Secretion of matrix metalloproteinase-2, matrix metalloproteinase-9 and tissue inhibitor of metalloproteinases into the intrauterine compartments during early pregnancy

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Matrix metalloproteinases (MMPs) are important enzymes in tissue remodelling, a key event for the development of the fetal membranes and placenta and establishing the feto–maternal interface during early pregnancy. This study has examined the secretion of the gelatinases, MMP-2 (72 kDa) and MMP-9 (92 kDa), and the endogenous tissue inhibitors of metalloproteinases (TIMPs) into extra-embryonic coelomic and amniotic fluids, the two principal intra-uterine compartments of the first trimester, and compared them to amniotic fluid collected later in gestation. In extra-embryonic coelomic fluid, gelatin zymography demonstrated that MMP-2 (72 kDa) was the predominant gelatinase, with some MMP-9 present. A broad range of TIMPs corresponding to TIMP-1 and TIMP-2, glycosylated and unglycosylated TIMP-3 and TIMP-4 was detected in this compartment by reverse zymography and immunoblot analyses. There was little gelatinase or TIMP activity in amniotic fluid in the first trimester. In amniotic fluid from the second trimester after fusion of the membranes obliterating the extra-embryonic coelom, and at term elective caesarean section, MMP-2 is the predominant gelatinase present, with a broad spectrum of TIMPs. These findings demonstrate that predominantly MMP-2 and also MMP-9, regulated by a range of TIMPs, are involved in intra-uterine tissue remodelling during the establishment of pregnancy.

Key words: fetus.matrix metalloproteinase/placenta/TIMP/trophoblast

Introduction

Successful pregnancy is dependent upon invasion of trophoblast into the decidua at implantation, and subsequently the further invasion of extravillous trophoblast into the walls of the maternal spiral arterioles (Fisher et al., 1985; Librach et al., 1991; Cross et al., 1994). These events require the breakdown of extracellular matrix and cellular migration. Little is known about the specific collagenase enzymes involved. The matrix metalloproteinases (MMPs) are an important family of zinc-dependent enzymes with a broad range of substrate specificities capable of the breakdown of extracellular matrix (Hulboy et al., 1997). The activity of secreted MMPs is regulated by specific endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), of which four have been identified (Hulboy et al., 1997). These TIMPs have a broad specificity for all MMPs and form tight stoichiometric non-covalently bound complexes with the activated MMPs to inhibit their enzymatic activity (Woessner, 1991).

In the uterus, a role for MMPs and TIMPs in the cyclical remodelling of the endometrium during the menstrual cycle has been established (Hampton and Salamonson, 1994; Rodgers et al., 1994; Zhang and Salamonson, 1997). During early pregnancy, MMPs are implicated in the invasion of trophoblast cells at implantation and during the establishment of the placental bed (Bishof et al., 1991; Graham and Lala, 1991; Shimonowitz et al., 1994; Huppertz et al., 1998). However, the intrauterine secretion of MMPs and TIMPs during human pregnancy has been poorly characterized. In the first trimester, within the uterus the extra-embryonic coelom is the space between the amnion and chorion. It becomes obliterated by the start of the second trimester as the amnion and chorion fuse. The amniotic sac and the extra-embryonic coelom are likely to accumulate locally secreted enzymes and inhibitors released by the surrounding amnion and chorion layers of the fetal membranes. Sampling of these fluid spaces offers a convenient window on in-vivo enzyme secretion by the fetal membranes in early pregnancy. We have chosen to examine the secretion of the gelatinases MMP-2 and MMP-9 as these enzymes have substrate specificity for type IV collagen, a major component of basement membranes. At the same time we have also characterized the secretion of TIMPs into the same fluid cavities.

