

# Expression of matrix metalloproteinases 2 and 9 in the mouse uterus during implantation and oil-induced decidualization

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During implantation, matrix metalloproteinases are believed to play roles in the tissue remodelling that accompanies decidualization in the endometrium and in embryo invasion. The objective of this study was to characterize further the expression of matrix metalloproteinases 2 and 9 in the mouse uterus during early pregnancy and oil-induced decidualization. mRNA encoding matrix metalloproteinase 2 was detected in pregnant uteri and uteri undergoing oil-induced decidualization by northern blot analyses. The steady-state concentrations of mRNA encoding matrix metalloproteinase 2 did not change significantly in implantation compared with inter-implantation areas on days 5–8 of pregnancy but were significantly lower in stimulated compared with non-stimulated uterine horns during artificially induced decidualization. mRNA encoding matrix metalloproteinase 9 was also detected in uteri undergoing oil-induced decidualization but not in pregnant uteri. Its concentration was significantly greater in uterine horns undergoing oil-induced decidualization compared with control horns. Immunoreactive matrix metalloproteinases 2 and 9 were detected in the uterus during early pregnancy and oil-induced decidualization by immunohistochemistry, localized to the endometrial stroma, but the staining progressively became weaker and was absent in areas that had undergone decidualization. By day 8 of pregnancy and 72 h after the induction of decidualization, matrix metalloproteinase 2 and 9 proteins remained mainly in the region of non-decidualized stromal cells adjacent to the myometrium. In implantation segments, they were also localized to the region of the trophoblast giant cells. The second objective of the present study was to determine whether endometrial stromal cells isolated from uteri sensitized for decidualization express matrix metalloproteinases 2 and 9. Northern blot analyses and gelatin zymography showed that these cultured cells expressed matrix metalloproteinase 2 and 9, and that transforming growth factor  $\beta$ 1 significantly increased matrix metalloproteinase 9 expression. The results of the present study further characterize matrix metalloproteinases 2 and 9 expression in the uterus during implantation and artificially induced decidualization.

## Introduction

Extracellular matrix (ECM) degradation occurs in the uterus during decidualization and blastocyst invasion (Graham and Lala, 1992; Abrahamson and Zorn, 1993). Decidualization involves the differentiation of the fibroblast endometrial stromal cells into large polyploid decidual cells. During this process, there is a transformation of the ECM from an interstitial-type (containing types I, III, V, VI collagens and fibronectin) to a basement membrane-type (containing components such as laminin, enactin and type IV collagen) (Wewer *et al.*, 1986; Glasser *et al.*, 1987; Glasser, 1990; Mulholland *et al.*, 1992). Blastocyst invasion of the endo-

metrium in rodents also involves ECM degradation, as the embryo invades the endometrial stroma after displacement of the uterine epithelium (Weitlauf, 1988). Since ECM degradation involves proteinases, many studies have been carried out to describe the expression of specific proteinase and proteinase inhibitor genes in the rodent endometrium during implantation. Examples of such studies in the uteri of rodents include matrix metalloproteinases (MMPs) and their inhibitors (Nuttall and Kennedy, 1999; Waterhouse *et al.*, 1993; Harvey *et al.*, 1995; Reponen *et al.*, 1995; Alexander *et al.*, 1996; Leco *et al.*, 1996; Das *et al.*, 1997), kallikriens (Chan *et al.*, 1999), cathepsins (Afonso *et al.*, 1997), plasminogen activator and their inhibitors (Wang *et al.*, 1996) and neuropsin (Chen *et al.*, 1998).

The MMPs are a family of genes (Massova *et al.*, 1998; Nagase and Woessner, 1999) that may play a role in ECM degradation during blastocyst invasion and tissue remodelling during

Received 15 December 1999.

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decidualization. Previous studies show that matrix metalloproteinases 2 (MMP-2) and 9 (MMP-9) are expressed in the endometrium during implantation as determined by *in situ* hybridization, Northern blot analysis, gelatin zymography and immunohistochemistry (Waterhouse *et al.*, 1993; Alexander *et al.*, 1996; Leco *et al.*, 1996; Das *et al.*, 1997). Since these studies dealt mainly with characterization of MMP-2 and -9 expression in segments of the uterus in which the embryo is implanting, one objective of the present study was to evaluate the differences in concentrations of mRNA MMP-2 and -9 and protein localization between implantation and inter-implantation areas of the uterus on days 5–8 of pregnancy. In addition, MMP-2 and -9 mRNA concentrations and protein localization were examined in non-stimulated and stimulated uterine horns during oil-induced decidualization to determine their expression in the uterus during decidualization but in the absence of an embryo. The results showed that maternal expression of MMP-9 increases in the uterus during decidualization. Therefore, it was possible to determine whether cultured mouse endometrial stromal cells from uteri sensitized for decidualization express MMP-9 and whether MMP expression by these cells is modulated by various cytokines expressed in the endometrium during implantation and decidualization.

## Materials and Methods

### *Animals and tissue collection*

All procedures involving animals were carried out in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the University of Calgary Animal Care Committee. CD1 mice (6–8 weeks old, 22–25 g) were obtained from Charles River Breeding Laboratories (Lachine, Quebec) and were housed under temperature- and light-controlled conditions (lights on from 07:00 to 19:00 h) with free access to food and water.

Females were placed with fertile males and the day a vaginal plug was observed was considered day 1 of pregnancy. Mice were killed by cervical dislocation and their uteri were dissected at 10:00 h on days 4–8 of pregnancy. On days 5–8 of pregnancy, segments (endometrium and myometrium) of the uterus containing implanting embryos (implantation segments) were dissected from those segments that did not contain embryos (inter-implantation areas). Evan's blue dye (0.1 ml; Sigma, St Louis, MO) solution (2% (w/v) in 0.9% (w/v) sodium chloride) was injected intravenously (tail vein) 5 min before mice were killed to identify implantation segments of the uterus on day 5.

