Reduced expression of tissue inhibitor of metalloproteinase (TIMP)-2 in gestational trophoblastic diseases

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To elucidate the involvement of type IV collagenases [matrix metalloproteinase (MMP)-2 and MMP-9] and their tissue inhibitors (TIMP-1 and TIMP-2) in the development of gestational trophoblastic disease (GTD), we quantified their levels in hydatidiform mole and choriocarcinoma tissues using specific enzyme-linked immunosorbent assays, and the results were compared with those from normal first trimester placenta. Levels of pro-MMP-2 were increased in hydatidiform mole, and they were further elevated in choriocarcinoma. Levels of pro-MMP-9 in choriocarcinoma and those of TIMP-1 in both hydatidiform mole and choriocarcinoma were also increased. In contrast, TIMP-2 levels were markedly decreased in both hydatidiform mole and choriocarcinoma. Similar results were obtained by the tissue culture of first trimester placenta and hydatidiform mole. Gelatin zymography indicated that the levels of both pro- and activated forms of MMP-2 and MMP-9 were higher in hydatidiform mole and choriocarcinoma. The decreased expression of TIMP-2 in hydatidiform mole and choriocarcinoma was confirmed by Western blot, Northern blot and immunohistochemistry, with the decrease being more pronounced in choriocarcinoma. Taken together, the present study shows that both TIMP-2 mRNA and protein levels are markedly decreased in GTD and the imbalance of MMP–TIMP production, shifted toward greater MMP activity, may be involved in the pathogenesis of GTD.

Key words: choriocarcinoma/hydatidiform mole/MMP/TIMP/trophoblastic disease

Introduction

Placental development requires a series of complex, coordinated interactions involving highly specialized fetal and maternal cells. The trophoblast plays a pivotal role in this process, which is characterized by the breaching of multiple basement membranes (including those of the endometrial epithelium, glands, blood vessels and decidual cells) and the degradation of the interstitial extracellular matrix of the endometrium (Lala and Graham, 1990). This invasive activity peaks around week 12 of pregnancy and declines rapidly thereafter.

The invasive growth of the trophoblast is similar to that of malignant tumours in many respects. The phenotypic change from carcinoma in situ to invasive carcinoma occurs when tumour cells acquire the ability to penetrate an epithelium basement membrane and invade the underlying stroma (Liotta et al., 1986). Unlike tumour invasion, however, trophoblast invasion is precisely regulated, confined spatially to the uterus and temporally to early pregnancy. In gestational trophoblastic diseases (GTD), this control mechanism seems to be impaired and an abnormal proliferation of the trophoblast occurs with varying propensities for local myometrial invasion and distant metastases.

Matrix metalloproteinases (MMPs) are a group of enzymes involved in matrix degradation. MMPs are grouped by their ability to degrade different types of extracellular matrix components, including collagen. Type IV collagen, which is a major component of the basement membrane and constitutes an important barrier to tumour cell invasion, is degraded by both MMP-2 (72 kDa, gelatinase-A) and MMP-9 (92 kDa, gelatinase-B). Several studies have documented the key roles of MMP-2 and -9 in the high invasive capacity of first trimester trophoblast (Librach et al., 1991; Polette et al., 1994; Shimonovitz et al., 1994; Hurskainen et al., 1996; Huppertz et al., 1998). Tissue inhibitors of metalloproteinases (TIMPs) bind 1:1 to MMPs to specifically inhibit their activities (Mignatti and Rifkin, 1993) and it has been suggested that successful implantation and placentation could result from a balance between MMPs and TIMPs (Lala and Graham, 1990; Graham and Lala, 1992). TIMP-1 inhibits the active form of all MMPs and the latent form of MMP-9 (pro-MMP-9) and is the most widely distributed TIMP (Goldberg
et al., 1992). TIMP-2 binds to both forms of MMP-2, while its inhibitory effect over the other MMPs is significantly lower (Stetler-Stevenson et al., 1989).

