Reduction in reproductive lifespan of tissue inhibitor of metalloproteinase 1 (TIMP-1)-deficient female mice

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Tissue inhibitor of metalloproteinase 1 (TIMP-1) is a multifunctional protein expressed in the ovary and uterus of females of several species including mice, rats and humans. Mice deficient in TIMP-1 protein display altered reproductive parameters, which include reduced serum progesterone concentrations and abnormal uterine morphology. As these abnormalities could lead to altered reproductive performance, the objective of this study was to examine whether TIMP-1 deficiency is associated with a reduction in the reproductive lifespan of female mice in which the TIMP-1 gene is disrupted. Wild-type ($n = 50$) and TIMP-1 null mice ($n = 90$) were mated with males of proven fertility from the same genotype for 12 months during which the number of litters delivered and the number of pups per litter were determined. Significantly fewer TIMP-1 null females achieved pregnancy (47 of 90

or 52%) compared with the wild-type mice (39 of 50 or 78%; $P < 0.05$). TIMP-1-deficient female mice that achieved and maintained pregnancy had significantly fewer litters during the 12 months (2.9 ± 0.8 versus 3.5 ± 0.8 ; $P < 0.01$) and significantly fewer pups per litter (5.7 ± 1.3 versus 4.7 ± 1.1 ; $P < 0.05$) than did wild-type mice. Females of both genotypes produced two consecutive litters, after which significantly fewer TIMP-1 null mice became pregnant. Mating of females with males of proven fertility from the other genotype confirmed that these abnormalities were not due to the inability of TIMP-1 null males to produce offspring. These data indicate that the absence of TIMP-1 is associated with a reduction in the reproductive lifespan of female mice, which may be manifested at the ovary, uterus or both organs.

Introduction

Tissue inhibitors of metalloproteinases (TIMPs) are expressed in the uteri of females in a variety of species including humans (Hampton and Salamonsen, 1994; Rodgers *et al.*, 1994; Zhang and Salamonsen, 1997), non-human primates (Brenner *et al.*, 1996), sheep (Hampton *et al.*, 1995), pigs (Menino *et al.*, 1997) and mice (Waterhouse *et al.*, 1993; Das *et al.*, 1997). In both humans and non-human primates (Hampton and Salamonsen, 1994; Rodgers *et al.*, 1994; Brenner *et al.*, 1996; Zhang and Salamonsen, 1997), TIMPs are postulated to control tissue remodelling and shedding of the endometrium during the menstrual period. However, TIMPs are also expressed beyond the period of menses within the uterus of these primate species, as well as within the uterus of females that do not show a period of menses (Waterhouse *et al.*, 1993; Hampton and Salamonsen, 1994; Rodgers *et al.*, 1994; Hampton *et al.*, 1995; Brenner *et al.*, 1996; Das *et al.*, 1997; Menino *et al.*, 1997). As TIMPs are multifunctional proteins (for a review, see Gomez *et al.*, 1997), they could control a variety of cell functions that may play paramount roles in uterine physiology. For example, TIMP-1 has been shown to

regulate cell proliferation (Bertaux *et al.*, 1991; Hayakawa *et al.*, 1992, 1994), programmed cell death (Alexander *et al.*, 1996; Guedez *et al.*, 1998), angiogenesis (Tagikawa *et al.*, 1990; Johnson *et al.*, 1994) and steroidogenesis (Boujard *et al.*, 1995; Nothnick *et al.*, 1997; Nothnick, 2000). Maintenance of a balance between cell proliferation and cell death, as well as uterine angiogenesis, and the role of the ovarian steroids within the uterus for the establishment of a successful pregnancy are essential. As such, TIMP-1 may contribute to the reproductive processes at a variety of cellular or biochemical levels.

