A Null Mutation in the Inflammation-Associated S100 Protein S100A8 Causes Early Resorption of the Mouse Embryo

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A Null Mutation in the Inflammation-Associated S100 Protein S100A8 Causes Early Resorption of the Mouse Embryo

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S100A8 (also known as CP10 or MRP8) was the first member of the S100 family of calcium-binding proteins shown to be chemotactic for myeloid cells. The gene is expressed together with its dimerization partner S100A9 during myelopoiesis in the fetal liver and in adult bone marrow as well as in mature granulocytes. In this paper we show that S100A8 mRNA is expressed without S100A9 mRNA between 6.5 and 8.5 days postcoitum within fetal cells infiltrating the deciduum in the vicinity of the ectoplacental cone. Targeted disruption of the S100A8 gene caused rapid and synchronous embryo resorption by day 9.5 of development in 100% of homozygous null embryos. Until this point there was no evidence of developmental delay in S100A8−/− embryos and decidualization was normal. The results of PCR genotyping around 7.5–8.5 days postcoitum suggest that the null embryos are infiltrated with maternal cells before overt signs of resorption. This work is the first evidence for nonredundant function of a member of the S100 gene family and implies a role in prevention of maternal rejection of the implanting embryo. The S100A8 null mouse provides a new model for studying fetal-maternal interactions during implantation. The Journal of Immunology, 1999, 163:2209–2216.

The S100 proteins are low m.w. calcium-binding proteins belonging to the EF hand superfamily (1). One member of this family, S100A8, is a secreted protein and acts as a potent pure chemoattractant for mouse and human neutrophils and macrophages (2, 3). At least two other members of the S100 family also possess chemotactic activity (4, 5). Intradermal injection of the S100A8 protein into rats or mice elicits a sustained inflammatory response closely resembling delayed-type hypersensitivity responses (2, 3). The S100 gene family also includes S100α, S100β, calbindins, and calcyclins (6) and forms a cluster of linked genes in the mouse and human genomes (1). Human S100A8 (also known as MRP8, calgranulin A, L1 light chain, cystic fibrosis Ag, and calprotectin) is the most closely related member of the human S100 family to mouse S100A8, although the level of homology is <60%, and the human protein lacks chemotactic activity (3). In both species, S100A8 forms complexes with S100A9 (MRP14) in the cytoplasm of neutrophils and macrophages in various stages of differentiation and activation and has been implicated in calcium-dependent regulation of myeloid cell function (7). In other studies in the laboratory we have found that S100A8 mRNA and protein is massively induced in the lung in response to i.v. injection of bacterial LPS (our unpublished data).

The first isolation of the mouse S100A8 and S100A9 gene reported upon the restricted expression of the two genes in the fetal liver (8). More recently, we have demonstrated that both genes are expressed exclusively in this location from around 10.5 days of embryological development (10.5 days postcoitum (dpc)3). Expression was not observed during yolk sac hematopoiesis and was also absent from mature macrophages that leave the liver in very large numbers during development (9). This pattern was consistent with in vitro studies in which both S100A8 and S100A9 mRNAs were induced transiently in bone marrow cultures stimulated with the macrophage-specific growth factor, CSF-1 (10). In this study we sought to define the possible roles of S100A8 by targeted disruption of the mouse gene. Given the very restricted pattern of expression of S100A8 during embryogenesis and its biological activities, we anticipated that a targeted disruption of the gene might cause a late embryonic lethality if macrophage/granulocyte production by the liver was perturbed, or it might compromise some aspect of inflammation or LPS responsiveness in adult animals if fetal expression was redundant. Because of the large size of the S100 family, it was possible that the function of S100A8 might be substituted by some other family member and no phenotype would be evident. A more extensive examination of sites of expression of S100A8 during embryogenesis herein revealed that the gene is also expressed in extra-embryonic tissues immediately following implantation, where it might regulate fetal-maternal interactions. In keeping with this hypothesis, we show that 100% of null embryos are resorbed by the mother at exactly the time that S100A8 is expressed.