In our previous report, we demonstrated that active secretion of MMP-2 and -9 from villous tissue in the first trimester and constant production of TIMPs (especially TIMP-2) throughout the gestational period are characteristic of placentation development (Niu et al., 2000). Concerning GTD, however, there are limited data describing MMPs and TIMPs using clinical tissue specimens. In the present study, we performed quantitative analysis of MMP-2, MMP-9, TIMP-1 and TIMP-2 to compare their levels in the tissues of GTD with those in first trimester placenta, and we found that TIMP-2 expression was markedly reduced in GTD.

Materials and methods

Tissue collection and preparation

Normal placental tissues were obtained from 15 women undergoing elective termination of pregnancy at gestational weeks 9–14. All pregnancies were assessed to be normal based on clinical history and gestational age was confirmed by ultrasound measurement of crown–rump length or biparietal diameter. Ultrasonographically and histologically verified complete hydatidiform molar tissue samples were obtained from 28 patients at 9–14 weeks gestation (19 patients had subsequent spontaneous remission and nine patients developed persistent GTD). Tissue specimens were also obtained from five patients with gestational choriocarcinoma with histological confirmation, and from two patients with invasive hydatidiform mole who underwent a hysterectomy, all of whom received no prior chemotherapy. Tissue samples were also obtained from two patients with gestational choriocarcinoma with histological confirmation, and from two patients with invasive hydatidiform mole who underwent a hysterectomy, all of whom received no prior chemotherapy. Tissue samples were collected immediately after delivery or underwent a hysterectomy, all of whom received no prior chemotherapy. Tissue samples were also obtained from two patients with gestational choriocarcinoma with histological confirmation, and from two patients with invasive hydatidiform mole who underwent a hysterectomy, all of whom received no prior chemotherapy.

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Quantification of MMP-2, MMP-9, TIMP-1 and TIMP-2

Concentrations of MMP-2, MMP-9, TIMP-1 and TIMP-2 in the tissue homogenates and the culture media were measured by the corresponding enzyme-linked immunosorbent assay (ELISA) systems obtained from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). The MMP-2 assay detects both the free form of pro-MMP-2 and the complexed form of pro-MMP-2 with TIMP-2, but not active MMP-2. The MMP-9 assay detects the free form of pro-MMP-9 and the complexed form of pro-MMP-9 with TIMP-1. The TIMP-1 assay measures free TIMP-1 and that complexed with MMPs. The TIMP-2 assay detects free TIMP-2 and the complexed form of TIMP-2 with active MMPs, but not the complexed form with pro-MMP-2. All assays were performed in duplicate.

Gelatin zymography

Analysis of MMP-2 and MMP-9 in the tissue homogenates was performed using sodium dodecyl sulphate (SDS)–polyacrylamide gel zymography. Samples (20 μg of proteins for each lane) were electrohoresed on SDS–polyacrylamide gels containing 0.3% gelatin. Following electrophoresis, gels were washed three times for 30 min in 2.5% Triton X-100 to remove SDS. After overnight incubation in reaction buffer (50 mmol/l Tris–HCl, pH 7.4, containing 5 mmol/l CaCl₂ and 0.02% NaN₃), gels were stained with Coomassie Brilliant Blue and destained in 20% methanol and 10% acetic acid. Proteinase activity was observed as a clear band of digested gelatin.

Western blot analysis of TIMP-2

Expression of TIMP-2 in the tissue homogenates was analysed by Western blot. Samples (50 μg of proteins for each lane) were separated on 12.5% SDS–polyacrylamide gels and proteins were transferred to the nitrocellulose transfer membranes (Amersham Pharmacia Biotech) using a semi-dry electrophoretic transfer cell system (TRANS-BLOT® SD BioRad; BioRad, Richmond, CA, USA). Monoclonal mouse anti-TIMP-2 primary antibody (Fuji Chemical Industries, Takaoka, Toyama, Japan) was used at a 1:500 concentration diluted in T–PBS. After 1 h incubation with the primary antibody, membranes were rinsed, exposed for 1 h to a 1:5000 dilution of horseradish peroxidase-linked anti-mouse antibody (Jackson Immuno-Research Lab. Inc., West Grove, PA, USA). Positive bands were visualized by enhanced chemiluminescence (ECL; Amersham) and exposure to Fuji film. Recombinant hTIMP-2 (Fuji Chemical Industries) was used as the positive control.

