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Tissue inhibitor of metalloproteinases from human bone marrow stromal cell line KM 102 has erythroid-potentiating activity, suggesting its possibly bifunctional role in the hematopoietic microenvironment

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In this study, we demonstrated that tissue inhibitor of metalloproteinases (TIMP) produced by human bone marrow stromal cell line KM-102 had erythroid-potentiating activity (EPA) which stimulates the proliferation of erythroid progenitor cells. We, then, propose a scheme for the bifunctional role of TIMP/EPA in hematopoietic microenvironment, that is, the maintenance of the integrity of bone marrow matrix and the proliferation of erythroid progenitor cells proceeding on the matrix.

Tissue inhibitor of metalloproteinases (TIMP); Erythroid-potentiating activity (EPA); Bone marrow stromal cell line (KM 102)

1. INTRODUCTION

Recently, Docherty et al. [1] reported the amino acid sequence of human TIMP predicted from its complementary DNA, and found that it is identical to a 28 kDa protein referred to as EPA and produced by the human T-lymphoblast cell line Mo [6]. This EPA stimulates colony formation by early and late erythroid-committed stem cells (BFU-E and CFU-E, respectively) from human bone marrow [3] and by the K562 human erythroleukemia cell line [4]. On the other hand, studies utilizing the long-term bone marrow cultures indicated that adherent stromal cells (fibroblastoid cells, adipocytes, endothelial cells, and macrophages) play a significant role in bone marrow hematopoiesis [5,6]. In this culture system, fibroblastoid stromal cells are a major population of the adherent layer and most of them are suggested to be derived from adventitial reticular cells situated in hematopoietic cords of the bone marrow [7]. Primary adherent cells or established fibroblastoid cell lines from human bone marrow stroma demonstrated a burst-promoting activity (BPA) that stimulates the growth of BFU-E [8–12]. BPA resides in granulocyte-macrophage colony-stimulating factor [13–15], interleukin-3 [16] and EPA [3]. Thus, a question arose as to whether or not TIMP derived from bone marrow stroma has EPA activity. Here, we describe the purification of TIMP from the supernatant of serum-free cultures of the human bone marrow fibroblastoid stromal cell line KM-102 and the presence of EPA in the purified material.

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2. MATERIALS AND METHODS

2.1. *Culture of human bone marrow stromal cell lines*

The human bone marrow stromal cell lines were maintained as described previously [8]. For preparation of serum-free conditioned medium, cell cultures were maintained for 7 days after a confluent layer had developed. Then the cells were washed 3 times in Dulbecco's modified phosphate-buffered saline (Sigma) and placed in serum-free Iscove's medium (Boehringer-Mannheim) containing phorbol myristate acetate (PMA) (10 ng/ml; Sigma) and calcium ionophore A23187 (0.2 μM; Sigma) for an additional 48 h. After harvesting of the conditioned medium, cells were re-fed with 5% fetal bovine serum (Hy-Clone)-Iscove's medium and incubated for 2 days, and then serum-free medium was again employed and collected again as in the aforementioned way. Pooled KM-102 culture medium was centrifuged (3000 rpm, 30 min), filtered (0.45-μm filter), and stored at -20°C until used.

2.2. *Assay for EPA*

EPA assay was performed with human peripheral blood cells obtained from a normal donor as described previously [8] except for several modifications as follows: nonadherent bone marrow cells (2.5×10^4/0.5 ml/well) were cultured in 24-well tissue culture plates; each well contained 500 μl of methylcellulose (Dow Chemicals) mixture. Cultures were incubated for 8 days; then 0.75 U/ml of erythropoietin was added to each well. Erythroid bursts were scored at 16 days.

2.3. *Purification of TIMP*

TIMP was purified in a homogeneous form KM-102 cell-conditioned medium by use of a monoclonal-antibody affinity column [17]. TIMP concentration was measured by a sandwich enzyme immunoassay [18].

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2.4. *Assay of collagenase and TIMP activities*

The activities of collagenase and TIMP were determined by the method of Terato et al. [19] using [^{14}C]glycine-labelled collagen. 1 U of collagenase activity is the amount of protein necessary to degrade 1 μg collagen/min at pH 7.5 and 35°C. 1 U of TIMP activity is defined as the amount of protein required for 50% inhibition of 2 U of collagenase.

