Platelet-derived collagenase inhibitor: Characterization and subcellular localization
(protease inhibitors/collagen degradation/α-granules)

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ABSTRACT Purified human platelets were found to contain a collagenase inhibitor that is immunologically, functionally, and chromatographically identical to that produced by human skin fibroblasts. None of the other formed elements of the blood (erythrocytes, granulocytes, mononuclear cells) possessed detectable quantities of this protein. Virtually all the collagenase inhibitor contained within platelets was released following platelet activation with thrombin. Similarly, platelet activation accompanying blood clotting also resulted in the release of this protein, the ratio of plasma to serum inhibitor levels being ~0.5. When platelets were subjected to subcellular fractionation, essentially all of the platelet-associated collagenase inhibitor was found to be located in the α-granule. Studies with radiolabeled inhibitor failed to detect uptake of inhibitor by platelets. Furthermore, immunologically reactive protein of similar quantity to that found in platelets was identified in human megakaryocyte lysates. Thus, the data suggest that the collagenase inhibitor is endogenously produced and stored within platelet α-granules. The platelet-derived collagenase inhibitor was antigenically identical to the collagenase inhibitor from human skin fibroblasts in double immunodiffusion and, like its fibroblast counterpart, inhibited collagenase on a 1:1 stoichiometric basis. When subjected to several of the chromatographic procedures utilized to purify the fibroblast protein, the platelet inhibitor behaved in an indistinguishable manner. Platelet factor 4, previously reported to be a collagenase inhibitor, was found to be immunologically unrelated to the platelet-derived collagenase inhibitor. Furthermore, platelet factor 4 displayed no collagenase inhibitory activity. Although the function of platelet-derived collagenase inhibitor is unknown, such a protein released by activated platelets may serve to regulate collagen turnover during the early stages of the inflammatory process.

At sites of vascular injury, numerous physiologic events occur that may be initiated or modulated by substances released from platelets. In vitro studies have shown that platelet products can mediate specific effects on target cells; for example, β-thromboglobulin (β-TG) is chemotactic for fibroblasts, whereas platelet-derived growth factor (PDGF) and platelet factor 4 (PF4) are chemotactic for monocytes and neutrophils as well as fibroblasts (1–3). Evidence also exists that PDGF is mitogenic for smooth muscle cells and fibroblasts (4).

Among the many physiologic processes that occur during tissue injury, one of great interest is the elaboration and control of collagenase. Since this enzyme initiates collagen degradation (5), regulation of collagenase activity may be important in both normal and pathologic states. Control of active collagenase in the extracellular matrix may be exerted through the action of collagenase inhibitors, of which three classes are generally distinguished. a2-Macroglobulin, the principal serum anti-collagenase, is a nonspecific protease inhibitor which accounts for >95% of the collagenase-inhibitory activity present in human serum (6, 7). Yet its high Mr (~720,000) and irreversible mechanism of inhibition (6) raise doubt concerning its actual in vivo role in the regulation of collagen degradation at the tissue level. A second group consists of tissue-derived glycoproteins of Mr 25,000–30,000 that are stable to extremes of pH and temperature and appear to specifically inhibit metalloproteases of connective tissue origin (8–11). Lastly, cationic proteins of Mr ~10,000 extracted from various tissues have also been reported to possess collagenase-inhibitory activity (12).

Human skin fibroblasts synthesize and secrete a Mr 28,500 glycoprotein that inhibits human skin collagenase in a stoichiometric manner on a 1:1 molar basis (8). This inhibitor has been purified to homogeneity and characterized biochemically (13). A specific antibody was developed (13) and led to comparative studies in which immunologically and functionally identical inhibitor protein was demonstrated in fibroblast lines derived from various human connective tissues, in smooth muscle cells and osteoblasts, and in serum and amniotic fluid (14). We now have identified a collagenase inhibitor in platelets which is functionally and immunologically identical to human skin fibroblast collagenase inhibitor. This inhibitor is contained within platelet α-granules and is released during platelet activation. It is proposed that the platelet-derived inhibitor may be a significant modulator of collagenase activity following acute tissue injury.