Northern blot analysis of TIMP-2

Total RNA was isolated from the frozen tissues by the guanidium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987). RNA was quantified by spectrophotometry at 260 nm and integrity was checked on 1% agarose gel. Total RNA samples (15–25 µg) were denatured by heating at 65°C for 15 min in buffer containing 50 mmol/l Sodium dodecyl sulphate (SDS)–4-morpholinepropanesulphonic acid (MOPS) and 17% formaldehyde. RNAs were fractionated on 1% agarose gel containing 2.2 mol/l 4-morpholinepropanesulphonic acid (MOPS) and 17% formaldehyde. RNAs were fractionated on 1% agarose gel containing 2.2 mol/l formaldehyde, blotted onto nylon membranes (Hybond-N; Amersham) by capillary transfer and fixed under a 150 mJoule UV cross-linker (GS Gene Linker; BioRad). The cDNA probe for TIMP-2 was a 585 bp fragment containing the sequence between nucleotides 349–933 of the coding region of human TIMP-2 cDNA (Stetler-Stevenson et al., 1990), which was generated by means of PCR amplification of poly(A)⁺ RNA from a week 7 human placenta, and labelled with [α-³²P]dCTP (Amersham). Hybridization was performed at 42°C for 18 h in the buffer composed of 5× saline–sodium phosphate–EDTA (SSPE), 5×Denhardt’s solution, 0.5% SDS, 50% deionized formamide and 200 ng/ml denatured salmon sperm DNA. The membranes were then washed in 2×SSPE–0.1% SDS for 10 min at 42°C, twice in the same buffer for 10 min at 55°C and 0.1×SSPE–0.1% SDS for 50 min at 55°C. The washed membranes were exposed to X-ray film (Kodak X-Omat AR5) with intensifying screens for 2 days at ~80°C. The membranes were subsequently reprobed with TIMP-2 expression in gestational trophoblastic diseases
a 32P-labelled human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA fragment to check the amount of total RNA present on the membranes.

**Immunohistochemistry of TIMP-2**

Formalin-fixed, paraffin-embedded tissue sections were cut 4 µm thick, deparaffinized in xylene, and dehydrated through graded ethanol series. Endogenous peroxidase activity was blocked by a 5 min treatment with 3% hydrogen peroxide. Sections were preincubated with 10% normal rabbit serum to minimize non-specific staining. The slides were then incubated with the primary anti-TIMP-2 mouse monoclonal antibody (Fuji Chemical Industries) at 4°C overnight, washed with PBS and incubated with biotinylated rabbit anti-mouse immunoglobulin G (10 µg/ml; Nichirei, Tokyo, Japan) at room temperature for 30 min. After washing with PBS, the slides were incubated with streptavidin–biotin–peroxidase complex (100 µg/ml; Nichirei). Diaminobenzidine was used as a chromogen for colour development. The slides were counterstained with haematoxylin. Negative control staining was performed using the primary antibody pretreated with an excess of recombinant TIMP-2.

**Statistical analysis**

Kruskal–Wallis analysis of variance was used to determine statistical significance of differences of MMP and TIMP levels among the groups, with Scheffé’s F-test used for between-group comparisons. Findings of $P < 0.05$ were considered significant.

**Results**

**Protease levels in the tissue homogenates**

Levels of pro-MMP-2, pro-MMP-9, TIMP-1, and TIMP-2 were determined and compared in the tissue homogenates of first trimester placenta, hydatidiform mole with subsequent spontaneous resolution, hydatidiform mole with subsequent persistent GTD, and choriocarcinoma tissues (Figure 1). An increase in pro-MMP-2 levels was observed in hydatidiform mole compared with those in the first trimester placenta (~1.7-fold increase in the mean value), and they were further elevated in choriocarcinoma (8.5-fold increase). No significant difference was observed in pro-MMP-9 levels between the first trimester placenta and hydatidiform mole, but a significant increase was observed in choriocarcinoma (7.3-fold increase). TIMP-1 levels were increased both in hydatidiform mole and choriocarcinoma as compared with those in the first trimester placenta (2.2- and 4.6-fold increase respectively). In contrast, TIMP-2 levels were markedly decreased in both hydatidiform mole and choriocarcinoma as compared with those in the first trimester placenta (3.5- and 7.6-fold decrease respectively). No significant differences in these four proteases were observed between hydatidiform mole with subsequent spontaneous resolution and those with subsequent persistent GTD.

