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Expression and implications of tissue inhibitor of metalloproteinases-4 in mouse embryo

Jian Zhang[1], Yun-Ge Zhao[2], Yu-Jing Cao[1], Qing-Xiang Amy Sang [2] and En-Kui Duan[1,3]

[1]State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, People's Republic of China and [2]Department of Chemistry and Biochemistry and Institute of Molecular Biophysics, Florida State University, Tallahassee, FL 32306, USA

[3]To whom correspondence should be addressed. E-mail: duane@panda.ioz.ac.cn

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Matrix metalloproteinases (MMP), tissue inhibitors of metalloproteinases (TIMP), and MMP–TIMP interactions may contribute to the highly programmed process of embryo implantation. The loss of the delicate MMP–TIMP balance may lead to abnormal implantation. The role of TIMP-4 in mouse implantation has not been reported. This study examined mRNA and protein expression levels of TIMP-4 in the blastocyst and uteri of pregnant mice. We also investigated the effects of a specific TIMP-4 antibody on embryo outgrowth and on the gene and protein expression levels of two gelatinases. High levels of TIMP-4 mRNA and protein were detected in day 3–5 embryos and in the trophoblast cells of mice blastocysts, suggesting that TIMP-4 may be involved in embryo implantation. Furthermore, TIMP-4 antibody promoted blastocyst outgrowth in a dose-dependent manner, but had no effect on blastocyst adhesion to extracellular matrix. A specific TIMP-4 antibody also increased mRNA and protein expression levels and the enzymatic activities of gelatinase A (MMP-2) and gelatinase B (MMP-9). This study suggests that TIMP-4 may restrict mouse blastocyst outgrowth and embryo implantation by inhibiting the activities of MMP-2 and -9.

Key words: blastocyst/implantation/matrix metalloproteinases/mouse embryo/TIMP-4

Introduction

Mouse embryo implantation is a well-organized process regulated by multiple factors (Tabibzadeh *et al*., 1995). There is an increasing interest in the biological mechanisms that allow for controlled growth and invasion of the embryo into the maternal endometrium during implantation and placentation. Cell adhesion molecules (such as integrins) and growth factors participate in both cell–cell and cell–matrix interactions (Harvey *et al*., 1995; Stewart *et al*., 1997). Meanwhile, plasminogen activators (PA) and matrix metalloproteinases (MMP) degrade the extracellular matrix (ECM) during the process of blastocyst invasion (Behrendtsen *et al*., 1992). Successful implantation depends on cooperation between the invasive blastocyst and the receptive endometrium.

The matrix metalloproteinases (MMP) are a family of enzymes that may play a role in ECM degradation and tissue remodelling during implantation. Controlled remodelling of the ECM is an essential aspect of the process of successful implantation, but uncontrolled remodelling has been shown to play a role in the aetiology of many diseases (Salamonsen *et al*., 1999; Fata *et al*., 2000). The overproduction and unrestrained activity of MMP has been linked to the malignant conversion of tumour cells. The down-regulation of MMP may occur at the transcription level through regulation of the gene products by non-activation of secreted proenzymes, and through interaction with specific inhibitor proteins such as tissue inhibitors of metalloproteinases (TIMP). TIMP are secreted as multifunctional proteins that play pivotal roles in the regulation of ECM metabolism. Their most widely known action is to inhibit the activities of MMP. Thus, the net MMP activity in the ECM during implantation is the result of a delicate balance between activated MMP levels and TIMP levels.

Four mammalian TIMP have been characterized at the sequence level: TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (Welgus *et al*., 1983; Stetler-Stevensen *et al*., 1989; Apte *et al*., 1995; Greene *et al*., 1996). The proteins are classified based on structural similarity to each other as well as their ability to inhibit MMP. Although TIMP are interchangeable in their capabilities as inhibitors of active MMP with few exceptions, they are distinguished by the formation of specific complexes with different pro-MMP. Secreted pro-MMP-2–TIMP-2 and pro-MMP-9–TIMP-1 complexes may represent an additional function for TIMP in controlling activation of specific latent MMP (Goldberg *et al*., 1989). Unlike TIMP-3, which has a unique association with the ECM, TIMP-4 binds to the COOH-terminal haemopexin-like domain of human gelatinase A in a manner similar to TIMP-2 (Stratmann *et al*., 2001). In addition, TIMP-1, -2 and -3 have distinct distributions in the implantation site and unique physiological roles during implantation. Both TIMP-1 and TIMP-2 are expressed in undifferentiated zones, and TIMP-2 has a relatively wide expression. TIMP-3 is expressed in the primary decidual cells surrounding the trophoblast cells (Leco *et al*., 1997). However, the distribution and the role of TIMP-4 in embryo implantation are unknown.

To better understand the role of TIMP-4 in embryo implantation, we have investigated the expression profiles of TIMP-4 and its effects on mouse embryo attachment and outgrowth.

