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EXPRESSION AND CHARACTERIZATION OF HUMAN TISSUE INHIBITOR OF METALLOPROTEINASES-1 IN A BACULOVIRUS-INSECT CELL SYSTEM

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SUMMARY: The baculovirus expression system was used to overexpress human tissue inhibitor of metalloproteinases-1 (TIMP-1). Approximately 5 mg of recombinant TIMP-1 was produced per 10^9 Sf9 insect cells infected with the recombinant baculovirus. The optimum time point for the production of biologically active rTIMP-1 was 20 hours postinfection. TIMP-1 activity was demonstrated by a soluble collagenase inhibition assay and reverse zymography. The baculovirus system has the advantage over previously described methods for generating human rTIMP-1 in that it generates large amounts of a fully glycosylated and active protein.

Tissue inhibitors of metalloproteinases (TIMPs) are involved in normal tissue remodeling, maintaining a balance between extracellular matrix deposition and degradation by matrix metalloproteinases (MMPs) (1). TIMP-1 is a highly disulfide constrained protein which is extensively glycosylated to yield a functional molecule of approximately 30 kDa (2). TIMP-1 inactivates collagenases by forming a noncovalent complex which is essentially irreversible (3). There is strong evidence suggesting that TIMP-1 regulates the invasive and metastatic potential of cells through MMP inhibition (4,5). Although TIMP-1 is primarily regarded as a modulator of proteolytic activity (6-8), it has also been reported to promote the growth of a variety of cell types (9,10).

In the present study we describe, for the first time, the expression of active recombinant human TIMP-1 in a baculovirus expression system. Although bacterial expression of human TIMP-1 has been reported previously (11), as has expression in mammalian cells (12), the final product is often inactive or the yield disappointingly low. The baculovirus vector system has been used successfully for the high-level production of many eukaryotic proteins (13), since it provides an environment suitable for the proper post-translational modifications and folding of the proteins.

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MATERIALS AND METHODS

Insect Cells: Recombinant virus was grown and generated in Spodoptera frugiperda (Sf9) cell monolayers or suspension cultures using IPL-41 supplemented with 2.6 g/l of tryptose phosphate broth (GIBCO, Grand Island, NY) and 5% fetal bovine serum (FBS) (Biofluids, Rockville, MD).

Construction of Recombinant Transfer Vectors: A 0.8 kb cDNA clone for human TIMP-1 was isolated from a human endothelial cell cDNA library as previously described (14). The TIMP-1 cDNA fragment was ligated into the baculovirus transfer vector pVL1393.

Recombinant Virus Production: To produce recombinant virus, Sf9 cells were cotransfected with a mixture of linearized baculovirus DNA (Pharmingen, San Diego, CA) and recombinant transfer plasmid DNA for 4 h. After incubation at 27°C for 4 days, the recombinant viruses were screened and selected by a plaque assay and dot-blot hybridization, using a ^{32}P-labelled TIMP-1 specific probe. A recombinant baculovirus was amplified to obtain a high titer stock solution (2 x 10^8 virus particles per ml).

Analysis of Polypeptides from Recombinant Virus-Infected Cells: Monolayers of Sf9 cells were infected with recombinant virus at a multiplicity of infection of 5-10 PFU per cell and incubated at 27°C. Cells and conditioned medium were collected for up to 5 days post-infection (pi). The conditioned medium was concentrated 20-fold with Centricon 10 (Amicon, Beverly, MA) and the cell pellets were lysed in 0.5 ml of extraction buffer (50 mM Tris HCl pH 7.5, 100 mM NaCl, 1% Nonidet P-40), containing either 10 μg/ml APMSF or 3.3 mM diisopropylfluorophosphate. Western blot analysis was carried out following protein separation by SDS polyacrylamide gel electrophoresis (PAGE). Partially purified rTIMP-1 preparations, obtained from conditioned medium of infected insect cells, were treated with 10 mM EDTA, pH 3, in order to partially dissociate the rTIMP-1 from enzyme complexes (15). After incubation at 37°C for 30 min, the samples were adjusted to neutral pH and 10 mM $CaCl_2$ was added before electrophoresis.

Antibody Production: A unique 15 amino acid sequence was selected from the published sequence of TIMP-1 (12), synthesized (MPS, San Diego, CA), and used as antigen for the production of an antipeptide antibody. A second anti-human TIMP-1 polyclonal antibody, used as a control, was a gift from Dr. Yves DeClerck (Children's Hospital, Los Angeles, CA).

