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Tissue inhibitor of metalloproteinase-2 (TIMP-2) has erythroid-potentiating activity

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Tissue inhibitor of metalloproteinase (TIMP) was purified and molecularly cloned on the basis of its erythroid-potentiating activity (EPA). TIMP/EPA appears to be a bifunctional molecule with both growth factor and anti-enzymatic activity. Recently, a second TIMP-related molecule was identified and we have investigated its possible erythroid-potentiating activity. Native, purified human TIMP-2 was assayed for erythroid-potentiating activity using an in vitro erythroid burst formation assay and was compared with that of previously characterized recombinant EPA/TIMP-1. The results demonstrate that both members of the tissue inhibitor of metalloproteinase family, TIMP-1 and TIMP-2, possessed erythroid potentiating activity which was inhibited by antibodies developed to neutralize EPA. These results suggest that TIMP-2 shares a common structural domain with EPA/TIMP-1 that is responsible for the erythroid-potentiating activity of these inhibitors. Therefore, TIMP-1 and TIMP-2, with both anti-protease activity and growth factor activity, join a family of bifunctional molecules such as fibroblast growth factor and thrombin which have both enzymatic and growth factor activity.

Metalloproteinase inhibitor; Collagenase inhibitor; Erythropoiesis; CFU-E; BFU-E

1. INTRODUCTION

Although erythropoietin is the primary regulator of erythropoiesis, several other proteins have been identified which are capable of erythropoiesis in vitro. The stimulating effects of these non-erythropoietin molecules were identified by in vitro erythroid colony assays to detect either burst-promoting activity (BPA) or enhancement of colony formation by more mature erythroid precursors (CFU-E) [1–5]. While early studies were conducted with unpurified preparations, several of the cloned myeloid colony-stimulating factors (CSFs) have been shown to enhance erythropoiesis in vitro, and in some cases in vivo. These CSFs include interleukin-3 (IL-3), granulocyte-macrophage CSF (GM-CSF) and granulocyte CSF (G-CSF) [6–8]. Also, platelet-derived growth factor (PDGF), activin, fibroblast growth factor (FGF) growth hormone, insulin and insulin-like growth factor-1 (IGF-1) augment erythropoiesis in vitro [9–15]. We identified an erythroid-potentiating activity (EPA) in the supernatant of the HTLV-II-transformed human T-lymphblast cell line (Mo), and purified the 28 000 Da glycoprotein molecule to homogeneity [5,16]. Subsequent cDNA cloning demonstrated that EPA was identical to tissue inhibitor of metalloproteinases (TIMP, TIMP-1) [17,18], which can be isolated from a variety of human tissues and body fluids and which has been shown to inhibit the collagenase family of enzymes [19–21]. The purified natural EPA and, subsequently, the recombinant EPA were shown to augment colony formation by human CFU-E, BFU-E and K-562 cells [16,17,22,23]. We were also able to demonstrate specific binding of radioiodinated recombinant EPA to the K-562 erythroleukemia cells [23].

Recently, we have isolated and characterized a novel protease inhibitor which upon direct amino acid sequencing appears closely related to EPA/TIMP-1 [24,25]. This inhibitor, which we refer to as TIMP-2, was initially isolated as a complex with the 72 000 Da type IV collagenase/gelatinase proenzyme from the conditioned media of human melanoma cells [17]. This complex has also been isolated from endothelial cells [26]. Dissociation of this complex and subsequent purification demonstrated that TIMP-2 is a 21 000 Da non-glycosylated protein that is dependent on internal disulfide bridges to maintain its inhibitory effect on collagenolytic activity. Subsequent cDNA cloning of TIMP-2 revealed that direct amino acid sequencing was correct in 95% of the residue assignments [25]. Comparison of the deduced amino acid sequence of TIMP-2 with that of EPA/TIMP-1 revealed 37% identity and 67% overall similarity. The positions of all 12 cysteine residues were conserved, as were 3 out of 4 tryptophan residues. Also there were regions of the primary structure of these proteins where the homology exceeded the average value of 67%, while there were other regions