Mice were ovariectomized under methoxyflurane (Metaflane<sup>tm</sup>) anaesthesia (MTC Pharmaceuticals, Cambridge, Ontario) and allowed at least 6 days to recover to obtain uteri sensitized for a decidualogenic stimulus. Oestradiol or progesterone, or both, in 0.1 ml sesame oil (Sigma) was then administered s.c. at 09:00 h over 8 days, essentially as described by Milligan and Mirembé (1985). Briefly, the animals received 100 ng oestradiol on days 1–3, then 1 mg progesterone plus 10 ng oestradiol on days 6–8. On the morning of day 8, when the uteri are optimally sensitized for

a decidualogenic stimulus, the mice were used for oil-induced decidualization or the uteri were dissected for endometrial stromal cell isolation. Fifteen microlitres of sesame oil was injected into the lumen of one uterine horn (stimulated horn) between 11:00 and 13:00 h on day 8 to obtain oil-induced decidualization. The other (non-stimulated) uterine horn was not injected with oil and served as a control. From day 9 onwards, the mice were injected with 1 mg progesterone. At 24, 48 and 72 h after the unilateral intraluminal oil injection, the mice were killed and the uterine horns were removed by dissection.

### *Immunohistochemistry*

Day 4 pregnant uterine horns plus non-implantation and implantation segments from days 5–8 pregnant uteri were placed in fixative (40 g paraformaldehyde l<sup>-1</sup> buffered in 0.01 mol phosphate buffer l<sup>-1</sup>, pH 7.2). Uterine horns were dissected into segments of approximately 0.5 cm and placed in fixative for use in oil-induced decidualization experiments. After 16 h of gentle agitation, the fixed tissues were rinsed and then placed in 70% ethanol. The next day, the fixed tissues were processed and blocked in paraffin wax using standard procedures.

After deparaffinization in xylene and rehydration, the 5–10 µm sections were incubated in blocking solution (PBS containing 5% normal donkey serum) for 1 h to prevent non-specific binding, and incubated for 1 h with affinity purified anti-MMP-2 or MMP-9 IgG (1 mg l<sup>-1</sup>; Santa Cruz Biotechnology, Santa Cruz, CA) in blocking solution. After three 10 min washes in PBS, the sections were incubated with biotinylated donkey anti-goat IgG (1.8 mg l<sup>-1</sup>, Zymed Laboratories, Mississauga, Canada) in blocking solution. After three 5 min washes with PBS, the sections were incubated with 1 mg peroxidase–streptavidin conjugate l<sup>-1</sup> (Zymed Laboratories) for 45 min. After four 5 min washes in PBS and one wash in water, the sections were incubated in substrate (3-amino-9-ethyl-carbazole) solution (AEC Substrate Kit, Zymed Laboratories) for 5–10 min. Positive staining was a red deposit and its specificity was verified by the absence of staining after using anti-MMP IgG pre-adsorbed with immunogen (Santa Cruz Biotechnology) or using goat IgG (1 mg l<sup>-1</sup>, Sigma Chemical Company) in place of the anti-MMP IgG (data not shown). Sections were lightly counterstained with haematoxylin (Fischer Scientific Company, Fair Lawn, NJ).

### *Reverse transcription–polymerase chain reaction (RT–PCR)*

The embryo, ectoplacental cone, decidua and uterine sheath (entire uterus minus embryo, ectoplacental cone and decidua) were dissected from day 8 pregnant uteri. RNA isolated from these tissues was subjected to reverse transcription–polymerase chain reactions (RT–PCR) as described by Harvey *et al.* (1995). The primers used were: 5'-CACCTACACCAAGAAGACTTCC-3' plus 5'-AACACAGCCTTCTCCTCTG-3' for MMP-2 and 5'-TTGAGTCCGGCAGACAATCC-3' plus 5'-CCTTATCCACGCGAATGACG-3'

for MMP-9. The expected sizes of the amplicons for MMP-2 and -9 were 332 bp and 433 bp, respectively. The sequences of the amplicons were confirmed by DNA sequencing (data not shown).

#### Cell isolation and culture

Uterine horns from uteri optimally sensitized for a deciduogenic stimulus were dissected and split longitudinally. They were then placed in Hank's balanced salt solution (HBSS; containing 100 U penicillin l<sup>-1</sup>, 0.1 g streptomycin l<sup>-1</sup>, 1.25 mg fungizone l<sup>-1</sup>) (all from Gibco BRL, Burlington, Ontario) containing 6 g l<sup>-1</sup> dispase II (Boehringer Mannheim, Laval, Quebec) and 25 g pancreatin l<sup>-1</sup> (Gibco) for 1 h at 4°C, 1 h at room temperature, and then 10 min at 37°C. The tubes were gently mixed to remove the endometrial epithelial cells, and the supernatant was discarded. The partially digested uterine horns were then washed twice in HBSS and placed in HBSS containing 0.5 g collagenase l<sup>-1</sup> (Sigma). After incubation for 45 min at 37°C, the tubes were mixed for 10–12 s until the supernatant became turbid with dispersed endometrial stromal cells. The supernatant was removed, placed in a sterile tube, centrifuged at 450 g for 10 min at room temperature and the pellet of cells was rinsed once with HBSS.

Cells (pooled from 10–25 uteri) were suspended in Dulbecco's Modified Eagle's Medium-F12 medium (DMEM-F12; with 100 U penicillin l<sup>-1</sup>, 0.1 g streptomycin l<sup>-1</sup> and 1.25 mg fungizone l<sup>-1</sup>) containing 10% heat-inactivated charcoal-stripped fetal calf serum (Gibco). The cell suspension was filtered through nylon mesh (70 µm) to remove glands and clumps of epithelial cells and plated at 5 × 10<sup>5</sup> cells (in 0.5 ml culture medium) in 24-well plates (Becton-Dickson, Lincoln Park, NJ). After an initial incubation period of 2 h at 37°C under 5% CO<sub>2</sub>:95% (v/v) air to allow differential attachment of the stromal cells, the medium and free-floating cells were removed and placed in serum-free DMEM-F12. These cell cultures were free of epithelial cells, as indicated by absence of positive immunocytochemical staining for cytokeratin (data not shown).

After 24 h in serum-free medium, the cells were cultured with or without cytokine in serum-free DMEM-F12. Human recombinant TGFβ1 (Gibco), epidermal growth factor (EGF) (Gibco), interleukin 1α (IL-1α) (kindly provided by the National Cancer Institute, Frederick, MD) and mouse recombinant leukocyte inhibitory factor (LIF) (Cedarlane Laboratories, Hornby, Ontario) were used at final concentrations of 5, 40, 10 and 5 ng ml<sup>-1</sup>, respectively. The concentrations of the cytokines used reflect physiological concentrations of these molecules (Schultz and Heyner, 1993) and are concentrations that have been shown to have biological activity in other culture studies in mice or rats (Paria and Dey, 1990; Harvey *et al.*, 1995; Bany *et al.*, 1998).

