Effect of inhibition of matrix metalloproteinases on endometrial decidualization and implantation in mated rats

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Implantation of the embryo into the endometrium is a highly regulated event that is critical for establishment of pregnancy. Molecules involved in this process provide potential targets for post-coital contraception. The aims of this study were to determine whether matrix metalloproteinases (MMPs) are present at implantation sites in rats and whether administration of a broad-based inhibitor of MMPs could inhibit embryo implantation. Uterine extracts from non-pregnant rats and from rats on days 3–9 of pregnancy were examined for the presence of MMPs. Doxycycline (5 or 15 mg day⁻¹) was administered by gavage to rats from the day of mating (day 0) to day 7 of pregnancy and the uterus was examined for implantation sites. A number of MMPs were present in all uterine samples. MMP-2 reached a peak on day 3, whereas the highest expression of MMP-7 occurred on day 7. MMP-13 and MMP-3 were present in smaller amounts. MMP-9 was detectable only on day 9. Treatment of rats with doxycycline had no effect on the number of implantation sites or on the total uterine mass. However, in treated rats, the process of decidualization was impaired and both the width and length of the decidual zone was reduced, resulting in a decrease in total decidual area from 1.20 ± 0.07 to 0.91 ± 0.07 mm² (mean ± SEM, controls versus doxycycline treated, P < 0.02). It is concluded that administration of MMP inhibitors during early pregnancy retards decidual development, but does not block implantation.

Introduction

Implantation of the embryo into the endometrium is a highly controlled event that is critical for the establishment of pregnancy. Understanding the molecular basis of this process is therefore important for the regulation of fertility. The failure of embryos to implant is a major limiting factor in the success of reproductive technologies and one or more of the molecules involved in the implantation process could provide a suitable target for post-coital contraception.

In species with haemochorial placentation, including rats, mice and primates, the trophoblast penetrates the uterine epithelium and invades deep into the stroma to make contact with the maternal blood supply. Such aggressive invasion is otherwise observed only with malignant cells. Trophoblast invasion shares many of the features of tumour invasion and metastasis and these include a central role for matrix-degrading proteinases. The principal difference between the two processes is that trophoblast invasion is tightly controlled within the uterus by proteinase inhibitors that are produced locally, whereas tumour invasion progresses without restraint (for review, see Salamonsen, 1999).

There are three classes of proteinases that are postulated to be of importance in trophoblast invasion because of their role in cleavage of extracellular matrix components: cysteine proteinases, serine proteinases and matrix metalloproteinases (MMPs) (Werb, 1989). The MMPs are the most critical enzymes in matrix degradation as their substrate specificities collectively allow them to degrade virtually all the components of both extracellular matrix and basement membranes. The MMP family includes the matrix metalloproteinases, collagenases, gelatinases and stromelysins together with a subgroup of membrane-bound MMPs. The actions of these enzymes are opposed by a family of tissue inhibitors of metalloproteinases (TIMPs). The role of MMPs in tumour invasion and metastasis is well documented (Powell and Matrisian, 1996) and they have also been implicated in the implantation process in a wide range of species (for review, see Salamonsen, 1999).

In addition to a direct role in trophoblast invasion, MMPs are likely to have a central role in the considerable remodelling of the endometrium that occurs during implantation and the early stages of placental development. In rats, stromal differentiation in response to the presence of a blastocyst leads to the formation of decidual tissue and is accompanied by loss of fibrillar collagen. As implantation proceeds to day 8, there is a progressive reduction in the concentration of total collagen at implantation sites compared with non-implantation sites (Myers et al., 1990), presumably due to the action of MMPs. Individual components of extracellular matrix have reduced or very low expression in decidual or decidualizing tissue and these include: fibronectin (Grinnell et al., 1982; Glasser et al., 1987); collagen

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type 1 (Clark et al., 1993), type VI (Mulholland et al., 1992), and types III and V (Hurst et al., 1997); and tenasin (Michie and Head, 1974). The initial clearance of these components from the stromal compartment would also require MMPs.