Materials and methods

Animals

Adult (virginal; 22–25 g) mice of the outbred Kunming white strain were purchased from the Experimental Animal Center, Institute of Zoology, Chinese

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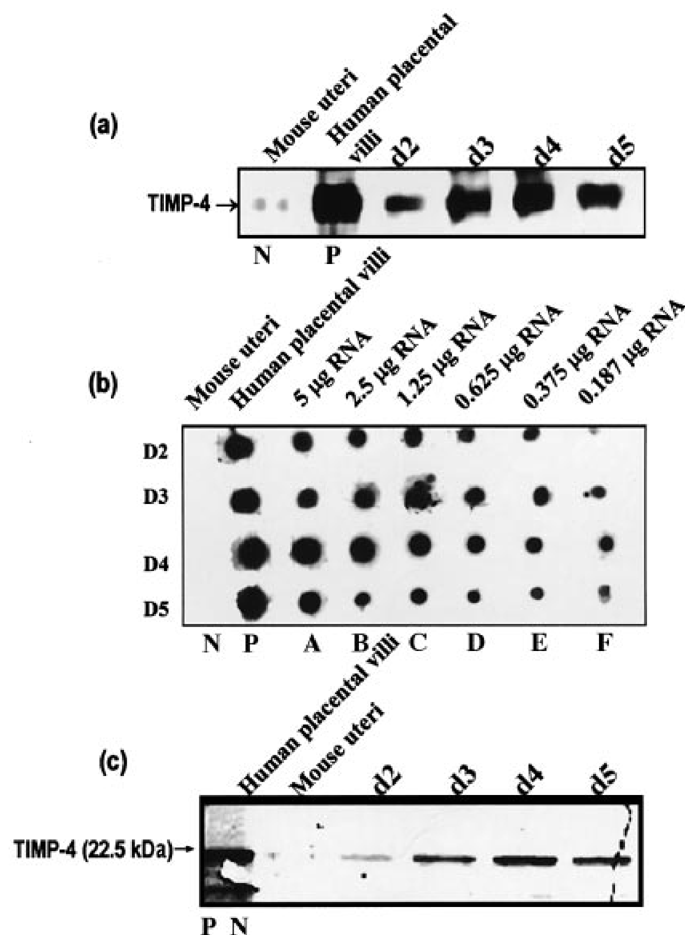

Figure 1. Expression of TIMP-4 in mouse uteri after ovulation. (**a**) Northern blot. (**b**) Dot hybridization. (**c**) Western blot. In (**a**) and (**b**), lane N: 5 μg uterine RNA of 12-week old virgin mice as a negative control; lane P: 5 μg human placental villi RNA as a positive control; lane d2, d3, d4, d5: day 2, day 3, day 4, day 5 after ovulation respectively in pregnant mice. In (**c**), 10 μg human placental villi (P) as positive control, 10 μg uterine protein of 12-week old virgin mice as a negative control; lanes A–F: mouse uterine total RNA concentration from 5, 2.5, 1.25, 0.625, 0.375 and 0.187 μg respectively.

Academy of Sciences, and raised at 25°C in a constant photoperiod (14 h light:10 h dark cycle). The Guidelines for the Care and Use of Animals in Research were followed. They were allowed free access to water and food. Virgin female mice were mated with fertile males of the same strain. The morning of finding a virginal plug was designated as day 1 of pregnancy.

Northern blot and dot hybridization analysis

Uteri from day 2 (day 2) to day 5 (day 5) mice were homogenized in a TRIzol solution (Gibco-BRL) and total RNA was isolated. The total RNA was quantified by absorbance at 260 nm and by ethidium bromide staining after electrophoresis of agarose gels. RNA was cross-linked to Hybond-N membranes by UV irradiation (0.12 J/cm^2) and was subjected to Northern blot or dot hybridization to detect TIMP-4 mRNA expression.

The TIMP-4 plasmid was kindly provided by Dr Kevin Leco (Department of Pharmacology and Therapeutics, University of Calgary, Calgary, Canada). The TIMP-4 probes were labelled using a Random Primer DNA labelling System (Gibco–BRL, Gaithersburg, MD, USA) in the presence of [^{32}P]dCTP and then purified using Nick Columns according to the manufacturer's instructions.

As for the dot hybridization, RNA was dotted onto the nylon membrane as detailed in the legend to Figure 1. Northern blot analysis was performed as previously described (Zhang *et al*., 2002). Subsequently, membranes were incubated in prehybridization buffer [1 mol/l NaCl, 50 mmol/l, Tris, 2.2 mmol/l sodium pyrophosphate, 10 g/l sodium dodecyl sulphate (SDS), 1×Denhardt's reagent, 10 mg/l denatured salmon sperm DNA, pH 7.5] for 3 h at 65°C. Labelled probe (25 ng, specific activity ~1 Ci/mg), denatured by placing in boiling water for 5 min and then snap-cooling on ice, was added and incubated overnight at 65°C. After hybridization, the membranes were washed twice in wash buffer 1 [1 g/l SDS, 2× standard saline citrate (SSC)] for 15 min at 25°C, then in wash buffer 2 (1 g/l SDS, 0.2× SSC) at 65°C. After autoradiography at –70°C for 3 days, the X-ray films were developed.

