Fetal and Maternal Transforming Growth Factor-β1 May Combine to Maintain Pregnancy in Mice

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ABSTRACT

One of the mysteries of pregnancy is why a mother does not reject her fetuses. Cytokine-modulation of maternal-fetal interactions is likely to be important. However, mice deficient in transforming growth factor-β1 (TGFβ1) and other cytokines are able to breed, bringing this hypothesis into question. The phenotype of TGFβ1 null-mutant mice varies with genetic background. We report here that, in outbred mice, the loss of TGFβ1-deficient embryos is influenced by the parity of their mother. This is consistent with the loss of mutants being due to immune rejection. An inbred line of TGFβ1−/− mice that supported TGFβ1-deficient fetuses had high levels of TGFβ1 in their plasma. Analysis of the amniotic fluids in this line indicated that biologically relevant levels of maternal TGFβ1 were present in the TGFβ1−/− fetuses. These data are consistent with maternal and fetal TGFβ1 interacting to maintain pregnancy, within immune-competent mothers.

INTRODUCTION

We are immunologically distinct from our mothers. Consequently, successful pregnancies involve mechanisms that prevent the mother’s immune system from rejecting her conceptus. The placenta has a primary role here as it separates the maternal and fetal blood supplies, thus limiting the exposure of the fetus to the mother. The placenta, however, is not a perfect barrier. Fetal cells enter the maternal blood supply [1] and maternal T cells and antibodies can be detected in viable fetuses [2]. Other mechanisms must therefore be acting in concert with the placenta to suppress fetal rejection.

Immune rejection in the adult is regulated by complex interactions involving multiple cytokines. These interactions are also likely to be important in pregnancy, with successful pregnancy being dependent on an appropriate balance of cytokines at the maternal-fetal interface. T-helper (Th) 2/3-type cytokines, such as transforming growth factor-βs (TGFβs), interleukin-10 (IL-10), and colony-stimulating factor-1 (CSF-1), appear to promote pregnancy whereas proinflammatory Th1 cytokines, such as tumor necrosis factor-α and IL-2, are detrimental [3–5].

The TGFβs are a small family of proteins with three mammalian members, all of which are potent regulators of the immune system. In most circumstances, the TGFβs suppress the activities of immune cells [6]. The three TGFβ isoforms are present in the uterus [7, 8] and concepti [9], but have distinct spatial and temporal patterns. TGFβ2 is produced by the placenta [7, 10] and has been implicated in creating immune tolerance of the placenta [5]. The distribution of TGFβ2 within the fetus is, however, comparatively limited [11], making it unlikely that it is primarily responsible for suppressing immune cells that pass through the placenta. TGFβ1 is a better candidate for this role, as it is ubiquitously present in fetal tissues [12, 13]. TGFβ1 could also be important for maintenance of the placenta, as decidual cells produce TGFβ1, as well as TGFβ2 [7, 8].

The phenotype of TGFβ1−/− mice is complex and varies with genetic background [14, 15], but to date, there have been no reports of maternal rejection of TGFβ1-deficient concepti (fetus plus extraembryonic membranes). However, all of the TGFβ1-deficient colonies studied have inbred background that limits the immunological distinction between the mother and her fetuses. We have therefore undertaken a retrospective examination of the breeding records of an inbred and an outbred colony of TGFβ1−/− mice, seeking evidence of maternal rejection of fetuses.

As a result of the limited exchange of material between the mother and her conceptus, immune-related conditions such as Rh hemolytic disease are rare during a first pregnancy [16]. This provides a means to assess the physiological role of cytokines in preventing immune-mediated abortion. Simply, if TGFβ1 prevents immune rejection of fetuses in vivo, then the loss of TGFβ1-deficient fetuses should increase with the parity (number of pregnancies) of their mother. We report here that the loss of TGFβ1−/− and TGFβ1−/− fetuses in outbred mice follows this pattern.

MATERIALS AND METHODS

Animals

All experiments were approved by The University of Otago’s Animal Ethics Committee. The majority of the mice were originally bred for experiments that are unrelated to the present study [17, 18].

Swiss Webster mice were obtained from the University of Otago’s colony.

The inbred TGFβ1−/− colony was a derivative of Prof. T. Doetschman’s [14] and was established with mice purchased from The Jackson Laboratory (Bar Harbor, ME). These mice had a mixed 129Sv × C57BL/6j background. The colony was maintained by mating TGFβ1−/− studs and dams. The genotypes of the mice were determined by polymerase chain reaction, as previously described [17].

