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Natural human monocyte gelatinase and its inhibitor

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Gelatinases produced by stimulated peripheral blood monocytes were detected by substrate zymography and were compared with those derived from tumor cells. Stimulated monocytes were found to produce an 85 kDa gelatinase which co-migrated upon electrophoretic separation and cross-reacted in immunoprecipitation experiments with a phorbol ester inducible metalloprotease from human tumor cells. The intact natural gelatinase (85 kDa), a high molecular weight and complexed gelatinase as well as a proteolytic fragment (25 kDa) were purified by substrate- and antibody-affinity chromatography techniques. Aminoterminal sequence analysis showed that natural monocyte gelatinase occurs as a truncated form of tumor cell gelatinase/type IV collagenase. Furthermore, peripheral blood monocytes were found to also produce a tissue inhibitor of metalloproteases (TIMP). TIMP was co-purified with gelatinase on gelatin sepharose and identified by microsequencing. The balanced and regulated production of gelatinase and TIMP might be important in monocyte migration and tissue remodeling.

Monocyte; Macrophage; Gelatinase; Type IV collagenase

1. INTRODUCTION

Human white blood cells are a rich source of proteinases such as plasminogen activators, elastase, collagenase, proteoglycanase and type IV collagenase/gelatinase [1–8]. The secreted enzymatic activity is balanced by the paracrine or autocrine co-production of specific enzyme inhibitors, e.g. plasminogen activator inhibitors (PAIs), tissue inhibitor of metalloproteinases (TIMP) [9–11]. Regulation of secreted proteinases is also achieved by activation of the proenzymes [12]. Furthermore, localised proteolysis is believed to play a key role in the control of extracellular matrix degradation [13,14]. Localisation of such proteolysis can be achieved by the presence of cellular binding sites for specific proteinases [15]. The proteases, their inhibitors and their binding sites are under the genetic control of local, often paracrine factors, e.g. cytokines [9,16]. Thus, chemotactic factors can influence the production of proteases and facilitate the directional migration of normal leukocytes [17]. The migration of tumor cells in invasion and metastasis is believed to be controlled by similar mechanisms [18,19].

Metalloproteases are primarily associated with the remodelling of extracellular matrix molecules (collagens, proteoglycans, gelatins) and most studied in bone and cartilage tissues [20,21], in tumor cells [22,23] and also in neutrophilic granulocytes [24]. Here we describe for the first time the purification to homogeneity of natural monocyte gelatinase as well as one of its inhibitors. Amino acid sequence analysis shows that the monocytic enzyme is a truncated form of tumor cell-derived gelatinase.

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2. MATERIALS AND METHODS

2.1. *Reagents*

Phorbol 12-myristate 13-acetate and gelatin were from Sigma, St. Louis, MO, USA. Polyriboinosinic·polyribocytidylic acid (PIC) was purchased from P-L Biochemicals, Milwaukee, WI, USA. Sodium dodecyl sulphate and ovalbumin (5 times cristallised) were from Serva, Heidelberg, Germany. IL-1β was purified from human buffy coat supernatants as described [25]. Concanavalin A (Con A) was from Calbiochem, San Diego, CA, USA and endotoxin from *E. coli* (0111.B4) was from Difco, Detroit, MI, USA.

2.2. *Isolation, culture and induction of cells*

Human Malavu cells [26] were grown in stationary culture flasks (Falcon Plastics, Oxnard, CA, USA) in Eagle's minimum essential medium with Earle's salts (EMEM) (Gibco, Paisley, Scotland), supplemented with 10% (v/v) fetal calf serum. Human mononuclear cells from heparinized human peripheral blood from single or multiple donors (Belgian Red Cross, Blood Transfusion Centers of Leuven and Antwerp) were purified by removal of erythrocytes (hydroxyethyl starch sedimentation) and of neutrophils (Ficoll/sodium metrizoate centrifugation) as previously described [27]. From the total mononuclear cell fraction monocyte cultures were obtained by adherence to plastic in serum-free EMEM (90 min, 37°C) and extensive washing. For the induction of gelatinases, 24 multi-well plate cultures (Nunc, Roskilde, Denmark) were treated with different inducers and cell culture supernatants were harvested after the indicated time intervals.