In Situ Hybridization: Infected or control Sf9 cells were placed on poly-L-lysine coated microscopic slides and fixed. A 274 base pair fragment of human TIMP-1 cDNA was inserted into the plasmid pGEM 7Zf. Single-stranded sense and antisense ^{35}S-labelled riboprobes were synthesized from this plasmid using T7 and SP6 polymerases, in an *in vitro* transcription system. *In situ* hybridization was carried as described by Angerer and Angerer (16).

Analysis of Inhibitory Activity of rTIMP-1: rTIMP-1 was partially purified from infected cell lysates by concanavalin-A sepharose chromatography. The collagenase inhibitory activity of the partially purified rTIMP-1 was assessed using [^{3}H] proline-labelled type I collagen (New England Nuclear, Boston, MA) as previously described (17). HUVEC conditioned medium was used as a source of collagenase which was activated with trypsin (0.01%) for 15 min followed by trypsin inactivation with SBTI (0.05%), and the collagenase activity was measured in the presence or absence of the partially purified rTIMP-1. Reverse zymography was carried out as previously described (14). Briefly, samples were analyzed by 14% SDS PAGE containing 0.1% casein and 30% (v/v) serum-free conditioned medium from the MDA-MB-231 cell line as a source of caseinolytic activity. After electrophoresis, the gels were washed three times in 2% Triton X-100 and incubated at 37°C for 72 h in a collagenase buffer. The gels were stained using 0.1% Coomassie blue in a mixture of acetic acid:methanol:water (1:3:6) and destained in the same mixture without the dye.

RESULTS AND DISCUSSION

The baculovirus expression system has become a useful method for producing large quantities of biologically active eukaryotic proteins (13). In this study, we report for the first time, on the production of functional human rTIMP-1 in the baculovirus insect cell system. TIMP-1 cDNA was ligated into the baculovirus transfer vector, pVL1393, and was cotransfected with the linearized baculovirus DNA into Sf9 cells. Using a plaque assay and dot blot hybridization, a recombinant baculovirus with the TIMP-1 cDNA incorporated in its genome was isolated. At 12 hours postinfection (pi), an increase in TIMP-1 mRNA expression in Sf9 cells, was first noted by *in situ* hybridization. The TIMP-1 expression peaked at 24 h and high expression was maintained for 72 h pi (Fig. 1). Hybridization of uninfected Sf9 cells with the antisense probe and of infected cells with a sense probe did not result in labelling above background.

rTIMP-1 was found to accumulate mainly within the cells. In mammalian cell lines, TIMP-1 is secreted and is mainly found in the culture medium. However, the secretory pathway for some glycoproteins has been shown to be compromised in the baculovirus infected Sf9 cells (18). This would explain why so little of the highly glycosylated TIMP-1 protein was secreted. The rTIMP-1 was analyzed by SDS-PAGE and Western blots, using a specific antibody. At 20 h pi, immunoreactive bands of 29 and 31 kDa were present in the cell lysate under reducing and nonreducing conditions (Fig. 2A). The two bands may represent different degrees of glycosylation of the TIMP molecule. No immunoreactive bands were detected in mock infected and uninfected Sf9 cells. Partially purified rTIMP-1

