Cell growth-promoting activity of tissue inhibitor of metalloproteinases-2 (TIMP-2)

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SUMMARY

Human tissue inhibitor of metalloproteinases-2 (TIMP-2) has a potent growth-promoting activity for wide range of human, bovine and mouse cells, having an optimal concentration (10 ng/ml, 0.46 nM) that is ten-times lower than that of TIMP-1 (Hayakawa et al. (1992) FEBS Lett. 298, 29). Neither TIMP-1 complexed with progelatinase B nor TIMP-2 complexed with gelatinase A, both of which have full inhibitory activity against active forms of matrix metalloproteinases (MMPs), showed any cell growth-promoting activity. On the contrary, both reductively alkylated TIMPs had no MMP inhibitory activity, but significantly stimulated cell proliferation. These facts clearly indicate that the cell-proliferating activity of TIMPs is independent of MMP inhibitory activity. We also demonstrated that [3H]thymidine was significantly incorporated into Raji cells, a Burkitt lymphoma cell line, in the presence of either 4 ng/ml of TIMP-1 or 0.1 ng/ml of TIMP-2. Under steady-state conditions at 4°C, high-(Kd=0.15 nM) and low-(35 nM) affinity binding sites for TIMP-2 were identified on Raji cells with 20,000 and 1.4×10^5 sites/cell, respectively. Both high- and low-affinity binding of 125I-TIMP-2 to Raji cells were competitively inhibited by unlabeled TIMP-2 but not by unlabeled TIMP-1, suggesting the presence of receptors for TIMP-2 independent from those for TIMP-1. TIMP-2 seems to be another new TIMP cell-growth factor in serum, besides TIMP-1.

Key words: cell-growth-promoting activity, tissue inhibitor of metalloproteinases-2 (TIMP-2), cell-surface receptor

INTRODUCTION

Two distinct tissue inhibitors of metalloproteinases, TIMP-1 and TIMP-2, have been isolated and characterized (Cawston, 1986; DeClerck et al., 1989; Stetler-Stevenson et al., 1989; Golderg et al., 1989; Kishi et al., 1991; Umenishi et al., 1991; Ward et al., 1991). Both TIMPs interact with active forms of matrix metalloproteinases (MMPs), such as interstitial collagenase, gelatinases A and B, and stromelysin 1, with a 1:1 stoichiometry. Kd values of around 10^{-10} (Docherty and Murphy, 1990), and their ability to inhibit MMPs, suggest that they may play an important role in the regulation of MMP activity, effectively controlling the amount of connective-tissue breakdown. TIMPs have also been shown to form complexes with progelatinases in a 1:1 stoichiometry, thereby inhibiting the autoactivation step of progelatinase activation (Ward et al., 1991; DeClerck et al., 1991; Howard et al., 1991; Okada et al., 1992).

We recently reported that human TIMP-1 has a potent growth-promoting activity for a wide range of cells and published some findings that support the proposition that TIMP-1 is a new cell-growth factor in serum (Hayakawa et al., 1992).

In this study, we report that TIMP-2 also has a potent growth-promoting activity for a wide range of cells, having an optimal concentration (10 ng/ml, 0.46 nM) that is ten-times lower than that of TIMP-1. The cell-proliferating activity of TIMP-2 seems to be a result of its direct interaction with the cell membrane and appears to be unrelated to its MMP inhibitory activity.

MATERIALS AND METHODS

Materials

Materials used and their sources were obtained as follows: carrier-free Na^{251}I (NEZ-033A, 364.08 mCi/ml) from Du Pont (Wilmington, DE); [methyl-3H]thymidine from Amersham International plc. (Buckinghamshire, England); Sepharose 4B, gelatin-Sepharose, and Sephadex G-25 (PD-10) column from Pharmacia Fine Chemicals (Uppsala, Sweden); Ultrogel AcA 54 from IBF Biotechnics (Villeneuve-la-Garenne, France); bovine serum albumin (Fraction V, BSA) and lactoperoxidase from Sigma Chemical Co. (St Louis, MO); anti-human fibronectin antibody from Cappel Organon Teknika Corp. (Westchester, PA); recombinant human interleukin-1 α (IL-1α) and tumor necrosis factor α (TNF α) from Genzyme (Cambridge, MA); p-aminophenylmercuric acetate (APMA) from Aldrich Chemical Co. (Milwaukee, WI); 6-week-old mice from Becton Dickinson and Co. (Lincoln Park, NJ).