2.3. *Detection of gelatinase activity by zymography*

Gelatinase activity in cell culture supernatants and column fractions was determined by sodium dodecyl sulphate/polyacrylamide gel

electrophoresis (SDS-PAGE) zymography according to a modification of the method of Heussen and Dowdle [26,28].

Gelatinase activity was quantified by computerised image analysis through two-dimensional scanning densitometry, and gelatinase activity was expressed as the scanning area under the curves, which is an integration ratio that takes into account both brightness and width of the substrate lysis zone relative to that of a positive control [17].

2.4. *Purification of monocyte gelatinase and amino acid sequence analysis*

For the production of peripheral blood monocyte gelatinase, about 10^{10} mononuclear cells from pooled human buffy coats (equivalent to 100 liters of human blood) were treated for 48 h with 2 μg/ml of Con A and 2 μg/ml endotoxin in serum-free culture medium. After 48 h incubation at 37°C, cell supernatants (1.8 liters) were collected and filtered (Whatman paper, 3MM, Whatman, Maidstone, England and 0.45 micron filter, Sterivex-HV, Millipore, Bedford, MA, USA, sequentially). Crude material was purified by substrate-affinity chromatography on gelatin sepharose [17] and antibody-affinity chromatography. To verify that the protein A-purified IgGs, used for antibody-affinity chromatography, recognised both the 65- and the 85-kDa gelatinases, all fractions of the protein A affinity chromatography were tested with a constant amount of a gelatinase laboratory standard by immunoprecipitation analysis as described below.

After gelatin sepharose affinity chromatography, bound enzyme was recovered with an elution buffer containing 50 mM Tris-HCl, pH 7.6, 10 mM EDTA, 0.02% NaN_3, 0.01% Tween 20, 1 M NaCl and 5% (v/v) DMSO. Four fractions (12 ml each) contained more than 98% of the eluted gelatinolytic activity. These were pooled and applied to the antibody-affinity column equilibrated with PBS. The column was eluted with 0.1 M citrate, 0.5 M NaCl, 0.01% (v/v) Tween 20, pH 2. Four fractions (5 ml each) containing approximately 50% of the eluted gelatinolytic activity were supplemented with 100 μg ovalbumin in 5 ml of bidistilled water and extensively dialysed against bidistilled water. The addition of ovalbumin enhanced protein recoveries after dialysis and did not interfere with subsequent aminoterminal sequence analysis since ovalbumin is NH_2-terminally blocked. After dialysis, the volume was reduced to 2 ml by freeze-drying in siliconised glass tubes and the remaining material again dialysed against bidistilled water. After concentration by lyophilisation, the sample (50 μl) was separated by SDS-PAGE in a 10% gel with a 4% stacking gel. The proteins in the gel were electrotransferred in a semi-dry blotting apparatus [29] for 4 h at 200 mA onto a Pro-blott membrane (Applied Biosystems, Foster City, CA) following the recommendations of the manufacturers. The membrane was then stained with Coomassie blue R-250. Fragments of the filter showing the stained protein bands were excised and the aminoterminal sequences of the corresponding proteins were determined in a modified version of the sequence cycle (BLOTT-1) on an automated gas-phase sequencer (Applied Biosystems, model 477 A with on-line analyser model 120 A). The adapted cycle for use in pulsed liquid chemistry yields higher sequencing efficiency with Pro-blott membranes in the absence of polybrene.

2.5. *Immunoprecipitation of gelatinases*

Gelatinolytic activity was immunoprecipitated with a polyclonal antiserum against the 67- and 85-kDa gelatinases from Malavu cells [17]. Briefly, gelatinases were reacted with the antibody and the immune complexes precipitated by binding to protein A on pretreated Staphylococcus bacterial cells. After extensive washing of the precipitates, the complexed molecules were dissociated with SDS and separated by SDS-PAGE. Reactivated gelatinase activity was determined by zymography as described [26].