The nude (Whn−/−) TGFβ1−/− colony was maintained by breeding Whn+/−, TGFβ1−/− dams with Whn+/−; TGFβ1−/− studs and are referred to as outbred/nu. The mothers therefore produced T cells and TGFβ1. The founder nude TGFβ1−/− mice were generated by breeding TGFβ1−/− dams with nude (Whn−/−) studs. The nude mice had an outbred Swiss Webster background (C57CD-IuBR) and were from a local colony, Charles River Laboratories (Wilmington, MA). Nude dams were periodically introduced to the colony to maintain its outbred character.
Timed pregnancies were generated by mating TGFβ1−/− males and females at 1700 h. The females were examined the following morning at 0700 h for the presence of copulatory plugs. The males were removed to prevent copulation during the daytime. Noon on the day of detection of a plug was defined as E0.5.

An outbred/wt colony was established by crossing inbred TGFβ1−/− studs with Swiss Webster dams. The male TGFβ1−/− pups resulting from these crosses were then crossed with Swiss Webster dams. The F2 TGFβ1−/− dams and studs were mated to analyze the survival of TGFβ1−/− fetuses in outbred Swiss Webster mice, lacking the nude mutation.

Collection of Amniotic Fluid

Pregnant dams were anesthetized with pentobarbitone and their abdomens opened. The amniotic fluid of each conceptus was collected by inserting a syringe with a 16-gauge needle through the chorion and amnion. The amniotic fluid was centrifuged at 12,000 g for 15 min at 4°C to remove cells and immediately snap frozen in liquid nitrogen. The samples were stored at −80°C. The tail of each fetus was collected and used for genotyping [17].

Collection of Platelet-Deficient Plasma

Platelet-deficient plasma was prepared using a standard technique. Mice were anesthetized with diethyl ether and 400 µl of their blood removed by cardiac puncture using a 16-gauge needle that had been washed with 2% EDTA. The blood was added to a tube containing 40 µl of 2% EDTA at 4°C and then spun at 12,000 × g for 15 min at 4°C.

ELISA

The concentrations of TGFβ1 in amniotic fluid and plasma were measured using the Promega Emax Immunoassay system (Promega, Madison, WI) according to the manufacturer’s instructions. Each sample was acidified with HCl to a pH of 1.6 for 15 min, neutralized with NaOH, and diluted with the manufacturer’s sample buffer to ensure that all measurements were made in the middle of the range of the ELISA (31–1000 pg/ml). The intraassay variation was 7.7%. The ELISA was specific to TGFβ1, with a cross-reactivity of less than 5% with TGFβ2 and TGFβ3 at 10 ng/ml (Promega).

RESULTS

Loss of Mutant TGFβ1 Concepti Is Related to Parity

Retrospective analysis of the breeding records of two colonies of TGFβ1−/− mice was undertaken to assess whether the loss of TGFβ1-deficient fetuses was related to parity. The frequency of TGFβ1−/− surviving to birth in the outbred/nu colony was strongly linked to parity. The loss of TGFβ1−/− concepti was not related to the age of the mother, indicating that the association with parity was not an indirect consequence of mothers being young during their first pregnancy.

We then examined whether experienced outbred/nu mothers rejected a proportion of their wild-type and heterozygous concepti, along with the mutants. The number of wild-type pups per litter averaged 3.3 and was not correlated with parity (not illustrated). The expected ratio of heterozygous to wild-type concepti was two, which was observed in the litter from monoparous mothers. The ratio with experienced mothers (two or more pregnancies) was 1.5, which is significantly less than expected (P < 0.01, chi-squared). The extent of this loss was maximal with the second litter and relatively constant thereafter (Fig. 2). This suggests that experienced outbred/nu mothers reject approximately one in four of their TGFβ1−/− concepti.

TGFβ1−/− concepti were also lost in the inbred colony (Table 1). However, in marked contrast with the outbred/nu colony, the extent of this loss was unrelated to parity, with mothers able to carry a proportion of their TGFβ1−/− embryos to term, even after 11 pregnancies (Table 1).
ratio of heterozygous to wild-type pups was also normal in the inbred colony for all parities (Table 1).