Materials and Methods

Location of S100A8 mRNA

Whole mount in situ hybridization using digoxigenin-labeled (DIG) riboprobes was performed on embryos at the stages indicated as described previously (11). S100A8 (CP10/MRP8) and S100A9 (MRP14) cDNA plasmids are detailed in Hu et al. (12). In each case sense and antisense mRNA probes were produced; any signal detected with the sense probes is noted in the figure legends. For the analysis of the implantation sites, individual deciduals were hemisected along the longest axis using a scalpel under a microscope (12). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Abbreviations used in this paper: dpc, days postcoitum; CSF-1, macrophage colony-stimulating factor; ES cell, embryonic stem cell; EPC, ectoplacental cone.
dissecting microscope before fixation in 4% paraformaldehyde and processing as described previously for embryos (11). Following hybridization and extensive washing, the DIG was detected using alkaline-phosphatase-conjugated anti-DIG Abs followed by histochemical staining which yields a blue-purple color. The pattern of expression of S100A8 and c-fms observed in Fig. 2 was observed in at least four independent litters at each developmental age; the approximate gestational age (dpc) was confirmed by examination of the stage of development of the embryos. Typically, multiple litters of each gestational age were combined, then 8–10 embryos were stained for expression of each marker examined. The timing of gestational age is based upon the assumption that coitus occurred at midnight on the evening before confirmation of a vaginal plug.

**Targeted disruption of the S100A8 gene**

The S100A8 targeting vector described in Fig. 1 is based upon a positive-negative selection strategy in which cells are initially selected for recombination using G418 resistance and then nonhomologous insertions are selected against because of their retention of the viral thymidine kinase gene using ganciclovir (13). Following homologous recombination at the S100A8 locus, the coding sequence is replaced by 1.8 kb of neomycin phosphotransferase (*neo*) gene driven by the phosphoglycerate kinase 1 (pgk1) promoter. Transfection of R1 embryonic stem (ES) cells (a gift from Dr. A. Nagy; Samuel Lunenfeld Research Centre, Toronto, Canada) by electroporation, selection of candidate homologous recombinants, screening by Southern blotting, and generation of chimeras by the morula aggregation method were performed as described by Monkley et al. (14). We selected two independent targeted ES cell lines (clone 52 and clone 79) for further study.

**Genotyping of S100A8 null mice**

Identification of the targeted allele in mice was based upon detection of the *neo* gene using the primers described (14), and identification of the wild-type, S100A8 allele using the primers 5’-GCTCCTGCTTCAAGA CATCGT-3’ (+22 to +41) and 5’-GCGGTTTCCTTGGAGATGCC-3’ (+898 to +880). For genotyping of preimplantation embryos, the uterus was flushed at 3.5 dpc and blastocysts were placed into 200 µl microwells in DMEM + 10% FBS for up to 7 days. During this time the blastocysts hatched, and trophoblast migration was observed. Genotyping was performed on DNA isolated from hatched blastocyst cells.

**Immunolabeling of disaggregated decidual cells**

For immunostaining, embryos were removed and decidua were washed in PBS, cut into small pieces, and drawn through a 23-gauge needle. Decidua were incubated in Petri dishes in 0.1% collagenase, 0.2% dispase (Boehringer Mannheim, Indianapolis, IN), 20% FCS in PBS for 60 min at 37°C. The digest was drawn through the 23-gauge needle, pelleted, and washed. The single cell suspension was blocked with 5% rat serum, then incubated with PE-conjugated anti-Mac-1 Ab (Caltag, South San Francisco, CA), washed, and analyzed on a FACSCalibur (Becton Dickinson, Mountain View, CA) flow cytometer.

**Transfer of embryos into lacZ-expressing mothers**

For the investigation of infiltration of embryos by maternal cells, 3.5-dpc embryos were collected from S100A8 (+/−) matings and transferred into pseudopregnant 253 strain female mice, which express nuclear lacZ in all cells under the control of the HMG-CoA reductase promoter (15). The mice were sacrificed at 6.5–9.5 dpc. Decidua and embryos were bisected as for in situ hybridization, fixed, and stained for lacZ expression as described (15). They were then embedded in paraffin wax, sectioned at 8 µ and counterstained with Neutral Red.