Recent studies using TIMP-1-deficient mice have indicated that TIMP may play an important role in the reproductive process. TIMP-1 null mice have altered oestrous cycles, uterine morphology and systemic steroid concentrations (Nothnick, 2000). Alterations to these parameters could lead to reproductive difficulties. The aim of the present study was to determine whether the absence of TIMP-1 affects the ability of female mice to achieve pregnancy. Female mice that were unable to express a functional TIMP-1 gene product (that is, TIMP-1 knockout) were used to evaluate the effect of TIMP-1 deficiency on the long-term reproductive potential in TIMP-1 null female mice. Wild-type and TIMP-1 null mice were mated over 12 months, and the number of litters and pups per litter were calculated for each genotype during this period.

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Materials and Methods

Animals

TIMP-1 null and wild-type mice were used in all studies. TIMP-1-deficient mice (SVter 129 background) were generated by homologous recombination of a *neo*-containing gene-targeting vector in mouse embryonic stem cells. Transmission of the mutant allele and the genotype of mice were determined by PCR analysis of the *neo* sequences in genomic tail DNA. TIMP-1 deficiency was confirmed at the transcription and protein level by northern blot analysis and protease inhibitor assays, respectively (Nothnick *et al.*, 1997).

Mice were housed within environmentally controlled conditions under the supervision of a licensed veterinarian. All animal procedures for these experiments were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee. Mice were maintained on a 14 h light:10 h dark cycle and were provided with water and mice chow *ad libitum*.

Female mice of each genotype ($n = 50$ wild-type and $n = 90$ null mice, aged 7–8 weeks) were used and all mice were fully inbred. Two to four littermates were selected randomly from each of 20 wild-type and 35 null litters. Females were mated with males of proven fertility of the same genotype and were checked twice a week for pregnancy. Pregnant females were removed and housed in separate cages. Females that did not become pregnant within 30 days after initial exposure to the males were excluded from further study and considered infertile. Pregnant females were allowed to nurse their pups for 21 days after which the pups were weaned and used for other experiments not related to this study. Once the pups were weaned, females were mated again with males of proven fertility of the same genotype and the procedure described above was repeated. This mating schedule continued until female mice failed to achieve pregnancy within 30 days of exposure to the males or until five consecutive litters were delivered. A period of approximately 12 months was termed the 'reproductive lifespan' of female mice. The percentage of fertile wild-type and TIMP-1 null mice (those that became pregnant within 30 days of exposure to the male) was calculated, in addition to the average number of litters per lifespan and the number of pups per lifespan. In addition, the number of pups born in each litter and the percentage of females producing pups with each consecutive litter were calculated between genotypes.

A separate study was conducted using 20 wild-type and 20 TIMP-1 null female mice to verify that any changes in reproductive lifespan were due to absence of the TIMP-1 gene product in the females (and not in the males). All of the females had previously delivered two litters and for the third mating, females were mated to males of proven fertility of the other genotype. The number of females that became pregnant and the number of pups born in each litter were then calculated and compared between genotypes of the females. The third mating period was chosen to assess

Fig. 1. The percentage of female mice of each genotype producing pups in each litter. The percentage of female mice delivering pups for each consecutive litter was calculated from the 39 wild-type ($\blacksquare$, $n = 39$) and 47 tissue inhibitor of metalloproteinase 1 (TIMP-1) null mice ($\square$, $n = 47$) that became pregnant and delivered pups after the first mating. Asterisks indicate significant differences within each litter between wild-type and null mice using Fisher's exact test: * $P < 0.05$; ** $P < 0.005$.

reproductive performance of the females on the basis of the outcome of the first experiment, which demonstrated that there was a significant reduction in the percentage of null females achieving pregnancy from the third mating.

Statistical analysis

The percentages of female mice producing pups with each litter were compared within the litter number between genotypes using the Fisher's exact test. The number of pups per litter, the number of litters per lifespan and the number of pups per lifespan were compared using the unpaired *t* tests. A comparison of the number of pups per litter within genotype across the number of litters born was made using one-way ANOVA. When a significant *F* value was obtained, the Student–Newman–Keuls test was used for post-hoc analysis. Significance was set at $P < 0.05$ for all studies and all data are displayed as the mean $\pm$ the standard deviation (SD) from the mean.

