Endothelial-cell-stimulating angiogenesis factor (ESAF) activates progelatinase A (72 kDa type IV collagenase), prostromelysin 1 and procollagenase and reactivates their complexes with tissue inhibitors of metalloproteinases: a role for ESAF in non-inflammatory angiogenesis

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Endothelial-cell-stimulating angiogenesis factor (ESAF) has been shown to activate procollagenase and reactivates complexes of collagenase and gelatinase A with tissue inhibitor of metalloproteinase (TIMP)-1. In the present paper we show a purification protocol for bovine pineal ESAF and that purified ESAF activates progelatinase A and prostromelysin-1. Unlike the activation of procollagenase by plasmin/plasminogen activator, which requires the presence of stromelysin for full activation, ESAF is able to activate fully all three proenzymes. Purified ESAF is also shown to reivate the complexes of gelatinase A, collagenase and stromelysin-1 with TIMP-2. Once separated, both enzyme and inhibitor are active; however, ESAF binds to the enzyme in a manner preventing it from further inhibition by TIMP. ESAF is the only physiological molecule able to reactivate the TIMP/enzyme complex.

INTRODUCTION

The three matrix metalloproteinases (MMPs), interstitial collagenase, stromelysin-1 and gelatinase A (72 kDa gelatinase), are secreted as zymogens or proenzymes requiring extracellular activation [1]. Together they degrade interstitial and basement-membrane collagens and are involved in both pathological breakdown and normal remodelling of connective tissues [2]. After activation these enzymes can be inhibited effectively by tissue inhibitors of metalloproteinases (TIMPs) [3]. The activation and inhibition of these MMPs is therefore a key step in the regulation of connective-tissue degradation.

Several stages of activation of these proenzymes have been described. Partial activation can be achieved with concurrent loss of molecular mass by enzymes such as plasmin, plasminogen activator or kallikrein [4,5]. This partial activation is followed by autocatalytic cleavage to a lower-molecular-mass form that is the fully active enzyme.

This schema only applies to procollagenase and prostromelysin-1, as progelatinase A cannot be activated to the initial intermediate form by enzymes such as plasmin and kallikrein [4,5]. However, a novel membrane-bound proteinase has recently been shown to activate gelatinase A [6].

p-Aminophenyl mercuric acetate (APMA) is able to activate the pro-forms of all three enzymes, although it is more effective with procollagenase and progelatinase A than with prostromelysin-1, where a longer time is required for full activation [7]. Stromelysin-1 is itself an activator of procollagenase as well as enhancing activation of procollagenase with other enzymes [8], and it has been suggested as a rate-limiting step in the full activation of interstitial procollagenase.

The major inhibitors of the active MMPs are the TIMPs [3]. TIMP-1 is a 28.5 kDa glycoprotein [9] capable of inhibition of all the major MMPs. TIMP-2 shares a significant similarity with TIMP-1 and is thought to be more selective towards the inhibition of gelatinase A [10]. It has been shown to be associated with and to co-purify with progelatinase A [11]. TIMP-2 has also been shown to inhibit the autoactivation of procollagenase initiated by APMA by forming a complex with the proenzyme [12]. Recently a third inhibitor, TIMP-3, has been identified that like TIMPs -1 and -2 inhibits collagenase, stromelysin and gelatinases A and B [13]. Both TIMPs -1 and -2 form tight complexes with the active MMPs, and until recently these complexes have been thought of as essentially irreversible.

