Genetically engineered mice demonstrate that adenosine deaminase is essential for early postimplantation development

Michael R. Blackburn1,*, Thomas B. Knudsen2 and Rodney E. Kellems1

1Verna and Marrs McLean Department of Biochemistry, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA
2Department of Pathology, Anatomy and Cell Biology, Jefferson Medical College, 1020 Locust Street, Philadelphia, PA 19120, USA

*Author for correspondence

SUMMARY

Adenosine deaminase (ADA) is an essential enzyme of purine metabolism that is enriched at the maternal-fetal interface of mice throughout postimplantation development. During early postimplantation stages Ada is highly expressed in both maternally derived decidual cells and zygotically derived trophoblast cells. For the current study we utilized genetically modified mice to delineate the relative contribution and importance of decidual and trophoblast ADA at the maternal-fetal interface. In females genetically engineered to lack decidual ADA a striking pattern of expression was revealed in giant trophoblast cells that surround the early postimplantation embryo. Embryos within gestation sites lacking both decidual and trophoblast ADA died during the early postimplantation period, whereas expression in trophoblast cells alone was sufficient for survival through this period. Severe disturbances in purine metabolism were observed in gestation sites lacking decidual ADA, including the accumulation of the potentially toxic ADA substrates adenosine and 2′-deoxyadenosine. These experiments provide genetic evidence that Ada expression at the maternal-fetal interface is essential for early postimplantation development in mice.

Key words: adenosine deaminase, 2′-deoxyadenosine, adenosine, decidua, trophoblast cells, placenta, transgenic mice

INTRODUCTION

Elucidating expression patterns of essential genes in the mammalian embryo has provided valuable information into mechanisms of normal and abnormal development. An area of research that has received considerably less attention is the study of genes that are highly expressed in extraembryonic and uterine tissues that constitute the maternal-fetal interface. Amongst the key cell types found at the maternal-fetal interface are zygotically derived trophoblast cells and maternally derived decidual cells (Peel and Bulmer, 1977). Trophoblast cells play a leading role in implantation, placental development, maternal regulation of pregnancy and metabolic protection of the conceptus (Cross et al., 1994; Guillemot et al., 1994; Soares et al., 1991; Blackburn et al., 1997). Decidual cells in direct contact with invading trophoblast cells may play an active role in the regulation of implantation chamber morphogenesis and the maintenance of pregnancy (Parr and Parr, 1989). Since mammalian development is ultimately dependent on the concerted expression of genes that control cellular processes in the decidua and placenta, an important question pertains to the identification and functional analysis of genes that play a primary role in the support of embryo development and pregnancy.

Adenosine deaminase (ADA) is an essential enzyme of purine metabolism that is enriched at the maternal-fetal interface of mice throughout development (Knudsen et al., 1988, 1991; Witte et al., 1991; Blackburn et al., 1992). During early postimplantation stages, Ada expression in the gestation site is predominantly maternal in origin. Expression is evident in the secondary deciduum as early as 6.5 days postcoitum (dpc) (Knudsen et al., 1991), and the level of expression in this tissue increases through 9.5 dpc and then wanes with the regression of the secondary deciduum, a process that is largely complete by 13.5 dpc (Welsh and Enders, 1985). Ada expression is also found in some subsets of trophoblast cells during the early postimplantation period; however, the high level of expression in the adjacent deciduum has made it difficult to delineate the pattern and level of zygotic Ada expression during this period. Ada expression appears in secondary giant trophoblast cells as they differentiate at the ectoplacental cone starting on day 7.5 pc and in the polyploid giant trophoblast cells that surround the implantation site (Knudsen et al., 1991). The number of Ada-expressing trophoblast cells increases as development proceeds. In the mature fetal placenta, ADA is enriched in the spongiotrophoblasts of the junctional zone, in syncytiotrophoblasts of the labyrinthine zone and in secondary trophoblast giant cells (Knudsen et al., 1991; Witte et al., 1991). High-level expression of Ada at the maternal-fetal interface suggests an important role in nucleoside metabolism during postimplantation development.
Determining the functional role of ADA in the fetal placenta has been aided by genetic studies in mice (Wakamiya et al., 1994; Blackbum et al., 1995). ADA-deficient fetuses generated by targeted mutagenesis died perinatally and also showed severe purine metabolic disturbances and hepato cellular damage (Wakamiya et al., 1994; Migchielsen et al., 1995). Considering that greater than 95% of the ADA found in the gestation site during fetal stages resides in trophoblast cells, it was hypothesized that trophoblast ADA is important for fetal development. To test this hypothesis, Ada expression was restored to the placenta of otherwise ADA-deficient gestation sites (Blackburn et al., 1995). Rescue transgenesis was accomplished by intercrossing mice heterozygous for the null Ada allele with transgenic mice carrying an ADA transgene under the control of Ada regulatory elements that target expression to trophoblast cells (Winston et al., 1992; Shi et al., 1997). Restoring ADA to trophoblast cells was sufficient to prevent most of the metabolic consequences and hepato cellular damage seen in ADA-deficient fetuses lacking placental expression, and was sufficient to rescue these fetuses from perinatal lethality (Blackburn et al., 1995). These genetic studies suggest that placental ADA plays an important role in protecting the fetus from endogenous purine nucleoside (adenosine and 2′-deoxyadenosine) intoxication during prenatal development.