Inbred TGFβ1+/− were then bred to Swiss Webster mice to produce an outbred TGFβ1+/− colony that is wild type for the nude mutation (outbred/wt, colony). The number of mutant pups in this colony was also strongly linked to parity (Fig. 3), indicating that the Swiss Webster background is sufficient for the loss of TGFβ1-deficient concepti to occur.

Maternal TGFβ1 Is Present in Concepti

The amount of maternal TGFβ1 in concepti was assessed by comparing the levels of TGFβ1 protein in the amniotic fluids of TGFβ1+/+, TGFβ1+/−, and TGFβ1−/− fetuses within a common TGFβ1+/− mother (see Table 2). The concentration of TGFβ1 in the amniotic fluids of the mutant fetuses was approximately half that of their wild-type littermates (Fig. 4B), indicating that significant maternal transfer of TGFβ1 protein had occurred. Heterozygotic concepti had TGFβ1 levels that were intermediate between their mutant and wild-type littermates (Fig. 4B). A gene dose relationship was also observed for the concentration of TGFβ1 in maternal plasma (Fig. 4A).

Plasma TGFβ1 Is Constant During Pregnancy

The concentration of TGFβ1 in the plasma of humans rises during pregnancy [19]. In contrast with this, the level of TGFβ1 in the TGFβ1−/− dams used in the present study was not different from that of their nonpregnant counterparts (Fig. 4A). The possibility that maternal TGFβ1 levels fluctuate during pregnancy was then further examined using Swiss Webster mice. No significant variation in plasma TGFβ1 was observed at any stage of pregnancy (Fig. 5).

DISCUSSION

Parity-Related Loss of TGFβ1-Deficient Concepti

The frequency of TGFβ1−/− and TGFβ1+/− mice surviving to birth was strongly linked to parity in the outbred/nu colony. This phenomenon was also observed in the outbred/wt colony, indicating that the presence of the nude (Whn−/−) mutation is not essential for parity-related loss of concepti.

The loss of TGFβ1-deficient concepti in the outbred col-

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**FIG. 4.** The figure illustrates the concentration of TGFβ1 in the plasma of adult inbred mice (A) and amniotic fluids of 16-day-old concepti in inbred mice (B). The bars representing wild-type, heterozygous, and null mutant animals are shaded in black, gray, or white, respectively. The sex of the mice in (A) is indicated beneath the bar using standard symbols or Pr to designate that the females were pregnant. The genotype in (B) represents that of the concepti. All of the mothers in (B) were TGFβ1+/− and had been mated with TGFβ1−/− studs. Each value is the mean ± the standard error of the mean of either eight (A) or five (B) mice. The values for the heterozygous and mutant mice are significantly different from those of the wild type (Student t; *P < 0.05; **P < 0.01; ***P < 0.001). In (A), the values of the heterozygous mice are not significantly different from 50% of the wild-type value. That is, the amount of TGFβ1 appears to be correlated with gender dosage.

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**TABLE 1.** Frequency of genotypes versus parity in the inbred TGFβ1−/− colony.*

| Parity | % +/+ | % +/− | % −/− | n   |
|--------|-------|-------|-------|-----|
| 1      | 28.7  | 64.8  | 6.6   | 335 |
| 2      | 29.7  | 63.8  | 6.5   | 185 |
| 3      | 30.5  | 66.3  | 3.2   | 95  |
| 4      | 26.0  | 66.0  | 8.0   | 50  |
| 5–12   | 29.8  | 62.0  | 8.3   | 121 |
| All    | 29.1  | 64.4  | 6.5   | 786 |

* There is no relationship between parity and the proportion of either TGFβ1−/− or TGFβ1+/− pups.

**TABLE 2.** Theoretical contribution of maternal and fetal TGFβ1 to amniotic TGFβ1.

| Conceptus genotype | Relative fetal TGFβ1 | Relative maternal TGFβ1 |
|--------------------|----------------------|-------------------------|
| TGFβ1+/+           | 1.0                  | 0.5                     |
| TGFβ1+/−           | 0.5                  | 0.5                     |
| TGFβ1−/−           | 0.0                  | 0.5                     |

* The values for the fetal component are normalized to those of the wild-type conceptus.
† The relative maternal TGFβ1 values are normalized to a wild-type mother; all maternal values are 0.5, as the mothers were TGFβ1−/−.
onies was not associated with maternal age, indicating that the association with parity is not an indirect consequence of more experienced mothers being older. Parity-related reproductive failure is rare and is usually indicative of the involvement of the maternal immune system. With the present study, this is particularly likely, as the fetuses being lost are deficient in a potent suppressor of immune responses.