Culture media

Dulbecco’s modified Eagle’s minimal essential medium (D-MEM) and fetal calf serum (FCS) were purchased from Gibco Laboratories.
Reductive alkylation

Anti-TIMP-1 monoclonal antibody-Sepharose 4B

HT1080 cells by use of an anti-fibronecctin antibody-Sepharose and homogeneity from the conditioned medium of TNF-α

anti-TIMP-2 monoclonal antibody (clone 67-4H11)-Sepharose 4B affinity and homogeneity from the conditioned medium of IL-1

minutes at 35°C

TIMP-1 or TIMP-2, and then the mixture was incubated for 30 minutes and then with 0.22 M iodoacetate for 20 minutes. The reaction mixture was dialyzed overnight against 30 mM Tris-HCl buffer, pH 7.8, containing 5 mM CaCl₂ to remove the excess of reagents for reductive alkylation.

Radioactive iodination of TIMP-2

rTIMP-2 was radioiodinated with carrier-free Na¹²⁵I by the lactoperoxidase method (Niinobu et al., 1990) with some modifications. Briefly, 5 µg of rTIMP-2 was placed in a tube and then diluted with 20 µl of 0.05 M sodium bicarbonate. Tris-HCl buffer (20 µl each of 0.05 M and 0.5 M, pH 7.4), 1 µCi of carrier-free Na¹²⁵I, and 5 µg of lactoperoxidase were added to the iodination vial. The iodination reaction was initiated by the addition of H₂O₂ diluted 1:75,000 with distilled water. After 2 minutes, the reaction mixture was applied to a Sephadex G-25 column that had been previously equilibrated with 0.05 M Tris-HCl buffer, pH 7.4, and elution was conducted with the same buffer. Radioactivity was measured in an automatic gamma counter with a counting efficiency of 70%, and TIMP-2 concentration was determined by a one-step sandwich EIA for TIMP-2 (Fujimoto et al., 1993a). TIMP-2 was radiolabeled to a specific activity of 3×10⁶-6×10⁷ cpm/µg.

Assay for ¹²⁵I-TIMP-2 binding to Raji cells

Raji cells were propagated in RPMI 1640 supplemented with 10% FCS at 37°C under an atmosphere of 5% CO₂ and 95% air. Before binding studies, the culture medium was changed to RPMI 1640 containing 0.2% BSA and 0.025 M Hepes buffer, pH 7.4, and the cultures were incubated for 2 days. The cells were then collected and washed three times with PBS.

For the time-course experiments, the cells were seeded into 6-well plastic plates at a cell density of 2.2×10⁶ cells/well in 1 ml of RPMI 1640 containing 10 ng (37,000 cpm) of ¹²⁵I-TIMP-2 with or without a 100-fold excess amount of unlabeled rTIMP-2.

For equilibrium binding experiments, serial dilutions (0.1-25.6 ng/ml) of ¹²⁵I-TIMP-2 having a specific activity of 57,170 cpm/ng were incubated at 4°C for 5 hours with 2.2×10⁶ cells in 1 ml of RPMI 1640 as a total volume. For determination of non-specific binding, a 100-fold excess of unlabeled TIMP-2 was added to the culture medium.

The assays were terminated by centrifuging the binding mixtures, and the cells collected were then washed twice with 2 ml of ice-cold 0.05 M Tris-HCl buffer, pH 7.4. The cells were next solubilized in 1 ml of 0.5 M NaOH for 20 minutes at room temperature and the radioactivity was counted in a gamma scintillation counter at 70% efficiency.

The residual radiolabel associated with the cells in the non-specific binding experiment was subtracted from the total bound fraction found in the absence of unlabeled TIMP-2 to give specific binding. All binding experiments were done with duplicate incubations.

For Scatchard analysis, data from the equilibrium binding experiments were analyzed according to the method of Munson and Rodbard (1980).