A non-enzymic, non-protein but naturally occurring low-molecular-mass factor has been shown to be capable of activation of procollagenase [14,15] and recently has also been shown to be capable of reactivating the complexes of collagenase and TIMP-1 and complexes of gelatinase A and TIMP-1 [16,17]. This factor, endothelial-cell-stimulating angiogenesis factor (ESAF), has a molecular mass of approx. 600 Da and stimulates the proliferation of microvascular endothelial cells in culture [18] both alone and synergistically with basic fibroblast growth factor (bFGF) [19], but has no effect on aortic or large vein endothelial cells [20]. ESAF has also been shown to produce an angiogenic response on the chick chorioallantoic membrane (CAM) [21]. ESAF has been identified and extracted from a variety of tissue sources [22], such as the bovine pineal gland, which is a major source of the factor [23], and the epiphyseal growth plate [24]. In the case of the growth plate this probably reflects the neovascularization occurring during transformation of avascular cartilage to vascular bone [24]. ESAF has also been demonstrated in measurable amounts in serum from patients with pathological conditions where active neovascularization is occurring, for example in proliferative retinopathy and fracture healing [25-27].

In the present study we report the purification of bovine pineal ESAF by HPLC and the activation by pure ESAF of both prostromelysin-1 and progelatinase A over a physiological time

Abbreviations used: APMA, p-aminophenyl mercuric acetate; CAM, chorioallantoic membrane; ESAF, endothelial-cell-stimulating angiogenesis factor; bFGF, basic fibroblast growth factor; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

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frame. Pure ESAF is also shown to reactivate the complexes formed between all of the three major MMPs and their inhibitors. This reactivation leads to the liberation of free fully active enzymes and TIMPs. We also show that ESAF binds to the liberated active enzymes, preventing their subsequent re-inhibition by TIMPs.

MATERIALS AND METHODS

Preparation of human skin fibroblast collagenase

Collagenase and procollagenase were prepared from fibroblasts obtained from skin donated by normal volunteers. Skin punch biopsies (5 mm diam.) were taken from the forearm and explanted into culture flasks. Released fibroblasts were further cultured in medium 199/10% foetal calf serum with the addition of 100 units of penicillin and 100 µg of streptomycin per ml of medium in a standard humid environment of 5% CO₂ in air. Cells were cultured until passage six. At this passage and when the fibroblasts were judged to be pre-confluent, the medium was removed and the cells were washed three times with PBS. The cells were then incubated for 48 h in serum-free medium 199 to which 1 µg of cytochalasin B per 500 ml of medium had been added. The conditioned medium was collected and immediately concentrated. Stromelysin-1, free collagenase and procollagenase were prepared from these concentrates as described by Suzuki et al. [28], using a green dye matrix column and gel-filtration chromatography techniques.

Extraction of ESAF from bovine pineal glands

In order to separate ESAF from its carrier protein, 100 bovine pineal glands were homogenized in 200 ml of 50 mM NH₄HCO₃ buffer containing 2 M MgCl₂, pH 7.6, and centrifuged at 40000 g for 1 h at 4 °C. The resulting supernatant was filtered through glass wool, before being dialyzed in 800 ml of NH₄HCO₃ buffer containing 2 M MgCl₂, which enabled separation of ESAF from its carrier protein, using an Amicon 3 kDa Spiral Filter. The resulting 450 ml of the filtrate was applied to a 1 cm x 10 cm ‘DIAION’ ion-exchange chromatography column (Daiichi Associates). The column was washed with 50 ml of distilled water, followed by 50 ml each of 50, 75, 90 and 100% HPLC-grade ethanol. ESAF activity was assayed by its ability to activate procollagenase and was found in the 90 and 100% fractions. These fractions were partially lyophilized to a volume of 1 ml by centrifugal evaporation (Gyrovap; V. A. Howe Ltd.) and purified using reverse-phase HPLC.

Purification of ESAF by HPLC

Concentrate (100 µl) was injected on to a 5 µ silica Hypermisil C18 ODS reverse-phase HPLC column equilibrated in a mobile phase of 60:40 acetonitrile:water. ESAF was eluted with a linear gradient of 60:40 acetonitrile:water to 100% acetonitrile over 20 min with a flow rate of 1 ml/min. ESAF eluted at approx. 70% acetonitrile at 17 min. Acetonitrile was removed by centrifugal evaporation at 4 °C, and the ESAF was aliquoted into 1 ml fractions, which were further purified using a 5 µ Hypersil C18 ODS reverse-phase column with an isocratic elution of 70% acetonitrile in water over 30 min with a flow rate of 1 ml/min. ESAF eluted from this column at 12 min. Fractions were collected, lyophilized to remove acetonitrile, and tested for activation of procollagenase before being further purified.