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where the homology between these proteins was reduced. Thus it was postulated that specific domains within the structure of the TIMP could account for the common properties of these proteins, namely inhibition of collagenolytic activity, as well as domains unique to each protein that could account for unique properties such as the ability to bind to the latent forms of specific members of the collagenase gene family [25]. In the present study we compare directly the erythroid-potentiating activity of recombinant EPA/TIMP-1 and native TIMP-2. The results demonstrate that like EPA/TIMP-1, TIMP-2 possesses erythroid-potentiating activity. Furthermore, this activity resides in a portion of the molecule that shares a common epitope with EPA and can be blocked by anti-EPA/TIMP-1 antibodies.

2. MATERIALS AND METHODS

2.1. *Preparation of TIMP-2*

Native human TIMP-2 was purified from the culture medium of human A2058 melanoma cells [24] as previously described. Briefly, the latent 72 kDa, type IV collagenase/gelatinase–TIMP-2 complex was isolated by gelatin affinity chromatography of the concentrated melanoma cell conditioned media. The purified complex was then dissociated and its components purified using reverse phase HPLC over a RP-300 column (Pierce Chemical Co.). The TIMP-2 containing peak was recovered, lyophilized, resuspended in distilled water and relyophilized to remove residual trifluoroacetic acid and/or acetonitrile. Material from this second freeze-drying step was then resuspended in 20 mM Tris-HCl, pH 7.5, and stored frozen at −80°C. The concentration of purified TIMP-2 protein solutions were initially determined using amino acid analysis and subsequently by the Bio-Rad method.

2.2. *Preparation of recombinant EPA*

Recombinant EPA was prepared as previously described [17] from medium conditioned by stably transfected Chinese hamster ovary cells, which constitutively express high levels of EPA from integrated copies of the p91023 (B) vector containing the human EPA cDNA clone. Briefly, serum-free medium was collected and the proteins concentrated and fractionated by gel filtration and reverse phase liquid chromatography using a Vydac (Western Analytical, Los Angeles) C-4 column. The concentration of purified protein was determined by amino acid composition analysis and subsequently using the Bio-Rad method.

2.3. *Bioassay for EPA*

Human peripheral blood BFU-E was assayed as described [14,16] by plating 3×10^5 cells/ml peripheral blood nucleated cells in methylcellulose in 0.1 ml/microtiter well. Erythroid bursts containing a minimum of 50 hemoglobinized cells were scored at 7–14 days with an inverted microscope. Control wells typically contained 40–45 bursts. Purified recombinant EPA or native TIMP-2 were diluted prior to assaying for EPA activity in 0.02% bovine serum albumin to give final concentrations between 0.02–1.8 nM. Diluent alone was added to control wells.

2.4. *Preparation of EPA-neutralizing antibodies*

Antibodies to recombinant EPA were raised in rabbits and screened for their ability to neutralize EPA activity in vitro [22]. Two polyclonal antibody preparations, Ab-A and Ab-B, which recognize EPA/TIMP-1 on Western blotting and which neutralize the in vitro stimulation of erythroid colony formation, were utilized in these experiments (gifts of Dr. Judith Gasson). 1 μl aliquots of these antibodies were added to wells containing either EPA/TIMP-1 or TIMP-2. The amount of antibody remained constant despite varying concentrations of ei EPA/TIMP-1 or TIMP-2 (see Table I).