MATERIALS AND METHODS

Reagents. [14C]Serotonin was a product of New England Nuclear. Thrombin, rotenone, 2-deoxyglucose, ATP, soybean trypsin inhibitor, and imidazole were obtained from Sigma. Nagarse (subtilisin B) was purchased from Enzyme Development, New York.

Preparation of Blood-Cell Lysates. Purified subpopulations of human blood cells were obtained by centrifugation of 10 ml of acid/citrate/dextrose (ACD)-anticoagulated blood through Ficoll-Hypaque gradients (Sigma) and subsequent differential centrifugation (15). Platelets, mononuclear cells, granulocytes, and erythrocytes were separated; each pool was >95% pure by direct microscopic examination. Cells were suspended in phosphate-buffered saline, pH 7.5, which was free of Ca2+. The four purified cell populations were freeze−thawed and sonicated, and the volume of each was adjusted to give a final protein concentration of 2 mg/ml.

Preparation of Plasma and Serum. Plasma was prepared by the method of Ludlam and Cash (16). Serum was obtained by allowing blood to clot overnight, followed by centrifugation at 2100 × g for 20 min. No significant release from platelets was observed (β-TG levels <1% of serum values).

Abbreviations: β-TG, β-thromboglobulin; PDGF, platelet-derived growth factor; PF4, platelet factor 4.
Preparation and Radio labeling of Human Skin Fibroblast Collagenase Inhibitor. Collagenase inhibitor was purified to homogeneity from the culture medium of normal human skin fibroblasts as described (13). Collagenase inhibitor was 3H-labeled in vivo by the reductive alkylation method of Rice and Means (17); a specific activity of 8,000 cpm/μg of protein was achieved.

Collagenase Inhibitor Assays. Collagenase inhibition was quantitated by a functional assay (14) in which the inhibition of the degradation of native reconstituted [14C]glycine-labeled guinea pig collagen (25,000 cpm/mg) (18) substrate gels by pure human skin collagenase (19) was determined. Inhibition was measured by comparison to a standard titration curve developed using electrophoretically pure inhibitor. The buffer used for all collagenase assays was 0.05 M Tris Cl, pH 7.5, containing 0.01 M CaCl2 and 0.15 M NaCl.

Human collagenase inhibitor was quantitated immunologically by ELISA (14).

Other Assays. β-Glucuronidase was assayed by the modified method of Fishman et al. (20). β-TG was determined by radioimmunoassay (Amersham). Protein concentrations were determined by the method of Lowry et al. (21). Gel diffusion was performed in 1.0% Jonagar as described by Ouchterlony (22).

In Vitro Studies of Platelet Release. Blood obtained from healthy volunteers was mixed with 0.1 volume of fresh acid/citrate/dextrose (15). Platelet-rich plasma was prepared by centrifuging the sample at 240 x g for 10 min; the plasma then was incubated with [14C]serotonin (0.03 μCi/μl; 1 Ci = 37 GBq) for 15 min at 37°C, after which the platelets were washed in platelet washing buffer (23) and suspended in platelet resuspension buffer (23) at ∼2.0 x 10^9 per ml. Platelet release was initiated by addition of thrombin (final concentration 3 units/ml) and incubation for 15 min at 37°C; the reaction was stopped with an equal volume of cold platelet resuspension buffer followed by centrifugation at 2100 x g. The supernatant was analyzed for [14C]serotonin in a liquid scintillation spectrometer and for collagenase inhibitor by ELISA.

Chromatography. Chromatography was performed at 4°C and column effluents were monitored at 230 nm. Platelets obtained by platelethhoresis were activated by thrombin (3 units/ml for 15 min at 37°C) and after centrifugation at 2100 x g, 200 ml of this supernatant was dialyzed versus 0.05 M Tris Cl, pH 7.5. The sample was then applied to a phosphocellulose column (P-1, Whatman; 1.6 x 10 cm) equilibrated with 0.05 M Tris Cl, pH 7.5, and the column was washed overnight. The bound protein was eluted with the same buffer but containing 1.0 M NaCl. The eluted protein containing virtually all the ELISA-reactive activity, was applied to a 1.6 x 10 cm Ultrogel AcA-44 column (LKB) equilibrated with 0.05 M Tris Cl, pH 7.5/0.5 M CaCl2/0.1 M (NH4)2SO4 and was eluted at 25 ml/hr.