**Results**

*S100A8 but not S100A9 is expressed in the vicinity of the ectoplacental cone (EPC) at 6.5 to 7.5 dpf*

Previous studies indicated S100A8 is expressed for the first time in the mouse embryo together with S100A9 in presumptive myeloid cells in developing liver around 11.5 dpc (8, 9). As a prelude to performance of targeted disruption of the gene, we examined the expression of S100A8 mRNA at earlier stages of development. This study revealed a second site of S100A8 expression in extraembryonic tissue. Following initial attachment and implantation
of the mouse embryo, the polar trophoderm proliferates to form the EPC and extraembryonic ectoderm, from which the various differentiated secondary trophoblast cell types arise. In many respects, EPC-derived trophoblasts resemble granulocytes and macrophages. They are capable of extensive phagocytosis (16) and like macrophages, express the gene encoding the receptor for CSF-1, the \textit{c-fms} protooncogene (11). Whole mount in situ hybridization on hemisected decidua at 6.5–8.5 dpc provides a three-dimensional perspective of gene expression in the implantation site. The application of this method, which is used routinely on mouse embryos, to gene regulation in the implantation site has not been reported previously to our knowledge. The \textit{c-fms} mRNA was detected within primary trophoblasts infiltrating surrounding tissues from the full perimeter of implanted embryos at 6.5 dpc, throughout the EPC and extending outwards from that pole into the deciduum. By contrast, S100A8 mRNA was only detected in very small subsets of cells that seemed to delineate the external perimeter of the EPC (not shown). By 7.5 dpc, the \textit{c-fms} positive cells were more extensively infiltrated into the deciduum surrounding the embryo, particularly around the EPC (Fig. 2A). S100A8 was clearly restricted to a halo of cells surrounding the EPC. The distribution apparently partly overlaps the distribution of \textit{c-fms} but the method is not amenable to double-labeling to confirm identity (Fig. 2B). Sections of in situ-stained embryos confirmed that the S100A8-positive cells flanking the EPC were large mononuclear cells with abundant cytoplasm and macrophages. They express high levels of S100A8 mRNA.

![FIGURE 2. Expression of S100A8 and c-fms mRNA in sites of implantation. Sites of expression of mRNA were detected by whole-mount in situ hybridization using digoxigenin-labeled RNA probes as described in Materials and Methods. Dark blue/purple formazan product indicates the sites of gene expression. For A–C, decidua were removed from the uterus and cut sagitally just lateral to the midline to expose the embryo. (Bar in A, B, D, and E = 300 \textmu m). A, Expression of \textit{c-fms} mRNA in 7.5-dpc embryo; note the expression of the gene in cells completely surrounding the embryo (the primary trophoblasts) as well as extensive expression in the ectoplacental cone (epc). Occasional staining of the embryo itself (middle embryo in B) is due to nonspecific trapping and was also observed with the sense probe. B, Expression of S100A8 mRNA in a subset of cells surrounding the embryo at 7.5 dpc. Unlike \textit{c-fms}, S100A8 mRNA is not expressed in the cells of the EPC, but is restricted to cells apparently infiltrating the deciduum. C, The 7.5-dpc embryos stained for S100A8 mRNA were embedded in paraffin, sectioned, and counterstained with neutral red. Blue stain denoting S100A8 expression flanking the EPC is restricted to large mononuclear trophoblast-like cells. D and E, Localization of \textit{c-fms} mRNA (D) in the placenta (stripped of decidual tissue) of an 11.5-dpc embryo, viewed from the maternal side, reflect the very high levels of expression in trophoblast giant cells observed previously (8). By contrast, S100A8 mRNA is undetectable except for a reticular pattern confined to the surface of the maternal face (E). Sections indicate this staining is associated with the vasculature (data not shown).]
expression in the vicinity of the EPC was no longer detectable by in situ hybridization or Northern blot analysis (data not shown). Whereas c-fms mRNA was maintained at very high levels in trophoblasts and trophoblast giant cells (Fig. 2D) at later stages of embryonic development (10.5–11.0 dpc), S100A8 mRNA expression was only detected in cells associated with the vasculature at the maternal face of the placenta (Fig. 2E). In contrast to the situation in the liver, where S100A8 was co-expressed with S100A9 in myeloid cells (14), S100A9 mRNA was not detected in the vicinity of the EPC at any stage except in decidual neutrophils (data not shown). Although most studies in human and mouse have indicated coexpression of the two S100 proteins, at least one other precedent for expression of secreted S100A8, in the absence of S100A9, occurs in mature macrophages responding to bacterial LPS (9). The cells expressing S100A8 and S100A9 in the fetal liver are also not necessarily coincident and appear morphologically distinct (8), although double labeling is required to confirm this proposal.