Materials and methods

Collection of samples

Matched samples (n = 7) of extra-embryonic coelomic and amniotic fluids were collected by ultrasound guided needle aspiration from women between 8–11 weeks gestation, prior to the termination of pregnancy by general anaesthesia, as described in detail previously (Wathen et al., 1991). Fetal viability and gestational age were confirmed by ultrasound. Amniotic fluid samples collected prospect-
ively in the second trimester by amniocentesis were obtained from the regional cytogenetics laboratory. All samples used had normal karyotypes. At term, amniotic fluid samples were collected at the time of elective caesarean section. Samples were stored at −20°C prior to performing zymography. Ethical approval for the collection of all samples used in these studies was granted by the appropriate St Bartholomew’s Hospital, and the Lothian Research Ethical Committees.

Detection and measurement of MMP-2 and MMP-9 by gelatinase zymography

MMP-2 and MMP-9 activities were detected by gelatinase zymography, as described previously (Rawdanowicz et al., 1994) with minor modifications. Briefly, amniotic and extra-embryonic coelomic fluid samples (the same volume of 5 µl was consistently used) were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE; 7.5% gels; Minigel apparatus; BioRad, Hemel Hempstead, UK) containing gelatin (1 mg/ml) with non-reducing conditions. Caseinolytic activity was also assayed by substituting casein (1mg/ml) for gelatin as substrate. The presence of SDS in the gel activates the latent forms of MMPs, as well as dissociating active forms from inhibitors and so permitting their detection. Gels were washed (twice, 2.5% (v/v) Triton X-100) then incubated in zymography digestion buffer (200 mM NaCl, 50 mM Tris, 5 mM CaCl2, 1 mM ZnCl2, 0.02% (v/v) Brij-35, pH 7.6; all chemicals from Sigma Chemical Co, St Louis MO, USA, except where specified) for 18 h at 37°C. Gels were immersed in staining solution (0.5% Coomassie Blue R250 in 30% methanol/10% glacial acetic acid in H2O) for 3 h at 23°C, then destained (staining solution with Coomassie Blue omitted) to reveal discrete areas where gelatin substrate has been hydrolysed by gelatinase activity.

Detection and measurement of TIMPs by reverse zymography

Detection of TIMPs was performed by reverse zymography using methods described previously, using a commercial kit (University Technologies Inc, Calgary, Canada; Hampton et al., 1995) with some minor modifications. Briefly, samples of extra-embryonic and amniotic fluids (same volume of 7.5 µl used consistently) were separated by PAGE using 12% gels containing 1 mg/ml gelatin and an MMP digestion buffer (2.5 mM; data not shown). Another unidentified band of latent form of MMP-2 (Figure 1). Lesser amounts of active MMP-2 (66 kDa) were detected. Amounts of the latent form of MMP-2 were significantly higher (P < 0.05; t-test) than the barely detectable activity in the matched amniotic fluid samples (Figure 1). Low levels of gelatinase activity were also detectable in extra-embryonic coelomic fluid at 92 kDa, corresponding to latent MMP-9 with virtually undetectable amounts in the amniotic fluid. The gelatinase activities at 72 and 92 kDa were further characterized as MMP activated proteins by inhibition with EDTA (5 mM) or o-phenanthroline (2.5 mM; data not shown). Another unidentified band of gelatinase activity of ~115 kDa was also present. The presence of MMP-2 in extra-embryonic coelomic fluid and amniotic fluid was confirmed by Western blot analysis (Figure 2a) using a specific antibody which demonstrated a predominant band at 72 kDa, corresponding to the latent form. The abundance of MMP-2 protein by Western blotting was similar to the zymography findings, demonstrating MMP-2 protein in its lowest abundance during the first trimester of pregnancy in amniotic fluid, with higher amounts present in extra-embryonic coelomic fluid and in amniotic fluid from the second and third trimesters. Using zymography with casein as substrate, no caseinolytic activity was detected in extra-embryonic coelomic or amniotic fluids from the first trimester (data not shown).