ESAF fractions resulting from the isocratic elution were applied to a gel-permeation HPLC column, Phenogel (Phenomenex) and eluted in 100% acetonitrile. This column has a molecular-mass separation range of 50–1000 Da. ESAF eluted from this column at 4.2 min. The flow rate was 700 µl/min, and the void volume was 1.4 ml. Fractions were collected, lyophilized and assayed for activation of procollagenase before further purification. Intermediate analysis of fractions purified from gel-permeation chromatography by electron ionization MS showed contaminating amounts of a co-purifying sugar later determined by NMR analysis to be fructose. The contaminating fructose in the ESAF preparation was removed by chromatography, using a Concanavalin-A–Sepharose 4B column (5 cm x 1 cm) equilibrated in HPLC-grade water. Aliquots (5 ml each) of ESAF were applied to the column and washed with 30 ml of water, lyophilized and tested for ESAF activation of procollagenase. The contaminating sugar remained on the column, which was discarded.

To remove solvent impurities due to concentration of organic solvents in the chromatography stages, 20 ml of ESAF solution was extracted with 50 ml of 100% ether in a standard extraction flask. The aqueous fraction containing ESAF was lyophilized and tested for ESAF activity. The ether fraction was discarded. Further purification of ESAF fractions was achieved using a 5 µ Hypersil C18 ODS column eluted isocratically with 70% acetonitrile in water with 100 µl injections of ESAF over 30 min and a flow rate of 1 ml/min. ESAF eluted as a single peak at 12 min and was lyophilized and tested using the activation of pro-collagenase.

This single peak was applied to a 1 mm internal diam. HPLC C18 microbore column and eluted isocratically with 75% acetonitrile in water over 20 min, using a microbore HPLC system and detector. The ESAF peak eluted at 7 min and was separated from minor impurities.

Finally, 20 µl (40%) of this pure ESAF peak was applied to a 4 mm internal diam. gel-permeation (Phenogel) column linked to a microbore HPLC system (Kontron Analytical) and eluted with 100% acetonitrile at a flow rate of 700 µl/min to produce a single chromatographic peak (Figure 1). The pure ESAF fraction is currently undergoing molecular structure studies.

ESAF assay

ESAF was routinely assayed using its ability to activate pro-collagenase [14]. A unit of ESAF is defined as the amount required to activate fully 1 unit of procollagenase.

Assay of MMPs

Collagenase and gelatinase A were assayed according to the method of McLaughlin et al. [16], using substrates of radio-labelled bovine type I collagen and type I gelatin respectively. Stromelysin-1 was assayed by the method of Galloway et al. [29], using a substrate of radiolabelled casein. The proenzymes were assayed after the addition of various concentrations of ESAF or 1 mM APMA. After the addition of TIMPs -1 and -2 to the active enzymes, activity was measured before and after the addition of ESAF. All incubations were carried out for 4 h at 36 °C.

A unit of collagenase degrades 1 µg of type I collagen/min at 36 °C [30]. A unit of gelatinase A degrades 1 µg of gelatin/min at 36 °C [29], and 1 unit of stromelysin-1 degrades 1 µg of casein/min at 36 °C [31].

The procollagenase was passed through a Sephacryl S-200 superfine gel-filtration chromatography column to improve its final purity.

Recombinant TIMP-1 and TIMP-2 were a gift from Dr. T. Cawston, Department of Rheumatology, Addenbrookes Hospital, Cambridge, U.K. We are also grateful to Professor Hideaki
Preparation and activation of MMP–TIMP complexes

Samples (200 µl) of purified active collagenase, gelatinase A or stromelysin were preincubated with TIMPs -1 or -2 for 30 min at 36 °C to form an enzyme–inhibitor complex in the ratio of 1 unit of TIMP to 1 unit of active MMP. A range of ESAF units from 0.0002 to 2.0 was added to the inhibitor complex and preincubated for 2 h at 36 °C before the normal assay appropriate for each enzyme. Controls in which ESAF was replaced with Tris/HCl assay buffer were included.