Pharmacological studies have suggested that ADA is also important during the early postimplantation period (Knudsen et al., 1989, 1992; Airhart et al., 1993). Treatment of pregnant mice with the potent ADA inhibitor 2′-deoxycoformycin on days 7.5 and 8.5 pc resulted in a near total loss of embryo viability. Use of embryo culture systems have further suggested that early embryos are particularly sensitive to 2′-deoxyadenosine intoxication (Gao et al., 1994; Wubah et al., 1996). Given the nature of the pharmacological studies, it has been difficult to assess the relative role of maternally derived decidual ADA and zygotically derived trophoblast ADA at these critical stages. However, genetically engineered mice that have been produced recently (Blackburn et al., 1995) can now be utilized to assess the relative expression and importance of ADA in these different cell types during early postimplantation stages. ADA-deficient mice rescued by placental expression of an ADA transgene lack ADA in all tissues outside the gastrointestinal tract (Blackburn et al., 1996), including the secondary deciduum (current study). These mice have provided us with the opportunity to clearly delineate the relative contributions of Ada expression in uterine stromal cells and in trophoblast cells at the maternal-fetal interface. We were also able to genetically assess the physiological and reproductive outcome of gestation sites that were deficient in decidual ADA, trophoblast ADA, or both. Our findings provide genetic evidence that Ada expression in the murine gestation site is essential for development during early postimplantation stages.

**Fig. 1.** Trophoblast Ada expression in 7.5 dpc gestation sites engineered to lack decidual ADA. Transverse sections through 7.5 dpc gestation sites were reacted with sheep antiserum to murine ADA followed by peroxidase detection (A-C; scale bar, 1 mm). (A) Wild-type gestation site exhibiting abundant ADA immunoreactivity in the secondary deciduum (d) as well as in cells of the ectoplacental cone (ec) and giant trophoblast cells (gc). (B) Gestation site from an ADA-deficient female lacking decidual ADA and containing an embryo expressing Ada from one wild-type Ada allele, the ADA transgene, or both. In the absence of decidual expression (d), prominent ADA immunoreactivity in the gc surrounding the embryo (e) was evident. (C) Gestation site from an ADA-deficient female containing an ADA-deficient embryo. Sections adjacent to those used for ADA immunolocalization were stained with hematoxylin and eosin to better monitor embryo morphology (D-F; scale bar, 100 μm). (D) 7.5 dpc wild-type implantation chamber showing a gastrulating embryo. d, secondary deciduum; ec, ectoplacental cone; 1, amniotic cavity; 2, exocoelom; 3, ectoplacental cavity. (E) Implantation chamber from a gestation site lacking decidual ADA but containing ADA in the giant trophoblast cells surrounding the embryo (as in B). The d and ec appear normal, whereas the embryo proper is developmentally delayed with only two cavities present, similar to that seen in a normal 6.5 dpc embryo. (F) Implantation chamber from a gestation site lacking both decidual and trophoblast ADA. The deciduum is fully developed and trophoblast cells are found (not shown here). However, the embryo itself is severely degenerate (*), with no distinguishable features.

**MATERIALS AND METHODS**

**Mice**

All mice were hybrids between 129/SV mice (used in the generation of Ada null mice) and FVB/N mice (used in the generation of transgenics harboring an ADA transgene). The original null Ada allele was defined as ada<sup>m1</sup> (Wakamiya et al., 1995), but will be referred to here as m1. Mice heterozygous for the null Ada allele (m1/+ and hemizygous for the ADA transgenic locus (Tg) (Blackburn et al., 1995) were intercrossed to generate rescued mice that were homozygous for the null Ada allele (Tg-m1/m1) (Blackburn et al., 1996). Tg-m1/m1 mice will be referred to as ADA deficient. Genotypes were determined by Southern blot analysis of genomic DNA obtained from tails at
sheep antiserum monospecific for murine ADA. The using a Rapid Chrome staining kit (Shandon). Standard procedures. Serial transverse sections (7 μm) were collected on poly-L-lysine coated microscope slides. Hematoxylin and eosin staining was carried out (Et al, 1991), using a VECTASTAIN ABC peroxidase kit. The procedure was essentially that described by Knudsen et al (1991), gestation sites were dehydrated, cleared and embedded in paraffin according to standard procedures. Serial transverse sections (7 μm) were collected on poly-L-lysine coated microscope slides. Hematoxylin and eosin staining was carried out using a Rapid Chrome staining kit (Shandon).

ADA immunolocalization was conducted using sheep antiserum monospecific for murine ADA. The procedure was essentially that described by Knudsen et al (1991), using a Vectastain ABC peroxidase kit (Vector Labs) with the addition of a 30 minute incubation in 0.3% H2O2 in PBS, to prevent nonspecific peroxidase activity. All photographs were generated using an Olympus BX60 Microscope with bright-field illumination. Images were processed for publication using an ES-1200C color scanner (Epson) at 300 dpi and Adobe Photoshop (4.0) software.