In-situ hybridization

In-situ hybridization was performed as previously described (Wang *et al*., 2001). Briefly, embryos were treated with proteinase K, prehybridized, and hybridized overnight with digoxigenin-labelled antisense transcripts from a TIMP-4 cDNA. The TIMP-4 antisense probe was a 600 bp fragment from nucleic acid bases 13 to 612. After hybridization, RNase treatment and three stringent washes were performed. Sections were incubated with mouse antidigoxigenin antibodies (Boehringer, Mannheim, Germany), followed by incubation with biotin-conjugated secondary goat anti-mouse antibodies (Dako, Hamburg, Germany). The colorimetric detection was performed using a standard, indirect streptavidin–biotin immunoreaction method with a Dako universal labelled-streptavidin-biotin (LSAB) kit according to the manufacturer's instructions. Parallel experiments were performed using a sense probe as negative control.

Immunocytochemistry

Specific polyclonal antibodies against TIMP-4 were produced and characterized as discussed in our previous reports (Liu *et al*., 1997; Hurst *et al*., 2001; Zhang *et al*., 2002). The final concentration of purified IgG used in the experiments was 10 μg/ml. Embryos were cultured in chamber slides. After 48 h in culture, embryos were fixed for 30 min in freshly prepared 4% paraformaldehyde (Sigma, St Louis, MO, USA) containing 0.2% Triton X-100. After rinsing several times in 0.01 mol/l phosphate-buffered saline (PBS; pH 7.4), embryos were incubated in 5% bovine serum albumin (BSA) for 45 min at room temperature to block non-specific binding of the antibodies. The BSA solution was then aspirated with filter paper, and embryos were incubated with the primary antibody against TIMP-4 diluted 1:100 in PBS at 4°C overnight. After rinsing in PBS, embryos were incubated in fluoroscein isothiocyanate-conjugated secondary antibody (Boehringer, Mannheim, Germany) at 37°C for 1 h, then rinsed in PBS. Finally, embryos were viewed under a fluorescent microscope (Leica, Heidelberg, Germany). Parallel experiments were performed with embryos using the preimmune IgG of the same rabbit as a negative control.

Western blot analysis

Proteins obtained from mouse uteri, embryos and conditioned media from cultured embryos were subjected to Western blot analysis to detect TIMP-4, MMP-2, MMP-9 and $\alpha_v\beta_3$ integrin expression using specific polyclonal antibodies (TIMP-4 antibody from our laboratory, MMP-2, MMP-9 and $\alpha_v\beta_3$ integrin antibodies from Gibco). Briefly, proteins obtained from cell lysates were boiled in SDS/β-mercaptoethanol sample buffer and 10 μg of protein samples were loaded onto each lane of 12% polyacylamide electrophoretic gels. The proteins were separated by electrophoresis and transferred onto nylon membranes in 25 mmol/l Tris, 192 mmol/l glycine buffer, pH 8.3. The membranes were blocked in 10% non-fat milk for 1 h. Primary antibodies were diluted 1:100 (to a final concentration of 0.5 μg/ml) in TTBS (30 mmol/l Tris, pH 7.4, 150 mmol/l NaCl, 0.1% Tween-20). After incubation with the primary antibodies overnight at 4°C, the blots were washed 4×15 min in TTBS, then incubated for 1 h in goat anti-rabbit IgG (Promega Corp., Madion, WI, USA) diluted 1:500 in TTBS. The blots were washed 4×15 min in TTBS and 2×15 min in TBS. The bands were visualized using the enhanced chemiluminescence method.

In-vitro attachment and outgrowth assays

Blastocysts were collected from mated, superovulated mice (96 h after 7.5 IU pregnant mare's serum gonadotrophin and 5 IU hCG administration).

Blastocysts were cultured in Ham's F-12 medium (Gibco-BRL, Gaithersburg, MD, USA) as reported by Zhang *et al*. (2001). Blastocysts that

