Potentiating effect of heparin in the activation of procollagenase by a low-M_r angiogenesis factor

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A low-M_r freely dialysable endothelial cell-stimulating angiogenesis factor (ESAF) from conditioned medium of a mouse lymphoma cell line has previously been shown to activate latent skin fibroblast procollagenase. Activation comparable with the maximum that can be achieved with trypsin is obtained with chemically undetectable amounts of the factor. We now show that when even smaller amounts of ESAF are used heparin is able to potentiate its action in this system. The relationship between this activity and the mechanism of angiogenesis, which is itself potentiated by heparin, is discussed.

Procollagenase *Heparin* *Angiogenesis* *Endothelial cell* *Fibroblast* *Lymphoma*

1. INTRODUCTION

We have recently reported the extraction of a low-M_r tumour angiogenesis factor, which induces neovascularization on the chick chorioallantoic membrane (CAM) [1] and stimulates the proliferation of cultured capillary endothelial cells [2]. The factor has also been obtained from non-tumour sources, and is called endothelial cell stimulating angiogenesis factor (ESAF). We have also shown that ESAF activates skin fibroblast procollagenase [3]. A unifying mechanism of action for a factor which is able to induce both endothelial cell proliferation and directional growth, which are properties of angiogenic substances [4], is difficult to understand. However, the ability of ESAF to activate a latent form of collagenase may be a highly relevant aspect.

It has been observed that mast cells accumulate at the leading tip of growing capillaries and it has been suggested that angiogenesis does not occur unless these cells are present. Heparin, a mast cell product, potentiates the effect of other angiogenic factors [5] as well as our own (unpublished) observations on the chick CAM bioassay. For this reason we have studied the effect of heparin on ESAF activation of skin fibroblast procollagenase.

2. MATERIALS AND METHODS

2.1. *Low-M_r angiogenesis factor (ESAF)*

ESAF was prepared from medium conditioned by a cloned mouse lymphoma cell line [6]. The angiogenesis factor was freely dialysable and eluted from a gel filtration column (Bio-Gel P2, 44 × 4.4 cm run in 10%, v/v, propan-2-ol/water, 45 ml/h) in a position corresponding to a M_r of 300–600.

2.2. *Human skin fibroblast procollagenase*

Procollagenase was prepared from serum-free human skin fibroblast conditioned medium using a modification of the method in [7]. Serum-free conditioned medium was applied to a Sephacryl S200 column (80 × 2.5 cm) and eluted with Tris–HCl buffer (pH 7.6) containing 0.2 M NaCl and 0.01 M $CaCl_2$. The enzyme was activatable by Mersalyl (table 1) and the metalloproteinase nature of the preparation was confirmed by the EDTA inhibition of the Mersalyl and ESAF activated proenzyme (table 1). Analysis of the degradation products from the enzyme-catalysed hydrolysis of Type-I collagen by SDS–polyacrylamide gel electrophoresis revealed the ¼ and ¾ collagen fragments characteristic of the action of mammalian collagenase [8].

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Table 1

Effect of ESAF, heparin and mersalyl on the activation of skin fibroblast procollagenase

Assay mixture	μg Type-I collagen degraded/16 h
Enzyme	0
+ mersalyl	10.7 ± 2.5
+ ESAF	1.7 ± 0.16
+ heparin	0.2 ± 0.05
+ heparin + ESAF	4.3 ± 0.32
Heparin alone	0.2 ± 0.1
ESAF alone	0.3 ± 0.08

Controls and EDTA blanks have been subtracted from the results

2.3. *Tritium-labelled Type-I collagen substrate*

Type-I collagen was prepared from foetal calf skin as in [9]. The collagen was shown to be pure by SDS–polyacrylamide gel electrophoresis and was labelled by a modification of the method in [10] whereby [^{3}H]acetic anhydride was used instead of ^{14}C-labelled reagent. Complete hydrolysis of 50 μg Type-I collagen catalysed by bacterial collagenase (from *Clostridium histolycum*, Sigma, London) yielded 6124 cpm.

2.4. *Collagenase assay*

Type-I collagen (50 μg) was dissolved n 25 μl 0.05 M Tris–HCl buffer (pH 7.6) containing 0.2 M NaCl and 0.01 M $CaCl_2$ (Tris buffer) and preincubated for 30 min at 36.5°C. During this time the collagen gelled. Mersalyl (25 mM, Evans Medical, Liverpool) and ESAF, both with and without EDTA (25 mM), and bacterial collagenase (1 mg/ml) were dissolved in Tris buffer and an aliquot (200 μl) was added to the collagen substrate together with procollagenase in Tris buffer (200 μl). The final volume was made up to 500 μl with Tris buffer and the mixtures were incubated at 36.5°C for 16 h. Following incubation, 250 μl of 0.05 M Tris–HCl buffer (pH 7.6) containing 6 M NaCl was added to each assay tube to precipitate unreacted collagen. The addition of 6 M NaCl resulted in low blanks. The tubes were incubated at 36.5°C for a further 30 min before centrifugation at 1720 × *g* for 30 min at 25°C. Aliquots (200 μl) of supernatant were counted in 5 ml scintillant (toluene containing 5%, w/v, PPO and 0.1%, w/v, POPOP:Triton X-100 (6:3, v/v) the whole mixture containing 1% perchloric acid). Each test was carried out in duplicate and each sample was counted in duplicate. The appropriate control (all reaction constituents with the enzyme substituted by 200 μl Tris buffer) was carried out for each assay.