ESAF binding studies

In experiments to determine whether ESAF bound to any of the three MMPs, the MMPs were incubated in a ratio of 1:30 ESAF:enzyme units for 2 h at 36 °C. Subsequently the mixtures were dialysed against three changes of 10 vol. of Tris/HCl assay buffer. The resulting dialysates and retentates were assayed for the presence of enzymes and ESAF. To determine whether ESAF bound to TIMPs, ESAF was incubated with either TIMP-1 or TIMP-2 in a ratio of 1:100 units of ESAF:TIMP for 2 h at 36 °C before dialysis against three changes of Tris/HCl assay buffer. The retentates and dialysates were assayed for enzyme, TIMP and ESAF activity.

TIMP-1 and TIMP-2 were assayed using their ability to inhibit collagenase and gelatinase A respectively [16]. A unit of TIMP-1 inhibits 2 units of collagenase by 50 % [31]. ESAF (0.2 unit) was subsequently incubated independently with 6 units of all three active MMPs, preincubated at 36 °C for 2 h and dialysed to remove any free ESAF. Either TIMP-1 or TIMP-2 (2 units each) was added to the retentate, further incubated for 2 h at 36 °C and assayed for the ability of TIMPs to inhibit active collagenase as described by McLaughlin et al. [16]. In the present study we have defined 1 unit of TIMP-2 as inhibiting 2 units of gelatinase A by 50 %. Free enzymes were treated with ESAF in the absence of inhibitors in a ratio of 1:60 ESAF:enzyme units as a control.

Comparison of activation of procollagenase by (1) ESAF alone, (2) ESAF with stromelysin, (3) trypsin alone, and (4) trypsin with stromelysin

Procollagenase (0.5 unit) was incubated with 0.25 µg of trypsin for 2 h at 36 °C. The trypsin was then inhibited with a five-fold excess of soya-bean trypsin inhibitor. In some experiments 1 unit of stromelysin-1 was also added, and the mixture was reincubated for 2 h. In other experiments, activation with trypsin was replaced with activation by 0.01 unit of ESAF with and without the addition of stromelysin-1. Collagenase was assayed as described above.

RESULTS

Activation of proMMPs by ESAF

Samples (2 units each) of all three proenzymes, procollagenase, progelatinase A and prostromelysin-1, were activated to 100 % or more of their potential activation with APMA, by between 0.01 and 0.2 unit of ESAF within 4 h. Typical activation curves are shown in Figure 2. Prostromelysin-1 was more fully activated by ESAF than by APMA in this time frame (Figure 2c). This was due to APMA not achieving its full activation capacity within 4 h. Equivalent activation to that achieved by ESAF took 12 h.
The time course for activation varied for each enzyme, taking approx. 20 min to reach 50% activation for procollagenase, 60 min for 50% activation for progelatinase A and 30 min for 50% activation for prostromelysin-1 (Figure 3).

Effect of inhibitors on the activation of proMMPs by ESAF

Addition of proteinase inhibitors, soya-bean trypsin inhibitor, PMSF, trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane and pepstatin, had no effect on the processing of the proMMPs by ESAF. However, the processing was inhibited by the metal chelator EDTA (Table 1).

Effect of ESAF on the molecular mass of proMMPs

Activation of the three proenzymes, procollagenase, progelatinase A and prostromelysin-1, with bovine pineal ESAF resulted in a loss of molecular mass that was identical to that obtained by activation with APMA. Typical SDS/PAGE patterns are shown in Figure 4 (procollagenase, 55 kDa to 45 kDa; progelatinase-A, 72 and 70 kDa to 68 and 66 kDa; and prostromelysin, 57 kDa to 45 kDa). TIMP-2-free progelatinase-A migrated as a 72 kDa band, presumably due to the presence of activation products.