[³H]Thymidine incorporation assay

Raji cells, cultured for 24 hours in serum-free culture medium (RPMI 1640), were collected and washed three times with PBS. The cells were seeded at a density of 2×10⁵ cells/well in 1 ml of culture medium containing the indicated amounts of TIMPs. Following the incubation for 20 hours at 37°C, 1 µCi/ml of [³H]thymidine was added. The cells were further incubated for the indicated times and washed three times with 1 ml of ice-cold PBS. After leaving in ice-cold 5% trichloroacetic acid for 20 minutes, the cells were washed twice with 1 ml of the same trichloroacetic acid solution. The cells were then solubilized in 0.5 M NaOH, and [³H]thymidine incorporated was determined by use of a liquid scintillation spectrometer at 40% efficiency.

Determination of DNA concentration

DNA contents were determined by the method reported by Higarder (1971).
RESULTS

Effects of different concentrations of TIMP-2 on cell proliferation
We first examined the effects of TIMP-2 addition on the growth of Gin-1 cells and Raji cells cultured in basal medium and compared these effects with those of TIMP-1 (Fig. 1). The cells were not able to grow in the basal medium alone. However, they grew significantly in the basal medium supplemented with either highly purified human natural (n) or recombinant (r) TIMP-2, with the maximal effect being at 10 ng/ml (0.46 nM). We obtained essentially the same result with K-562 cells cultured in ASF 104, an albumin-free enriched culture medium (data not shown).

Effect of TIMP-2 on the growth of other cells
We next examined the effect of nTIMP-2 on the proliferation of several other human, bovine and mouse cells cultured in ASF-104. It appeared that the growth of all the cells examined was significantly stimulated by TIMP-2 (Fig. 2).

Effects of reductive alkylation and complex formation with MMPs on cell-proliferating activity of TIMPs
Neither alkylated TIMPs nor TIMPs complexed with MMP-1 showed any inhibitory activity against MMP-1. However, both proMMP-2/TIMP-2 and proMMP-9/TIMP-1 complexes showed full inhibitory activity (Table 1). The alkylated TIMPs, however, showed some cell-proliferating activity that was statistically significant. But TIMPs complexed with either MMP-1 or proMMPs had no cell-proliferating activity at all (Fig. 3).

Stimulation of DNA synthesis by TIMPs
We first examined the dose-dependent stimulation of DNA

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**Fig. 1.** Effect of the addition of different concentrations of TIMP-1 and TIMP-2 on the proliferation of human gingival fibroblasts (Gin-1) (A) and Burkitt lymphoma cell line Raji (B) in serum-free basal medium. DNA content at day 7 with Gin-1 and day 3 with Raji was determined. Results are the means of three experiments, along with s.d.

**Fig. 2.** TIMP-2 dependency of cell proliferation of several human, bovine and mouse cells. Gin-1, human gingival fibroblasts; Raji, Burkitt lymphoma cell line; K-562, human erythroleukemia cells; WI-38VA13, SV40-transformed human lung cells; BAC, bovine articular chondrocytes; 3T3, Swiss albino mouse embryo fibroblasts; J774-1, mouse lymphocyte-like cells. DNA content was determined at day 3 for Raji and K-562, at day 4 for BAC and J774-1, at day 5 for 3T3, and at day 7 for Gin-1 and WI-38VA13. Results are the means of three experiments, along with s.d. *P<0.05 against ASF 104 group.
synthesis following the addition of either TIMP-1 or TIMP-2 to Raji cells at high cell density, where minimal cell proliferation was observed, and got the maximal stimulation at 4-5 ng/ml of TIMP-1 and 0.1 ng/ml of TIMP-2 (data not shown).

We next examined the time courses of [3H]thymidine incorporation following the addition of TIMP-1, TIMP-2 or 10% FCS as a positive control to Raji cells at the high cell density. The cells showed a significant increase in [3H]thymidine incorporation in the presence of either 4 ng/ml of TIMP-1 or 0.1 ng/ml of TIMP-2 (Fig. 4).

Binding of [125I]-TIMP-2 to Raji cells

Binding of [125I]-TIMP-2 to Raji cells was examined at 4°C, and the equilibrium binding was attained at 2 hours (data not shown). Analysis of the equilibrium binding data using the Ligand program (Munson and Rodbard, 1980) yielded a Scatchard plot that was most consistent with the presence of two classes of receptors: 12.5% of the sites (2×10⁴ sites/cell) having a high affinity with a K_d of 0.15 nM, and the rest of the sites (1.4×10⁵ sites/cell) having a low affinity with a K_d of 35 nM (Fig. 5).