3. RESULTS

3.1. *Comparison of gelatinases produced by human monocytes and tumor cells*

Confluent cultures of human Malavu cells or adherent peripheral blood monocytes were stimulated in serum-free medium for 24 h. Culture fluids of untreated and treated cells were tested for the production of gelatinases by a substrate conversion assay. Fig. 1A shows these zymographic analyses. The Malavu cells spontaneously secreted high levels of a 65-kDa gelatinase. These cells could be stimulated by phorbol ester to produce a 85-kDa gelatinase. Human monocytes treated with IL-1β or the double-stranded RNA PIC, secreted exclusively a 85-kDa gelatinase.

This cytokine-induced gelatinase from monocytes resembles the phorbol ester-induced gelatinase from human tumor cells as shown by co-migration on SDS-PAGE as well as by immunoprecipitation experiments (Fig. 1B). The polyclonal antiserum immunoprecipitated all forms of tumor cell gelatinase, whereas the preimmune serum did not react with any of these. Furthermore, the immunoprecipitated amounts of gelatinase were sufficient to yield enzymatic activity in zymographic assays.

The immune antiserum reproducibly precipitated an active enzyme from monocyte culture fluids with an apparent molecular weight of 85 kDa (Fig. 1B). When larger volumes of culture fluid were used it became apparent that monocytes also produce lesser amounts of another gelatinase that had a molecular weight of 65 kDa. Most probably this is the homologue of tumor cell gelatinase with an apparent molecular weight of 67 kDa (Fig. 1B). Similarly, the human myelomonocytic cell line, THP-1, when stimulated with IL-1β or

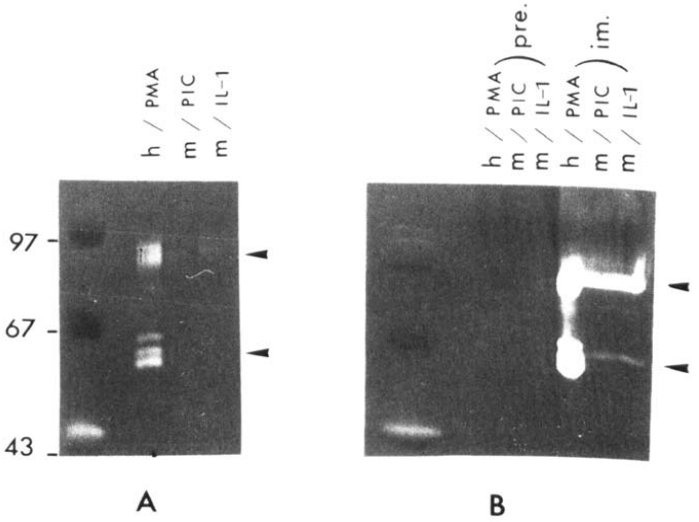

Fig. 1. Relationship of IL-1-induced monocytic gelatinase with the 85-kDa gelatinase from phorbol ester-stimulated tumor cells. (A) Zymographic analysis. Samples of IL-1β-stimulated (100 U/ml) or poly(rI)·poly(rC)-treated (PIC, 100 μg/ml) human monocytes (m) and phorbol ester-treated (PMA, 100 ng/ml) Malavu cells (h) were subjected to zymography for migration analysis. (B) Immunoprecipitation of monocytic gelatinase by a polyclonal antiserum directed against Malavu cell gelatinases. Symbols are as in panel A. Pre. indicates the zymographic analysis after precipitation with preimmune and im. with immune serum.