Trypsin had no action against progelatinase-A (results not shown).

Activation of procollagenase by stromelysin-1, trypsin and ESAF

Over a 4 h assay incubation time, activation of procollagenase in the presence of trypsin alone resulted in around 90–92% activation, as compared with 100% activation with 2 mM APMA. Activation in the presence of stromelysin-1 alone resulted in approx. 70–74%, activation. However, activation of procollagenase by ESAF in the presence or absence of stromelysin-1 and activation of procollagenase in the presence of trypsin and stromelysin always resulted in activations of approx. 114 and 112%, respectively (Table 2).

Reactivation of MMP–TIMP complexes by ESAF

In this case full reactivation of all the enzymes required the same amount of ESAF, i.e. 0.01 unit of ESAF for full reactivation of 1 unit of enzyme complexed with 1 unit of TIMPs. Reactivation of the collagenase–TIMP-1 complex started at a lower concentration of ESAF than for the other two enzymes, but this had no bearing on the final result (Figure 5). The same results were obtained using TIMP-2 (Figure 6).

Binding studies of active MMPs and TIMPs -1 and -2 with ESAF

ESAF itself is freely dialysable; however, it bound to all the three enzymes and could not then be recovered by dialysis against Tris/HCl buffer (Table 3). Neither ethanol nor 2 M MgCl₂, both of which dissociate ESAF from its carrier protein in serum and
tissue, could dissociate the bound ESAF from the enzymes. When ESAF was mixed with TIMP-1 or TIMP-2, both ESAF and TIMPs were fully recovered, ESAF in the dialysate and TIMPs in the retentate, indicating that no binding of ESAF to TIMP had occurred (Table 4).

MMPs to which ESAF was bound retained their activity but were found not to respond to inhibition with TIMPs -1 or -2, whereas untreated enzyme under the same conditions was readily inhibited (Table 5).

**DISCUSSION**

The general concept for the mechanism of activation of the proMMPs has been focused on the serine proteinases of the fibrinolytic system, plasmin and plasminogen activator [4]. Indeed plasmin has been shown to achieve 20% activation of pro-collagenase, but full activation of this enzyme by plasmin requires the additional presence of stromelysin-1 [34].

A similar situation exists where plasma kallikrein is used as an activator of procollagenase [31]. Stromelysin-1 itself has been shown capable of complete activation of procollagenase, but the process is amazingly slow, complete activation only being achieved after 3–4 days of incubation with a 20-fold molar excess of stromelysin-1 over procollagenase. When stromelysin-1 is added to either plasmin- or kallikrein-treated procollagenase, complete activation is achieved in 1 h; however, neither plasmin nor kallikrein is capable of activating progelatinase A either alone or in combination with stromelysin-1. In contrast, ESAF is able to activate fully all three of the major proMMPs, interstitial procollagenase, progelatinase A and prostromelysin-1, within 90 min. In this context it is interesting that full activation of prostromelysin-1 with organomercurials such as APMA does not occur immediately, but requires approx. 12 h [30].

ESAF activation requires amounts of ESAF that are so small as to be extremely difficult to quantify, but the amounts required for full activation of 0.5 unit of procollagenase reflect approx. 3% of the activity present in a single pineal gland. All the activations of the proMMPs result in lower-molecular-mass

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**Table 2 Activation of procollagenase by ESAF, trypsin and stromelysin-1**

Activation of 0.5 unit of procollagenase by treatment with combinations of 2.5 µg of trypsin, 1.0 unit of stromelysin-1 or 0.01 unit of ESAF. Percentage activation is that compared with total activation with 2 mM APMA over a 4 h incubation period.