Subcellular Fractionation. Platelets from 140 ml of blood were washed, incubated with [14C]serotonin (0.03 μCi/ml at 37°C for 15 min) and 1 mM acetylsalicylic acid (24), and then treated with 20 mM rotenone and 5 mM 2-deoxyglucose at 37°C for 20 min. Three milligrams of Nagarse and 10 mg of ATP were added at 20°C, followed after 5 min by 20 mg of soybean trypsin inhibitor. The platelets were collected by centrifugation and resuspended in 10 ml of 0.01 M Tris Cl, pH 7.4/0.25 M sucrose/1 mM EDTA, and the suspension was then homogenized by ninja blender with a Tekmar blender (head speed 2700 rpm) (27) at 1°C for 15 min (25). Differential centrifugation resulted in the following fractions: F1, pellet after 1000 x g for 22 min (whole platelets, large fragments); F2, pellet after 12,000 x g for 20 min (mitochondria, granules); F3, pellet after 100,000 x g for 60 min (membranes); and F4, 100,000 x g supernatant (all soluble material).

To subfractionate the granule fraction (24, 26), F2 was re-suspended in 2.0 ml of 10 mM Tris Cl, pH 7.4/0.25 M sucrose/1.0 mM EDTA; 1.5 ml of this suspension was layered on an 11-ml continuous 30-60% sucrose gradient in 5 mM TrisCl, pH 7.4/0.1 mM EDTA and centrifuged 100,000 x g for 2 hr (Spinco SW41 swinging-bucket rotor), after which nine fractions were visually identified by their light scattering properties and then sequentially removed from the top of the tube. The fractions were assayed for collagenase inhibitor, β-TG, and [14C]serotonin after treatment with 0.2% Triton X-100. Protein determinations were performed without the addition of detergent. For assay of β-glucuronidase, each fraction was diluted 5- to 8-fold with 0.25 M sucrose and centrifuged at 100,000 x g for 1 hr. The pellets were resuspended in 0.2 ml of 2 mM imidazole, pH 7.4/0.15 M NaCl.

Platelet Uptake of Collagenase Inhibitor. Platelet-rich plasma from 20 ml of blood was incubated with 3H-labeled inhibitor (1 μg/ml; 8000 cpm/μg) at 37°C for 1 hr. The platelets were washed in platelet washing buffer (23), suspended in platelet resuspension buffer (23), and then divided into two aliquots. One sample was treated with thrombin (3 units/ml) at 37°C for 15 min, while the other served as control. The platelets were then collected by centrifugation and the radioactivity in the supernatant fractions was measured. A similar experiment was also performed with unlabeled inhibitor. Platelets were incubated in buffer containing collagenase inhibitor at a 20-fold greater concentration (10 μg/ml) than that present in plasma. Following washing and the addition of thrombin, the platelets were sedimented, and inhibitor release was quantitated by ELISA.

PF4 and Megakaryocyte Lysate. Pure PF4 (27) was kindly supplied by J. S. Huang and T. F. Deuel (The Jewish Hospital of St. Louis, Washington University Medical Center). Human megakaryocyte lysate was generously provided by E. Rabellino (Cornell Medical School).

RESULTS

Collagenase inhibitors that are functionally and immunologically identical to that secreted by human dermal fibroblasts have been identified in a number of human connective tissues and in amniotic fluid and serum (14). Although the serum inhibitor may be derived from numerous sources, the descriptions of a collagenase inhibitor in platelets (28) and polymorphonuclear leukocytes (29) suggested that at least a fraction of this serum collagenase inhibitor might originate from the formed elements of the blood. Using a sensitive and specific ELISA for human collagenase inhibitor (14), we examined cell lysates of purified platelets, mononuclear cells, granulocytes, and erythrocytes for the presence of immunoreactive protein. Only the platelet lysate contained such material, at 225 ng/ml of cell protein; the other cell lysates contained no measurable quantities of inhibitor.