A broad spectrum of TIMP activity was detected by reverse zymography predominantly in extra-embryonic coelomic fluid (Figure 3). Three bands of gelatinase inhibitory activity were present at 27–30 kDa (these correspond to the molecular weight of TIMP-1, glycosylated TIMP-3 and TIMP-4), 24 kDa (corresponding to unglycosylated TIMP-3) and 21 kDa (corresponding to TIMP-2). These bands aligned with standards to

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In extra-embryonic coelomic fluid, analysis by zymography demonstrated that the predominant gelatinase activity was identified at 72 kDa molecular weight, corresponding to the latent form of MMP-2 (Figure 1). Lesser amounts of active MMP-2 (66 kDa) were detected. Amounts of the latent form of MMP-2 were significantly higher (P < 0.05; t-test) than the barely detectable activity in the matched amniotic fluid samples (Figure 1). Low levels of gelatinase activity were also detectable in extra-embryonic coelomic fluid at 92 kDa, corresponding to latent MMP-9 with virtually undetectable amounts in the amniotic fluid. The gelatinase activities at 72 and 92 kDa were further characterized as MMP activated proteins by inhibition with EDTA (5 mM) or o-phenanthroline (2.5 mM; data not shown). Another unidentified band of gelatinase activity of ~115 kDa was also present. The presence of MMP-2 in extra-embryonic coelomic fluid and amniotic fluid was confirmed by Western blot analysis (Figure 2a) using a specific antibody which demonstrated a predominant band at 72 kDa, corresponding to the latent form. The abundance of MMP-2 protein by Western blotting was similar to the zymography findings, demonstrating MMP-2 protein in its lowest abundance during the first trimester of pregnancy in amniotic fluid, with higher amounts present in extra-embryonic coelomic fluid and in amniotic fluid from the second and third trimesters. Using zymography with casein as substrate, no caseinolytic activity was detected in extra-embryonic coelomic or amniotic fluids from the first trimester (data not shown).
TIMP-1, TIMP-2 and glycosylated and unglycosylated TIMP-3. In amniotic fluid samples from the first trimester, very low to undetectable amounts of TIMP activity were present. Reverse zymography is unable to distinguish precisely between the TIMP isoforms of 27–30 kDa molecular weight. The presence of TIMP-4 was, therefore, determined by Western blot analysis (Figure 2b) which demonstrated only low abundance in extra-embryonic coelomic and amniotic fluids from the second trimester and at term. Little TIMP-4 was detectable in amniotic fluid collected during the first trimester.

**MMP-2, MMP-9, and TIMPs in amniotic fluid during the second trimester**

In amniotic fluid samples collected in the second trimester MMP-2 activated protein corresponding to the latent (72 kDa) form was the predominant gelatinase activity present (Figure 4). There was a less intense band at 66 kDa, corresponding to the active form of MMP-2. Significantly greater (*P* < 0.05; *t*-test) amounts of MMP-2 (latent) compared to MMP-9 (latent) were present in the second trimester amniotic fluid. There was significantly higher (*P* < 0.05; *t*-test) secretion of latent MMP-2 into second trimester amniotic fluid compared with samples collected in the first trimester. Active MMP-2 was also present but in lower amounts than the latent isoform. The pattern of TIMPs activity was similar to that present in the extra-embryonic coelom in the first trimester, with three main bands of activity visualized (Figure 5).

**Discussion**

These studies demonstrate the presence of MMP-2 and MMP-9 gelatinases in first trimester extra-embryonic coelomic fluid, as well as in the amniotic fluid at all gestations. The predominant MMP activated protein was the latent form of MMP-2 which was present in increasing concentrations in amniotic fluid from the first to the second trimester. The same fluid compartments contained TIMP activities corresponding to TIMP-1, TIMP-2, glycosylated and unglycosylated TIMP-3, and TIMP-4 at all gestations. The TIMP activity in the amniotic fluid also increased with gestation.