<table>
<thead>
<tr>
<th>Activation (%)</th>
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<tr>
<td>Procollagenase + trypsin</td>
</tr>
<tr>
<td>Procollagenase + trypsin + stromelysin-1</td>
</tr>
<tr>
<td>Procollagenase + stromelysin-1</td>
</tr>
<tr>
<td>Procollagenase + ESAF</td>
</tr>
<tr>
<td>Procollagenase + ESAF + stromelysin-1</td>
</tr>
</tbody>
</table>

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**Figure 4 SDS/PAGE of proMMPs with ESAF**

Activation of 0.5 unit of each of the three major proMMPs by 0.03 unit of ESAF or 1 mM APMA for 4 h at 37 °C. (A) Procollagenase: alone (lane 1), with APMA (lane 2) and with ESAF (lane 3). (B) Progelatinase-A: alone (lane 1), with APMA (lane 2) and with ESAF (lane 3). (C) Prostromelysin: alone (lane 1), with APMA (lane 2) and with ESAF (lane 3). Molecular-mass markers (kDa) are shown in each lane labelled Mr.

**Figure 5 Reactivation of the MMP–TIMP-1 complex**

Reactivation profiles on a log scale of the complexes of 1 unit of TIMP-1 with 1 unit of collagenase (a), gelatinase A (b) or stromelysin-1 (c) by a range of ESAF units from 0.0002 to 0.2.
forms of the enzymes, and activation by ESAF is no exception. In fact the molecular mass of the final active conversion product of progelatinase A is similar if not identical to that obtained from APMA. Furthermore, active products of conversion of procollagenase and prostromelysin have molecular-mass forms equivalent to those obtained with APMA (Figure 4).

Very little consideration has been given to the possibilities of different mechanisms for activation of proMMPs in different physiological conditions associated with angiogenesis. For example, in inflammation, where kallikrein, plasmin and plasminogen activator will be present, activation is more likely to be via these enzymes. However, in non-inflammatory conditions such as bone growth, where controlled activation of proMMPs is associated with angiogenesis [24], and similarly in proliferative retinopathy of diabetes, ESAF is likely to be the physiological activator. Evidence for the presence of ESAF in these and other non-inflammatory angiogenic conditions has been reviewed by Odedra and Weiss [15].

Inhibition of MMPs with the specific inhibitors of these enzymes has been considered to be irreversible within a physiological context. Although dissociation of these enzyme–inhibitor complexes has been achieved chemically by acetic acid, this resulted in the liberation of an inactive enzyme [35].

Activation of the TIMP1–collagenase complex with ESAF, leading to recovery of fully active collagenase, has been reported previously [16]. The results reported here show that ESAF is also capable of reactivating the complexes of gelatinase A and stromelysin-1 with both TIMPs -1 and -2 and of collagenase with TIMP-2. In all these cases the reactivation gave rise to fully active enzymes and functional inhibitors. However, rather surprisingly, ESAF bound to the dissociated active MMPs in a manner rendering them no longer susceptible to inhibition by either of the TIMPs.

The time required for both activation of proenzymes and reactivation of the enzyme–inhibitor complexes by ESAF is relatively short, consistent with a physiological time frame.

It is possible that ESAF binds to the enzyme at the TIMP-binding site and has a greater affinity for this site than for the inhibitor. Alternatively ESAF may bind to the enzyme in such a way as to prevent TIMP binding to its own site. Since ESAF could not be removed from the enzyme with 2 M MgCl₂ or ethanol, it is more likely to be a covalent rather than an ionic binding, though this needs further study. However, it is apparent

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**Figure 6 Reactivation of the MMP–TIMP-2 complex**

Reactivation profiles on a log scale of the complexes of 1 unit of TIMP-2 with 1 unit of collagenase (a), gelatinase A (b) or stromelysin-1 (c) by a range of ESAF units from 0.0002 to 0.2.

**Table 3 Binding studies of ESAF and active MMPs**

Percentage recovery of both active enzyme and ESAF, showing ESAF bound to the active MMPs from the retentate and dialysate of dialysed mixtures of 6 units each of collagenase, gelatinase A or stromelysin-1 with 0.2 unit of ESAF.