The long-term aim is to identify molecules that are critical for implantation and can be targeted for contraceptive purposes (Nie et al., 1997). Given the importance of MMPs for invasive and remodelling processes, the objectives of the present study were to establish whether MMPs and their inhibitor TIMP-3 are produced during early pregnancy in rats and whether oral administration of a broad-based inhibitor of MMPs during very early pregnancy could inhibit or limit the implantation process. Such studies provide fundamental information regarding the importance of these enzymes in implantation, which could be applied in a primate model.

Materials and Methods

Animals

Parous 70–80 day-old female Sprague–Dawley rats (body weight 230 ± 23 g, mean ± SD) were mated with fertile males and examined for vaginal plugs. The day of mating was designated as day 0. For studies across early pregnancy, animals (n = 3–5 per group) were killed by CO₂ asphyxiation and decapitation on the morning of days 3, 5, 7 and 9 after mating or were un-mated (non-pregnant control). The uterus was excised, trimmed and where appropriate (days 5, 7 and 9) implantation sites were separated. The tissue was snap-frozen in liquid N₂ and stored at −80°C. For treatment with an inhibitor of MMPs, rats were randomly assigned into vehicle and doxycycline treatment groups (n = 7 rats per group). Animals were weighed at the start and completion of the treatment. Doxycycline hydrochloride (Sigma Chemical Co., St Louis, MO), which is a broad-based inhibitor of MMPs (Golub et al., 1991) was prepared at 5 mg ml⁻¹ and 1 ml was administered to rats by gavage once a day from day 0 to day 6. On the morning of day 7, the rats were killed, the uteri were collected and weighed, and the number of implantation sites was counted. Some implantation sites were fixed overnight in phosphate-buffered formalin, washed thoroughly with PBS (pH 7.4) and embedded in wax. Other implantation sites were snap-frozen in liquid N₂. Control rats were similarly treated but water (vehicle) was administered by gavage. In a second experiment, the doxycycline was prepared in acidified 2% (w/v) carboxymethyl cellulose, as it was possible that this vehicle would allow a different pharmacokinetic profile of rate of distribution (Golub et al., 1991). This doxycycline was administered to five rats at a dose of 15 mg ml⁻¹ day⁻¹. Otherwise, the protocol was exactly the same as for the initial experiment. All animal experimentation was approved by the Institutional Animal Ethics Committee at the Monash Medical Centre.

Extraction of MMPs

MMPs were extracted from samples of rat uterus as described for ovary (Curry et al., 1992). Briefly, weighed tissue was homogenized on ice in 0.01 mol CaCl₂, I⁻ with 0.25% Triton-X100 (1:20, w/v) and centrifuged at 9000 g for 30 min, and the supernatant (Triton extract) was frozen. The pellet was resuspended in 10 volumes of high calcium Tris buffer (0.1 mol CaCl₂, I⁺, 0.05 mol Tris 1⁺, 0.15 mol NaCl 1⁺, pH 7.5), heated to 60°C for 6 min, re-centrifuged and the supernatant (heat extract) was frozen. This second extraction dissociates any metalloproteinases bound to substrate. Extracts were subjected to gelatin and casein gel zymography for analysis of MMP content.

Zymography

Proteinase activity in both Triton and heat extracts was analysed by zymography on 10% (w/v) sodium dodecyl sulfate (SDS)-polyacrylamide gels containing 1 mg gelatin ml⁻¹ (all reagents from BioRad, North Ryde) or 1 mg casein ml⁻¹ (Sigma) under non-reducing conditions (Rawdanowicz et al., 1994; Salamonsen et al., 1997). Loading of samples was normalized such that a volume representing the same wet weight of tissue was applied to each lane. Gelatinase or caseinase activity was visualized by negative staining and bands were identified by comparison with known human MMPs. Standard preparations of pure human MMP-1 and -3 were used (H. Nagase, University of Kansas, Kansas City, KS, USA). Culture medium from baby hamster kidney (BHK) cells that had been stably transfected with human MMP-9 (D. Edwards, University of East Anglia, Norwich, UK) was used for MMP-2 and MMP-9 standards. These standards have all been described (Rawdanowicz et al., 1994; Salamonsen et al., 1997). MMP identity of all bands was confirmed by incubation of parallel gels in the presence of EDTA (5 mmol l⁻¹). Relative activities of gelatinase bands were semi-quantitated by densitometric analysis of zymograms (Kleiner and Stetler-Stevenson, 1994) using the Hewlett-Packard Scanjet II with Deskscan software (Hewlett-Packard, Palo Alto, CA) set on a black and white photograph with 256 grey shades. The area of the bands was analysed using the NIH Image Version 1.54 equipped with gel plotting macros by measuring the area beneath the peaks plotted through the lane profile for the appropriate enzyme. Comparisons were made only among samples run on the same gel. Previous analyses of doubling dilutions of samples on each type of zymogram verified the semi-quantitative nature of these analyses, whereas analysis of the same samples for MMP-1 by casein zymography and ELISA substantiated changes in MMP-1 measured by this method (Salamonsen et al., 1997).