#### Northern blot analysis

Tissue samples or cells were homogenized into a guanidinium isothiocyanate solution and total RNA was isolated using methods described by Chomczynski and

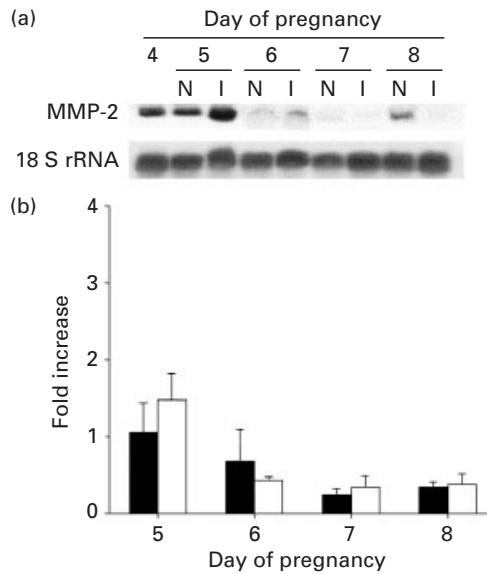
Sacchi (1987). Total RNA was quantified by absorbance at 260 nm as well as by ethidium bromide staining after electrophoresis through agarose gels. Samples of total RNA (10 µg) were subjected to denaturing agarose gel electrophoresis and then transferred to Hybond-N membranes (Amersham, Oakville, Ontario) by capillary transfer as described by Sambrook *et al.* (1989). The RNA was cross-linked to the membranes by UV irradiation (0.12 J cm<sup>-2</sup>).

Fragments of mouse MMP-2 (HindIII/ScaI), MMP-9 (nucleotides 2209–2430) and 18 S rRNA (nucleotides 1300–1504) cDNAs, subcloned into plasmid Bluescript SK vector, were kindly provided by D. Edwards (University of East Anglia, Norwich). The 240 bp MMP-2, 222 bp MMP-9 and 205 bp 18S rRNA cDNA fragments (25 ng) were labelled using a Random Primer DNA Labeling System (Gibco) in the presence of <sup>32</sup>P-dCTP (Amersham) and then purified using Nick Columns (Pharmacia Biotech, Baie d'Urfé, Quebec) according to the manufacturers instructions. Membranes were incubated in prehybridization buffer (1 mol NaCl l<sup>-1</sup>, 50 nmol Tris l<sup>-1</sup>, 2.2 nmol sodium pyrophosphate l<sup>-1</sup>, 10 g SDS l<sup>-1</sup>, 1 × Denhardt's reagent (Sambrook *et al.*, 1989), 10 mg denatured salmon sperm DNA l<sup>-1</sup>, pH 7.5) for 3 h at 65°C. Labelled probe (25 ng; specific activity approximately 1 Ci mg<sup>-1</sup>), denatured by placing in boiling water for 5 min and then snap-cooling on ice, was added and hybridization was carried out overnight at 65°C. After hybridization, the membranes were washed for 15 min twice in wash buffer 1 (1 g SDS l<sup>-1</sup>, 2 × SSC) then twice in wash buffer 2 (1 g SDS l<sup>-1</sup>, 0.2 × SSC) at 65°C. After autoradiography at -70°C with X-Omat AR or Biomax-MS film (Eastman Kodak, Rochester, NY), probes were removed from the membranes as described by Sambrook *et al.* (1989).

The relative amounts of RNA loaded into each lane and transferred to the membranes were determined by probing the blots with <sup>32</sup>P-labelled cDNA for mouse 18 S rRNA. MMP-2 and MMP-9 mRNA along with 18 S rRNA signals were quantified by densitometry. The relative concentrations of the signals for the mRNAs on the autoradiograms were calculated as the ratios of mRNA signal to 18 S rRNA signal.

#### Gelatin zymography

Gelatin zymography was performed as outlined by Behrendtsen *et al.* (1992) to determine whether the cells secrete MMP-2 and MMP-9. Briefly, samples of culture medium (10 µl) were diluted in sample buffer (final: 2 mmol urea l<sup>-1</sup>, 20 g SDS l<sup>-1</sup>, 0.125 mol Tris l<sup>-1</sup>, pH 6.8, containing bromophenol blue) then subjected to electrophoresis on a 10% (w/v) polyacrylamide gel polymerized in the presence of 60 mg gelatin l<sup>-1</sup>. Gels were rinsed in 2.5% (w/v) Triton X-100, incubated in CAB buffer (0.05 mol Tris l<sup>-1</sup>, 0.2 mol NaCl l<sup>-1</sup>, 5 mmol CaCl<sub>2</sub> l<sup>-1</sup>, 0.5 g Brij 35 l<sup>-1</sup>, 0.2 g Na<sub>3</sub> l<sup>-1</sup>, pH 7.2) for 24 h and stained using Coomassie blue stain. Clear bands represent gelatinolytic activity present in the samples of culture medium. In some experiments, the gels were incubated in CAB buffer containing 50 mmol EDTA l<sup>-1</sup> to determine whether the activities required calcium.



**Fig. 1.** Northern blot analysis of steady-state concentrations of mRNA encoding matrix metalloproteinase 2 (MMP-2) in the mouse uterus during the peri-implantation period. (a) Representative autoradiographs of a membrane containing RNA from days 4–8 pregnant uteri (N, inter-implantation area; I, implantation segment). (b) Mean  $\pm$  SEM ( $n = 4$ ) fold increase in mRNA:18 S rRNA signals for MMP-2 relative to those on day 4 of pregnancy ( $\square$ , inter-implantation area;  $\blacksquare$ , implantation segment).