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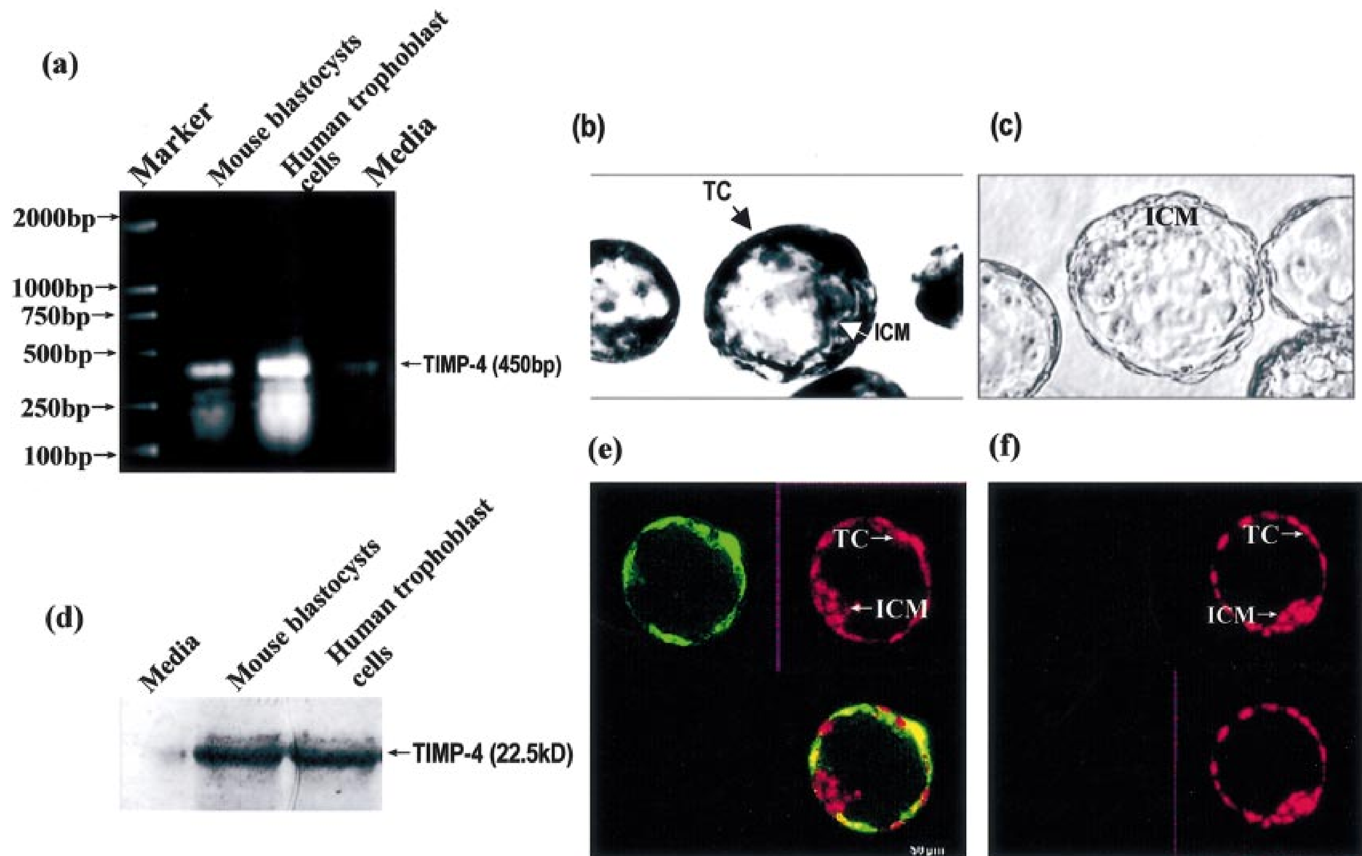

Figure 2. Expression of TIMP-4 mRNA and protein in mouse blastocyst. (**a**) RT–PCR. (**b**) In-situ hybridization with antisense probe. Black staining indicates positive signal. (**c**) In-situ hybridization with sense probe. (**d**) Western blot. (**e**) Immunofluorescence with TIMP-4 antibody. The green colour represents TIMP-4 protein staining and the red colour indicates nucleus staining. The yellow colour represents the overlap of green and red. Note that positive signals of TIMP-4 were observed in the cytoplasm of trophoblast cells. (**f**) Immunofluorescence with preimmune IgG. Lanes: human trophoblast cells as a positive control; culture media as negative control. ICM = inner cell mass; TC = trophoblast cells. Scale bar = 50 µm.

developed consistently were selected and ready to be studied after 24 h preculture in culture medium. Culture dishes were precoated with 10 µl fibronectin (1 µg/µl; Sigma). Fifty blastocysts were cultured in droplets of conditioned medium under mineral oil and incubated at 37°C, in 5% CO_2 in a humidified chamber. Control group (C): F-12 with 50 µg/ml preimmune IgG; A1, A2 and A3 groups were treated with 10, 50 and 100 µg/ml TIMP-4 antibody respectively. All experiments were repeated more than three times.

The attachment or outgrowth was observed at 24 h or 48 h after culture using phase-contrast microscopy. The blastocyst was designated as 'attachment', if it had remained in its original position. If any movement was detected, the blastocyst was designated as 'non-attachment'. After attachment, the blastocyst began to grow outwards. When primary giant trophoblast cells were visible around the attachment site of the attached blastocysts, we designated the blastocyst as 'outgrowth'.