Immunohistochemistry

Formalin-fixed implantation sites were investigated by immunohistochemistry using primary rabbit antisera specific for human TIMP-3 (Triple Point Biologics, Forest Grove, OR) and mouse MMP-9 (L. Moons, Centre for Transplantation Technology and Gene Therapy, Leuven, Belgium). Briefly, the wax was removed from 5 μm sections and non-specific binding sites were blocked with 10% normal goat serum in Tris-buffered saline (pH 7.6, NGS–TBS)
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for 20 min and with primary antiserum diluted in the same buffer at 1:300 and 1:100. For negative controls, rabbit IgG, diluted to the same final protein concentration in each case, was substituted for the primary antiserum. Slides were incubated for 60 min at room temperature and washed successively with TBS, TBS with 0.6% (v/v) Tween 20, and TBS. Biotinylated goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA) diluted at 1:200 in 10% fetal calf serum in TBS was applied for 30 min at room temperature, the slides were washed as described earlier and treated with components of the strepABC-alkaline phosphate system (Vector) according to the manufacturer’s instructions. Sections were counterstained with Harris haematoxylin (1:10), dehydrated and mounted. Microscopy was performed using an Olympus BH2 microscope and photographed using an Olympus camera with ND and LBD2N filters.

Western blot analysis

Equal volumes of extracts from two uteri or implantation sites from each of the non-pregnant and day 3, 5, 7 and 9 pregnant rats were pooled. Each sample (10 μl) together with molecular mass standards (kaleidoscope prestained standards; BioRad, Regent’s Park, NSW) was subjected to SDS-PAGE on a 12% gel under reducing conditions and the proteins were transferred to Hybond-P™ membranes (Amersham Life Science, Sydney). After blocking non-specific sites with 10% (w/v) skim milk powder in TBS with 0.6% (v/v) Tween 20 for 1 h at room temperature, blots were incubated overnight at 4°C with a 1:1000 dilution of rabbit antiserum to rat MMP-7 (E. Woessner, University of Miami, FL, USA) in 5% (w/v) skim milk powder in TBS with 0.3% (v/v) Tween 20. The blot was incubated with a 1:3000 dilution of donkey anti-rabbit IgG conjugated to horseradish peroxidase (Amersham) and the ECL Plus Kit (Amersham). Blots were exposed to Kodak XOMAT AR film for 30 s to 5 min, and developed.

Staining and morphometric assessment of implantation sites

Formalin-fixed implantation sites were sectioned at 5 μm and displayed sequentially on slides. The mid-line of each implantation site was determined and six sequential cross-sections at this point were stained with haematoxylin and eosin. Measurements were made using an Olympus BX-50 microscope with an OC-M micrometer reticle. Length and width of the implantation site, length and width of the endometrium and length and width of the decidual zone were measured in ten and 12 individual implantation sites from seven control and seven doxycycline-treated rats, respectively. Analysis was performed after the image was captured with a Pulinen TMC-6 video camera coupled to a Pentium PC computer using a Screen Machine II FAST multimedia video adaptor (FAST Multimedia AG, Munich) to determine the number of cells per unit area in the decidual zone. A software package (Olympus DK CASTGRID V1.0) was used to generate a counting frame (14565 μm²) directly on to the video screen. Fields to be counted were selected using a systematic uniform sampling scheme generated by the CASTGRID computer program with the aid of a motorized stage (Multicontrol 2000, ITK, Ahornweg). The number of cells in at least seven and up to 13 squares (to a total of at least 250 cells) was counted for each section and the number of cells per ten squares was calculated. Cell counting was performed in a single implantation site from each of seven animals in both treatment and control groups.