ADA enzymatic assay
Gestation sites from various matings were collected on day 9.5 pc, dissected from the myometrium and separated into antimesometrium (together with giant trophoblast cells), mesometrium (containing the developing chorioallantoic placenta) and the yolk sac containing the embryo proper. The latter were examined morphologically before use in allele type analysis. Antimesometrial and mesometrial samples were quick-frozen in liquid nitrogen and stored at −70°C. ADA enzymatic activity was assayed for in crude supernatants under saturating adenosine substrate conditions at 30°C using a spectrophotometric assay (Winston et al., 1992; Blackburn et al., 1996). The decrease in absorbance at 265 nm resulting from the deamination of adenosine to inosine was continuously monitored in a Beckman DU-50 spectrophotometer, and the rate of decrease was calculated at linearity. Specific activities are presented as nmoles adenosine deaminated per minute per mg protein.

Zymogram analysis
Zymogram analysis was performed as previously described (Knudsen et al., 1991). Gels were loaded with 1 μg protein of crude homogenate for ADA activity, and with 2 μg of protein to monitor purine nucleoside phosphorylase activity as a positive control (Blackburn et al., 1996).

Analysis of nucleosides
Gestation sites from various matings were collected on day 8.5 pc under ice-cold PBS, then quick-frozen in liquid nitrogen for extraction and analysis of nucleosides (Knudsen et al., 1992; Blackburn et al., 1995, 1996). The HPLC system consisted of two 510 pumps with control module, a Rehodyne injector, a 486 tunable absorbance detector under the control of Millenium software (Waters). Separation was through a reversed-phase (C18) Customsil ODS column (4.6×254 mm) (Custom LC Inc) protected by a NovaPak C18 Sentry Guard Column (Waters). The mobile phase was 0.2 M NH4H2PO4 (pH 5.1) with a superimposed methanol gradient.

Histology and ADA immunohistochemistry
Individual gestation sites, defined as the embryo proper and its extraembryonic membranes contained within the maternal deciduum, were dissected from pregnant females of various matings (described above) on days 7.5 or 9.5 pc. After fixation for 1 hour at 25°C in 70% ethanol, 10% formalin, and 5% acetic acid, (Knudsen et al., 1991), gestation sites were dehydrated, cleared and embedded in paraffin according to standard procedures. Serial transverse sections (7 μm) were collected on poly-L-lysine coated microscope slides. Hematoxylin and eosin staining was carried out using a Rapid Chrome staining kit (Shandon). ADA immunolocalization was conducted using sheep antiserum monospecific for murine ADA. The procedure was essentially that described by Knudsen et al (1991), using a Vectastain ABC peroxidase kit (Vector Labs) with the addition of a 30 minute incubation in 0.3% H2O2 in PBS, to prevent nonspecific peroxidase activity. All photographs were generated using an Olympus BX60 Microscope with bright-field illumination. Images were processed for publication using an ES-1200C color scanner (Epson) at 300 dpi and Adobe Photoshop (4.0) software.

ADA enzymatic assay
Gestation sites from various matings were collected on day 9.5 pc, dissected from the myometrium and separated into antimesometrium (together with giant trophoblast cells), mesometrium (containing the developing chorioallantoic placenta) and the yolk sac containing the embryo proper. The latter were examined morphologically before use in allele type analysis. Antimesometrial and mesometrial samples were quick-frozen in liquid nitrogen and stored at −70°C. ADA enzymatic activity was assayed for in crude supernatants under saturating adenosine substrate conditions at 30°C using a spectrophotometric assay (Winston et al., 1992; Blackburn et al., 1996). The decrease in absorbance at 265 nm resulting from the deamination of adenosine to inosine was continuously monitored in a Beckman DU-50 spectrophotometer, and the rate of decrease was calculated at linearity. Specific activities are presented as nmoles adenosine deaminated per minute per mg protein.

Zymogram analysis
Zymogram analysis was performed as previously described (Knudsen et al., 1991). Gels were loaded with 1 μg protein of crude homogenate for ADA activity, and with 2 μg of protein to monitor purine nucleoside phosphorylase activity as a positive control (Blackburn et al., 1996).

Analysis of nucleosides
Gestation sites from various matings were collected on day 8.5 pc under ice-cold PBS, then quick-frozen in liquid nitrogen for extraction and analysis of nucleosides (Knudsen et al., 1992; Blackburn et al., 1995, 1996). The HPLC system consisted of two 510 pumps with control module, a Rehodyne injector, a 486 tunable absorbance detector under the control of Millenium software (Waters). Separation was through a reversed-phase (C18) Customsil ODS column (4.6×254 mm) (Custom LC Inc) protected by a NovaPak C18 Sentry Guard Column (Waters). The mobile phase was 0.2 M NH4H2PO4 (pH 5.1) with a superimposed methanol gradient.