Two indexes were adopted to measure the invasive capacity of blastocysts on fibronectin-precoated dishes: (i) percentage of embryos attaching, which is the ratio of embryos attached to fibronectin versus the total number of embryos hatched; (ii) percentage of blastocysts with outgrowth, which is the ratio of embryos with primary giant trophoblast cells versus the total number of embryos attatched.

RT–PCR

Total RNA isolated from embryos cultured in F-12 media was subjected to RT–PCR using mouse TIMP-4-specific primer (Leco *et al*., 1997) (sense primer: 5′-ACTGGGATCCGTCATCCGAGCCAAA; anti-sense primer: 5′-GCATAAGCTTCCATCCACAAGCAGTG) to detect TIMP-4 mRNA expression. Secondly, RNA isolated from embryos cultured in different media was subjected to semi-quantitative RT–PCR to detect MMP-2, MMP-9 and α_v integrin mRNA expression. The gene-specific primers of MMP-2 and MMP-9 used for amplification by the PCR were synthesized according to Bany *et al*. (2000). MMP-2 sense primer: 5′-CACCTACACCAAGAACTTCC-3′; MMP-2 antisense primer: 5′-AACACAGCCTTCTCCTCCTG-3′; the estimated fragment is 332 bp. MMP-9 sense primer: 5′-TTGAGTCCGGCAGACAATCC-3′; MMP-9 antisense primer: 5′-CCTTATCCACGCGAATGACG-3′; the estimated fragment is 433 bp. α_v Integrin primers: sense primer: 5′-CCGCCGGTGCCAGCCCATTGAG-3′; anti-sense primer: 5′-GCTACCA-GGACCACCGAGAAGT-3′; the estimated fragment is 337 bp (Illera *et al*., 2000). β-Actin sense primer: 5′-GTGGGGCGCCCCAGGCACCA-3′; β-actin antisense primer: 5′-CTTCCTTATTGTCACGCACGATTTC-3′; the estimated fragment is 540 bp.

RNA was reverse-transcribed (RT) by oligo $(dT)_{15}$ priming and AMV reverse transcriptase (Promega Corp.). PCR amplification was carried out on 5 µl of the RT product (~20 ng RNA). PCR cycles were as follows: 95°C for 5 min followed by 35 cycles for cell samples of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min. PCR products (10 µl) were visualized under UV light on 1% agarose gels containing 1 µg/ml ethidium bromide.

Gelatin zymography

The activity of MMP-2 or -9 in different media was demonstrated by gelatin zymography. The different harvested culture media (C, A1, A2 and A3) were cultured for 48 h respectively, and were standardized according to the protein content of the supernatant, which was measured according to the method of Bradford (1976). Thus, 10–20 µl medium, equivalent to 6 µg protein of cell lysates, was loaded into each lane for zymography. The medium was mixed 5:1 (v:v) with sample buffer (0.3 mol/l Tris–HCl, pH 6.8, 0.15% phenolphthalein, 30% glycerol, 10% SDS) and then applied to gels for electrophoresis without boiling under non-reducing conditions in 12.5% acrylamide gels containing 1 mg/ml gelatin (Sigma). After electrophoresis, the gels were washed at room

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temperature for 1 h in 2.5% Triton X-100, 50 mmol/l Tris–HCl, at pH 7.5, to remove SDS, and were then incubated overnight in buffer (150 mmol/l NaCl, 5 mmol/l $CaCl_2$, and 50 mmol/l Tris–HCl, pH 7.6) at 37°C. Thereafter, gels were stained with 0.1% (w:v) Coomassie Brilliant Blue R-250 (Gibco-BRL) in 30% (v:v) isopropyl alcohol, 10% glacial acetic acid for 60 min and destained in 10% (v:v) methanol, 5% (v:v) glacial acetic acid. The gelatinolytic activities were detected as clear bands on a uniform blue background.

Statistics

Scheffé's correction of analysis of variance was performed for multiple comparisons. Data represent the mean ± SD from three experiments, and differences with $P \leqslant 0.05$ were considered significant.

Results

Expression of TIMP-4 mRNA and protein in mouse uteri after ovulation

Northern blot, dot hybridization and Western blot were used to detect TIMP-4 mRNA and protein expression in pregnant mouse uteri from day 2 to day 5 after ovulation. Our results showed that expression of a 450 bp TIMP-4 mRNA and a 22.5 kDa protein were weak in uteri of day 2. The expression levels of TIMP-4 mRNA were higher in day 3 to day 5 uteri, reaching their highest level at day 4 (Figure 1). Neither TIMP-4 mRNA or protein expression were not detected in the uterus of virgin mice (Figure 1).