**Protease levels in the culture media**

To investigate the secretion potential of pro-MMP-2, pro-MMP-9, TIMP-1 and TIMP-2 by the molar tissue, the amounts of these proteases were measured in the culture media and were compared with those of the first trimester placenta (Figure 2). According to the time course study, the protease levels in the culture media increased until 2–4 h of incubation, after which they reached a plateau (data not shown). Thus, the incubation time was set at 4 h. Also, during this incubation period, alteration of the villous structure was not seen; neither outgrowth of the fibroblasts nor suspension of the less viable trophoblasts was observed, suggesting that this culture condition could reflect the in-vivo secretion potential. Levels of pro-MMP-2 secreted by the samples of molar tissue were significantly higher than those in the first trimester placenta (2.5-fold increase). Unlike pro-MMP-2, no significant difference in pro-MMP-9 levels was seen between the first trimester placenta and hydatidiform mole. Concerning TIMPs, TIMP-1 levels were not significantly different between the first trimester placenta and hydatidiform mole. In contrast, secreted TIMP-2 levels were dramatically decreased in hydatidiform mole (5.8-fold decrease). As with the tissue homogenates, no significant differences were observed between secretions of hydatidiform mole with subsequent spontaneous resolution and those with subsequent persistent GTD. Thus, the results of the organ culture seem to be consistent with the data obtained by the tissue homogenates.

**Zymography analysis of MMPs**

Gelatin zymography analysis for the tissue homogenates showed MMP-2 and MMP-9 proteins, and the active forms as
TIMP-2 expression in gestational trophoblastic diseases

Figure 2. Quantitative analysis of (A) pro-MMP-2, (B) pro-MMP-9, (C) TIMP-1 and (D) TIMP-2 in the culture media of the first trimester placenta (P), hydatidiform mole with subsequent spontaneous resolution (M), and hydatidiform mole with subsequent persistent GTD (PTD). The incubation was performed for 4 h. Bars indicate mean values. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 3. Gelatin zymography analysis of the tissue homogenates. Lane 1, week 7 placenta. Lane 2, week 9 placenta. Lane 3, week 11 placenta. Lane 4, hydatidiform mole with subsequent spontaneous resolution. Lane 5, hydatidiform mole with subsequent persistent GTD. Lanes 6 and 7, choriocarcinoma. The bands corresponding to pro-MMP-2 (72 kDa), active form of MMP-2 (%), pro-MMP-9 (92 kDa), and active form of MMP-9 (★) are indicated.

Western blot analysis of TIMP-2

To confirm a marked decrease in TIMP-2 levels of hydatidiform mole and choriocarcinoma, Western blot analysis was performed. As shown in Figure 4, an immunoreactive band (22 kDa) corresponding to TIMP-2 was identified in the samples of first trimester placenta, and its intensity was greatly decreased in hydatidiform mole and choriocarcinoma.

Northern blot analysis of TIMP-2

Two species of TIMP-2 mRNAs were detected by Northern blot analysis of total RNA of the tissues from the first trimester placenta, hydatidiform mole and choriocarcinoma (Figure 5).
The sizes of the mRNAs were estimated as ~3.5 and 1.0 kb, consistent with a previous report (Stetler-Stevenson et al., 1990). The levels of TIMP-2 mRNA were lower in hydatidiform mole than in the first trimester placenta, and they were further decreased in choriocarcinoma.