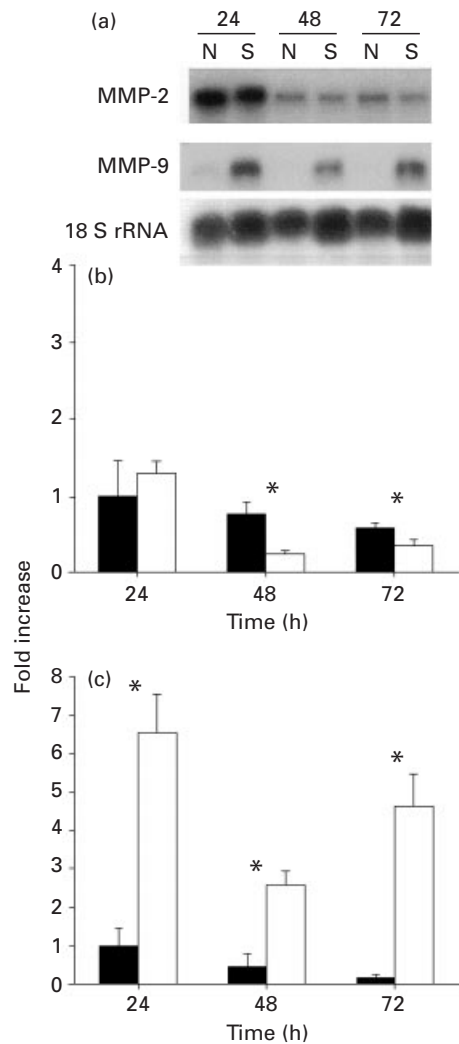
### Statistical analysis

Analysis of variance was used to compare the relative concentrations of the mRNAs between non-implantation and implantation segments or non-stimulated and stimulated uterine horns. The significance of the effects of day of pregnancy or time after stimulation and the site of sampling on the relative concentration of each mRNA was determined by analysis of variance with tissue site as a repeated measure. Where appropriate, paired Student's *t* tests or Duncan's multiple range tests were used to determine differences between means. The significance of treatment effects on the relative concentrations of mRNAs encoding MMP in the cultured cells were determined by Student's *t* tests. Heterogeneity of variance was reduced by logarithmically transforming the data before statistical analysis. All statistical analysis was carried out using SAS Software (SAS Institute, Cary, NC).

## Results

### mRNAs encoding MMP-2 and MMP-9 in uterine tissues

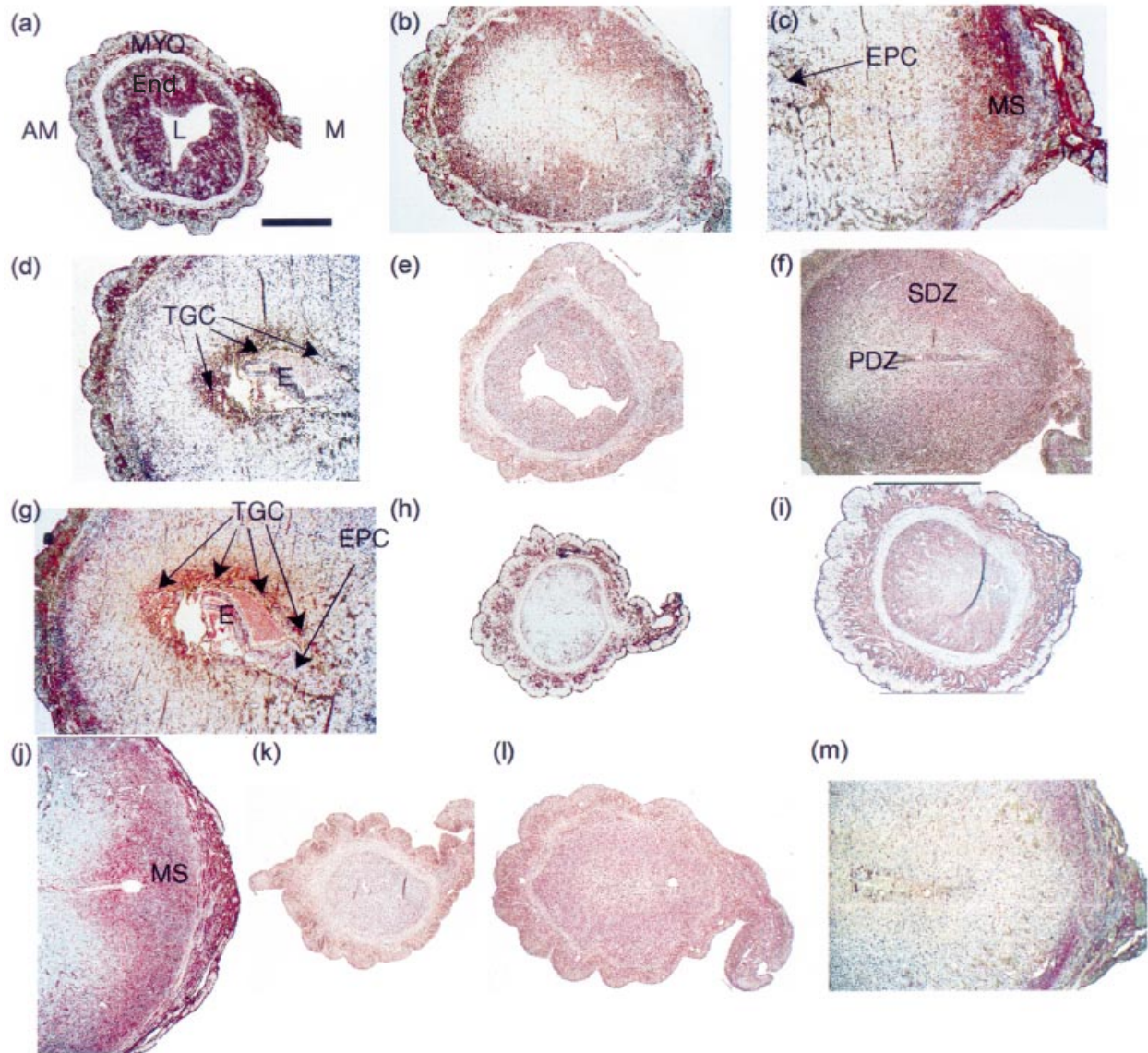
mRNA encoding MMP-2, but not mRNA encoding MMP-9, was detected in the uterine samples on days 4–8 of pregnancy by northern blot analyses (Fig. 1a). The steady-state concentrations of mRNA encoding MMP-2 were not significantly ( $P > 0.05$ ) different between non-implantation and implantation segments of the uteri on days 5–8 of pregnancy (Fig. 1b). However, there was a significant ( $P < 0.05$ ) effect of day of pregnancy on steady-state