Fig. 2. ADA immunolocalization in 9.5 dpc gestation sites from mice containing various combinations of decidual and trophoblast ADA. Transverse sections through 9.5 dpc gestation sites were reacted with a sheep antiserum to murine ADA (scale bar, 1 mm). (A) Gestation site from a female heterozygous for the null Ada allele (m1/+), containing a m1/+ or wild-type embryo. Intense ADA immunoreactivity was seen in the secondary deciduum (d) as well as giant trophoblast cells (gc), syncytiotrophoblasts of the developing labyrinthine zone (lz) and spongiotrophoblasts of the junctional zone (jz), e, embryo. (B) Gestation site from a m1/+ female containing a m1/m1 embryo. Intense ADA immunoreactivity was found in the secondary deciduum (d) but was absent from the trophoblast cells and embryo. (C) Gestation site from a ADA-deficient female containing an embryo with expression from one wild-type Ada allele (m1/+), the ADA transgene, or both. Removing expression from the deciduum (d) enabled the visualization of intense ADA immunoreactivity in secondary giant trophoblast cells (gc) that completely surround the embryo (e). Expression was maintained in trophoblast cells of the lz and jz as well. (D) Gestation site from a Tg-m1/m1 female containing a m1/m1 embryo. No ADA immunoreactivity was found in the deciduum (d) or trophoblast cells (t), and the implantation chamber was devoid of embryonic material (*).
(Knudsen et al., 1992). Flow rate was 1.5 ml/minute and the injection volume 200 µl. Absorbance was continuously monitored at a wavelength of 254 nm and peaks were identified and quantitated based on coretention of known amounts of external standards (Sigma). Peaks of interest were verified by enzymatic shift assay.

RESULTS

The abundance of ADA in trophoblast cells is clearly evident in gestation sites that are genetically deficient in decidual ADA

During early postimplantation stages of development in mice, both the secondary deciduum and trophoblast cells express high levels of Ada (Knudsen et al., 1991); however their close proximity to one another has made it difficult to delineate the relative pattern and levels of expression from these cells types. Assessing the abundance and distribution of ADA in trophoblast cells was made possible by investigating the pattern of Ada expression in gestation sites of rescued ADA-deficient females (Blackburn et al., 1995). These gestation sites lacked expression of Ada in the deciduum and thus highlighted trophoblast expression. Shown in Fig. 1A-C are transverse sections through 7.5 dpc gestation sites from various matings that were reacted with sheep antiserum to murine ADA. Intense ADA immunoreactivity was abundant in the secondary deciduum on day 9.5 pc, while there were gestation sites that lacked detectable ADA immunoreactivity in the deciduum as well as trophoblast cells (Fig. 1C). These results provide direct evidence that there is an abundance of ADA provided by trophoblast cells surrounding the 7.5 dpc embryo.

Ada expression reaches peak levels in the secondary deciduum on day 9.5 pc, while expression continues to increase in all trophoblast lineages (Knudsen et al., 1991; Blackburn et al., 1992). To better portray the expression of Ada in trophoblast cells at this stage of development, gestation sites deficient in decidual ADA were examined using ADA immunohistochemistry. Fig. 2A demonstrates the pattern of Ada expression in a heterozygous (m1/+e) female harboring either a wild-type or m1/− embryo. There was prominent expression in the secondary deciduum, and in all trophoblast lineages. Within these same litters were gestation sites that maintained decidual expression but did not exhibit expression in trophoblast cells (Fig. 2B). In contrast to this, 9.5 dpc gestation sites from ADA-deficient females showed no expression in the deciduum (Fig. 2C,D), whereas expression was found in all trophoblast cells (Fig. 2C). This pattern of expression was seen in 14 gestation sites from 3 litters. Based on predicted Medelian inheritance, 75% of these gestation sites should contain expression from the wild-type Ada allele alone or together with expression from the Tg locus, and 25% should contain expression from the Tg locus alone. In all cases expression was seen in all trophoblast lineages, confirming previous results showing that the regulatory elements can recapitulate the wild-type Ada expression pattern (Shi et al., 1997). Also present at this stage of development were gestation sites that lacked Ada expression in the deciduum as well as trophoblast cells (Fig. 2D). These results clearly delineate trophoblast cell populations that express an abundance of Ada during early postimplantation stages of development, and demonstrate that while Ada expression is prominent in the deciduum, trophoblast cells provide an enriched source of this enzyme at the maternal-fetal interface.

Examination of gestation sites lacking decidual ADA allows for the quantitation of ADA enzymatic activities in trophoblast cells

It has been difficult to accurately quantitate the levels of ADA enzymatic activity in trophoblast cells during early postimplantation stages, because of the close proximity of the deciduum, and the inability to dissect these tissues apart. This problem was overcome by examining the levels of ADA enzymatic activity in gestation sites lacking decidual ADA (Fig. 3). Shown in Fig. 3A are the levels of ADA enzymatic activity found in wild-type gestation sites. The highest enzyme specific activity resided in the antimesometrium (526 nmole/minute/mg protein), presum-
ably associated with enriched expression in the secondary deciduum. Less activity (29 nmoles/minute/mg protein) was associated with trophoblast cells in mesometrial samples. Levels of ADA enzymatic activity in gestation sites lacking decidual Ada expression were substantially lower than those observed in wild-type gestation sites (Fig. 3B). Of these gestation sites, the highest levels of ADA enzymatic activity were found in the antimesometrial half of gestation sites containing Tg-m1/+ embryos (19 nmoles/minute/mg protein; Fig. 3B). This activity represents trophoblast Ada expression from one wild-type Ada allele together with expression from one ADA transgenic locus. Gestation sites that expressed Ada from the ADA transgenic locus alone exhibited detectable but low levels of ADA enzyme specific activity (7 and 3 nmoles/minute/mg protein for antimesometrial and mesometrial halves respectively, Tg-m1/m1, Fig. 3B). As expected, there was no ADA enzymatic activity detected in gestation sites lacking functional Ada alleles (m1/m1, Fig. 3B). By subtracting values obtained from Tg-m1/+ antimesometria from those of Tg-m1/m1 antimesometria it was determined that one wild-type allele provides a specific activity of 12 nmoles/minute/mg. Therefore, giant trophoblast cells in the wild-type antimesometrium provide approximately 24 nmoles/minute/mg, or 5% of that found in the secondary deciduum. Similar calculations suggest that trophoblast cells of the mesometrial half contain 6 nmoles/minute/mg. Through these studies we have been able to quantitate the relative levels of ADA enzymatic activity provided by the deciduum and trophoblast cells in the early postimplantation gestation site.