Expression of TIMP-4 mRNA and protein in mouse embryo

A 450 bp TIMP-4 band was detected in mouse embryo by RT–PCR (Figure 2a). The PCR product was sequenced to confirm that it was the TIMP-4 gene (data not shown). In-situ hybridization showed that TIMP-4 mRNA was localized in the trophoblast cells, but not in the inner cell mass of mouse embryos (Figure 2b). In addition, Western blot analysis showed a 22.5 kDa band in the embryo lysates (Figure 2d). Immunohistochemistry results revealed that TIMP-4 protein was in the cytoplasm of trophoblast cells, not the inner cell mass (Figure 2e). TIMP-4 protein localization was coincident with its mRNA expression.

Effects of anti-TIMP-4 IgG on the adhesion and outgrowth of mouse blastocysts

To test the hypothesis that TIMP-4 is involved in the attachment and outgrowth of the mouse blastocyts, in-vitro attachment and outgrowth assays were performed using an anti-TIMP-4 antibody. The results showed that after 24 h of culture, the percentage of embryos attaching did not change, regardless of the dose of TIMP-4 antibody (Figure 3). However, the presence of TIMP-4 antibody increased the percentage of blastocysts showing outgrowth after 48 h culture at 50 and 100 µg/ml ($P < 0.05$ and $P < 0.01$ respectively).

Anti-TIMP-4 IgG up-regulates the mRNA and protein expression of MMP-2, MMP-9, and their activities

RNA isolated from mouse embryos cultured in the presence of anti-TIMP-4 IgG was subjected to RT–PCR and Northern blot to detect levels of MMP-2, MMP-9 and α_v integrin mRNA expression. Our results show that every dose of anti-TIMP-4 IgG tested increased the mRNA expression of MMP-2 and MMP-9, but had no effect on α_v integrin mRNA expression (Figure 4a,b). There was a positive correlation between the mRNA expression of MMP-2 and the dosage of TIMP-4 antibody used.

Protein obtained from these mouse embryos was subjected to Western blot analysis to detect levels of MMP-2, -9 and $\alpha_v\beta_3$ integrin protein expression. All doses of anti-TIMP-4 IgG tested increased the protein expression of MMP-2 and MMP-9 (Figure 4c,d), but had no effect on the protein expression of $\alpha_v\beta_3$ integrin. There was a positive correlation between the protein expression of MMP-2 and the dosage of TIMP-4 antibody used.

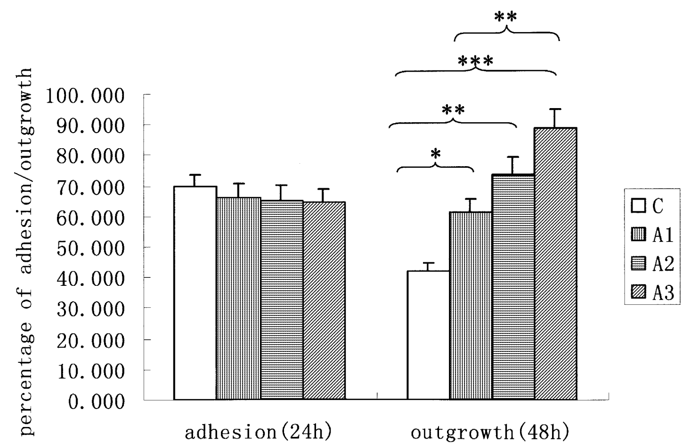

Figure 3. Effects of TIMP-4 antibody on the adhesion and outgrowth of mouse blastocysts. C, control groups. A1, A2 and A3: treated with 10, 50 and 100 µg/ml of anti-TIMP-4 IgG, respectively. $*P < 0.05$, $**P < 0.01$. 24 h and 48 h refers to time of culture.

The activity of MMP-2 or MMP-9 in different media was determined by zymography. All doses of anti-TIMP-4 IgG tested increased the activity of MMP-2 and MMP-9 (Figure 4e,f). The activity of active MMP-2 was increased significantly. At the same time, there was a positive correlation between the activity of MMP-2 and dosage of TIMP-4 antibody used. Hence, we conclude that the stimulatory effect on MMP-2 activity by TIMP-4 antibodies is dose-dependent. In addition, all doses of TIMP-4 antibody moderately increased the activity of MMP-9.

Discussion

Mouse embryo implantation is a highly regulated process with cooperation by different factors. Numerous studies in mice (Waterhouse *et al*., 1993), primates (Blankenship *et al*., 1994) and humans (Hurskainen *et al*., 1996) have shown that MMP and TIMP are key regulators of blastocyst implantation. Generally, MMP promote invasion by trophoblast cells, while TIMP hinder it.