The loss of TGFβ1−/− concepti in the inbred line was unrelated to parity. Several factors may combine here to create this phenomenon. First, the capacity of the inbred mothers to mount an immune attack against their concepti may be very limited. In inbred lines, the mother and her fetuses are genetically similar, which may attenuate (but not abolish) the ability of the mother to recognize her fetuses as foreign. Additionally, the inbred mice analyzed in this study had C57BL/6 ancestry, which can lead to diminished immune responses in some circumstances [20, 21] (see also www.informatics.jax.org/external/festing/search_form.cgi).

Second, the inbred mice had higher plasma levels of TGFβ1 than outbred mice (cf., Figs. 4A and 5), which may make the survival of their offspring less dependent on fetal sources of TGFβ1 (see below). Last, successful pregnancy is likely to depend on multiple genes, which may be differentially expressed in the inbred and outbred mice. This could lead to different strains of mice having different dependencies on TGFβ1 (see below).

Loss of TGFβ1-Deficient Concepti in Monoparous Mother

Some TGFβ1−/− concepti die early in development due to inadequate development of the yolk sac [14, 22]. The variability of this early death is due to allelic variation in a region of chromosome 5: TGFβ1−/− concepti survive when the chromosome is from NIH/Ola or 129 mice but not when it is from C57BL/6 mice [15, 23]. The inbred colony analyzed in this study had a mixed C57BL/6 × 129 background. The loss of TGFβ1−/− concepti that occurred in the inbred colony is thus probably due to the selective elimination of the mice with the unfavorable C57BL/6 allele. The loss of TGFβ1−/− concepti in the monoparous outbred mothers is suggestive that the unfavorable C57BL/6 allele is common in outbred colonies. However, we do not discount the possibility that immune rejection may also contribute to the loss of TGFβ1−/− concepti in monoparous outbred litters.

Dual Sources of TGFβ1

The TGFβ1 in amniotic fluid was observed to be of dual maternal and fetal origins. Amniotic fluid is a mixture of fetal urine and lung secretions [24]. The presence of maternal TGFβ1 within the amniotic fluid is thus indicative that maternal TGFβ1 passes through the fetus. Consistent with this, TGFβ1 protein is bound to the connective tissues of TGFβ1 null mutant concepti [18, 25] and intact iodinated TGFβ1 is recoverable from fetuses after injection into the mother [25]. Thus, all fetal tissues appear to be bathed in TGFβ1 from both the mother and from sources within the fetus (see [26] for the locations of fetal TGFβ1 mRNA).

In the inbred line, the amniotic fluid from TGFβ1 mutant fetuses had half the level of TGFβ1 protein of their wild-type equivalents (see Fig. 4B and Table 2). This implies that the amniotic fluid from the wild-type concepti contained very similar amounts of maternal and fetal TGFβ1. This method may, however, underestimate the maternal contribution, as the dams used in this study were TGFβ1−/−,
sufficient to suppress autoimmune attack in TGFβ1−/− pups [14, 33]. Both of these phenomena are, however, in marked contrast with the TGFβ1-dependent development of the yolk sac, which appears to be entirely under the control of fetal TGFβ1 [32, 34]. This is not surprising, as the defect in the yolk sac is lethal before extensive development of the placenta has occurred. The ability of maternal TGFβ1 to reach the conceptus would thus be limited.

The situation with the inbred mice also contrasts with that of the multiparous outbred mothers where fetal TGFβ1 production is an important determinant of survival: wild-type concepti survive; TGFβ1−/− concepti have reduced survival; TGFβ1−/− concepti do not survive, even though their amniotic fluids contain approximately 50% of TGFβ1 of the wild-type littersmates. This suggests that the TGFβ1-dependent suppression of parity-related loss of concepti only occurs if the level of TGFβ1 exceeds a threshold, as in the deficits observed in TGFβ1−/− adults [27].

In summary, the data reported here show that concepti are exposed to significant levels of both fetal and maternal TGFβ1. The levels of fetal TGFβ1 appear to be a determinant of whether a conceptus is rejected or not, although the evidence clearly indicates that maternal TGFβ1 and other factors are also important. In at least some circumstances, these factors may be sufficient to fully compensate for a low level of fetal TGFβ1.

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