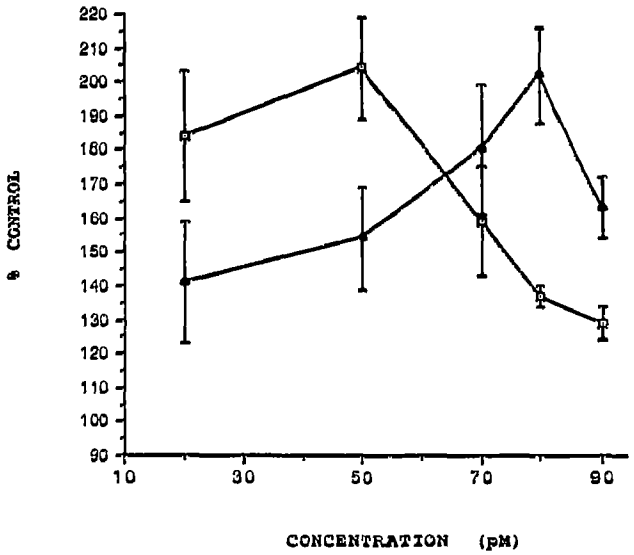

Fig. 1. Concentration dependence of erythroid-potentiating activit recombinant EPA/TIMP-1 (◆) and native, purified TIMP-2 (□). peak of TIMP-2 activity occurs at 50 pM compared with the p activity of recombinant EPA/TIMP-1 which occurs at 80 pM

3. RESULTS

3.1. *Erythroid-potentiating activity of TIMP-2*

The erythroid-potentiating activity of TIMP-2 compared with that of recombinant EPA/TIMP-1 experiments conducted in parallel. The results of th assays were normalized against the diluent control a were expressed as a percent of control value. The res shown in Fig. 1 demonstrate that TIMP-2 stimula the growth of erythroid burst-forming units (BFU over a concentration range that is similar to that recombinant EPA/TIMP-1. The activity of TIMP-2 these assays peaks at a concentration of 50 pM co pared with the maximum for EPA/TIMP-1 which curred at 80 pM. In these assays the number of colo peaked at 202% of controls for EPA/TIMP-1 and 20 of controls for TIMP-2. The biological activity of tive, purified EPA/TIMP-1 has previously b reported to be in the pM range [19,23]. The simila in the primary sequences, dilution profiles and pote of these preparations suggests that EPA/TIMP-1 TIMP-2 stimulate erythroid colony formation thro a similar mechanism.

3.2. *Inhibition of TIMP-2 erythroid-potentiating acti by EPA/TIMP-1 neutralizing antibodies*

To test if the erythroid-potentiating activity of th TIMP species was due to related epitopes we utili EPA neutralizing antibody preparations, Ab-A and B. Optimized concentrations of EPA/TIMP-1

Table I

Concentration (pM)	Control	+ NRS	+ Ab-A	+ Ab-B
EPA/TIMP-1				
0	100 ± 2	100 ± 1	99 ± 1	100 ± 1
20	141 ± 18	113 ± 1	85 ± 1	–
50	162 ± 33	121 ± 1	84 ± 1	–
70	180 ± 15	128 ± 1	89 ± 2	145 ± 1
80	202 ± 14	159 ± 6	92 ± 5	126 ± 2
90	163 ± 2	135 ± 6	87 ± 4	105 ± 3
TIMP-2				
0	100 ± 1	100 ± 1	100 ± 1	100 ± 1
20	184 ± 19	140 ± 14	87 ± 3	146
50	204 ± 15	157 ± 11	96 ± 4	113 ± 1
70	159 ± 16	138 ± 7	87 ± 3	112 ± 1
80	137 ± 3	114 ± 1	91 ± 5	–
90	129 ± 5	109 ± 1	98 ± 1	–

NRS, normal rabbit serum.

TIMP-2 were tested in the presence of 1 μl of these antibody preparations. These EPA/TIMP concentrations ranged from 20–90 pM, as determined in the experiments shown in Fig. 1. Control experiments utilizing normal rabbit serum were also conducted. The results of these studies are shown in Table I. In the control experiments addition of normal rabbit serum resulted in a slight reduction of erythroid-potentiating activity when compared to experiments in which only TIMP-2 or EPA/TIMP-1 was added. However, the addition of the antibody Ab-A resulted in inhibition of erythroid colony growth such that the values in these assays were always below that of the unstimulated control. This was evident in experiments in which both EPA/TIMP-1 and TIMP-2 were used to stimulate colony growth. Antibody Ab-B also inhibited the action of these agents but to a lesser degree. The ability of antibodies, developed to recognize and neutralize EPA/TIMP-1, to also neutralize TIMP-2 activity in these assays suggests that the erythroid-potentiating activity of these species is contained within a common domain shared by EPA/TIMP-1 and TIMP-2.