To date, 25 different MMP have been identified (Sternlicht *et al*., 2001). Previous studies have shown that, in the mouse uterus, during the peri-implantation period, MMP-2 participates in the early phase of decidualization and neovascularization required for placentation (Alexander *et al*., 1996). MMP-9 is expressed in stromal cells on day 5, and in trophoblast giant cells on day 8, and this is coupled with the expression of TIMP-3 in the stroma surrounding the embryo, so MMP-9 and TIMP-3 may act in concert to regulate trophoblast invasion in the mouse uterus (Das *et al*., 1997). Four mammalian TIMP have been characterized biochemically, and the expressions and physiological roles of TIMP-1, -2, and -3 during implantation have been reported. Zhao *et al*. reported that TIMP-1, -2 and -3 mRNAs were highly expressed in the antimesometrium primary decidual zone (pdz) surrounding the implanting embryo on day 6, and in whole decidualized stromal cells at the implantation site on day 7, which is similar to that of MMP-2 and -9 (Zhao *et al*., 2002). The implanting rat embryo induced the similar and simultaneous expression for MMP-2, -9, TIMP-1, -2, and -3 mRNA in the decidualized stromal cells at the implantation sites, indicating that TIMP acted as major inhibitors to regulate the extent of rat embryo invasion. Here we investigate the

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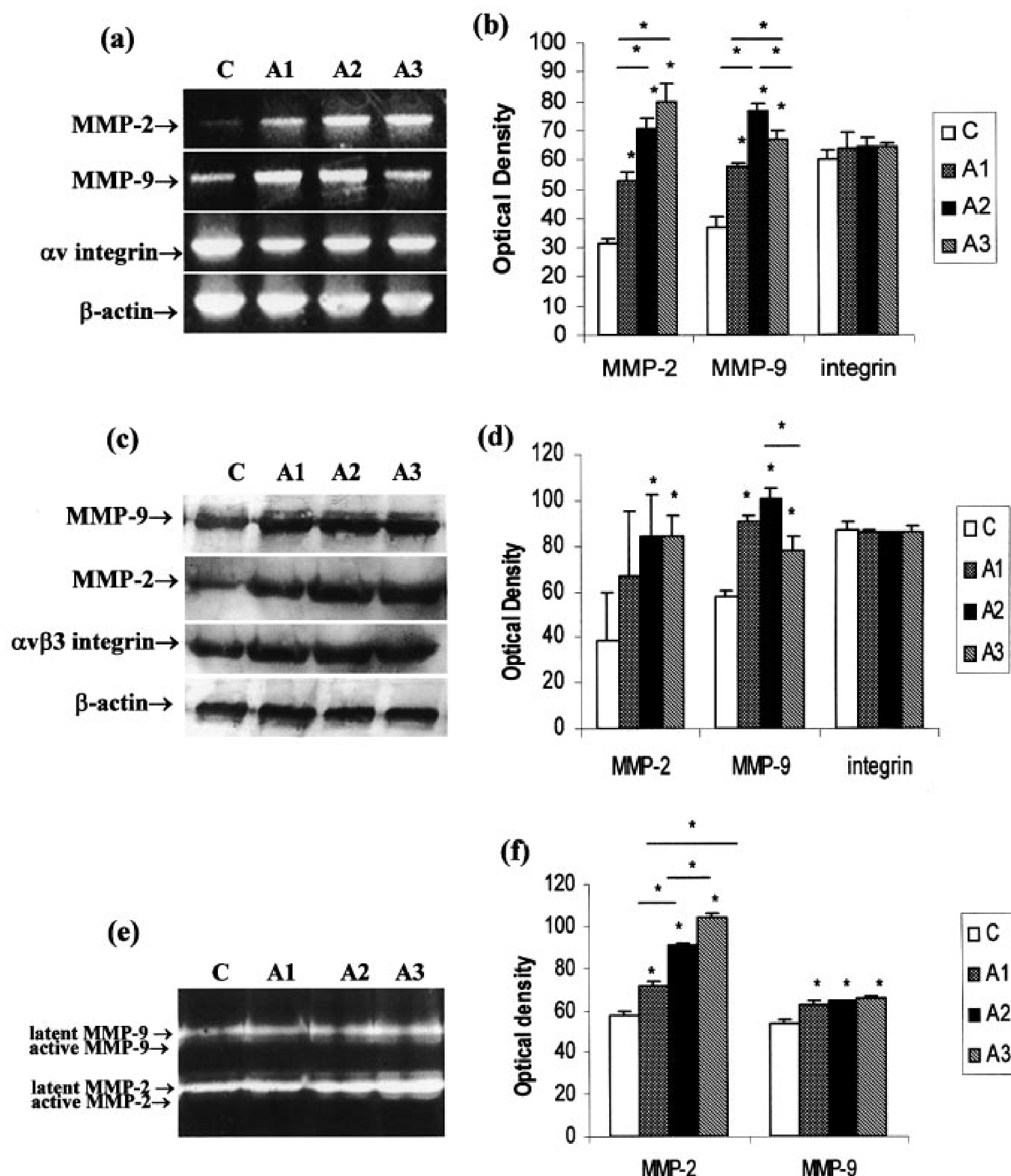