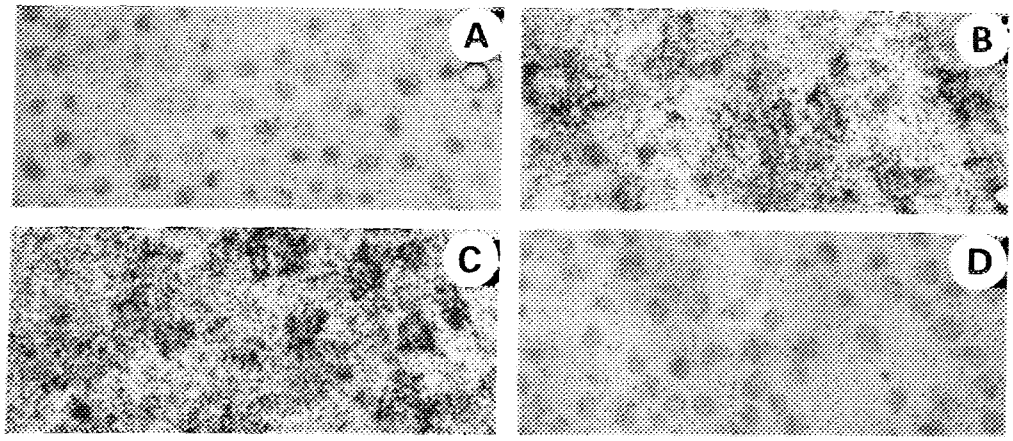

Figure 1. *In situ* hybridization of ^{35}S labelled antisense TIMP-1 riboprobe to pVL1393-TIMP-1 infected Sf9 cells. (A) 12 h pi; (B) 24 h pi; (C) 72 h pi; (D) uninfected 72 h control cells. The cells were stained with hematoxylin (x 32).

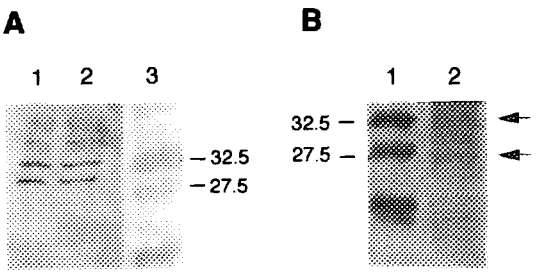

Figure 2. (A) Western blot analysis of a lysate prepared from pVL1393-TIMP-1 infected Sf9 cells, 20 h pi, in the presence (lane 1) and absence (lane 2) of 0.1 M 2-mercaptoethanol. M.W. markers (lane 3).
(B) Reverse casein zymogram of partially purified rTIMP-1 (lane 2). Arrows show the TIMP-1 activities found at 27 and 31 kDa. M.W. markers (lane 1). Dark bands were verified as inhibitory bands by comparison with the same samples separated by regular 14% SDS PAGE.

collected at 20 h effectively inhibited collagenase activity of HUVEC conditioned medium, producing almost a 75% inhibition as compared to the controls in a soluble collagenase assay (Table 1). Reverse casein zymography was used to further confirm the metalloproteinase inhibitory nature of this protein. Inhibition of caseinolytic activity was observed as two dark bands of 27 and 31 kDa representing TIMP-1 (Fig. 2B). Densitometric analysis revealed that cell lysates at 20 h pi contained approximately 5 mg of active inhibitor per 10^9 Sf9 infected cells.

At 24 h and later time points, there was progressive loss of the 29 and 31 kDa forms with the appearance of a 66 kDa immunoreactive band (Fig. 3A). This band was resolved to a 29 kDa form following treatment with 6 M urea and 0.1 M 2-mercaptoethanol (Fig. 3B). Other authors have described the formation of inactive aggregates of TIMP-1 of similar molecular weight in purified preparations of native TIMP-1 (19-21). A possible explanation for the appearance of a 66 kDa band may involve defective glycosylation. It has been shown that for some recombinant proteins produced in insect cells, glycosylation is only an efficient

Table 1 Inhibition of type I collagen degradation in a soluble assay

Sample	[^{3}H] Type I Collagen Degradation (cpm + SD)
CM	38861 ± 4682
CM + 10 mM EDTA	4314 ± 217
CM + 10 µl rTIMP-1	9472 ± 741

Conditioned media from TPA stimulated HUVEC (CM) was used as a source of collagenolytic activity. A representative of three separate experiments is shown here. The activity is expressed as cpm present in the soluble fraction. Each sample was analyzed in triplicate.