Gestation sites deficient in decidual ADA exhibit various developmental abnormalities

Pharmacological studies have shown that embryolethality is associated with ADA inhibition in the early postimplantation gestation site (Knudsen et al., 1989 and 1992; Airhart et al., 1993); however, these studies were not able to assess the relative importance of ADA in the deciduum and trophoblast cells. The availability of genetically engineered mice lacking decidual ADA has enabled us to assess the reproductive outcome of gestation sites that express Ada only in trophoblast cells, or are totally ADA deficient. Histological analysis at 7.5 dpc revealed distinct differences amongst gestation sites expressing Ada in varying locations (Fig. 1). At this stage, wild-type gastrulating embryos are easily distinguished as tripalaminar disks and by the presence of three clearly separable cavities, including the amniotic, exocoelomic and ectoplacental cavities (Fig. 1D). A large number of gestation sites (<10) from Tg-m1/m1 females that lacked Ada expression in the deciduum, but expressed Ada in trophoblast cells surrounding the implantation chamber, contained embryos that appeared to be developmentally delayed (Fig. 1E). The ectoplacental cone and giant trophoblast cells of these embryos were well established in a fully developed deciduum. The embryos themselves, however, did not display expanded amniotic and exocoelomic cavities (Fig. 1E), thus resembling 6.5 dpc embryos (Snell and Stevens, 1966). All gestation sites lacking both decidual and trophoblast ADA were severely degenerating (Fig. 1F). These findings indicate that ADA is critical for early postimplantation development, and suggest that expression in trophoblast cells that surround the developing embryo may provide significant protection from the harmful consequences of ADA deficiency as early as 7.5 dpc.

As gestation proceeds, ADA levels increase in both the deciduum and trophoblast cells (Knudsen et al., 1991). To determine the consequences of removing decidual and/or trophoblast ADA on the progression of both embryonic and placental development, gestation sites deficient in decidual ADA were examined at 9.5 dpc. In Fig. 2C it is shown that the placenta of gestation sites containing only trophoblast ADA developed normally (Fig. 2C). The majority of gestation sites examined (10 out of 14) that lacked decidual ADA and contained trophoblast ADA did not show a pronounced delay in embryo development as was seen at 7.5 dpc (compare Fig. 2C with Fig. 1E). Gestation sites that lacked decidual and trophoblast ADA were severely affected at 9.5 dpc (Fig. 2D). Implantation chambers were devoid of embryonic material, suggesting the embryo has been completely destroyed by this stage. This was associated with an increase in maternal blood flow in the gestation site and other signs of resorption. An interesting finding was that both the visceral and parietal yolk sacs appeared to be intact in gestation sites lacking Ada expression (Figs 2D, 4B). In addition, giant trophoblast cells were detected on the periphery of the implantation chamber, and an expanded ectoplacental cone devoid of vasculature, and lacking extraembryonic mesoderm and demarcated labyrinthine and junctional zones, was seen (Fig. 4B). These findings suggest that the normal pattern of trophoblast differentiation and placental development does not occur in gestation sites lacking ADA. However, trophoblast cells themselves appear to be less sensitive to the consequences of ADA deficiency than cells of the embryo proper.

Expression of Ada in the gestation site is critical for early postimplantation development

To more clearly assess the phenotype of embryos, as well as facilitate the genotyping of individual gestation sites, embryos were dissected out of gestation sites at 9.5 dpc, examined under a dissecting microscope, and then used as a source of genomic DNA for genotyping. The appearance of wild-type or m1/+ embryos that develop in gestation sites containing decidual ADA is shown in Fig. 5A. Representative embryos from gestation sites lacking decidual ADA, but containing trophoblast ADA are shown in Fig. 5B.C. Fig. 5B represents embryos from gestation sites containing expression in trophoblast cells provided by the ADA transgene as well as one wild-type Ada locus (Tg-m1/+). These embryos were typically smaller than wild-type embryos, but did not exhibit a severe developmental delay. Fig. 5C represents embryos from gestation sites with expression in trophoblast cells of the embryo proper. As was seen at 7.5 dpc (compare Fig. 2C with Fig. 1E). Gestation sites that lacked decidual and trophoblast ADA were severely affected at 9.5 dpc (Fig. 2D). Implantation chambers were devoid of embryonic material, suggesting the embryo has been completely destroyed by this stage. This was associated with an increase in maternal blood flow in the gestation site and other signs of resorption. An interesting finding was that both the visceral and parietal yolk sacs appeared to be intact in gestation sites lacking Ada expression (Figs 2D, 4B). In addition, giant trophoblast cells were detected on the periphery of the implantation chamber, and an expanded ectoplacental cone devoid of vasculature, and lacking extraembryonic mesoderm and demarcated labyrinthine and junctional zones, was seen (Fig. 4B). These findings suggest that the normal pattern of trophoblast differentiation and placental development does not occur in gestation sites lacking ADA. However, trophoblast cells themselves appear to be less sensitive to the consequences of ADA deficiency than cells of the embryo proper.