**Fig. 2.** Northern blot analysis of steady-state concentrations of mRNA encoding matrix metalloproteinase 2 (MMP-2) and 9 (MMP-9) in the mouse uterus during oil-induced decidualization. (a) Representative autoradiographs of a membrane containing RNA from non-stimulated (N) and stimulated (S) uterine horns of mice at 24, 48 or 72 h after the unilateral injection of oil into the uterine lumen. Mean  $\pm$  SEM ( $n = 3$ ) fold increase in mRNA:18 S rRNA signals for MMP-2 (b) and MMP-9 (c) relative to those for non-stimulated horns at 24 h ( $\square$ , non-stimulated horn;  $\blacksquare$ , non-stimulated horn).

concentrations of mRNA encoding MMP-2, indicating that these decreased over days 5–8 in both non-implantation and implantation segments.

mRNAs encoding MMP-2 and MMP-9 were detected in non-stimulated and stimulated uterine horns during oil-induced decidualization (Fig. 2a). Steady-state concentrations of mRNA encoding MMP-2 (Fig. 2b) were not significantly different between non-stimulated and stimulated uterine horns at 24 h. However, at 48 and 72 h, the steady-state concentration of mRNA encoding MMP-2 was significantly ( $P < 0.05$ ) greater in non-stimulated than in stimulated uterine horns. By contrast, the steady-state concentration of mRNA encoding MMP-9 (Fig. 2c) was



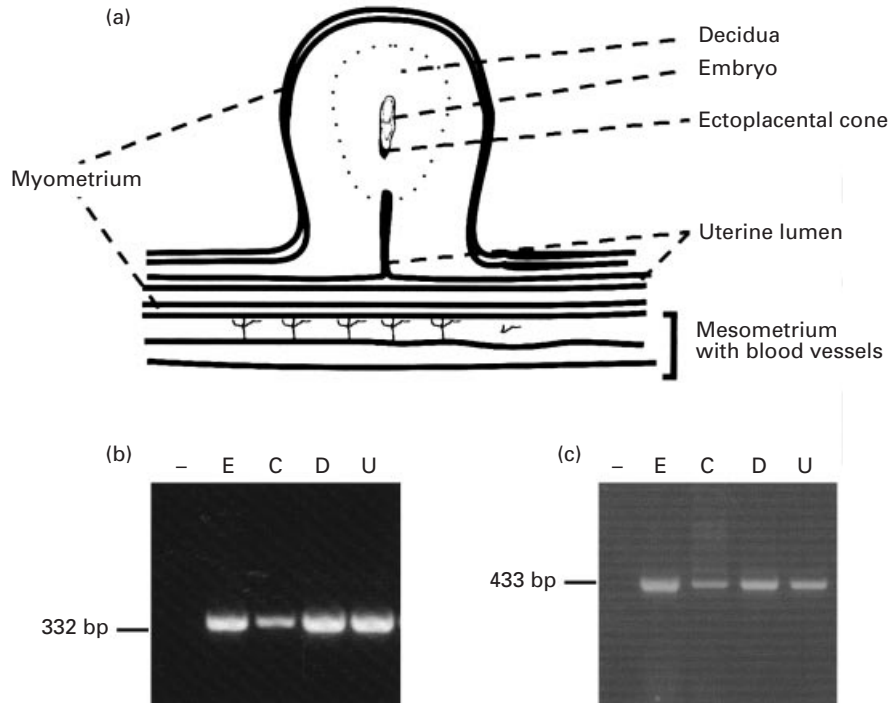
**Fig. 3.** Immunohistochemical localization of matrix metalloproteinase 2 (MMP-2) (a–d, h–j) and MMP-9 (e–g, k–m) during the peri-implantation period and oil-induced decidualization. Light micrographs showing immunolocalization in the uterus on day 4 (a, h) and implantation segments on days 6 (b, f) or 8 (c, d, g). Light micrographs showing immunolocalization in non-stimulated (h, k) and stimulated (i, l) uterine horns at 24 h, and stimulated uterine horns at 72 h (j, m). AM, antimesometrial; E, embryo; End, endometrium; EPC, ectoplacental cone; L, uterine lumen; M, mesometrial; MS, mesometrial endometrial stroma; MYO, myometrium; PDZ, primary decidual zone; SDZ, secondary decidual zone; TB, trophoblast; TGC, trophoblast giant cells. These results are representative of sections from at least three different mice. Scale bar represents 600  $\mu$ m.

significantly greater ( $P < 0.05$ ) in stimulated than in non-stimulated uterine horns at 24, 48 and 72 h.

#### MMP-2 and MMP-9 in uterine tissues

MMP-2 and MMP-9 were detected by immunohistochemistry in the endometrium of days 4–8 of pregnancy. On day 4, before the onset of implantation, immunoreactive MMP-2 was detected throughout the stroma of the endometrium (Fig. 3a). This pattern of staining remained the same in inter-implantation areas on days 5–8. Although

immunoreactive MMP-2 remained in the stroma in implantation segments, staining became weak or was absent in the zone of decidualization. On day 6, immunoreactive MMP-2 was seen mainly outside the decidualized zone in the endometrial stroma (Fig. 3b). By day 8, immunoreactive MMP-2 was detected mainly in the area of the primary trophoblast giant (PTG) cells, in the thin layer of non-differentiated stromal cells adjacent to the myometrium, and in the stromal cells at the mesometrial pole (Fig. 3c,d). Immunoreactive MMP-9 was also found in the endometrium on day 4 (Fig. 3e) and in inter-implantation areas from day 5 to day 8. By day 6 after implantation, immunoreactive



**Fig. 4.** Matrix metalloproteinase 2 (MMP-2) and MMP-9 RT-PCR. (a) Diagram of a longitudinal section through the implantation segment of a day 8 pregnant uterus. Photographs of ethidium bromide stained agarose gels of MMP-2 (b) and MMP-9 (c) RT-PCR products (amplicons). Lanes: control (-, no reverse transcription), embryo (E), ectoplacental cone (C), decidua (D) and uterine sheath (U) RT-PCR. Results are representative of three experiments.