Cell-proliferating activity of TIMP-2 in serum

It was very recently reported that TIMP-2 is another constitutive component of human serum together with TIMP-1 (Fujimoto et al., 1993a). By using the sandwich EIA for TIMP-2, we found that TIMP-2 was also a constitutive component of FCS as well as human serum, and its concentration was 131±66 ng/ml (mean±s.d., n=33). We assume that it exists in free form like that in human serum (Fujimoto et al., 1993a). Then we investigated whether the cells might grow in TIMP-2-free FCS. As shown in Fig. 6, the proliferation of Raji cells was remarkably suppressed in TIMP-2-free FCS. Almost complete suppression of cell growth was observed when both TIMP-1 and TIMP-2 were removed from the FCS. The growth was, however, significantly restored by the addition of the same amount of either TIMP-1 (18 ng/ml) or TIMP-2 (6 ng/ml), as was detected in the original 10% FCS. Furthermore, the growth was almost fully restored by the addition of both TIMPs. The cell-proliferating activity of TIMP-2 in FCS was general for other human, bovine and mouse cells as well as Raji cells, as shown in Fig. 7. The growth dependency on TIMP-2 in FCS was, however, different from one cell line to another.

DISCUSSION

TIMP-2 showed the maximal effect of its cell-proliferating activity at 10 ng/ml, which was a ten-times lower concentration than that for the maximal effect of TIMP-1 (Fig. 1). However, the fact that TIMP-2 has no growth-promoting activity at 100 ng/ml led us to a hasty and erroneous conclusion in our previous paper (Hayakawa et al., 1992).

The finding that human TIMP-2 stimulates mouse cells together with human and bovine cells is in contrast with the finding that human TIMP-1 does not stimulate mouse cells at all (Yamashita et al., unpublished data). This difference between the effects of these two human TIMPs on the proliferation of mouse cells might be ascribed to the difference in amino acid sequence homology between human and mouse.
TIMPs (Shimizu et al., 1992); that is, there is 72% homology between human and mouse TIMP-1, but 97% homology between human and mouse TIMP-2. We demonstrated that TIMP-2 also stimulates the proliferation of human erythroleukemia K-562 cells, as does TIMP-1 (Fig. 2). This is consistent with a previous report indicating that human rTIMP-2 has erythroid-potentiating activity and that its maximal effect occurs at a slightly lower concentration than that of TIMP-1 (Stetler-Stevenson et al., 1992). Recently it has been demonstrated that erythropoietin- or hexamethylene-bisacetamide-induced erythroid differentiation of a mouse erythroleukemia progenitor cell line, ELM-I-1-3, seems to be coupled with cell cycling that might occupy especially high-affinity cell surface receptors of the former (Murphy et al., 1991; DeClerck et al., 1993) suggests that the N-terminal domains of TIMPs might be related to the cell-growth-promoting activity of TIMPs. However, as we discussed above, TIMP-1/proMMP-9 and TIMP-2/proMMP-2 complexes have no cell-growth-promoting activity at all (Fig. 3), though they have full inhibitory activity against active forms of MMPs. So, it is not easy to speculate on what parts of TIMP structures are really responsible for their growth-promoting activity. Experiments are going on in our laboratory to narrow down the active domains by use of synthetic peptides that represent partial amino acid sequences of TIMP-1.

The cell-growth-promoting activity of TIMP-1 is suggested to be a direct cellular effect mediated by a cell surface receptor (Avalos et al., 1988; Bertaux et al., 1991), not occurring through the inhibition of MMPs. In this study we tried to demonstrate the presence of TIMP-2 receptors on cell surfaces. Most cells actually produce and secrete TIMPs in their culture medium. Avalos et al. (1988) speculated that these TIMPs might occupy especially high-affinity cell surface receptors and presented inconsistent results for the receptor binding assay. When we examined Raji cells, we found that they expressed neither TIMP-1 nor TIMP-2 at either mRNA or protein product level. By using these cells, we could clearly demonstrate the presence of both high- and low-affinity binding sites on the cells. The $K_d$ value of 0.15 nM for the high-affinity binding is close to half of the value (0.46 nM) of the
TIMP-2 concentration that gave the maximum stimulation for cell growth (Fig. 1). The presence of low-affinity binding sites on the cells might relate to the inhibitory effect of higher concentrations of TIMP-2 on cell proliferation. Either high- or low-affinity binding of 125I-TIMP-2 to the cells was competitively inhibited by unlabeled TIMP-2 but not by unlabeled TIMP-1, suggesting the presence of cell-surface receptors specific for TIMP-2.