Statistical analyses

Statistical significance of the difference between control and doxycycline-treated animals was analysed using the Student’s t test. Differences among days of pregnancy were analysed by ANOVA and Tukey’s post hoc multiple comparison test (least significant difference procedure). Differences were considered significant at P < 0.05.

Results

MMPs in the rat uterus during early pregnancy

Both Triton and heat extracts of tissue were tested initially on gelatin and casein gels. No differences were observed in bands of activity between the two extracts and therefore the heat extracts were used for analyses. On gelatin gels (Fig. 1a),
the major bands were at approximately 72 and 67 kDa and were similar to the bands for proMMP-2 and active MMP-2, respectively, in the standard. However, in the extracts from the rat uterus, the 72 kDa band appeared as a doublet and the 67 kDa band was stronger than that representing latent enzyme. These bands were present in all samples from non-pregnant uterus and in uterine samples from days 3, 5, 7, and 9 of pregnancy. An additional band that was similar to the MMP-9 standard was detectable only on day 9 and probably represents latent rat MMP-9. Densitometric analysis of total MMP-2 present in duplicate samples run on a single gel demonstrated increased concentrations of MMP-2 on day 3 of pregnancy, which decreased to a value lower than that measured in non-pregnant animals by days 7–9 of pregnancy (Fig. 1b). These differences were the same when latent and active bands were analysed separately (not shown). No differences were detectable among samples from animals on day 7 of pregnancy, either in untreated or doxycycline-treated groups (5 mg day⁻¹; each n = 6; Fig. 1a). The densitometric results were 100 ± 2.1% and 102 ± 4.4%, respectively, for latent MMP-2, and 100 ± 8.6% and 101 ± 2.7%, respectively, for active MMP-2. No bands of gelatinase activity were observed when the gels were incubated in the presence of EDTA, confirming that the activities were due to MMP action.

When samples from implantation sites on day 7, from either control animals or those treated with doxycycline, were subjected to zymography on casein gels, a number of faint bands of caseinolytic activity were observed (Fig. 2b). These were similar to the standards for human proMMP-1 (approximately 51 kDa), active human MMP-3 (49 and 45 kDa) and active MMP-1 (42 kDa). No activity coincident with the latent form of human MMP-3 (57 kDa) was apparent. Rat collagenase has calculated molecular masses of 51.3 and 42.2 kDa (Quinn et al., 1990), similar to those for human collagenase. A major band of a much lower molecular mass was also apparent on the casein gels. No differences could be detected between the control and treatment groups. This observation would be expected if doxycycline acts by binding to and at the active site of the enzyme; such binding would be unlikely to withstand the electrophoretic conditions of zymography. All bands could be inhibited with EDTA. Similar gels used to analyse extracts from across early pregnancy had bands that were too faint for analysis or reproduction.

Western blot analysis for MMP-7 showed a number of bands of approximately 60, 48, 28, and 18 kDa, presumably representing latent and active forms of the enzyme along with dimers of these enzymes (Fig. 3). Such bands have been identified in extracts of rat uterus (F. Woessner, personal communication). A marked increase in total MMP-7 was apparent in implantation sites on days 7 and 9 of pregnancy compared with non-pregnant uterus and days 3 and 5 of pregnancy.

MMP-9 was not stained strongly in any of the seven separate implantation sites examined and specifically was not found in the invading trophoblast cells at this stage, although very faint staining was observed in the decidua (Fig. 4a). However, cells of haematopoietic origin within the tissue stained positive (Fig. 4c), confirming the efficacy of the antiserum for rat MMP-9. Immunohistochemical detection of TIMP-3 in implantation sites on day 7 of pregnancy showed strong staining in the decidual cells surrounding the implantation site, but not in the endometrium (Fig. 4d,e). Staining was also present in the circular and longitudinal smooth muscle and was particularly strong in the vascular smooth muscle surrounding the blood vessels.