Resorption of S100A8 null embryos occurs between 9.5 and 13.5 dpc

To address the possible functions of S100A8 in myeloid and trophoblast cells, we created a disruption of the S100A8 gene in the mouse genome. The mouse S100A8 (MRP8) genomic DNA sequence reported by others (20) has been confirmed in our laboratory. The targeting strategy is outlined in Fig. 1. Because the gene is comparatively small, the introduced mutation removed the majority of the coding sequence and no functional protein product is comparatively small, the introduced mutation removed the majority of the coding sequence and no functional protein product was possible. The targeting vector was transfected into embryonic stem cells, and after positive-negative selection (for G418 resistance and ganciclovir-resistance respectively) the transfected clones were screened for correct targeting of the S100A8 gene. Two homologous recombinants were used to produce chimeras by morula aggregation, and male chimeras from both lines transmitted the overt genotype that can be detected by PCR, possibly presaging the overt resorption that becomes visibly obvious only 24 h later (9.5–10.5 dpc; Table II). Transplacental leukocyte infiltration occurs in normal pregnancies (21) but appears to be strictly controlled (22) and is presumably below the limits of detection by PCR in wild-type embryos.

To seek evidence that the apparent excess of heterozygotes detected by PCR was indeed due to infiltration by maternal cells, we transplanted 3.5-dpc embryos from heterozygous matings into mothers in which a nuclear lacZ transgene is expressed in all cells

Table II. Genotype/phenotype analysis of embryos derived from mating heterozygous S100A8 null mice

<table>
<thead>
<tr>
<th>Embryo Age (dpc)</th>
<th>Total Embryos</th>
<th>Genotype</th>
<th>Resorbing Embryos</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>(+/+)</td>
<td>(+/-)</td>
</tr>
<tr>
<td>13.5</td>
<td>33</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>12.5</td>
<td>27</td>
<td>2</td>
<td>23</td>
</tr>
<tr>
<td>10.5</td>
<td>15</td>
<td>7</td>
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<tr>
<td>9.5</td>
<td>56</td>
<td>14</td>
<td>42</td>
</tr>
<tr>
<td>8.5</td>
<td>98</td>
<td>18</td>
<td>76</td>
</tr>
<tr>
<td>6.5–7.5</td>
<td>109</td>
<td>16</td>
<td>75</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>27</td>
<td>5</td>
<td>15</td>
</tr>
</tbody>
</table>

* The distribution of genotypes at 8.5 dpc compared to that at 6.5–7.5 dpc using a χ² contingency test. The results indicate that the two sets of genotypes are independent; χ² = 7.613, 2 degrees of freedom, p = 0.02. Hence the apparent loss of S100A8 null homozygotes at 8.5 dpc is statistically significant.
heterozygous crosses (Fig. 4) on hemisected decidua from a series of 7.5-dpc embryos from S100A8 gene. Whole-mount in situ detection of S100A8 mRNA embryos from a heterozygous (+/−) cross. Extensive infiltration of the EPC and the embryo by lacZ-positive (blue) cells (arrows) is evident.