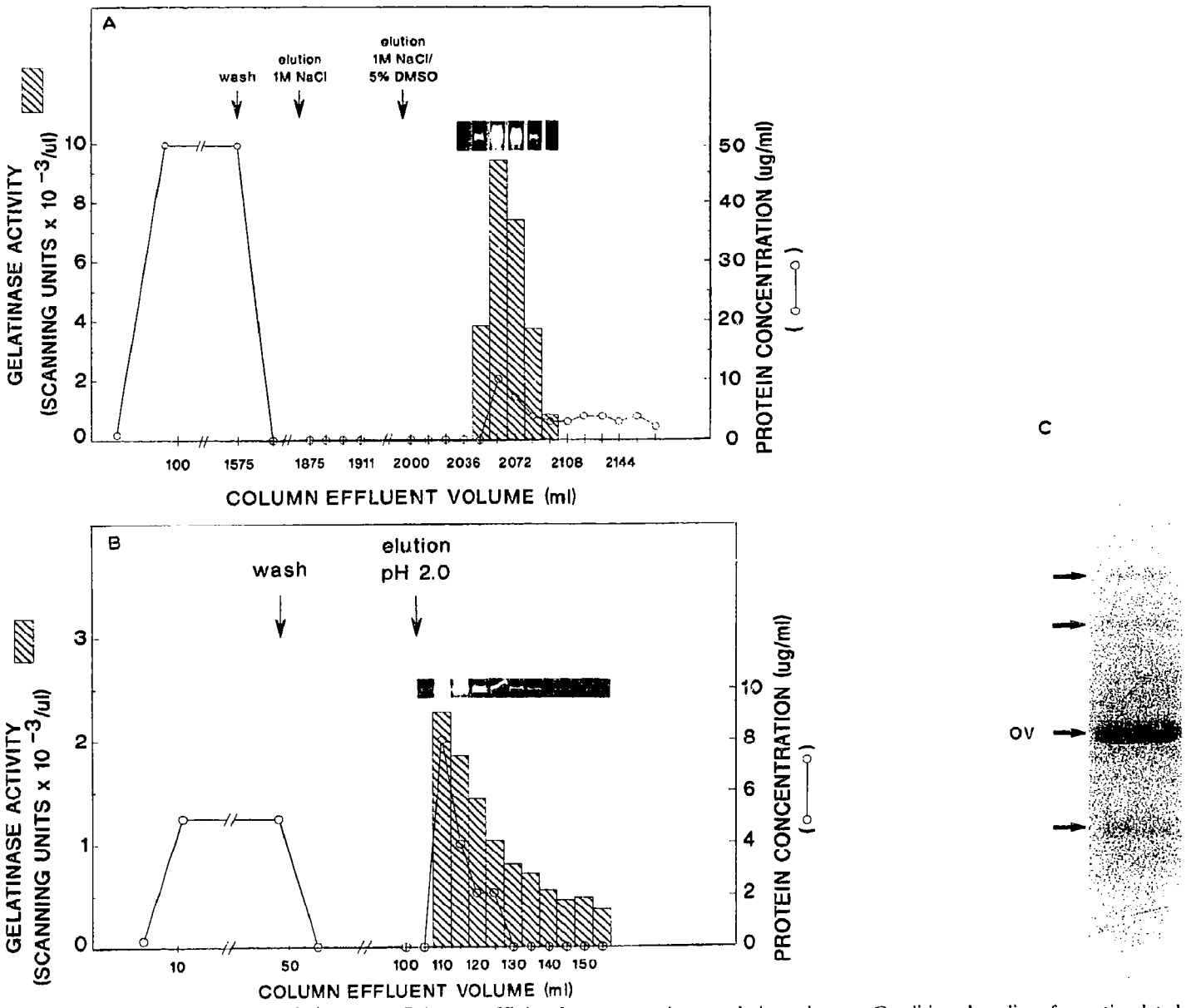

Fig. 2. Purification of monocyte gelatinase. (A) Substrate affinity chromatography on gelatin sepharose. Conditioned medium from stimulated monocytes was reacted with gelatin, coupled to sepharose. To prevent digestion of the substrate, the gelatinase was inhibited with EDTA. After extensive washing of the affinity resin, the bound enzyme was eluted and protein content as well as enzyme activity determined on all fractions. The insets show the zymographic analyses. (B) Affinity chromatography on IgG column. Monocyte gelatinase was purified by immunoadsorption to a polyclonal antibody against tumor cell-derived gelatinase. The bound enzyme was eluted at pH 2, neutralised and concentrated. Legends as in A. (C) Staining analysis of reduced monocyte gelatinase. Approximately 5 μg of purified gelatinase, supplemented with 5 μg of ovalbumin were separated by gel electrophoresis and stained with Coomassie blue. Arrows indicate the position of the 85 kDa, 65 kDa, ovalbumin (ov) and TIMP respectively.

phorbol ester, produced a gelatinase of approximately 90 kDa. This myelomonocytic gelatinase also reacted with the antibody directed against Malavu-gelatinase in immuno-precipitation experiments. However, THP-1 cells did not produce the 65 kDa gelatinase (data not shown).