Results

Within the first 30 days of exposure to males of proven fertility of the same genotype, significantly more wild-type females became pregnant (39 of 50 or 78%) compared with TIMP-1 null females (47 of 90 or 52%; $P < 0.05$). Wild-type females that became pregnant after the first mating and produced offspring produced up to five litters within 12 months. In particular, 100% of wild-type females produced offspring after the first two matings, but this decreased to 87%, 68% and 40% for the third, fourth and fifth matings, respectively (Fig. 1). Similarly, most TIMP-1-deficient mice produced offspring after the first two matings; however,

Fig. 2. Number of pups born per litter for each genotype. The average number of pups born with each consecutive litter was recorded for wild-type (■) and tissue inhibitor of metalloproteinase 1 (TIMP-1) null (□) mice. Data were obtained from 39 wild-type mice and 47 TIMP-1 null mice and are displayed as mean $\pm$ SD. Different letters indicate significant differences within each genotype across the number of litters (a,b for comparison within wild-type mice and a,b for comparison within TIMP-1 null mice) by one-way ANOVA. Asterisks indicate statistically significant differences within litter between genotypes using unpaired *t* tests: * $P < 0.001$; ** $P < 0.01$.

compared with wild-type mice, significantly fewer TIMP-1 null mice produced offspring after the third, fourth and fifth matings (51%, 21% and 0%, respectively; Fig. 1).

A similar pattern was also observed when the number of pups born per litter was assessed. Both wild-type and TIMP-1 null mice gave birth to a similar number of pups in each of the first two litters (Fig. 2). However, by the third litter, TIMP-1 null mice gave birth to significantly fewer pups (4.5 ± 1.2 versus 6.5 ± 1.2 for wild-type mice; Fig 2.). In addition, significantly fewer pups were born to TIMP-1 null mice in the fourth (3.9 ± 1.0 versus 5.4 ± 1.3 for wild-type mice) and fifth litters (0 ± 0 versus 5.0 ± 0.9 for wild-type mice; Fig. 2). Overall (from the first to fifth mating), TIMP-1 null mice had significantly fewer litters per lifespan (2.9 ± 0.78 versus 3.5 ± 0.76) and significantly fewer pups per lifespan (14.5 ± 4.7 versus 21.5 ± 7.6 ; $P < 0.001$) compared with wild-type mice.

Wild-type and TIMP-1 null female mice were mated with reproductively proven males of the other genotype to verify that the decrease in the reproductive lifespan (that is, the ability to achieve pregnancy and deliver pups) was not influenced by the genotype of the males (Fig. 3). Similar to the initial study in which male and female mice of the same genotype were mated, significantly more ($P < 0.05$) wild-type female mice became pregnant when mated with null males (16 of 20 or 80%) compared with TIMP-1 null females mated with wild-type males (9 of 20 or 45%; Fig. 3a). These wild-type female mice also delivered more pups compared with TIMP-1 null females (5.6 ± 1.0 versus 4.4 ± 1.3 ; Fig. 3b).

Fig. 3. The reduction in the reproductive lifespan of female tissue inhibitor of metalloproteinase 1 (TIMP-1) null mice is independent of the male genotype. Five- to six-month-old wild-type and TIMP-1 null female mice that had delivered two consecutive litters from previous matings with males of proven fertility from the same genotype were mated with males of proven fertility from the other genotype. Females were exposed to males for up to 30 days, after which they were excluded from the study. (a) The percentage of the wild-type and null females that delivered pups. (b) The number of pups born per litter resulting from mating of females and males of opposite genotypes. Different letters indicate significance by Fisher's exact test (a) and unpaired *t* test (b).