To assess the capacity of platelets to release collagenase inhibitor during activation, plasma and serum values from

Table 1. Plasma and serum levels of collagenase inhibitor

<table>
<thead>
<tr>
<th>Collagenase inhibitor, ng/ml</th>
<th>Plasma</th>
<th>Serum</th>
<th>Plasma/serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>456</td>
<td>980</td>
<td>0.47</td>
</tr>
<tr>
<td>2</td>
<td>395</td>
<td>868</td>
<td>0.46</td>
</tr>
<tr>
<td>3</td>
<td>277</td>
<td>688</td>
<td>0.40</td>
</tr>
<tr>
<td>4</td>
<td>622</td>
<td>1187</td>
<td>0.52</td>
</tr>
<tr>
<td>5</td>
<td>755</td>
<td>1451</td>
<td>0.52</td>
</tr>
<tr>
<td>6</td>
<td>338</td>
<td>1180</td>
<td>0.29</td>
</tr>
<tr>
<td>7</td>
<td>540</td>
<td>952</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Mean ± SD 483 ± 168 1044 ± 250 0.46 ± 0.09

Plasma and serum were obtained from normal healthy donors and analyzed by the ELISA for human fibroblast collagenase inhibitor.

seven healthy individuals were compared (Table 1). β-TG levels, determined for three of the plasma samples, were 62 ng/ml, 77 ng/ml, and 48 ng/ml (normal plasma, <52 ng/ml; normal serum, 15,000–25,000 ng/ml), demonstrating that the procedure for drawing plasma resulted in no significant platelet activation. The collagenase inhibitor (ELISA) values for plasma averaged ~46% of serum levels. Thus, half of the immunoreactive inhibitor protein present in serum can be attributed to its release from platelets during clotting. To show that activation of the clotting cascade per se does not result in inhibitor production, platelet-poor plasma was stimulated with thrombin. No increase in immunoreactive protein was noted after exposure to thrombin (i.e., 465 ng/ml with thrombin vs. 512 ng/ml for control).

The release of collagenase inhibitor from isolated platelets was next examined. Washed, [3H]serotonin-labeled platelets were stimulated with thrombin (3 units/ml at 37°C for 15 min). After removal of the platelets, the supernatant contained 13,100 cpm of [3H]serotonin and 202 ng of immunoreactive inhibitor protein per ml. In comparison, a non-thrombin-treated preparation had 1870 cpm and 41 ng of immunoreactive protein per ml of supernatant fraction. These data indicate that thrombin-induced serotonin release is accompanied by the release of material that is immunologically cross-reactive with human skin collagenase inhibitor.

Subcellular fractionation of platelets was performed to delineate the location of the collagenase inhibitor. Differential centrifugation yielded three pools, the crude granule fraction (F2), the membrane fraction (F3), and the soluble material (F4). The analysis of these fractions is summarized in Table 2. β-TG and serotonin, both known components of the platelet granules, were found predominantly in F2 (crude granule). More than 95% of the immunoreactive collagenase inhibitor was also located in this same crude granule fraction. Virtually none was associated with membranes (F3), and the small amount of inhibitor found in the soluble fraction (F4) most likely arose from granule rupture incidental to processing.

To determine which granule population contained the collagenase inhibitor, the F2 crude granule fraction was separated into nine subfractions by sucrose density gradient centrifugation (24, 26). The distributions of β-glucuronidase, serotonin, β-TG, and collagenase inhibitor are shown in Fig. 1. β-TG, a known α-granule component (30), was found in highest concentrations in subfractions 6–8. [3H]Serotonin was most concentrated in subfraction 9, consistent with its localization as a component of the dense granule (26). β-Glucuronidase had a broad distribution, characteristic of a third granule subfraction, lysosomal granules (26). As seen in Fig. 1, collagenase inhibitor is distributed in a pattern which corresponds to that of β-TG, indicating that it is a component of the platelet α-granule.