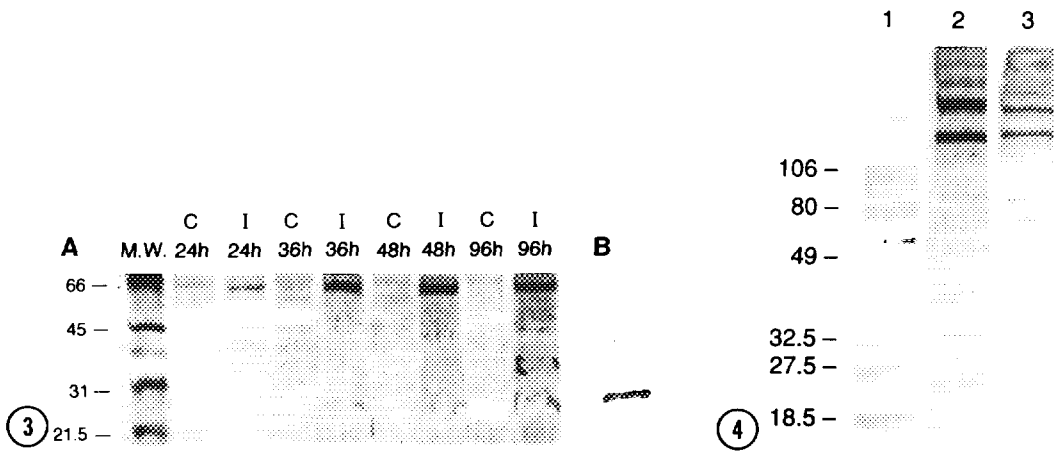

Figure 3. Western blot analysis using a TIMP-1 polyclonal antibody.
(A) Lysates prepared from control (C) and pVL1393-TIMP-1 infected (I) Sf9 cells, in the absence of reducing agents, using samples collected between 24 to 96 h.
(B) Lysate prepared from pVL1393-TIMP-1 infected Sf9 cells, 36 h pi, in the presence of 6 M urea and 0.1 M 2-mercaptoethanol.

Figure 4. Western blot analysis of partially purified conditioned medium from pVL1393-TIMP-1 infected Sf9 cells, 20 h pi, in the absence (lane 2) and presence (lane 3) of 10 mM EDTA, pH 3. M.W. markers (lane 1).

process early in infection. As recombinant protein synthesis increases and the host cell metabolic processes are shut down, the components required for glycosylation may become limited (22). Since oligosacharides may keep protein conformation in a biologically active state (23), defective glycosylation may cause the production of intermediate folding states, leading to aggregation. Aggregation of rTIMP-1 in the baculovirus system can be avoided by selecting an early (20 h) time point for harvesting.

Relatively small amounts of rTIMP-1 were secreted by the infected Sf9 cells. Upon passage of the conditioned medium through a concanavalin A column, two immunoreactive proteins of approximately 115 and 130 kDa were observed. When these partially purified samples were treated with 10 mM EDTA, at pH 3, a method that partially separates TIMP from enzyme complexes (15), two bands of the same molecular weight as observed in the cell lysates were identified, suggesting that the secreted rTIMP-1 was bound to metalloproteinases constitutively produced by Sf9 cells (Fig. 4). This was confirmed by the identification of two gelatinolytic metalloproteinase species of 72 and 92 kDa which possibly represent MMP-2 and MMP-9 in conditioned media from Sf9 cells (not shown). The presence of these enzymes should be remembered in this system when expressing recombinant proteins which are susceptible to metalloproteinase degradation.

The baculovirus system was used to generate nonglycosylated rTIMP-1. The Sf9 cells were infected with the recombinant TIMP-1 baculovirus in the presence of tunicamycin

which is known to prevent N-glycosylation. The molecular weight of the rTIMP-1 produced by the tunicamycin treated cells was approximately 23 kDa, whereas the untreated cells produced the 29 and 31 kDa forms as described above (not shown).

After lysis of the infected insect cells, rTIMP-1 was rapidly degraded in the absence of proteinase inhibitors. It has been reported that TIMP-1 is resistant to heat and acid treatment, but is destroyed by reduction, alkylation and high concentrations of trypsin (17), neutrophil elastase, and chymotrypsin (24). After trying various combinations of proteinase inhibitors we found that degradation of the rTIMP-1 was prevented by treatment with 3.3 mM diisopropylfluorophosphate or 10 μg/ml APMSF.

This study has demonstrated the feasibility of using the baculovirus system to express human rTIMP-1. Human rTIMP-1 has previously been expressed in other systems including mammalian cells (12) and *E. coli* (11). Murine TIMP-1 which presents 76% homology to human TIMP-1 has been recently expressed using a baculovirus expression vector (25). Although mammalian expression systems can generate functional proteins, the yield is usually very low. The *E. coli* system generates a high yield of recombinant protein, but the protein is not modified and may be difficult to purify in a nondenatured state. The main advantage of the baculovirus system over the other systems is its capacity to produce large amounts of functional TIMP-1. This expression system is also well suited for generation of modified (unglycosylated, mutated) forms of human TIMP-1 which will aid in the attempt to understand further the role of human TIMP-1 in physiological and pathological processes.

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