2.5. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting*

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli [20]. Molecular weight markers (Bio-Rad) were used as standards. After electrophoresis, the protein in the gel was transferred onto a nitrocellulose sheet. The sheet was then soaked in 4 μg/ml Fab' (clone 7-6Cl)-peroxidase conjugate. Staining was performed by the hydrogen peroxide system with 3,3'-diaminobenzidine.

3. RESULTS AND DISCUSSION

Collagenase activities and TIMP concentrations in media from both unstimulated and stimulated serum-free cultures of 5 human stromal cell lines were determined and are summarized in Table I. Stimulation by PMA and a calcium ionophore induced 2–15 times more production of collagenase in these cell lines with the exception of KM-105. The production of TIMP was, however, stimulated only rather slightly (1.1–1.7 times) in any of these cell lines. KM-102 cell-conditioned medium seemed to be the richest in TIMP among the conditioned media of the 5 cell lines. Collagenase was secreted essentially as a proenzyme that was activated after APMA treatment.

Highly purified TIMP was subjected to SDS-PAGE. The protein migrated as a single band visualized by either Coomassie brilliant blue or immunostaining and its mobility indicated a molecular weight of 27 kDa (Fig. 1a). Purified TIMP blocked the collagenase activity from the KM-102 stromal cell line together with the collagenases from chick skin [21], bovine dental pulp [22], and dog [23] and human [24] polymorphonuclear leukocytes, but not bacterial collagenase (data not shown). The blockage of KM-102 collagenase activity was confirmed by the detection of diminished degradation products (α^A and α^B) of collagen in the presence of TIMP (Fig. 1b).

Table I

Production of collagenase and TIMP by clonal human bone marrow stromal cell lines

Cell line	Unstimulated		PMA and Ca ionophore	
	Collagenase (mU/ml)	TIMP (μg/ml)	Collagenase (mU/ml)	TIMP (μg/ml)
KM-101	20.1(0)	0.41	285.9(1.3)	0.69
KM-102	4.3(0)	1.23	22.1(0)	1.73
KM-103	16.5(0)	0.38	46.0(0)	0.47
KM-104	6.1(0)	0.42	13.3(0)	0.48
KM-105	10.1(0)	0.23	8.5(0)	0.32

Enzyme activities without APMA activation are given in parentheses

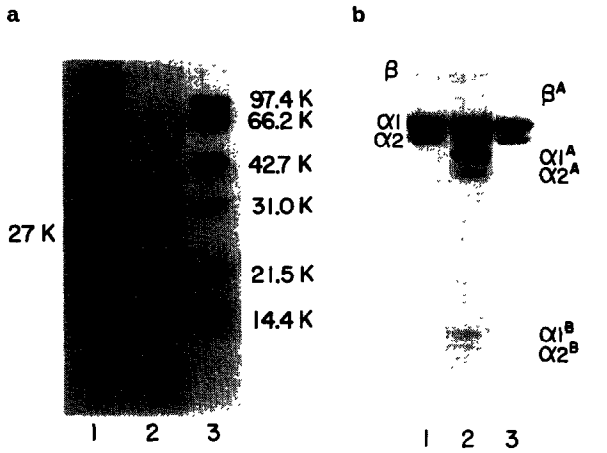

Fig. 1. Homogeneity and inhibitory activity of KM-102 cell TIMP. (a) SDS-12% PAGE of purified TIMP immunoblotted (1) or stained with Coomassie brilliant blue (2), or of standard proteins (3). (b) SDS-6% PAGE fluorography. [^{14}C]Glycine-labelled collagen (53 μg) was incubated alone (1), with 21 mU collagenase partially purified from KM-102 cell-conditioned medium (2), or with 13 ng (159 mU) KM-102 cell TIMP (3) at 21°C for 96 h. X-ray film was exposed to the gel for 4 days at −70°C.