**Effect of doxycycline treatment on implantation**

Doxycycline, a broad-based inhibitor of MMP action, was administered to rats to establish the importance of MMPs in
Aims.

Oral and intravenous treatment with either 5 or 15 mg doxycycline day⁻¹, from the day of mating until day 7 of pregnancy, had no significant effect on body weight of the animals during this time. Similarly, doxycycline did not affect the mass of the uterus or the total number of implantation sites compared with rats treated with vehicle (Table 1).

Effect of doxycycline treatment on decidualization

Given that there were no differences between animals in the first and second experiments (5 or 15 mg doxycycline day⁻¹ in different carriers), tissues from both experiments were included in this analysis. Morphometric examination of cross-sections of uterus from day 7 that were stained and cut through the mid-plane of the implantation sites, revealed that there were no significant differences in the mean length or width of the implantation site, the width of the decidual zone or the total width and length of the endometrium.

However, the decidual zone (mesometrial–antimesometrial) was shorter in length (P < 0.05), and there was a significant reduction in length (P < 0.05), width (P < 0.05) and total area (P < 0.02) of the decidual zone when expressed as a percentage of the equivalent measurement for total endometrium in each individual animal (Table 2, Fig. 5).

Within the implantation sites, given areas were sampled randomly and the number of cells was counted to establish any effect of doxycycline treatment on the number of cells per unit area of decidua. There were 2533 ± 174 cells mm⁻² in control animals (mean ± SEM, n = 7) and 2428 ± 192 cells mm⁻² in doxycycline-treated animals (n = 7; no significant difference, P > 0.05). Therefore, although inhibition of MMPs decreased the size of the decidual zone, this treatment did not affect the number of cells per unit area of this zone.

Discussion

The results of this study demonstrate that a number of MMPs,
both latent and active, are present in the non-pregnant uterus and in implantation sites in rats. Furthermore, administration of doxycycline, an inhibitor of MMPs, from the time of mating to day 7 of pregnancy, affects the extent of decidualization of the stroma that occurs in response to an implanting embryo. The role of MMPs in decidualization remains to be elucidated. It is possible that when degradation of matrix outside the decidual zone is impaired, decidualization is inhibited. Alternatively, MMPs may affect this process by proteolytic activation or degradation of precursor effector molecules which themselves contribute to decidualization. For example, MMPs degrade insulin-like growth factor binding protein 3 (Fowlkes et al., 1995) and interleukin 1β (Ito et al., 1996), and MMP-7 processes molecules such as TNFα precursor and urokinase-type plasminogen activator (Wilson and Matrisian, 1996). Doxycycline may also directly inhibit the actions of proteinases that are closely related to MMPs, such as the

Table 1. Uterine mass, number of implantation sites and increase in body weight in control and doxycycline-treated rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Increase in body weight (%)</th>
<th>Uterine mass (mg)</th>
<th>Number of implantation sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.8 ± 1.0</td>
<td>651.4 ± 24.8</td>
<td>13 ± 0.5</td>
</tr>
<tr>
<td>Doxycycline (5 mg day⁻¹)</td>
<td>8.7 ± 2.0</td>
<td>704.3 ± 50.5</td>
<td>13 ± 0.9</td>
</tr>
<tr>
<td>Doxycycline (15 mg day⁻¹)</td>
<td>5.9 ± 1.0</td>
<td>726.0 ± 61.1</td>
<td>14 ± 1.0</td>
</tr>
</tbody>
</table>

Values represent the mean ± SEM from seven or five animals per group.