Table 1. Analysis of 9.5 dpc embryos*

<table>
<thead>
<tr>
<th>Embryo genotype</th>
<th>Delayed†</th>
<th>Dead‡</th>
<th>Normal</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>m1/+</td>
<td>2</td>
<td>–</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>m1/m1</td>
<td>–</td>
<td>5</td>
<td>–</td>
<td>5</td>
</tr>
<tr>
<td>Tg-m1/+</td>
<td>3</td>
<td>–</td>
<td>10</td>
<td>13§</td>
</tr>
<tr>
<td>Tg-m1/m1</td>
<td>4</td>
<td>–</td>
<td>–</td>
<td>4</td>
</tr>
</tbody>
</table>

*Embryos from three matings between females lacking decidual ADA (Tg-

†Embryos were considered developmentally delayed if they had not yet turned.

‡Dead embryos were degenerated beyond recognition, however, extraembryonic membranes were intact.

§The large number of Tg-m1/+ embryos is due to one pregnant female being homozygous for the transgenic locus.
phoblast cells provided by the ADA transgene only (Tg-m1/m1). The majority of these embryos appeared to be developmentally delayed, as was evident by the absence of embryo turning. A representative 9.5 dpc embryo lacking decidual and trophoblast ADA is shown in Fig. 5D (m1/m1). Such embryos were severely degenerated while the extraembryonic membranes appeared intact. A summary of the phenotypes of various embryos is presented in Table 1. Collectively, these data suggest that Ada expression in the gestation site is critical for normal development. Furthermore, expression in trophoblast cells alone was sufficient for survival although it was associated with varying degrees of developmental delay.

Gestation sites deficient in decidual ADA exhibit severe disturbances in purine metabolism

ADA deficiency is often associated with disturbances in purine metabolism, including the accumulation of the ADA substrates adenosine and 2'-deoxyadenosine (Hershfield and Mitchell, 1995). To investigate the metabolic disturbances associated with the absence of decidual ADA, gestation sites were harvested on day 8.5 pc and analyzed for the levels of ADA substrates and products. In order to preserve the integrity of nucleosides, whole gestation sites were rapidly dissected free from the myometrium and quick frozen in liquid nitrogen. It was therefore not possible to identify the genotype of individual gestation sites. For this reason the levels of nucleosides in individual gestation sites within a litter are presented instead of mean values (Fig. 6). Inosine, a major product of the ADA reaction, was detected in all gestation sites of m1/+ females (containing decidual ADA) mated to m1/+ males (Fig. 6A, m1/+). The ADA substrate adenosine was readily detected in these gestation sites (Fig. 6B, m1/+), whereas the cytotoxic ADA metabolite 2'-deoxyadenosine was undetectable (Fig. 6C, m1/+). In contrast to these results, inosine levels were lower in gestation sites of Tg-m1/m1 females (lacking decidual ADA) mated to m1/+ males (Fig. 6A, m1/m1). Adenosine and 2'-deoxyadenosine levels were markedly increased in these gestation sites (Fig. 6B,C, m1/m1). It is interesting to note that the cytotoxic metabolite 2'-deoxyadenosine accumulated to very high levels in one sample (0.25 nmoles/mg from undetectable levels of ≤0.001 nmoles/mg). We speculate that 2'-deoxyadenosine accumulation may be associated with the loss of embryo viability seen in gestation sites that lack both decidual and trophoblast ADA. These results indicate that there were severe metabolic disturbances associated with the absence of decidual ADA, and suggest that expression in trophoblast cells alone may be sufficient to prevent the accumulation of 2'-deoxyadenosine to toxic levels.

DISCUSSION

During early postimplantation stages in mice (6.5-9.5 dpc), ADA is highly expressed in two genetically distinct cell populations in the gestation site: the maternally derived stromal cells of the secondary deciduum, and the zygotically derived trophoblast cells of the choriovitelline and chorioallantoic placentas (Knudsen et al., 1991). Because of the close proximity of giant trophoblast cells and the secondary deciduum, it has been difficult to assess the relative pattern and level of zygotically derived ADA in the gestation site. In the current study, we used a genetic approach to clearly delineate Ada expression and function in trophoblast cells during these critical stages of development. Giant trophoblast cells surrounding the implantation chamber exhibited a striking pattern of ADA immunoreactivity (Figs 1B, 2C). This was revealed in mice engineered