These studies, of course, cannot precisely identify the cellular origin of the various MMPs and TIMPs. Enzyme activity recovered from the extra-embryonic coelom may arise from mesenchymal cells lining either the chorion or amnion epithelium, from adjacent trophoblast, or even from more distantly placed decidual cells. Similarly, enzyme activity
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Figure 3. (a) Representative reverse zymography gel demonstrating the presence of tissue inhibitor of metalloproteinases activity (TIMPs; visualized as darker bands) in matched samples of extra-embryonic coelomic (e) and amniotic fluid (a) obtained during the first trimester of pregnancy. These TIMP activities align with standards (marked by arrows) to TIMP-1 (1; 30 kDa) and TIMP-2 (2; 21 kDa; standard 1+2), and TIMP-3 (g3 – glycosylated form of 30 kDa; ung3 -unglycosylated form of 24 kDa; standard 3). A sample of term amniotic fluid (af) was used as a positive control. Matrix metalloproteinase (MMP)-2 and MMP-9 activities are indicated. (b) Densitometric analysis (mean ± SEM, n = 5) of TIMP activities at 27–30 kDa (corresponding to TIMP-1, the glycosylated form of TIMP-3 and TIMP-4; open bars), 24 kDa (unglycosylated TIMP-3; hatched bars) and 21 kDa (TIMP-2; solid bars) in extra-embryonic coelomic (ee cf) and amniotic fluid (af).

recovered from the amniotic fluid may arise from the amnion epithelium or from the growing fetus. Furthermore, although it seems likely that enzyme present in these fluid compartments is present as overspill from various local tissues which are undergoing remodelling, it is equally possible that the enzymes have been directly secreted into these spaces to mediate specific functions. Gestation related changes in MMP and TIMP activities have been quantified by densitometric analysis of gelatin zymography. The lack of suitable standards, limited number of samples that can be examined at any one time (only gels run directly in parallel are comparable) and inability to compare absolute protein content or activity using this technique, only permits the demonstration of relative changes in amounts of activated gelatinase proteins (Martelli et al., 1993).

Figure 4. (a) Representative zymography gel showing gelatinase activity corresponding predominantly to the latent form of matrix metalloproteinase-2 (MMP-2; 72 kDa) in amniotic fluid samples collected during the first and second trimesters of pregnancy and at elective Caesarean section at term. Lesser amounts of the active form of MMP-2 (66 kDa) are also present. (b) Densitometric analysis (mean ± SEM, n = 6) of MMP-2 activated protein (latent form; 72 kDa; open bars) and MMP-9 (latent form; 92 kDa; solid bars). Low levels of MMP-2 activated protein are present in samples from the first trimester, with higher levels during the second trimester and at term.

Both MMPs and TIMPs are likely to be involved in the tissue remodelling that accompanies the rapid growth and structural changes of the fetal membranes in the first trimester.
This may be reflected in the increased amounts of the active form of MMP-2 in amniotic fluid during the second trimester. Proteases may also have an action to cleave inactive growth factor and cytokine precursors to active forms, and to liberate growth factors bound to extracellular matrix (Cross et al., 1994; Damsky et al., 1994). Interestingly, TIMPs stimulate proliferation and may play a direct role in the development of the intra-uterine structures (Hayakawa et al., 1994; Corcoran and Stetler-Stevenson, 1996). A complex of TIMP-1 with procathepsin-L has been shown to upregulate steroidogenesis in the testis (Boujrad et al., 1995) raising the possibility that TIMPs stimulate procathepsin-L activity and may play a direct role in the development of the extra-embryonic coelom, or if proteins of oestradiol 17-β, progesterone and 17α-progesterone found in the extra-embryonic coelom (Jauniaux et al., 1993, Atkinson et al., 1996).

In conclusion, these studies have demonstrated compartmentalization of MMPs and TIMPs within the uterus in the first trimester of human pregnancy. MMP-2 and -9, and TIMP-1, -2, -3 and -4 are present predominantly in the extra-embryonic coelomic fluid, with barely detectable amounts in amniotic fluid. When the extra-embryonic coelom becomes obliterated the concentrations of MMPs and TIMPs in the amniotic fluid increase. It is not clear if this compartmentalization of enzyme and endogenous inhibitors is related to specific functions within the extra-embryonic coelom, or if proteins have accumulated here as overspill from surrounding rapidly developing tissues.

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References


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