Table 2. Analysis of decidual cross-sectional area compared with endometrial cross-sectional area in control and doxycycline-treated rats

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Control</th>
<th>Doxycycline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Implantation site length (mm)</td>
<td>0.625 ± 0.172</td>
<td>0.467 ± 0.080</td>
</tr>
<tr>
<td>Implantation site width (mm)</td>
<td>0.102 ± 0.001</td>
<td>0.099 ± 0.005</td>
</tr>
<tr>
<td>Decidual width (mm)</td>
<td>1.498 ± 0.047</td>
<td>1.413 ± 0.100</td>
</tr>
<tr>
<td>Decidual length (mm)</td>
<td>1.989 ± 0.042</td>
<td>1.771 ± 0.022*</td>
</tr>
<tr>
<td>Endometrial width (mm)</td>
<td>2.182 ± 0.084</td>
<td>2.296 ± 0.109</td>
</tr>
<tr>
<td>Endometrial length (mm)</td>
<td>3.140 ± 0.057</td>
<td>3.027 ± 0.124</td>
</tr>
<tr>
<td>Decidua as percentage of total width (%)</td>
<td>1.715 ± 0.020</td>
<td>1.540 ± 0.036*</td>
</tr>
<tr>
<td>Decidua as percentage of total length (%)</td>
<td>1.592 ± 0.070</td>
<td>1.465 ± 0.078*</td>
</tr>
<tr>
<td>Decidual area as percentage of total endometrial area (%)</td>
<td>1.096 ± 0.067</td>
<td>0.906 ± 0.067**</td>
</tr>
</tbody>
</table>

Values represent the mean ± SEM from randomly selected implantation sites (10 and 12, respectively) from seven animals in each treatment group (control and doxycycline, randomly selected from Expts 1 and 2). For each implantation site, average measurements were taken from six cross-sections through the centre of the implantation site.

Fig. 5. Comparison of cross-sections through implantation sites from (a) control and (b) doxycycline-treated rats on day 7 of pregnancy. Scale bars represent 100 μm.
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adaminys, TNFα-converting enzyme, which is inhibited by hydroxamate-based MMP inhibitors (Black et al., 1997).

The MMPs detected within the rat uterus, particularly at implantation sites, include MMP-2, MMP-7, rat collagenase (MMP-13) and MMP-3. The presence of MMPs in non-pregnant endometrium is not unexpected, as this tissue is constantly remodelling. MMPs are involved in the cyclical changes that occur in the human endometrium, in which remodelling is more marked but occurs over a longer period (for review, see Salamonsen and Woolley, 1996). Active remodelling occurs at implantation sites in all mammals and studies in mice have demonstrated that MMPs, particularly collagenase and stromelysin-1, appear to be involved in tissue remodelling in and around the implantation site (Alexander et al., 1996).

An unexpected finding in the present study was the failure to detect strong focal staining of MMP-9 by immunohistochemistry at implantation sites on day 7, although gelatin zymography detected a faint band of MMP-9 in extracts on day 9 and there was faint immunostaining in the decidual zone. In mice, both mRNA and protein for MMP-9 have been localized at implantation sites, and specifically in giant trophoblast cells, from day 5.5 to day 8.5 of pregnancy (Reponen et al., 1995; Alexander et al., 1996; Das et al., 1997). It is possible that MMP-9 would also be detected in giant trophoblast cells in rats after day 7. In humans, cytotrophoblast obtained during the first trimester of pregnancy, during which time trophoblast invasion is greatest, also expresses MMP-9 (Fisher et al., 1989; Bischof et al., 1991). The efficacy of the antibody used was confirmed by strong positive staining of MMP-9 in leukocytes within blood vessels in the tissues examined.

MMP-7 (matrilysin) was first identified and subsequently characterized in the involuting rat uterus (Woessner and Taplin, 1988). MMP-7 is localized to epithelial cells in the endometrium, particularly those of the lumen although some mRNA is also found in the glands (Wilson and Matrisian, 1996), and its mRNA cannot be detected by in situ hybridization of embryos at various stages (Wilson et al., 1995). This is the first study to demonstrate the presence of MMP-7 during implantation. The increased MMP-7 in implantation sites on days 7 and 9, when much of the epithelium has degenerated, indicates there may be an additional cellular source, possibly the decidual cells as these represent a transition from a mesenchymal to an epithelial phenotype (Bell, 1985). In the present study, attempts to immunolocalize the enzyme in the tissues were not successful. However, MMP-7 was detected in leukocytes in blood vessels confirming the efficacy of the technique.