<table>
<thead>
<tr>
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<th>Recovery of active ESAF (dialysate) (%)</th>
<th>Recovery of enzyme (retentate) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenase + ESAF</td>
<td>6.9 ± 0.8</td>
<td>94.6 ± 2.4</td>
</tr>
<tr>
<td>Gelatinase A + ESAF</td>
<td>5.0 ± 0.4</td>
<td>95.5 ± 4.9</td>
</tr>
<tr>
<td>Stromelysin-1 + ESAF</td>
<td>3.3 ± 0.4</td>
<td>94.0 ± 6.2</td>
</tr>
</tbody>
</table>

**Table 4 Binding studies of ESAF and TIMPs**

Percentage recovery of ESAF and both TIMP-1 and -2 from dialysed mixtures of 2 units of TIMP-1 or 2 units of TIMP-2 with 0.02 unit of ESAF; distilled water is a control.

<table>
<thead>
<tr>
<th></th>
<th>Recovery of ESAF (dialysate) (%)</th>
<th>Recovery of TIMP (retentate) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESAF + water</td>
<td>99.4 ± 0.4</td>
<td>–</td>
</tr>
<tr>
<td>Water + TIMP-1</td>
<td>–</td>
<td>98.2 ± 0.3</td>
</tr>
<tr>
<td>Water + TIMP-2</td>
<td>–</td>
<td>99.0 ± 0.6</td>
</tr>
<tr>
<td>ESAF + TIMP-1</td>
<td>99.4 ± 0.1</td>
<td>95.3 ± 1.6</td>
</tr>
<tr>
<td>ESAF + TIMP-2</td>
<td>98.6 ± 0.9</td>
<td>97.9 ± 2.0</td>
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</table>

**Table 5 Binding studies of ESAF-treated MMPs and TIMPs**

Percentage inhibition of 6 units each of the active MMPs, collagenase, gelatinase A and stromelysin-1 with and without pre-treatment with 0.2 unit of ESAF by 2 units of TIMP-1 or 2 units of TIMP-2.

<table>
<thead>
<tr>
<th></th>
<th>Inhibition by TIMP-1 (%)</th>
<th>Inhibition by TIMP-2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenase</td>
<td>93.3 ± 3.2</td>
<td>92.4 ± 0.9</td>
</tr>
<tr>
<td>Collagenase*</td>
<td>3.4 ± 0.7</td>
<td>2.7 ± 0.6</td>
</tr>
<tr>
<td>Gelatinase A</td>
<td>94.4 ± 3.3</td>
<td>96.5 ± 2.1</td>
</tr>
<tr>
<td>Gelatinase A*</td>
<td>5.5 ± 1.0</td>
<td>0.8 ± 0.7</td>
</tr>
<tr>
<td>Stromelysin-1</td>
<td>96.7 ± 2.3</td>
<td>92.8 ± 1.7</td>
</tr>
<tr>
<td>Stromelysin-1*</td>
<td>2.9 ± 1.1</td>
<td>3.4 ± 0.6</td>
</tr>
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</table>

* Enzymes pre-treated with 0.2 unit of ESAF and dialysed.
that the binding site of ESAF in procollagenase that induces activation must be distinct from that in the active collagenase when dissociated from TIMP, since activation of the proenzymes does not lead to production of a non-inhibitable active enzyme. It is probable that in this instance ESAF binds preferentially to a site in the propeptide rather than to the enzyme itself. Once the structure of ESAF is fully determined, the nature of the TIMP-binding site in the enzyme may become clearer.

The dissolution of the capillary basement membrane is a prerequisite for the initiation of the angiogenic process, and the ability of ESAF to activate enzymes capable of achieving such dissolution may be of great importance in its function as an angiogenic factor. Although current wisdom appears to perceive plasmin or plasminogen activator as the major activators of proMMPs in angiogenesis, these enzymes are less obviously present in angiogenesis occurring in the absence of inflammatory cells. ESAF on the other hand is present in non-inflammatory conditions of angiogenesis such as bone growth and fracture healing [36], and we conclude that ESAF may be a major contributor to matrix degradation in these circumstances.

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33 Reference deleted

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