**Immunohistochemistry of TIMP-2**

In the first trimester placenta, prominent immunoreactivity for TIMP-2 was found in the syncytiotrophoblasts, and moderate immunoreactivity was seen in the cytotrophoblasts of the villi and the villous stromal cells (Figure 6A). Immunoreactivity was decreased in the extravillous trophoblasts. Less intense immunoreactivity was seen in hydatidiform mole as compared with that in the first trimester placenta (Figure 6B). In invasive hydatidiform mole, the syncytiotrophoblasts were moderately positive, and the cytotrophoblasts were scarcely positive for TIMP-2 (Figure 6C). No staining was seen in the uterine myometrium (data not shown). Similarly, the trophoblastic cells in choriocarcinoma were weakly positive for TIMP-2 and the uterine myometrium was negative (Figure 6D). Interestingly, strong immunoreactivity for TIMP-2 was observed in the stromal cells of the decidua from the first trimester placenta (Figure 6E). Semi-quantitative analysis of the staining intensity by two independent observers is summarized in Table I, showing that the staining intensity of TIMP-2 tended to be decreased in hydatidiform and choriocarcinoma as compared with that in the first trimester placenta.

**Discussion**

Although both normal and neoplastic cells produce MMPs and other proteinases, only malignant cells are invasive, and...
unregulated secretion of MMPs and TIMPs has been implicated in tumour invasion and metastasis. Therefore, it is likely that control of MMP activity by specific inhibitors such as TIMPs is a cause for the differential functioning of these enzymes in normal and neoplastic tissues. For example, uncontrolled secretion or constitutive activation of secreted MMPs with a concomitant decrease in TIMP production might be responsible for the abnormal proliferation of the trophoblast in GTD. In the present study, we tested this hypothesis by measuring levels of MMP-2, MMP-9, TIMP-1 and TIMP-2 in normal and neoplastic tissues. For example, uncontrolled secretion or constitutive activation of secreted MMPs with a concomitant decrease in TIMP production might be responsible for the abnormal proliferation of the trophoblast in GTD. In the present study, we tested this hypothesis by measuring levels of MMP-2, MMP-9, TIMP-1 and TIMP-2 in normal and neoplastic tissues.

One of the novel findings of the present study is that TIMP-2 levels were markedly reduced at both mRNA and protein levels in the tissues of GTD. In a qualitative study using immunohistochemistry by Vegh et al. (1996), MMP-2 expression was reported to be higher in hydatidiform mole and choriocarcinoma than in first trimester placenta (Vegh et al., 1999), consistent with the findings of the present study. However, to the best of our knowledge there have been no quantitative studies on the levels of MMPs and TIMPs using clinical tissue specimens of GTD. Expression of TIMP-2 has also not been examined. The present results demonstrate that the imbalance of MMP-2/TIMP-2 levels clearly distinguishes between normal placenta and GTD, choriocarcinoma in particular, and this disruption of the balance in favour of gelatinase activity may be a critical factor for trophoblast invasion.

Concerning MMP-9, no significant difference in its levels was observed between the first trimester placenta and hydatidiform mole. This may be due to the intrinsic characteristics of the first trimester trophoblast; it has been reported that first trimester trophoblasts secrete MMP-9 in larger amounts than MMP-2 (Shimonovitz et al., 1994; Niu et al., 2000). The importance of MMP-9 for trophoblast invasion has also been emphasized by other investigators (Librach et al., 1991; Morgan et al., 1998). However, MMP-9 levels were significantly elevated in choriocarcinoma. Therefore, although TIMP-1 levels were elevated in choriocarcinoma, MMP-9 may also contribute to increased invasiveness of trophoblastic cells in choriocarcinoma.