Now, we have found that both TIMP-1 and TIMP-2 are constitutive components of human serum and also of FCS. We demonstrated that cell proliferation was remarkably suppressed in TIMP-free FCS and was appreciably restored by the addition of TIMPs, suggesting that both TIMPs are potent growth factors in serum (Fig. 6).

The depletion of both TIMPs from FCS resulted in an unexpectedly dramatic reduction in the cell-growth-stimulating ability of FCS (Fig. 6). But this loss was not merely due to the co-depletion of other growth factors together with TIMPs, because the re-addition of rTIMPs was sufficient for full recovery of cell-growth-potentiating capability. However, it has been well recognized that FCS contains many other potent cell growth factors. One possible explanation towards reconciling these inconsistent findings, would be the synergistic function of TIMPs on cell proliferation with other growth factors in serum, like the combination of platelet-derived growth factor and somatomedins (Scher et al., 1979). Experiments with this idea in mind are under way in our laboratory. About one-third of both TIMP-1 and TIMP-2 in human serum exists as a complex with progelatinase B and A, respectively (Kodama et al., 1990; Fujimoto et al., 1993a,b, and unpublished data). As we discussed above, both TIMPs lose their cell-growth-promoting activity when they form a complex with their corresponding progelatinase. Therefore, progelatinases seem to act as a kind of regulator to control the growth factor activity of TIMPs, by forming a complex with these TIMPs. Although several chemically defined synthetic media have been developed, the requirement for serum still remains for many vertebrate cell lines, hinting that one or more further unknown serum factors may play key roles in regulating in vivo cell maintenance and proliferation. TIMPs may be such factors. The growth dependency on both TIMPs in FCS was, however, different from one cell line to another (Fig. 7). Growth might depend on the amount of TIMPs that those cell lines produce themselves, which would stimulate cell proliferation by an autocrine mechanism. It might also depend on the characteristics of the receptors of each cell line. The growth of mouse cells, however, could not be fully restored by the addition of human TIMP-2 to TIMP-2-free FCS, suggesting a difference between human and bovine TIMP-2s with respect to their growth-stimulating activities in mouse cells.

Recently one of the chicken inhibitors of metalloproteinases, ChlMP-3 (a 21 kDa protein), which was proposed to be a new, matrix-specific TIMP-3 (Pavloff et al., 1992), was reported to stimulate the proliferation of growth-retarded, nontransformed cells maintained under low serum conditions (Yang and Hawkes, 1992). These findings lead us to conclude that cell-growth-promoting activity is a common feature of members of the TIMP family.

Among other bifunctional molecules having both protease inhibitory and cell-growth-promoting activities, such as pancreatic secretory trypsin inhibitor/tumor-associated trypsin inhibitor (PSTI/TATI; McKeehan et al., 1986; Niinobu et al., 1990; Stenman et al., 1991), epidermal growth factor/urogastrone (EGF/UGO; Green and More, 1980; McKeehan et al., 1986) and urinary glycoprotein proteinase inhibitor (McKeehan et al., 1986), EGF/UGO is quite comparable with TIMPs in many respects. Both factors are fundamental and ubiquitous proteins in human beings, and both stimulate a wider range of cells. EGF/UGO, the amino acid sequence of which is homologous with that of PSTI/TATI (Hunt et al., 1974; Scheving, 1983; Yamamoto et al., 1985), actually has potent protease inhibitor activity.

During the preparation of this manuscript, a paper appeared suggesting that a growth-stimulatory protein from SV40-transformed human fibroblasts is identical to TIMP-2 (Nemeth and Goolsby, 1993).

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