3.2. *Purification of monocyte gelatinase and identification of sequence analysis*

Mononuclear cells from a large pool of buffy coats were stimulated for 48 h with Con A and endotoxin in serum-free medium. The supernatant fluids were subjected to substrate affinity chromatography on gelatin sepharose (Fig. 2A). All activity was recovered and the purification factor was 300-fold. The eluate from the substrate column was further processed by antibody-affinity chromatography (Fig. 2B) using the antibody against Malavu gelatinase. The purification factor was 2.8 and the recovery 75%. Staining analysis of the purified sample showed the presence of 3 protein bands at 85, 65 and 25–30 kDa respectively (Fig. 2C). The 85-kDa protein migrated to the same position as the ac-

Table I

NH_2-terminal sequence analysis of natural monocyte gelatinase and comparison with tumor-derived gelatinase[a]

Reduced natural monocyte gelatinase (85 kDa)		Unreduced natural monocyte gelatinase (≥100 kDa complex)	
L F P G D L R T . .	(m, 40%)[b]	L F P G – L R – N L T D . .	(30%)[b]
L V L F P G D L . .	(m, 60%)	L V L F P G D L R – N L . .	(70%)
A P R Q R Q S T L V L F P G D L R T . .	(t)		

[a] Peripheral blood monocytes (m) were stimulated with Con A (2 μg/ml) and *E. coli* endotoxin (2 μg/ml) for 48 h. Gelatinases from culture fluids were purified by affinity chromatography on gelatin-sepharose and specific antibody, followed by SDS-PAGE. After electroblotting to a solid support the transferred proteins were processed on a gas-phase microsequencer. The comparison of reduced monocytic (m) gelatinase and tumor cell-derived gelatinase (t) from fibrosarcoma cells [30] is shown. The sequence of monocyte gelatinase was at the 2.5 pmol level.

[b] % indicates relative amount of the variant form.

tive enzyme (determined by zymography analysis). After extensive dialysis and concentration, 55 μg of antibody-purified material was denatured and further separated by SDS-PAGE. The proteins in the gel were then electrotransferred to a solid support and stained with Coomassie blue. Parts from the blotting membrane containing the stainable bands were excised and the corresponding polypeptides were sequenced. The amino acid sequences of the 85-kDa gelatinase as well as the percentage occurrence of each sequence are summarised in Table I. The experimentally determined sequences from monocytic gelatinase could be aligned with those of fibrosarcoma type IV collagenase [30]. Comparison of secreted monocytic with tumor-derived gelatinase shows that the former lacks at least 8 NH_2-terminal amino acid residues. In addition, at least two NH_2-terminal variants were present in the monocyte-derived preparations, the most abundant (60%) species being two residues longer than the other species. Except for the truncation, the microsequencing analysis together with the serological crossreactivity indicates identity between the core polypeptide of the monocytic natural gelatinase and that of tumor cells.

In order to verify the amino acid sequence, a sample was processed by electroblotting transfer without prior reduction and denaturing. It is known that this procedure yields higher molecular weight complexes [5]. A sample so-obtained was processed as above and was found to yield the sequence shown in Table I. Amino-terminal truncation as well as relative abundancies were comparable to those obtained with the reduced sample.

3.3. *Purification and identification of a tissue inhibitor of metalloproteinases*

When the afore- mentioned 25–30 kDa protein, co-purified with gelatinase from monocytes, was visualised after electroblotting and the corresponding protein(s) analysed, two sequences were obtained (Table IIA). A weak signal was observed in the 3 pmol range corresponding perfectly to those of previous analyses of intact gelatinase. Therefore, the latter represents a fragment of intact gelatinase. This indicates that the gelatin-binding domain of monocyte gelatinase (and its fragments) are located in the amino terminal third of the intact molecule.

A second sequence signal was obtained in the 15 pmol range. This sequence, shown in Table IIB, could be aligned with that of purified erythroid-potentiating activity of T-lymphoblasts [31] and with that of the tissue inhibitor of metalloproteinases (TIMP) [32]. Thus, monocytes produce, in parallel with gelatinase, an inhibitor of this metalloproteinase.

