physiological responses by engaging cell surface receptors (18). Little, however, is known regarding its role in adenosine signaling during mammalian development. Our results show that placental correction of ADA enzymatic activity failed to prevent the accumulation of adenosine in the fetus, suggesting...
that elevated adenosine levels are not overtly detrimental to these fetuses. The other substrate, deoxyadenosine, is a cytotoxic metabolite that kills target cells by interfering with deoxynucleotide synthesis and/or disrupting cellular transmethylation reactions (17). Interference with deoxynucleotide synthesis is mediated by the phosphorylation of deoxyadenosine to dATP via nucleoside and nucleotide kinases (19). High concentrations of dATP inhibit ribonucleotide reductase and disrupt deoxynucleotide synthesis needed for DNA synthesis and repair (20, 21). Another route of deoxyadenosine cytotoxicity involves the inhibition S-adenosylhomocysteine (AdoHcy) hydrolase, leading to the inhibition of transmethylation reactions critical to cellular function (22, 23). The most striking reversal in purine metabolic disturbances seen in rescued fetuses pertained to deoxyadenosine metabolism, with both deoxyadenosine and dATP remaining at near normal levels (Fig. 4, B and C). High levels of nucleoside kinases and AdoHcy hydrolase are found in rodent livers (24, 25), suggesting that either of these metabolic pathways may be involved in the hepatocellular damage seen in ADA deficient fetuses. Recent studies have shown that AdoHcy hydrolase activity is inhibited in tissues of ADA-deficient fetuses and tissues of ADA-deficient human fetuses survive prenatal development and are not known to suffer significant hepatocellular damage, although mild liver findings have been noted (17). These differences may suggest that there is a greater need for ADA during mouse prenatal development. Genetic restoration of ADA in the placentas of ada<sup>mm</sup>/ada<sup>mm</sup> fetuses allows them to survive prenatal development, providing the opportunity to investigate whether postnatal mice develop phenotypes similar to those seen in humans. Preliminary observations suggest that rescued ADA deficient animals exhibit lymphopenia and immune deficiency.

ADA is expressed at high levels at three different places and times during the murine life cycle: first in the trophoblasts of the placenta during prenatal development (6, 7), next in the gastrointestinal epithelium of the adult (16), and then in the decidua of the pregnant uterus (6). The lack of ADA in the first of these places, the placenta, results in a phenotype that precludes our ability to investigate its importance in adult tissues. Placental expression of an ADA minigene on an ADA-deficient background allowed for survival through the prenatal phenotypic bottleneck and provided adult mice that can now be used to assess the role of ADA in adult tissues such as the gastrointestinal tract, the decidua, and the immune system.

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