Fig. 4. Histological analysis of ADA-deficient extraembryonic tissues on day 9.5 pc. Adjacent sections of 9.5 dpc gestation sites analyzed for ADA immunolocalization were utilized for histological analysis (scale bar, 500 μm). (A) Wild-type or m1/+ developing placenta showing extraembryonic mesoderm, a well vascularized labyrinthine zone (lz); an organized junctional zone (jz); and giant trophoblast cells (gc). md, mesometrial deciduum. (B) Gestation site of an ADA-deficient female containing a m1/m1 conceptus. The implantation chamber was devoid of embryonic material, however the parietal (pys) and visceral yolk sacs (vys) were intact. Trophoblast cells and extraembryonic ectoderm were present, however, there was no extraembryonic mesoderm and a lack of differentiation of the extraembryonic ectoderm into labyrinthine trophoblast cells. Giant trophoblast cells were detected while the junctional zone was unorganized.
to lack ADA in the deciduum. The expression level in giant trophoblast cells was estimated to be about 5% of that found in wild-type gestation sites at 9.5 dpc. However, given that the specific activities measured are inclined to be diluted by the large amount of decidual tissue in samples, the level of activity at the maternal-fetal interface provided by trophoblast cells is likely quite significant. The current studies have also allowed us to determine the cell-type specificity of the genetic regulatory elements used in the rescue of ADA-deficient fetuses (Blackburn et al., 1995). In this sense, the ADA transgene served as a reporter gene to monitor spatial and temporal expression capabilities of the placental enhancer from the murine Ada gene (Shi et al., 1997). In all cases, gestation sites of Tg-m1/m1 mothers showing Ada expression in trophoblast cells, exhibited expression in all three trophoblast lineages (Fig. 2C). Coincidently, 25% of these gestation sites contained expression from the ADA minigene only. Therefore, the placental regulatory elements used were capable of recapitulating the wild-type pattern of Ada expression in all trophoblast cell lineages. These experiments provide a new appreciation for the highly localized expression of Ada at the maternal-fetal interface.

Previous studies have shown that Ada expression in the fetal placenta is important for development (Blackburn et al., 1995). The animals resulting from that study provided us the opportunity to genetically assess the importance of ADA at the maternal-fetal interface during early postimplantation stages of development. Embryos residing in gestation sites deficient in decidual and trophoblast ADA were all dead or degenerating by 7.5 dpc. These findings are in good agreement with previous pharmacological studies using the ADA inhibitor 2′-deoxycoformycin, in which treatment on days 7.5 or 8.5 pc resulted a
near total loss of embryo viability by 10.5 dpc (Knudsen et al., 1989; Airhart et al., 1993). However, the death of embryos in genetically deficient gestation sites occurred up to 72 hours sooner than that seen following 2'-deoxycoformycin exposure. This is likely due to the total absence of ADA enzymatic activity throughout development in our genetic studies, as compared to relatively short periods of ADA inhibition following 2'-deoxycoformycin treatment.

It was not possible to distinguish the relative importance of decidual and trophoblast ADA using 2'-deoxycoformycin since the inhibitor could access both cell types. However, the ability to genetically engineer gestation sites lacking ADA in either decidual cells or trophoblast cells enabled us to assess the functional importance of ADA in these cell types. Embryos that are deficient in trophoblast ADA but develop in an implantation chamber containing decidual ADA (Fig. 2B), survive the early postimplantation period but ultimately die from liver damage as a result of placental ADA deficiency during fetal stages (Blackburn et al., 1995; Wakamiya et al., 1995). Embryos developing in gestation sites lacking decidual ADA but containing trophoblast ADA, develop beyond the early postimplantation period but displayed retarded development at 9.5 dpc. This delay did not appear to be embryolethal because ADA-deficient mothers consistently gave birth to viable Tg-m1/+ and Tg-m1/m1 pups (data not shown). Thus, it is essential to have ADA present at the maternal-fetal interface during early postimplantation stages. Furthermore, although there appears to be some benefit to having ADA in the deciduum, trophoblast ADA is sufficient to meet this early postimplantation requirement for ADA.

Phenotypes resulting from ADA deficiency are often associated with severe disturbances in purine metabolism (Hershfield and Mitchell, 1995; Knudsen et al., 1992; Wakamiya et al., 1995; Migchielsen et al., 1995). In the current study we observed that the absence of ADA in the deciduum was accompanied by severe disturbances in purine metabolism as well. These disturbances included the accumulation of the ADA substrates adenosine and 2'-deoxyadenosine (Fig. 6). Pharmacological inhibition of ADA in the gestation site by 2'-deoxycoformycin is also accompanied by an accumulation of adenosine and 2'-deoxyadenosine (Knudsen et al., 1992). It has been suggested that the accumulation of 2'-deoxyadenosine in particular leads to widespread p53-dependent apoptosis in the embryo and subsequent loss of viability (Gao et al., 1994; Wubah et al., 1996). Other studies report that 2'-deoxycoformycin treatment interferes with allantois development which in turns leads to embryo death (Airhart et al., 1996). It is likely that the embryolethality and developmental delay seen in genetically modified gestation sites in the current study are a result of the accumulation of adenosine and 2'-deoxyadenosine. These substrates are presumably readily produced at the maternal-fetal interface as a byproduct of apoptosis (Blackburn et al., 1997), which occurs in abundance at the maternal-fetal interface as the embryo implants and expands (Welsh and Enders 1985). These findings suggest that Ada expression at the maternal-fetal interface plays an essential role in protecting the early postimplantation embryo from the harmful metabolic consequences associated with massive tissue remodeling. Interestingly, Ada expression in the trophoblast cells alone, although not optimal, seems sufficient to provide adequate protection in the absence of decidual ADA. This suggests that Ada-expressing trophoblast cells that surround the developing embryo serve as a protective barrier from the harmful accumulation of ADA substrates.