2.5. *Heparin*

Heparin (sodium salt from porcine intestinal mucosa, Sigma, St Louis MO) was used. A sample of heparin was fractionated by gel filtration on a column (1 × 60 cm) of Bio-Gel P60 equilibrated in 0.05 M Tris–HCl buffer (pH 7.6) containing 0.2 M NaCl and 0.01 M $CaCl_2$. Heparin (13 mg) dissolved in the same buffer was applied to the column and eluted at a flow rate of 10 ml/h. The absorbance of the column effluent was monitored at 280 nm and 2.5-ml fractions were collected. Two major peaks of 206 nm absorbing material were obtained corresponding to M_r-values of 35000 and 16000. The concentration of glycosaminoglycan in each peak was determined by Alcian blue precipitation [11]. A 200 μl aliquot containing 5 μg heparin was used in the collagenase assay.

3. RESULTS AND DISCUSSION

The results for the mersalyl, ESAF and heparin activation of skin fibroblast procollagenase are given in table 1. Mersalyl is a synthetic organic mercurial compound which is routinely used to activate latent collagenolytic enzymes [12]. We have previously described the activation of skin fibroblast collagenase by low-M_r angiogenesis factor [3].

Incubation of heparin with the proenzyme resulted in a small activation of the enzyme. However, incubation of the proenzyme with both heparin and ESAF resulted in an activation greater than that achieved with either component alone. Treatment of the collagen with ESAF and heparin in the absence of enzyme resulted in no cleavage of the substrate.

The heparin used in these experiments was then fractionated into two major components with M_r-values of 35000 and 16000. Only the 16000 M_r component was effective in activating skin fibroblast procollagenase (table 2). This material was in the expected M_r range for heparin [13] and

Table 2
Effect of fractionated heparin on activation of skin fibroblast procollagenase

Assay mixture	μg Type-I collagen degraded/16 h
Enzyme[a]	0
+ mersalyl	11.7 ± 0.2
+ H_1	0.25 ± 0.05
+ H_2	0.3 ± 0.04
+ ESAF[c]	9.1 ± 0.6
+ ESAF + H_1	8.6 ± 0.4
+ ESAF + H_2	14.2 ± 0.7
H_1[b]	0
H_2[b]	0

[a] Skin fibroblast procollagenase
[b] H_1 = M_r 35000 heparin, H_2 = M_r 16000 heparin
[c] ESAF used in this experiment was a different batch from that used for results in table 1
Controls and EDTA blanks have been subtracted from the results

it is not clear what the high-M_r component represents, although our results show that it contains glycosaminoglycan.

We have previously reported that ESAF activates a skin fibroblast procollagenase [3]. Here we have shown that heparin potentiates the action of limiting amounts of ESAF. Furthermore, heparin alone gives slight activation of procollagenase whereas, like ESAF, it has no effect on the fully activated enzyme. The mild activating effect of heparin on mouse bone collagenase has been explained in [14,15] as being due to binding of the heparin to both collagen substrate and to collagenase, thereby increasing the concentration of enzyme at the surface of the fibril. However, it is unclear from their work whether the effect of the heparin was to activate a latent mouse bone collagenase or to enhance the activity of a previously activated enzyme. It would seem unlikely that the effect of heparin in our system is due to this mechanism as heparin alone has no effect on the fully active enzyme. Rather, the most significant effect of heparin is its ability to potentiate, in a synergistic manner, the activating effect of limiting amounts of ESAF on procollagenase. As mentioned above, heparin binds to both enzyme and substrate and we have shown that ESAF binds strongly to collagen. The significance of this observation is being investigated.

The mechanism of action of tumour angiogenesis factor is unclear. The requirements for an angiogenesis factor are that it can stimulate endothelial cells to proliferate and also cause them to migrate towards the source of the factor. The directional effect could be partially explained by the activation by angiogenesis factors of procollagenase present in the connective tissue matrix [3]. Since the collagen in the intracellular matrix is in a fibrillar form, digestion would be limited, as the insoluble collagen is a relatively poor substrate for the enzyme [16]. The appearance of mast cells during new capillary growth with their ability to release heparin has been described [5] and heparin has been shown to potentiate angiogenesis on the chick CAM [5]. An explanation for this observation might be that heparin could potentiate the action of ESAF locally resulting in a 'concentration' of collagenolytic activity. This in turn could lead to the formation of channels in the matrix. These channels would provide a route along which endothelial cells could migrate towards the source of angiogenesis factor. However, the question remains what the stimulus is for the appearance of mast cells? The overall process of angiogenesis which encompasses the initiation, growth and control of new blood vessel growth must involve a number of steps, possibly arranged to form a cascade of reactions. It is obvious that considerably more information must be obtained before the mechanism of angiogenesis is fully explained. However, the results presented here suggest that one effect of heparin in the process of angiogenesis may be to modulate ESAF activation of a procollagenase.

It would also be possible that a basement membrane-degrading enzyme, synthesized and secreted by endothelial cells themselves, could be activated in the same manner. Preliminary work in our laboratory has shown that ESAF is able to activate a partially purified basement membrane-degrading enzyme. We are currently studying this effect together with the possibility that heparin might also potentiate this activity.

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