The decidua of S100A8+/− embryos identified based upon the absence of S100A8 mRNA, and the embryos within them, were indistinguishable from the (+/?) embryos and decidua of littermates. No gross deficiency in the EPC was evident, and decidua were the same size and shape as those of wild-type embryos (Fig. 4A). It is possible to obtain further differentiation of trophoblasts in vitro by taking explant cultures of the EPC (23). A series of heterozygous matings was performed, and 7.5-dpc EPC explants were cultivated for 4–7 days as described without exogenous growth stimulus. All cultures formed adherent trophoblast-like cells, and there was no clear difference in the extent of growth or spreading that would identify a subset of putative S100A8 null EPC cultures.

To assess the decidual reaction further, we digested 29 individual decidua from three separate heterozygous matings with collagenase/dispase and identified myeloid cells by flow cytometry. A representative profile is shown in Fig. 4B. Two populations of cells of different size and light scattering properties expressed the type 3 complement receptor (CD11b, Mac-1 Ag) present on granulocytes and macrophages. There was remarkably little variation in the numbers or proportions of the two cell populations between littersmates (Fig. 4B). The data indicate that there is no gross effect of the null mutation on the initial myeloid cell infiltration of the decidua.

Discussion

The S100 family contains some 20 functionally and structurally related proteins, including at least two other members (calbindin and mts1) expressed in trophoblasts (24, 25). S100A8 has previously been detected only in myeloid cells by flow cytometry. A representative profile is shown in Fig. 4B. Two populations of cells of different size and light scattering properties expressed the type 3 complement receptor (CD11b, Mac-1 Ag) present on granulocytes and macrophages. There was remarkably little variation in the numbers or proportions of the two cell populations between littersmates (Fig. 4B). The data indicate that there is no gross effect of the null mutation on the initial myeloid cell infiltration of the decidua.
later stages of placental development and trophoblast differentiation. Activated decidual cells are also concentrated in the vicinity of the EPC, shown clearly by induced expression of the TIMP-3 metallo-proteinase inhibitor (34), and these cells are not readily distinguished morphologically from trophoblasts. Hence, on the basis of cell morphology and limited precedent, S100A8 expression could have been either fetal or maternal in origin. In this paper, we have shown that there are fetally derived cells infiltrating the deciduum in

![Image](https://via.placeholder.com/150)

**FIGURE 4.** Normal development and decidualization of the implantation site of the S100A8 null mouse to 7.5 dpc. *A*, Comparison of hemisected decidua from S100A8<sup>−/−</sup> (left) or S100A8<sup>+/−</sup> (right) implantation sites at 7.5 dpc. In both cases, S100A8 mRNA has been localized by whole-mount in situ hybridization. Identification of −/− embryos was based on the absence of detectable S100A8 mRNA in the vicinity of EPC. This phenotype was observed in 11/46 embryos. The staining at the very tip of the EPC was present in all embryos examined and was of maternal origin. Embryonic and decidual development in the S100A8<sup>−/−</sup> is indistinguishable from the normal. *B*, Representative flow cytometry profile for expression of CD11b (Mac-1 Ag) on isolated decidual cells. The two apparent classes of CD11b-positive cells separable on the basis of size (FSC-H) probably represent granulocytes (R1) and macrophages (R2). The range of values for percentages of cells within each of the gates is given at the right of the panel. Note that the range varied between litters, presumably reflecting minor differences in precise gestational age and rapidity of the decidual reaction. No clear outlying values were observed in any of the three litters (total, 29 embryos).
the vicinity of the EPC (Fig. 3), and the data in Fig. 4 confirm that the S100A8-positive cells are of fetal origin. Indeed, it would be difficult to see a mechanism for early embryonic loss in the S100A8 null if the cells expressing the gene were maternal. One gene that does resemble S100A8 in expression pattern is urokinase plasminogen activator, which is induced around 7 dpc surrounding the EPC, in a halo of cells presumed to be migrating trophoblasts (34).