Both adenosine and 2'-deoxyadenosine are potent bioactive nucleosides. Adenosine is an extracellular signaling molecule that can elicit many physiological responses through subclasses of membrane bound adenosine receptors (Stiles, 1992). 2'-Deoxyadenosine is a cytotoxic metabolite that is thought to provide the metabolic basis for the immunodeficiency seen in ADA-deficient humans and mice (Hershfield and Mitchell, 1995; Blackburn et al., 1996), as well as embryolethality and fetal liver damage associated with ADA deficiency in mice (Gao et al., 1994; Wakamiya et al., 1995; Blackburn et al., 1995). 2'-Deoxyadenosine cytotoxicity is likely to provide the metabolic basis for embryolethality in ADA-deficient gestation sites as well. Adenosine levels were markedly elevated in all gestation sites deficient in decidual ADA, suggesting trophoblast ADA could not prevent accumulation to saturating levels (Fig. 6). 2'-Deoxyadenosine levels were not as high as adenosine levels but displayed fluctuations (Fig. 6). This may reflect the ability of trophoblast ADA to prevent the accumulation of 2'-deoxyadenosine above threshold levels beyond which embryo viability is lost. 2'-Deoxyadenosine is thought to be cytotoxic by mechanisms that include the interference of deoxynucleotide metabolism and/or cellular transmethylation reactions utilizing S-adenosylmethionine as a methyl donor (Hershfield and Mitchell, 1995). There is evidence to suggest the involvement of both of these mechanisms in the embryolethality seen in ADA-deficient gestation sites. Direct exposure of early postimplantation mouse embryos to 2'-deoxyadenosine in culture, leads to an accumulation of dATP and massive apoptosis, suggesting disruptions in deoxynucleotide metabolism may be involved (Gao et al., 1994). Embryos that are genetically deficient in S-adenosylhomocysteine hydrolase, a key enzyme in S-adenosylmethionine metabolism, die during the early postimplantation period (Papaioannou and Mardon, 1983; Miller et al., 1994), suggesting an interference in cellular transmethylation reactions is a potential target of 2'-deoxyadenosine cytotoxicity in the ADA-deficient gestation site. The genetically modified mice used in this work will provide a model system to monitor specific metabolic pathways involved in 2'-deoxyadenosine cytotoxicity and embryolethality.

Embryos found in gestation sites modified to lack decidual ADA, and containing only trophoblast ADA, tended to be developmentally delayed (Figs 1E, 5C; Table 1). The mechanism for this phenomena is unknown; however, perturbations in adenosine signaling may be involved. Adenosine is known to arrest cleavage in the starfish embryo at the onset of blastulation (Tsuchimori et al., 1988), it can also contribute to the maintenance of meiotic arrest in mammalian oocytes (Miller and Berhman, 1986), and can suppress embryonic limb outgrowth (Knudsen and Elmer, 1987). Consistent with these observations, elevated adenosine levels in gestation sites lacking decidual ADA may disrupt adenosine signaling mechanisms and cause a delay in development. More knowledge into the expression pattern and function of adenosine receptors in the early postimplantation embryo will be required before such a mechanism can be tested. A unique finding in ADA-deficient gestation sites was the relative insensitivity of trophoblast cells to the cytotoxic consequences of ADA deficiency. Whereas embryos in ADA-deficient gestation sites were degenerate, extraembryonic membranes and trophoblast
cells persisted (Fig. 4B). This may be due to the resistance of these cell types to 2'-deoxyadenosine cytotoxicity, or trophoblast cells may be subject to protective effects of adenosine (Engler and Gruber, 1991). Assessing the expression of components of 2'-deoxyadenosine cytotoxicity and adenosine signaling in trophoblast cells will contribute to or understanding of the mechanisms involved in their insensitivity.

In summary, we have utilized mice that have been genetically modified to lack ADA in the maternal deciduum to delineate the level and pattern of zygotically derived ADA in trophoblast cells, and to assess the reproductive outcome of mice lacking decidual ADA, trophoblast ADA or both. We demonstrate that giant trophoblast cells that surround the embryo during early postimplantation stages of development are enriched in ADA. Severe metabolic disturbances were associated with the absence of decidual ADA; however, the abundant expression in trophoblast cells appeared to be capable of protecting the embryo from the accumulation of ADA substrates to toxic levels. This was not the case in completely ADA-deficient gestation sites, from the accumulation of ADA substrates to toxic levels. This trophoblast cells appeared to be capable of protecting the embryo

Severe metabolic disturbances were associated with the absence of ADA-deficient fetuses and placenta. We demonstrate that the level and pattern of zygotically derived ADA in trophoblast cells were modified to lack ADA in the maternal deciduum to delineate the mechanisms involved in their insensitivity.


(Received 3 June 1997)