Since platelets appeared to contain collagenase inhibitor within their α-granules, we investigated whether megakaryocytes, the progenitor cells of platelets, also possess this protein. A megakaryocyte lysate (from ~3 × 10⁵ cells per ml) was found to contain immunoreactive inhibitor protein at 140 ng/ml by ELISA. This same preparation contained β-TG at 4290 ng/ml, yielding inhibitor/β-TG ratio of 0.033, comparable to the ratio of 0.025 calculated for platelet lysates (Table 2).

Since PF4 has been reported to inhibit collagenase (28), we compared the immunologic and functional properties of this protein with platelet-derived collagenase inhibitor. In the ELISA for human fibroblast collagenase inhibitor, no immunologic cross-reactivity was found in a preparation of pure PF4 (27). When subjected to functional assay, PF4 (tetramer, Mr, 28,000) at a 7-fold molar excess over collagenase (Mr, 55,000) failed to display any anti-collagenase activity (Table 3). Thus, PF4 is structurally unrelated to the platelet-derived collagenase inhibitor discussed in this report.

### Table 2. Subcellular localization of platelet components

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Inhibitor, ng</th>
<th>β-TG, ng</th>
<th>Serotonin, cpm × 10⁻³</th>
<th>β-Glucuronidase, units*</th>
<th>Protein, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000 × g supernatant</td>
<td>2073</td>
<td>83,779</td>
<td>43.2</td>
<td>450</td>
<td>6.14</td>
</tr>
<tr>
<td>F2 (12,000 × g pellet)</td>
<td>2089</td>
<td>&gt;21,500</td>
<td>28.6</td>
<td>170</td>
<td>0.78</td>
</tr>
<tr>
<td>F3 (100,000 × g pellet)</td>
<td>98</td>
<td>4,376</td>
<td>2.4</td>
<td>115</td>
<td>0.43</td>
</tr>
<tr>
<td>F4 (100,000 × g supernatant)</td>
<td>343</td>
<td>3,688</td>
<td>7.3</td>
<td>97</td>
<td>3.54</td>
</tr>
</tbody>
</table>

Platelet homogenate was prepared from 140 ml of whole blood and subjected to initial centrifugation at 1000 × g. This supernatant was then separated into fractions F2, F3, and F4 by differential centrifugation. The 1000 × g supernatant values indicate total amounts prior to fractionation. The F2 fraction contained mitochondria and granules; F3, membranes; and F4, soluble material.

*Modified Sigma units.

![Fig. 1. Distribution of β-glucuronidase, serotonin, β-TG and collagenase inhibitor in sucrose density gradient subfractions of F2. Data are plotted using an analysis scheme described previously (26). Subfractions are numbered horizontally and the widths shown directly reflect their relative protein contents. The vertical column heights represent relative recoveries (percentage of β-glucuronidase, serotonin, β-TG, or inhibitor/percentage of total protein per subfraction). Collagenase inhibitor is distributed in a pattern which is virtually identical to that for β-TG, indicating that it is a component of the platelet α-granule.](image-url)
furthermore, does not appear to be a collagenase inhibitor. To further characterize the platelet-derived collagenase inhibitor, its chromatographic behavior was examined employing several of the steps used to purify inhibitor from fibroblast culture medium (13). Material for such analysis was obtained by thrombin-stimulated release from fresh platelets, followed by removal of the platelets by centrifugation. This step seemed advantageous because >80% of the platelet-associated inhibitor was released during thrombin stimulation (data not shown) and because large amounts of cellular protein were eliminated that would otherwise be present in a cell lysate. Platelet-derived inhibitor bound avidly to phosphocellulose (no inhibitor was detected by ELISA in the flow-through or wash fractions) and was eluted by 0.05 M Tris Cl, pH 7.5/1.0 M NaCl. The eluate was then applied to an Ultrogel AcA-44 column, which produced two large and distinct protein peaks (Fig. 2). When the column fractions were assayed for their functional ability to inhibit collagenase, a single peak of inhibitory activity was found, corresponding to the second protein peak (fractions 21-25). Similarly, by ELISA measurements, essentially all of the immunoreactive material appeared in these same fractions. An apparent Mr = 31,000 (Fig. 2) was derived from this gel filtration column, a value identical to the apparent Mr of fibroblast-derived collagenase inhibitor on this chromatographic matrix (8, 13). Furthermore, material from this inhibitor peak displayed an immunologic reaction of identity against pure fibroblast inhibitor in Ouchterlony gel diffusion (Fig. 3). When tested for functional activity, collagenase was inhibited on a 1:1 molar basis, using values for inhibitor concentration determined by ELISA (not shown). This stoichiometry is also characteristic of human fibroblast collagenase inhibitor (14).