Given the lack of any overt defect in either embryo or trophoblast development in vivo or in vitro before resorption of the S100A8−/− embryo we propose that S100A8 is secreted, as occurs in activated macrophages, and contributes to the regulation of fetal-maternal interaction. In support of this hypothesis, recent studies in our laboratory (S. Leung and C. L. Geczy, unpublished data) have shown that although abundant S100A8 protein can be detected in decidual extracts at 8.5 dpc, S100A8 apparently does not accumulate within cells in the vicinity of the EPC (as evidenced by immunocytochemistry in which decidual neutrophils provide a positive control). As noted in the Introduction, we have shown that S100A8 mRNA is massively inducible in mouse organs following i.v. LPS injection (our unpublished data) indicating that expression in mice, as in humans, is associated with inflammation. The decidual reaction to implantation is essentially a form of acute inflammation (17, 18, 35). The data in Fig. 4 indicate that the primary decidual reaction occurred normally in S100A8 null mice, not surprisingly because much of the process occurs in response to blastocyst hatching, well before S100A8 is expressed maximally at 8.5 dpc. S100A8 apparently does not accumulate within cells in the vicinity of the EPC (as evidenced by immunocytochemistry in which decidual neutrophils provide a positive control). As noted in the Introduction, we have shown that S100A8 mRNA is massively inducible in mouse organs following i.v. LPS injection (our unpublished data) indicating that expression in mice, as in humans, is associated with inflammation. The decidual reaction to implantation is essentially a form of acute inflammation (17, 18, 35). The data in Fig. 4 indicate that the primary decidual reaction occurred normally in S100A8 null mice, not surprisingly because much of the process occurs in response to blastocyst hatching, well before S100A8 is expressed maximally at 7.5 dpc.

The actual process of resorption in S100A8 null embryos must occur very rapidly, because among the hundreds of implantation sites we have examined (Table II), the one shown in Fig. 3B is the only one in which the embryo was not either completely normal, or completely destroyed. A similar pattern of resorption is observed in a natural model of early embryo loss, the CBA/J × DBA/2 cross. In this model, embryonic development is also completely normal until 8 dpc, and embryo loss has been attributed to activation of macrophages and/or NK cells, or to ischemia due to vascular thrombosis (36) and inflammation (36, 37). S100A8 could contribute to the prevention of such pathology in a normal implantation site. It is chemotactic at low concentrations and the macrophages elicited have a particular phenotype including efficiency of uptake of lipid (38) and a high phagocytic index (our unpublished data), properties similar to those described for circulating monocytes in normal pregnancy (39). Hence the S100A8 secreted in the vicinity of the EPC could regulate the state of macrophage activation and procoagulant activities, thus protecting the embryo from immune attack by maternal cells. Alternatively, or in addition, high levels of S100A8 may be protective by virtue of its ability to be readily oxidized by reactive oxygen intermediates including hypochlorite, the major oxidant produced by activated granulocytes (40). By analogy, the anti-oxidant defense provided by thioredoxin functions as an essential protective factor against oxidative stress to embryos, though the thioredoxin null mutant dies earlier than the S100A8 null (41). In the future it will be of interest to compare the S100A8 null with the CBA/J × DBA/2 cross as a model for early embryo loss, particularly once the null allele has been crossed onto the appropriate inbred genetic backgrounds. PGE2, CSF-1, leukemia inhibitory factor, FasL, IL-1β, TGF-β, and IL-10, have variously been attributed roles in regulating infiltration of the embryo by maternal cells and the latter two prevent resorption in the CBA/J × DBA/2 model (22, 36, 42).

Among these candidate regulators, S100A8, a known regulator of myeloid cell function and inflammation (2, 3, 38, 40, 43), is unique in being expressed at precisely the critical time and place where infiltration of the embryo by maternal cells must be regulated.

Acknowledgments
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References
21. Piotrowski, P., and B. A. Croy. 1996. Maternal cells are widely distributed in maternal-maternal interaction. In support of this hypothesis, recent stud- ies in our laboratory (S. Leung and C. L. Geczy, unpublished data) have shown that although abundant S100A8 protein can be de-