Figure 4. Effects of TIMP-4 antibody on MMP-9 and MMP-2 mRNA, protein and activity in mouse blastocysts. (**a**) RT–PCR of MMP-2, MMP-9 and αv integrin in mouse blastocysts. (**b**) Analysis of RT–PCR of MMP-2, MMP-9 and αv integrin in mouse blastocysts. (**c**) Western blot of MMP-2, MMP-9 and αv integrin in mouse blastocysts. (**d**) Analysis of Western blot of MMP-2, MMP-9 and integrin in mouse blastocysts. (**e**) Gelatin zymography of MMP-2 and MMP-9 in conditioned media from mouse blastocysts. (**f**) Analysis of activity of MMP-2 and MMP-9 in conditioned media from mouse blastocysts. 10 μg protein from conditioned media from mouse blastocysts were analysed for gelatinase activity in the presence of 5 mmol/l Ca^{2+}. Lane C: treated with 50 μg/ml preimmuno IgG as controls. Lanes A1, A2 and A3: treated with 10, 50 and 100 μg/ml of TIMP-4 antibody respectively. $^*P < 0.05$.

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expression of TIMP-4 and the relationship between MMP-2, -9 and TIMP-4 in developing mouse embryo.

Protein and mRNA expression of TIMP-4, and the mRNA expression and activities of MMP-2 and -9 after treatment with anti-TIMP-4 IgG in the mouse embryo are examined here for the first time. We detected strong TIMP-4 mRNA and protein expression in pregnant mice uteri at day 4 and day 5 after ovulation (implantation 'window'), but not in the uterus of virgin mice, suggesting that TIMP-4 may be involved in the regulation of mouse embryo implantation. TIMP-4 protein and mRNA were detected in the trophoblast cells by immunocytochemistry, in-situ hybridization, RT–PCR and Western blot analysis.

We hypothesize that the TIMP-4 antibody may block TIMP-4 anti-MMP function *in vitro* based upon the results that anti-TIMP-4 IgG could increase the percentage of blastocysts with outgrowth, but not adhesion, in a dose-dependent manner (Figure 3). These results suggest that neutralization of TIMP-4 activity with a specific antibody may promote blastocyst outgrowth.

We further hypothesized that the TIMP-4 antibody may also affect the mRNA and enzyme activity levels of MMP-2 and -9, which is considered to be the major contributor to cell invasion. Western blot analysis, gelatin zymography, semi-quantitative RT–PCR and Northern blot analysis showed that anti-TIMP-4 IgG could increase the protein levels, mRNA levels, and the enzymatic activities of

MMP-2 and -9. However, there was no effect on the expression of $\alpha_v\beta_3$ integrin, which is an important cell adhesion molecule that plays a pivotal role in mouse embryo implantation by regulating cell–cell and cell–matrix interaction. These results indicate that anti-TIMP-4 IgG may not only neutralize the TIMP-4 anti-MMP activity, but may also indirectly up-regulate MMP-2 and MMP-9 activities via unknown mechanisms.

Pro-MMP-2 is activated by membrane type-1 (MT1)-MMP, and previous studies have shown that TIMP-4, but not TIMP-1, blocks MT1-MMP-mediated progelatinase-A (MMP-2) activation in human umbilical vein endothelial cells (Bigg *et al*., 2001). In addition, thrombin, a critical enzyme in the coagulation cascade, has also been associated with angiogenesis and activation of the zymogen form of MMP-2 (gelatinase-A) (Lafleur *et al*., 2001). Thrombin inefficiently cleaved recombinant 72 kDa pro-MMP-2, but efficiently cleaved the 64 kDa MT1-MMP-processed intermediate form. Thrombin also rapidly (within 1 h) increased cellular MT1-MMP activity, and at longer time points (>6 h) it increased expression of MT1-MMP mRNA and protein. However, the effects of thrombin were blocked by tissue inhibitor of metalloproteinase-2 (TIMP)-2 and TIMP-4, but not TIMP-1 (Lafleur *et al*., 2001). Therefore we speculated that TIMP-4 might inhibit the activation pathway of pro-MMP-2 by blocking the effect of MT1-MMP or thrombin during mouse embryo implantation.

Our previous studies show that the IC_{50} values of TIMP-4 against six major MMP, MMP-1, -2, -3, -7, -9 and -26, were 19, 3, 45, 8, 83 and 0.4 nmol/l respectively (Liu *et al*., 1997; Zhang *et al*., 2002). TIMP-4 is therefore a physiological inhibitor of MMP in the ECM metabolism. Anti-TIMP-4 IgG blocked the effects of TIMP-4 by limiting embryo outgrowth and decreasing the inhibition of MMP-2 and -9. Hence the ability of trophoblast cells to invade into the ECM is increased as the activity of MMP-2 and -9 are increased.

In summary, this study suggests that TIMP-4 might play a role in the tissue-remodelling processes associated with embryo implantation by inhibiting the activity of MMP-2 and -9. It also suggests that anti-TIMP-4 antibodies may indirectly regulate MMP-2 and -9 gene transcription levels. The net effect of TIMP-4 may be the restriction of the uncontrolled growth of embryos.

Acknowledgements

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