MMP-9 was found mainly in the outside of the primary decidual zone with the greatest staining in the stroma adjacent to the myometrium (Fig. 3f). By day 8, the strong MMP-9 staining only remained in the thin layer of non-differentiated endometrial stromal cells adjacent to the myometrium and in the area of the trophoblast giant cells (Fig. 3g).

During oil-induced decidualization, immunoreactive MMP-2 and -9 were found in the endometrial stroma of non-stimulated horns at 24 (Fig. 3h,k), 48 and 72 h. Immunoreactive MMP-2 and -9 were also found in the endometrial stroma of stimulated horns at 24 h, and the intensity of staining appeared greater here than it did in non-stimulated horns (Fig. 3i,l). By 72 h, immunoreactive MMP-2 and -9 were found mainly in the thin layer of non-differentiated stromal cells adjacent to the myometrium (Fig. 3j,m). In addition, similar to day 8 of pregnancy, immunoreactive MMP-2 protein was also concentrated in the mesometrial pole of the stroma.

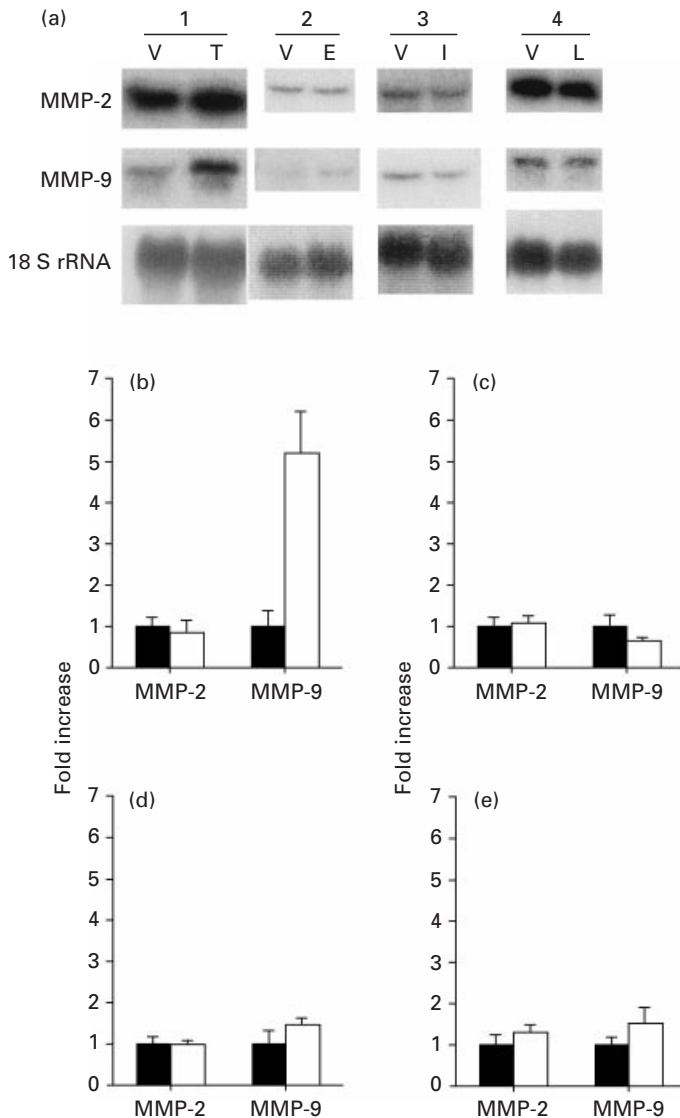
In all uterine sections, immunoreactive MMP-2 and -9 were also localized in the myometrium, predominantly in the tissue between the circular and longitudinal muscle layers. This localization did not change with pregnancy or during oil-induced decidualization.

#### MMP-2 and MMP-9 RT-PCR

RNA was isolated from the embryo, ectoplacental cone, decidua and uterine sheath from day 8 pregnant uteri (Fig. 4a). MMP-2 (Fig. 4b) and MMP-9 (Fig. 4c) transcripts were detected in RNA from the embryo, ectoplacental cone, decidua and uterine sheath by RT-PCR. Control RT-PCR reactions, in which the enzyme reverse transcriptase was omitted for the RT-PCR, showed no amplicons. Absolute purity of the cell types in these dissections cannot be guaranteed, especially for the ectoplacental cone, in which there is a high possibility of maternal cells. Nonetheless, this sensitive RT-PCR procedure demonstrates that mRNAs encoding both MMP-2 and MMP-9 are detectable and present in all regions of the embryo and uterus.

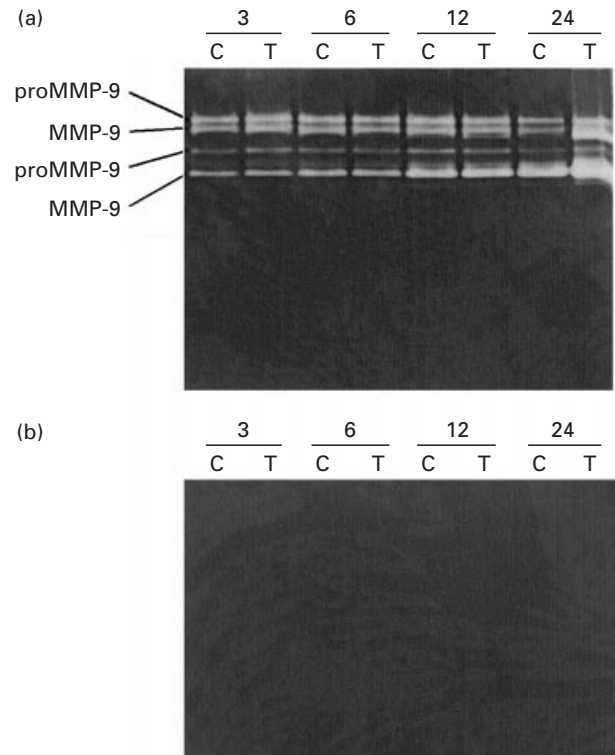
#### mRNA encoding MMP-2 and MMP-9 in cultured cells