4. DISCUSSION

Our findings indicate that, like EPA/TIMP-1, TIMP-2 can stimulate the growth of erythroid precursors. The maximal effect for both TIMP-2 and EPA/TIMP-1 was 204 and 202%, respectively. This is consistent with previous reports in which the addition of recombinant human EPA/TIMP-1 to cultures containing mononuclear human peripheral blood resulted in a near doubling of the number of BFU-E colonies [17,27]. In the present study TIMP-2 appears to be as effective, with the maximal effect occurring at a slightly lower concentration of 50 pM compared with EPA/TIMP-1 which had a maximal effect at 80 pM. Furthermore, based on the homology between these 2 proteins at the amino acid level, the similarity in their dilution profiles for erythroid-potentiating activity, and the similarity in the dose for maximum effect in these assays, we propose that TIMP-2 functions in a manner very similar to EPA/TIMP-1 in stimulating erythroid colony growth. This is supported by experiments in which antibodies which neutralized the stimulatory effects of EPA/TIMP-1 on erythroid colony growth also eliminated the effects of TIMP-2 on colony growth.

The relationship between the mitogenic activity and the protease inhibitory properties of EPA/TIMP-1 and TIMP-2 is presently unknown. The growth stimulation by these proteins is thought to be a direct cellular effect mediated by a cell surface receptor [23], and not through inhibition of metalloprotease activity, although the precise mechanism of the growth stimulation by EPA/TIMP-1 and TIMP-2 is yet to be defined. In other bifunctional molecules, such as basic fibroblast growth factor and thrombin, the domains responsible for growth factor activity are physically distinct from either the enzyme-inducing or the esterolytically active sequences [28,29]. By analogy, in the case of TIMP-1 and TIMP-2, we would expect the domains responsible for the growth factor activity to be distinct from the sequences responsible for their metalloproteinase inhibitory activity. Others have postulated that EPA may mediate its effect on erythropoiesis by modulating erythropoietin receptors [30]. Recent studies have demonstrated that EPA/TIMP-1 is effective when administered in vivo at stimulating erythropoiesis in anemic mice or mice treated with erythropoietin [31,32]. This suggests that despite the disparity between the in vitro estimates of maximally effective doses (50-80 pM) for stimulating erythroid colony growth and the measurement of human serum levels of EPA/TIMP-1 (5–20 nM) [33], the administration of exogenous recombinant EPA/TIMP-1 may act synergistically in the stimulation of erythropoietin-driven erythopoiesis.

In vivo administration of recombinant EPA/TIMP-1 has also been shown to reduce lung colonization by highly invasive *ras*-transformed rat embryo fibroblasts [34]; however, it is felt that this effect is directly related to the protease inhibitory activity of the EPA/TIMP-1 molecule as demonstrated in in vitro invasion assays [35]. TIMP-2 has also been shown to be effective in blocking tumor cell invasion in these assays [36,37]. Also, both EPA/TIMP-1 and TIMP-2 [38], as well as TIMP-related cartilage-derived inhibitors [39,40] have been shown to inhibit angiogenesis in vivo. Although the exact mechanism of this inhibition has not been shown, the TIMP-related inhibitors have been shown to inhibit both endothelial cell growth and endothelial cell migration in vitro [40]. These cartilage-derived inhibitors show striking similarity to both EPA/TIMP-1 [40] and TIMP-2 [39]. Whether these effects on endothe-

lial cells are mediated by direct binding to a cell-specific receptor or are the result of protease inhibition is not known. The diversity of these proposed roles for EPA/TIMP-1 and TIMP-2 indicates that evolutionary conservation has allowed a single molecule to subserve more than one physiologic activity.

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