Table II

NH_2-terminal sequence analysis of 25 kDa polypeptides[a]

Unreduced 25 kDa gelatinase fragment (3 pmol signal)		Unreduced 25 kDa monocyte TIMP (15 pmol signal)	
		5 10 15 20 25	
L – L F P G D L – T . .	(30%)[b]	– T – V P P H P Q T A F – N S D L V I – A – F – G T P E	
L F P G D L – T N L T D . .	(70%)	A T C V P P H – Q T A F C N S D L V I R A K F V G T	(EPO)
		C T C V P P H P Q T A F C N S D L V I R A K F V G T P E	(TIMP)

[a] Pooled buffy coat mononuclear cells were stimulated for 48 h with Con A and endotoxin and the culture fluids were processed as in Fig. 2. A protein band of 25–30 kDa was excised and the amino terminal residues sequenced. The left panel shows the relative presence of the gelatinase fragments. In the right panel the monocyte TIMP sequence was compared with the amino acid sequence of human erythroid-potentiating factor (EPO) [31] and MO T-lymphoblastic tissue inhibitor of metalloproteinases (TIMP) [32].

[b] % indicates relative amount of the variant form.

4. DISCUSSION

The present study describes the detection of gelatinase induced and secreted by stimulated human peripheral blood monocytes. This gelatinase has a molecular weight of approximately 85 kDa as assayed by SDS-PAGE using both zymography and staining analysis. The monocytic gelatinase is inducible by the cytokine IL-1 and by double-stranded RNA. The monocytic gelatinase was identified as the possible homologue of a phorbol ester-inducible gelatinase from human tumor cells by the following criteria: (a) co-migration on SDS-PAGE, (b) binding with and activity against the gelatin substrate, and (c) serological relationship as determined by immunoprecipitation and binding experiments. Compared to other cell types such as human diploid fibroblasts, melanoma, osteosarcoma and hepatoma-derived cell lines [26], which all secrete several different gelatinolytic enzymes, monocytes from peripheral blood preparations seem to produce mainly the 85-kDa type. Only after concentration of monocyte gelatinase by immunoprecipitation, an additional 65-kDa species was observed on zymography. Monocyte gelatinase preparations, concentrated by chromatographic purification also showed the presence of the 65-kDa enzyme on zymography (data not shown). Characterisation in terms of structural analysis and functional properties of the 65-kDa species is impaired by the low yields obtained.

All experiments were performed under serum-free conditions. This was found to avoid 'spontaneous' production of gelatinase by monocytes. Serum-free culture conditions were also necessary because of the presence of intrinsic gelatinases in all sera tested.

By the subsequent use of affinity chromatography on gelatin substrate and on antibody directed against purified tumor cell gelatinase, two major proteins were co-purified: gelatinase and a tissue inhibitor of metalloproteinases. Most likely TIMP was indirectly bound to the affinity resins via complex formation with gelatinase. Previous studies have already established the presence and regulation of TIMP in monocytes [10,11].

Gelatinases of 67–72 kDa and of 90 kDa have been isolated from bronchial lavage or alveolar macrophages [7,33]. Upregulation in alveolar macrophages of a 90-kDa gelatinase by *Escherichia coli* endotoxin, was recently described [33]. Similarly, EGF and IL-1 have been reported to induce type IV collagenase/gelatinase in tumor cells [30].

For more than a decade fruitless attempts have been made to purify (by multistep chromatographic procedures) the type IV collagenase/gelatinase from natural human monocytes. The use of large scale blood cell processing and of a convenient substrate-affinity chromatographical method has now enabled us to achieve this goal. It appears that the gelatinase from normal monocytes occurs as a truncated form of gelatinase/type IV collagenase previously isolated from tumor cells [30]. Furthermore, the monocytic gelatinase core enzyme is identical to that from neutrophils [17]. It remains to be determined which enzymes are involved in this truncation process and whether such biochemical differences can be exploited to discriminate between normal (leukocytic) and pathological (tumoral) cell migration.

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