Mouse endometrial stromal cells from uteri sensitized for decidualization were cultured in serum-free medium for 24 h and then incubated without or with TGF $\beta$ 1 (5 ng ml<sup>-1</sup>), EGF (40 ng ml<sup>-1</sup>), IL-1 $\alpha$  (10 ng ml<sup>-1</sup>) or LIF (5 ng ml<sup>-1</sup>) for 6 h. mRNAs encoding both MMP-2 and MMP-9 were detected in



**Fig. 5.** Northern blot analysis of the effects of cytokines on steady-state concentrations of mRNA encoding matrix metalloproteinase 2 (MMP-2) and mRNA encoding MMP-9 in cultured mouse endometrial stromal cells. (a) Representative autoradiographs of membranes containing RNA from cells incubated without (V) and with 5 ng TGFβ1 (T) ml<sup>-1</sup>, 40 ng EGF (E) ml<sup>-1</sup>, 10 ng IL-1α (I) ml<sup>-1</sup> or 5 ng LIF (L) ml<sup>-1</sup>. Mean ± SEM (*n* = 3–4) fold increase in the ratios of mRNA:18 S rRNA signals for MMP-2 and MMP-9 in response to TGFβ1 (b), EGF (c), IL-1α (d) or LIF (e) relative to untreated cells (□, without cytokine; ■, with cytokine).

the cells by northern blot analysis (Fig. 5a). The steady-state concentration of mRNA encoding MMP-9, but not mRNA encoding MMP-2, significantly ( $P < 0.005$ ) increased in response to TGFβ1 (Fig. 5b). The steady-state concentrations of mRNAs encoding MMP-2 and MMP-9 were not significantly ( $P > 0.05$ ) changed in response to EGF, IL-1α or LIF (Fig. 5c–e).



**Fig. 6.** Gelatin zymography of medium from mouse endometrial stromal cell cultures. Samples of medium (10 μl) from cells treated with or without TGFβ1 (5 ng ml<sup>-1</sup>) for 3, 6, 12 or 24 h were analysed for gelatinase activity in the presence of 5 mmol Ca<sup>2+</sup> l<sup>-1</sup> (a) and Ca<sup>2+</sup> plus 50 mmol EDTA l<sup>-1</sup> (b). Bands, which correspond to the relative molecular masses of MMP-2 and MMP-9, are shown and are representative of the results from three different experiments. MMP-9, unprocessed MMP-9; proMMP-9, processed MMP-9; proMMP-2, unprocessed MMP-2; MMP-2, processed MMP-2.

#### Gelatin zymography

Cells were cultured in serum-free medium for 24 h and then incubated with or without TGFβ1 (5 ng ml<sup>-1</sup>) for 3, 6, 12 or 24 h. Samples of the medium were subjected to zymographic analysis and four major bands of gelatinolytic activity that had relative molecular masses of 62, 72, 97 and 110 kDa were detected (Fig. 6a). These molecular masses correspond to the known sizes of processed MMP-2, proMMP-2, processed MMP-9 and pro-MMP-2, respectively (Alexander *et al.*, 1996). Gels incubated either without Ca<sup>2+</sup> or in the presence of EDTA (Fig. 6b) showed no bands of gelatinolytic activity.

#### Discussion

MMP-9 is expressed mainly in the PTG cells during implantation. In agreement with the results of Alexander *et al.* (1996), MMP-9 was localized to the region of the PTG cells during implantation in mice. The source of this protein is likely the primary trophoblast giant cells since they are the major site of localization of mRNA encoding MMP-9 in the

pregnant uterus, as determined by *in situ* hybridization (Harvey *et al.*, 1995; Reponen *et al.*, 1995; Alexander *et al.*, 1996; Leco *et al.*, 1996; Das *et al.*, 1997). Therefore, PTG cells are the major sites of MMP-9 expression in the uterus during implantation. As shown in the present study and others (Alexander *et al.*, 1996; Das *et al.*, 1997), mRNA encoding MMP-9 is undetectable, or barely detectable, in the endometrial stroma during implantation when northern blot methods are used. However, there is data to support the notion that the endometrial stroma is also a source of MMP-9 expression during implantation and oil-induced decidualization, albeit at relatively low concentrations. mRNA encoding MMP-9 is transiently present in a small subpopulation of endometrial stromal cells at implantation sites just after the onset of implantation (Das *et al.*, 1997). Furthermore, in the present study, mRNA encoding MMP-9 was detected in the decidual tissue and uterine sheath from day 8 pregnant uteri by RT-PCR, and in the uterus during oil-induced decidualization by northern blot analysis. Although it was not detected in the endometrial stroma by immunohistochemistry, Alexander *et al.* (1996) detected MMP-9 in samples of decidua and deciduoma by gelatin zymography. A different antibody to that used by Alexander *et al.* (1996) was used in the present study to detect MMP-9 in the endometrial stroma during implantation and oil-induced decidualization by immunohistochemistry. Taken together, these results show that mRNA encoding MMP-9 and MMP-9 are present in the endometrial stroma during implantation and oil-induced decidualization. However, it is possible that the source of the MMP-9 found in the endometrium is extrauterine, at least in part, (Alexander *et al.*, 1996). Nonetheless, the finding that mRNA encoding MMP-9 is detected in the endometrium indicates that at least some of the MMP-9 is expressed within the endometrium during implantation and oil-induced decidualization.

MMP-9 expression increases in the uterus during pregnancy and artificially-induced decidualization. mRNA encoding MMP-9 was not detected in the uterine segments from pregnant uteri using northern blot analysis. However, MMP-9 transcripts have been detected in RNA from decidua plus embryo on days 6–10 of pregnancy, with concentrations increasing between days 7 and 10 (Alexander *et al.*, 1996). Since this increase parallels the increase in the number of differentiated trophoblast giant cells, it is believed to be due to PTG cell MMP-9 expression (Alexander *et al.*, 1996). In the present study, steady-state concentrations of mRNA encoding MMP-9 were significantly greater in stimulated compared with non-stimulated uterine horns during artificially induced decidualization. Since embryo TGC cells are not present in this model, these results indicate that mRNA encoding MMP-9 is expressed by the uterus during oil-induced decidualization. However, the exact source of the increased mRNA concentrations remains to be determined. MMP-9 and the mRNA encoding it were detected in cultured endometrial stromal cells isolated from uteri sensitized for decidualization. Therefore, endometrial stromal cells are a possible source of the increased concentrations of mRNA encoding MMP-9 found during oil-induced decidualization.