TIMP-3 has been postulated as the inhibitor that is responsible for limiting the extent of trophoblast invasion in mice (Harvey et al., 1995; Alexander et al., 1996; Leco et al., 1996). In the present study, TIMP-3 was present in decidual cells surrounding the conceptus, within the implantation sites on day 7 and also in the myometrium and vascular smooth muscle. Thus in this respect, there is accord between mice and rats. TIMPs, including TIMP-3, are expressed strongly in human endometrium throughout the menstrual cycle (Zhang and Salamonsen, 1997) and all three TIMPs examined (TIMP-1, -2 and -3) were expressed maximally in decidualized stroma. It appears that these inhibitors play an important role in maintaining the integrity of endometrial tissue and of the blood vessels. Given that the TIMPs bind and inhibit MMPs with a 1:1 stoichiometry, tissue degradation will only occur at those focal points in which very high concentrations of MMPs are expressed. Expression of TIMPs in the decidual cells supports the observation that the maternal decidua has an unusual ability to limit the invasion of embryonic trophoblast (Abrahamsohn and Zorn, 1993).

Initially, the lack of effect of an MMP inhibitor on embryo implantation per se was unexpected. However, during this study, a similar lack of failure of embryo implantation after targeted disruption of the MMP-9 gene was described (Rinkenberger et al., 1997). Similarly, administration of the hydroxamate MMP inhibitor M1 to mice and overexpression of the gene for TIMP-1 (Alexander et al., 1996; Rinkenberger et al., 1997) did not decrease the number of live births. However, the extent of decidualization at implantation sites was limited and this resulted in misorientation of embryos. Inhibition with a synthetic inhibitor of the cathepsins, cysteine proteinases that have also been localized at implantation sites, had very similar effects on the decidual reaction in mice to those observed with MMP inhibitors (Alfonso et al., 1997). It is possible that interactions among multiple families of enzymes and inhibitors are necessary for successful implantation but that any one family is not critical.

Doxycycline has been used in a number of studies as an inhibitor of MMP action, but the specific mechanisms by which its effects are exerted are still unclear. Tetracyclines have direct inhibitory actions on a wide range of MMP activities, possibly acting through mechanisms that affect the active site zinc, which is essential for enzyme activity (Golub et al., 1983; Greenwald et al., 1994). However, no effects of doxycycline were seen on enzyme activity in zymograms, indicating that binding of the drug is probably interrupted by the electrophoretic conditions used (Petrinec et al., 1996), a proposal that is supported by the present study. Although doxycycline inhibits the activity of MMP-1,-2,-3 and -9 in vitro, there is evidence that it may affect the cellular production of MMP-9 but not MMP-2 in vivo (Petrinec et al., 1996). However, MMP-2 mRNA expression is decreased in skin keratinocytes cultured in the presence of the drug (Uitto et al., 1994). The present study did not aim to investigate the mechanisms of action of doxycycline, but to determine whether this drug could inhibit implantation; this was not the case. The observed effects of doxycycline on decidual development were unexpected and could be accounted for by doxycycline inhibiting the production or activity of some MMPs, or by reduction of other proteases or other proteins necessary for decidual growth. These mechanisms warrant further investigation.

Data localizing MMPs or their inhibitors at early implantation sites in primates is not yet available, although information from in vitro studies indicates there will be similarities with rats. Human trophoblast clearly has a high degree of invasive capacity as shown by the number of ectopic pregnancies in women. One likely difference between primates and rodents could be in the involvement of the gelatinase MMP-2 which, in addition to MMP-9, has been localized in many cells, including human trophoblast.
cells within implantation sites during the first trimester (Fisher et al., 1989; Bischof et al., 1991; Fernandez et al., 1992). Studies with marmoset trophoblastic tissue derived from blastocysts at implantation showed expression of MMP-2 but not MMP-9 in vitro (Franek et al., 1999). Whether MMPs have a role in the spontaneous decidualization that occurs in humans even in the absence of a conceptus remains to be established.

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