Discussion

The present study demonstrates that TIMP-1 null female mice have fewer litters and fewer pups during their reproductive lifespan than do wild-type females. By the third mating between TIMP-1 null females and male mice, there was a significant reduction in the number of TIMP-1 null females that became pregnant. This reduction did not occur in TIMP-1 wild-type mice, indicating that TIMP-1 may either preserve the reproductive potential in these mice or that the absence of TIMP-1 is somehow deleterious to reproduction. Mating female and male mice to the other genotype indicated that the reduction in reproductive lifespan was attributed to the female, as significantly more wild-type females mated to TIMP-1 null males achieved pregnancy compared with null females mated to wild-type males. The processes affected by the absence of TIMP-1 could be at the ovary or uterus. Fewer TIMP-1 null mice achieved pregnancy, and of those that did significantly more failed to become pregnant with successive matings. Sharpe-Timms *et al.* (1998) demonstrated that women with altered fertility, such as women with endometriosis or unexplained infertility (K. Sharpe-Timms, personal communication) have significantly lower peritoneal fluid and serum concentrations of TIMP-1. These findings indicate that, in humans, TIMP-1 may play a role in the reproductive process and regulation of fertility. However, the mechanisms by which this is manifested are unknown.

Assessment of uterine morphology in TIMP-1 null mice of reproductive age indicated that there are marked

alterations in the structure of the uterine lumen, which could influence implantation of the embryo (Nothnick, 2000). This alteration is associated with a significant increase in uterine matrix metalloproteinase (MMP) activity (W. Nothnick, unpublished), indicating that the absence of TIMP-1 leads to an imbalance in MMP activity. Increased expression of MMPs could influence a variety of biological functions within the uterus, which could have a negative effect on the reproductive process. Matrix metalloproteinases can regulate the bioavailability of cytokines (Gearing *et al.*, 1994; Schönbeck *et al.*, 1998) and growth factors (Fowlkes *et al.*, 1994; Manes *et al.*, 1997; Suzuki *et al.*, 1997; Wu *et al.*, 1999), which in turn can influence uterine cell proliferation (Shiraga *et al.*, 1997; Zhang *et al.*, 1998; Adesanya *et al.*, 1999) or cell death (Tanaka *et al.*, 1998; Lea *et al.*, 1999). The observation that the effect of the absence of TIMP-1 on the reproductive lifespan of female mice does not occur until after the second litter indicates that prolonged absence of TIMP-1 (and prolonged increases in MMP activity) is required for these deficiencies to be manifested.

As progesterone is an essential steroid for the reproductive process, alterations in production or action of progesterone could reduce reproductive lifespan. TIMP-1 has been demonstrated to regulate progesterone production *in vitro* (Boujard *et al.*, 1995) and TIMP-1-deficient mice of reproductive age have significantly lower concentrations of serum progesterone during oestrus, a time that coincides with the development of the early corpus luteum (Nothnick, 2000). Furthermore, subsequent studies using immature, gonadotrophin-primed TIMP-1 null mice confirmed the postulate that TIMP-1 null mice have reduced serum concentrations of progesterone during the period of corpus luteum development (W. Nothnick, unpublished). These significantly lower concentrations of progesterone may not be sufficient to allow for, or maintain, pregnancy in TIMP-1 null mice. As in the uterus, these alterations may be due to, and only become apparent after, prolonged absence of TIMP-1 (that is, after the birth of the second consecutive litter). In addition, improper periods or increased concentrations of cytokines resulting from the increased MMP activity could influence ovarian steroidogenesis. Cytokines, such as tumour necrosis factor α (Adashi *et al.*, 1990; Benyo and Pate, 1992; Best *et al.*, 1994) and interleukin 1 β (Best and Hill, 1995; Donesky *et al.*, 1998; Kohen *et al.*, 1999), suppress progesterone production *in vitro* and may influence progesterone production *in vivo* in TIMP-1 null mice.

In summary, the present study demonstrates that female TIMP-1 null mice have a shorter reproductive lifespan than do wild-type mice. This reduction may be due to insufficiencies at the uterus, ovary or both of the organs. Furthermore, insufficiencies may result from prolonged exposure to increased MMP activity, which occurs with the TIMP-1 null mice. Future studies will concentrate on determining the mechanisms and the level at which TIMP-1 influences the reproductive process in females.

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