MMP-2 is also expressed in the endometrium during early

pregnancy. The distribution of mRNA encoding MMP-2 in implantation segments has been studied using *in situ* hybridization (Das *et al.*, 1997). MMP-2 transcripts are found in the subepithelial stroma of the endometrium on days 4 and 5 and then primarily in the secondary decidual zone on day 6 of pregnancy. By days 7 and 8 of pregnancy, localization of mRNA encoding MMP-2 is concentrated in the non-decidualized stromal cells adjacent to the myometrium. On days 7 and 8, mRNA encoding MMP-2 is also localized to the mesometrial pole of the endometrium, the presumptive site of placenta formation (Alexander *et al.*, 1996; Das *et al.*, 1997). In the present study, the pattern of MMP-2 localization by immunohistochemistry parallels that described for mRNA encoding MMP-2. Although it is not the case for the mRNA (Das *et al.*, 1997), in the present study, MMP-2 was localized to the trophoblast giant cells on day 8 of pregnancy. The source of this MMP-2 may be the endometrium or the embryo since mRNA encoding MMP-2 was detected in both using RT-PCR. Therefore, MMP-2 appears to be constitutively expressed in the endometrial stroma just before implantation. This expression gradually decreases in endometrial stroma that has undergone decidualization.

The regulation of MMP-2 and MMP-9 expression in the uterus during implantation could be directed by signals of uterine or embryonic origin. In the present study, the expression of MMP-2 and MMP-9 in the pregnant uteri paralleled that in the uteri undergoing oil-induced decidualization in two ways. First, concentrations of mRNA encoding MMP-2 were not different between implantation and interimplantation areas or between stimulated and non-stimulated uterine horns. Second, the patterns of MMP-2 and MMP-9 localization in the endometrial stroma were similar between implantation segments and stimulated uterine horns. These results indicate that changes in the pattern of expression of MMP-2 and -9 in the endometrium during implantation can occur without direct signals from the embryo.

Formation of the deciduoma in response to an artificial stimulus provides a well-controlled model of decidualization. In the present study, mRNA encoding MMP-9 was detected easily in non-stimulated and stimulated horns during oil-induced decidualization by northern blot analysis. However, concentrations of mRNA encoding MMP-9 in the pregnant uterus appeared to be low, requiring the use of very sensitive RT-PCR methods for detection. These results indicate that the expression of mRNA encoding MMP-9 in the uterus differs substantially between pregnant uteri and uteri undergoing oil-induced decidualization. This difference may be a consequence of differences in the hormonal milieu between these uteri, or an embryo-derived factor may be inhibiting MMP-9 expression in the pregnant uterus. These and other potential explanations of why it is easy to detect mRNA encoding MMP-9 during oil-induced decidualization but not pregnancy warrants further study.

There are at least two processes occurring in the uterus during implantation that involve proteinases: embryo invasion and decidualization. The predominant MMP produced by blastocysts *in vitro* is MMP-9 and the use of neutralizing antibodies inhibits blastocyst invasiveness (Librach *et al.*, 1991; Behrendsen *et al.*, 1992). MMP-9 expression



occurs in the trophoblast giant cells *in vivo* (Alexander *et al.*, 1996; Leco *et al.*, 1996; Das *et al.*, 1997). These observations indicate that MMP-9 plays a role in blastocyst invasiveness. As discussed above, MMP-2 and MMP-9 are also present within the endometrial stroma during implantation. MMP-2 expression is abundant in the endometrial stromal cells when the blastocyst is about to implant, but once they begin to undergo decidualization, MMP-2 expression decreases. Inhibition of metalloproteinases *in vivo* using metalloproteinase inhibitors alters decidualization, resulting in smaller decidua (Alexander *et al.*, 1996; Rechtman *et al.*, 1999). This finding indicates that MMPs are required for normal decidualization in mice. However, since decidualization still occurs in the presence of inhibitors, it is apparent that MMPs are not absolutely required for decidualization. A broad-spectrum metalloproteinase inhibitor does not inhibit implantation in rats (Rechtman *et al.*, 1999). In addition, implantation still occurs in MMP-2- and MMP-9-deficient mice (Itoh *et al.*, 1997; Vu *et al.*, 1998). Other proteinases must be sufficient to compensate for the MMP deficiencies in these animals. Whether implantation and decidualization occur in a normal fashion in mice doubly deficient in MMP-2 and -9 remains to be determined.

TGF $\beta$ 1 and type II TGF $\beta$  receptor are expressed in the endometrium during early pregnancy (Akhurst *et al.*, 1990; Tamada *et al.*, 1990; Manova *et al.*, 1992; Roelen *et al.*, 1994). During the pre-implantation period, TGF $\beta$ 1 is expressed in the luminal and glandular epithelia and, after implantation, it is expressed mainly in the decidua. Although TGF $\beta$ 1 is expressed in the endometrium during implantation and decidualization, its potential role in regulation of MMP-2 and -9 gene expression in the mouse endometrium remains to be elucidated. In the present study, in mouse endometrial stromal cells, TGF $\beta$ 1 caused an increase in the concentration of mRNA encoding MMP-9, but not mRNA encoding MMP-2. Therefore, TGF $\beta$ 1 is a potential regulator of MMP-9 expression in the endometrial stroma during implantation and decidualization. Notably, other cytokines (EGF, IL-1 and LIF) that are found in the endometrium during implantation (Huet-Hudson *et al.*, 1990; Pollard, 1990; Stewart *et al.*, 1992; De *et al.*, 1993) did not have effects on MMP expression in the present study.

In conclusion, the present study further characterized MMP-2 and MMP-9 expression in the mouse uterus during implantation and artificially-induced decidualization. It also demonstrated that cultured mouse endometrial stromal cells express MMP-2 and MMP-9 and that the expression of MMP-9 is increased in these cells in response to TGF $\beta$ 1. Further work is required to determine how MMP-2 and -9 gene expression is regulated in the uterus during implantation and decidualization.

This research was supported by a grant from the Medical Research Council of Canada (GAS, grant MT-15138) and the Natural Science and Engineering Research Council of Canada. B. M. Bany was the recipient of Fellowships from the Lalor Foundation and the Alberta Heritage Foundation for Medical Research during this work.

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