**DISCUSSION**

This report documents the presence in platelets of a collagenase inhibitor immunologically, functionally, and chromatographically identical to that secreted by dermal fibroblasts and found in the culture medium of other human connective tissues, in serum, and in amniotic fluid (14). None of the other formed elements of the blood contained detectable quantities of this protein. Significant amounts of platelet-derived inhibitor are released during clotting, resulting in a 2-fold increase in inhibitor concentration in serum as compared to plasma. Although the origin of collagenase inhibitor in plasma is not known, the inhibitor is produced by mesenchymal cells throughout much of the body (14) and likely gains access into the bloodstream from capillaries that supply such tissues. Although platelets are another potential source, plasma inhibitor levels are ~50% of serum levels, in contrast to β-TG, for which plasma levels are <1% of serum levels. Thus, for platelets to contribute appreciably to the level of collagenase inhibitor present in plasma, platelet α-granules would have to preferentially "leak" this protein as compared to other constituents, such as β-TG, or the clearance of this inhibitor from serum would have to be very slow.

Since platelets exist in a plasma milieu which contains collagenase inhibitor (Table 1), it was crucial to determine whether the source of platelet-associated inhibitor was related to the exogenous uptake of this protein. After subcellular fractionation, virtually all of the platelet-associated inhibitor was found to be contained in the α-granule (Fig. 1), which also stores other platelet-derived proteins such as PDGF, β-TG, and PF4 (3). As would be expected from these data, thrombin stimulation caused release of nearly all the inhib-
tor contained in platelets. Furthermore, during the clotting process, platelet activation was accompanied by release of inhibitor, producing serum inhibitor levels of 1.0 μg/ml as compared to plasma levels of 0.5 μg/ml (Table 1). The finding that megakaryocyte lysates contain immunoreactive inhibitor protein also supports the concept that platelet-associated inhibitor is endogenously produced. Finally, when collagenase inhibitor was radiolabeled and platelets were incubated at a concentration of inhibitor comparable to that present in plasma, no exogenous uptake could be detected. Similarly, unlabeled inhibitor was not taken up by platelets even at concentrations 20-fold greater than those present in plasma (data not shown). Although biosynthetic studies will be required to definitively establish the source of the platelet collagenase inhibitor, the data in this study strongly suggest that its production is endogenous.

PF4 has previously been reported to be a collagenase inhibitor (28). Using preparations of PF4 purified to homogeneity by affinity chromatography on heparin-Sepharose followed by gel filtration on Sephadex G-100 (27), we were unable to show any collagenase inhibitory activity. The PF4 preparation used in the report (28) that described enzyme inhibition was obtained with a purification utilizing only the affinity step. Since fibroblast collagenase inhibitor binds avidly to fibroblast-Sepharose (unpublished observations), contamination with the platelet-derived collagenase inhibitor described in the present investigation may explain the reported collagenase inhibitory activity of PF4.

The function of the platelet-derived collagenase inhibitor is unknown. Collagenase is elaborated by fibroblasts (19), mononuclear cells (32), and polymorphonuclear leukocytes (29) at sites of tissue injury. Likewise, platelets are active participants in reactions occurring as a result of tissue injury and as part of the inflammatory response (1–4). Therefore, one mechanism for regulating collagenase activity during tissue injury may be the release of a specific collagenase inhibitor from platelets. Indeed, at sites of local injury, concentrations of collagenase inhibitor released by activated platelets may be significant and may regulate collagen turnover during the early stages of wound repair. In addition, various pathologic processes could conceivably involve platelet-derived collagenase inhibitor. For example, in the fibrosing diseases and even in atherosclerosis, the platelet inhibitor could promote the accumulation of collagen, thereby contributing to the pathogenesis of these complicated and multifactorial disorders.

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