The immunohistochemical results of the present study showed that TIMP-2 was abundantly present in the stromal cells of first trimester decidua, although quantification of MMPs and TIMPs in the decidua was not performed. Decidual tissue is known to have a protective role against invasion of the trophoblast, and regulation by the factors derived from the decidua has been emphasized by several investigations (Graham and Lala, 1991; Librach et al., 1991; Bischof et al., 1998). In fact, the invasion of the first trimester trophoblast is considered to be under dual regulation—in an autocrine way by the factors derived from the trophoblast itself and in a paracrine way by the uterine factors (Bischof et al., 2000). Thus, in hydatidiform mole before uterine evacuation, although the TIMP-2 level is decreased as compared with that in the first trimester placenta, the presence of the decidual tissue is likely to contribute to preventing an excessive penetration of the trophoblast into the myometrium through paracrine factors such as TIMP-2. On the other hand, in invasive hydatidiform mole it is possible that the absence of the decidua, together with decreased TIMP-2 production by the trophoblast itself, permits trophoblastic penetration deep into the myometrium, occasionally resulting in uterine perforation. Regulation by paracrine factors, such as TIMP-2, derived from the decidua may also explain the clinical observation that most of the choriocarcinoma cases associated with term pregnancy become manifest after delivery, and there are cases of asymptomatic intraplacental choriocarcinoma detected incidentally on the examination of the third trimester placenta (Barghorn et al., 1998; Lele et al., 1999).

One of the important questions in the management of patients with hydatidiform mole is, ‘Which mole will progress to persistent GTD?’ Several substances such as telomerase (Bae and Kim, 1999; Cheung et al., 1999), epidermal growth factor receptor and c-erbB-3 (Tuncer et al., 1999), and mm23-H1 (Iwase et al., 2001) have been reported to be associated with the development of persistent GTD. In the present study, no significant difference in the levels of MMP-2, MMP-9, TIMP-1 or TIMP-2 was observed between hydatidiform moles with subsequent spontaneous remission and those that progressed to persistent GTD, indicating that these proteinases may not be good predictors for the development of persistent GTD after mole evacuation. However, it is possible that significant differences exist in the levels of the other MMPs and/or TIMPs, and this merits further investigation.

Gestational trophoblastic tumours are one of the tumours most sensitive to chemotherapy, and even patients with wide-

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**Table I.** Staining pattern of TIMP-2

<table>
<thead>
<tr>
<th>Specimens</th>
<th>Staining intensity</th>
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<tr>
<td></td>
<td>Negative</td>
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<tr>
<td>Placenta (n = 10)</td>
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</tr>
<tr>
<td>CT</td>
<td>–</td>
</tr>
<tr>
<td>ST</td>
<td>–</td>
</tr>
<tr>
<td>EVT</td>
<td>–</td>
</tr>
<tr>
<td>Villous stroma</td>
<td>–</td>
</tr>
<tr>
<td>Decidua</td>
<td>–</td>
</tr>
<tr>
<td>Mole</td>
<td></td>
</tr>
<tr>
<td>M (n = 8)</td>
<td>–</td>
</tr>
<tr>
<td>CT</td>
<td>–</td>
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<tr>
<td>ST</td>
<td>–</td>
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<tr>
<td>EVT</td>
<td>–</td>
</tr>
<tr>
<td>PTD (n = 6)</td>
<td>–</td>
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<tr>
<td>Invasive mole (n = 2)</td>
<td>–</td>
</tr>
<tr>
<td>CT</td>
<td>–</td>
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<td>ST</td>
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<tr>
<td>EVT</td>
<td>–</td>
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<tr>
<td>Myometrium</td>
<td>2</td>
</tr>
<tr>
<td>Choriocarcinoma (n = 5)</td>
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</tr>
<tr>
<td>Trophoblastic cell</td>
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<tr>
<td>Myometrium</td>
<td>5</td>
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CT = cytotrophoblast; ST = syncytiotrophoblast; EVT = extravillous trophoblast; M = mole with spontaneous resolution; PTD = mole with subsequent persistent GTD.
spread metastases can be cured by multiple-agent chemotherapy. However, some cases are fatal despite surgical resection of chemo-resistant lesions and multiple chemotherapy regimens (Bower et al., 1997), and new approaches are required to overcome drug resistance cases. Since over-expression of TIMP-2 has been reported to reduce tumour growth, angiogenesis, invasion and metastasis in experimental models (Valente et al., 1998; Hajitou et al., 2001), agents capable of modifying activities of MMPs and TIMPs could be incorporated as candidates to help overcome chemoresistance in GTD.

References


Submitted on August 22, 2001; accepted on December 20, 2001