As shown in Table II, we demonstrated that the purified TIMP has EPA activity. That is, the number of colonies derived from erythroid stem cells increased by the supplementation with 6.25 ng/ml or more purified TIMP. Furthermore, the addition of anti-TIMP monoclonal antibody [17] neutralized the EPA activity of KM-102 cell-derived TIMP, as evidenced by the significant decrease in the number of BFU-E- or CFU-E-derived colonies compared with the number in the control cultures. This reduction was also observed in the control cultures when the antibody was added (data not shown), thus suggesting the presence of TIMP/EPA in the commercially obtained fetal calf serum used and its neutralization by the added antibody.

Generally, most connective tissue cells secrete at least 3 distinct matrix metalloproteinases, interstitial col-

Table II

Number of BFU-E and CFU-E colonies in methylcellulose cultures containing different doses of KM-102 TIMP

0 ng/ml	6.25 ng/ml	25 ng/ml	100 ng/ml
BFU-E colony number (mean ± SD, n = 4)			
16.7 ± 1.5	31.5 ± 5.0*	23.0 ± 2.0 (9.5 ± 3.5)	23.5 ± 2.0

0 ng/ml	6.25 ng/ml	12.5 ng/ml	25 ng/ml	50 ng/ml
36.3 ± 8.3	47.3 ± 4.9	52.8 ± 5.5	65.8 ± 5.4* (14.3 ± 3.4)	38.5 ± 8.2

Numbers in parentheses are mean colony numbers formed in the presence of anti-TIMP monoclonal antibody (clone 7-6Cl, 880 μg/ml; 1:10 dilution for BFU-E, 1:50 dilution for CFU-E). * Significant difference ($P < 0.01$) from the corresponding control

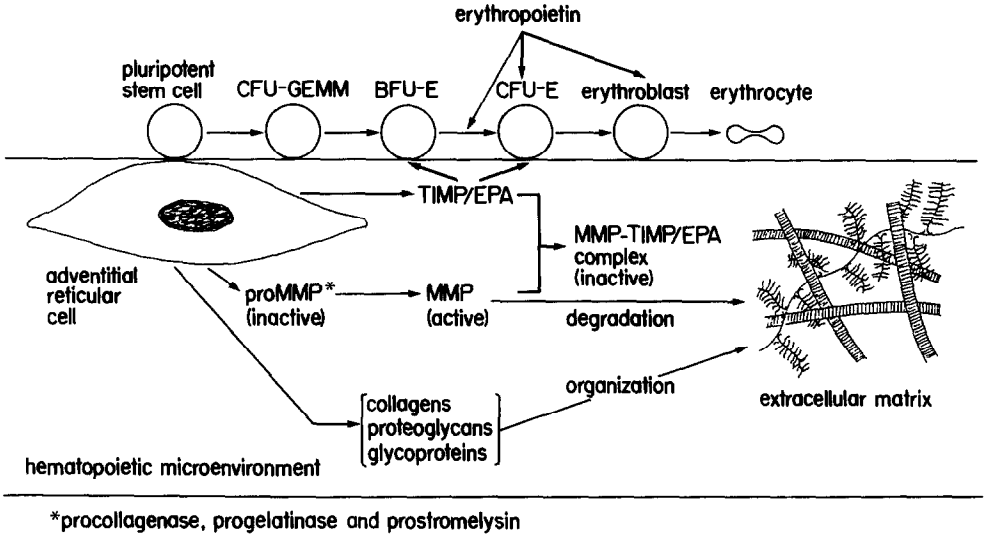

Fig. 2. Proposed scheme for bifunctional role of TIMP/EPA in hematopoietic microenvironment. MMP, matrix metalloproteinases.

lagenase, gelatinase (type IV collagenase) and stromelysin, which could mutually be responsible for the turnover of most protein components of extracellular matrix, that is, collagens I–V, VII, and X, gelatin, elastin, proteoglycan core protein, fibronectin, and laminin [25–36]. On the other hand, these connective tissue cells also produce a specific inhibitor of these matrix metalloproteinases called TIMP [37–42]. As a consequence, the balance between the activities of matrix metalloproteinases and TIMP is thought to be of central importance for the maintenance of connective tissue integrity. Essentially the same situation may apply in the bone marrow matrix (Fig. 2).

In conclusion, bone marrow stromal cells were demonstrated to produce a bifunctional protein that not only acts as a metalloproteinase inhibitor (TIMP) but also has EPA activity to stimulate the proliferation of erythroid progenitor cells.

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