Growth factors for human fibroblasts in the solute remaining after clot formation

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Summary

Fibroblasts adhere to, and readily grow into, fibrin clots that form as a result of the cleavage of fibrinogen by thrombin. Subsequent fibroblast replication is believed to be stimulated by mitogens released by entrapped platelets, such as platelet-derived growth factor. We suggest that the supernatant remaining after the fibrinogen–thrombin reaction could stimulate fibroblast replication, even in the absence of other blood components. To examine this hypothesis we expressed liquid from a fibrin clot and measured its mitogenic activity on human lung fibroblasts, in serum-free conditions, using a colormetric assay based on uptake and subsequent release of Methylene Blue. The clot supernatant caused a mitogenic response of 51±6% above control and was equivalent to about half that elicited by medium containing 10% newborn calf serum. On their own, both thrombin and fibrinopeptides A and B (small molecular weight cleavage products released from fibrinogen) showed some mitogenic activity, but there was also activity in higher molecular weight cleavage products, suggesting the presence of uncharacterised mitogens. It is proposed that these agents may play important roles in wound healing and diseases associated with vascular leakage and fibrosis, by stimulating fibroblast replication.

Key words: fibroblast, growth factors, fibrinogen, thrombin, fibrinopeptides

Introduction

The formation of a blood clot is important not only for haemostasis, but also for the initiation of repair following tissue injury. It is known that polymerised fibrin acts as a matrix for fibroblast growth (Brown *et al.* 1989; Fingert *et al.* 1987). Smaller molecules entrapped during clot formation could also have important biological effects. For example, thrombin has been reported to be mitogenic for fibroblasts derived from a variety of species (Chen and Buchanan, 1975; Pohl *et al.* 1979; Glenn *et al.* 1980). Fibrinopeptide B, a soluble product of the thrombin-induced conversion of fibrinogen to fibrin (Laki, 1951; Lorend, 1951), is chemotactic for fetal bovine fibroblasts (Senior *et al.* 1986). However, the possibility that fibrinopeptides A and B are mitogenic for fibroblasts has, as far as we are aware, not been examined.

We formed the hypothesis that the products of clot formation could stimulate fibroblast proliferation. To examine this we assayed the mitogenic potential of the soluble products remaining after clot formation. In addition to thrombin and fibrinopeptides A and B, at least two cleavage peptides were shown to have fibroblast mitogenic activity. These results suggest a novel mechanism for fibroblast replication associated with fibrin clot formation, which could have implications for wound healing and diseases associated with vascular leakage and fibrosis.

Materials and methods

Clot formation

The *in vitro* generation of a fibrin clot from fibrinogen has been described by several groups (Francis *et al.* 1979; Pohl *et al.* 1979; Sherman *et al.* 1974). In this study a clot was produced *in vitro* by the interaction of human thrombin (final concentration 2.1×10^{-9} M, Sigma Chemical Co., T7009) with human fibrinogen (final concentration 1.6×10^{-6} M, Sigma Chemical Co., F4883) in Dulbecco's modification of Eagle's medium (DMEM), pH 7.4. After incubation at 20°C for 30 min, the clot was separated into solid and liquid phases (termed clot and clot supernatant, respectively) by allowing the clot to retract and subsequently decanting the remaining liquid. To remove fragments the clot supernatant was passed through a 0.2 μm filter (Gelman Sciences, 6224192).

Cell culture

Cell lines used in this study were: FLF1 (a human fetal lung fibroblast line established in our laboratory), IMR90 and HFL1 (both from the American Tissue Culture Co., Rockville, MD, USA). Cells were maintained in DMEM supplemented with 10% newborn calf serum (NCS Imperial laboratories, Andover, UK) and 2.5 $\mu g\,ml^{-1}$ amphoteracin B (Fungizone, Gibco, Paisley, UK).

To set up microwell cultures, cells were washed with calcium- and magnesium-free phosphate-buffered saline, suspended in 0.02% EDTA/0.02% trypsin and resuspended in serum-free medium. Cells were counted in a haemocytometer, and the cell suspension was added in DMEM as described below.

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Assay for fibroblast replication

Cell replication was assessed using a rapid and convenient assay for counting cells cultured in microwell plates (Oliver *et al.* 1989). A 50 μl sample of test solution in serum-free medium was serially diluted across the central eight columns of a 96-well plate. A 50 μl sample of serum-free medium was also added to columns 2 and 11, which represented controls. A suspension of 6×10^3 cells in 50 μl of DMEM was subsequently added to each well of columns 2–11. Plates were then incubated for 48 h at 37°C in an atmosphere of 10% CO_2 and 100% humidity. The cell line routinely used was FLF1, although assays were also performed on both IMR90 and HFL1 cells.

To validate this assay as a means of estimating fibroblast number following replication, we also used morphometric techniques counting four areas in each well. In order to allow a correlation between the two techniques, cell counting was performed on cells that were subsequently assayed by the colormetric method.

Assay to determine the protein concentration of the clot supernatant

Protein concentration of the clot supernatant was determined using a method developed by Bradford (1976), based on the binding of Coomassie Brilliant Blue G-250 (Bio-Rad, product no. 500-0006). The method is sufficiently sensitive to allow the quantification of microgram quantities of protein, making it ideal for the measurement of protein in clot supernatant produced in this study.

Gel filtration chromatography

Separation of clot supernatant was accomplished by molecular sieve chromatography performed on 2 ml of clot supernatant. A column, 1.6 cm in diameter and 96 cm in length, was packed with Sephacryl S-300 (LKB Co., Uppsala, Sweden) and calibrated using standard molecular weight markers. Conditions used throughout these experiments were: eluent buffer 0.02 M $(NH_4)_2CO_3$, fraction size 2.5 ml and flow rate 1 ml min^{-1}. Fractions were freeze-dried and resuspended in 1 ml DMEM. Each fraction was tested for mitogenic activity in the fibroblast replication assay described above.

Data analysis

For the replication assays, 21 plates were assayed for clot supernatant, 11 for fibrinogen, 13 for thrombin, 8 for fibrinopeptide A, 7 for fibrinopeptide B and 6 for the combined peptides A and B. There was good agreement both within wells of a column (coefficient of variation (c.v.) 4.9±0.5%) and between the two control columns (c.v. 3.4±0.4%). To allow comparison between plates, mean optical density for each column (n=6) was expressed as % above or below the control wells (n=12). Values obtained for all plates were then used to calculate the means and standard errors at each concentration, and these are the values given in the text and figures.

Results

Fig. 1A shows that the clot supernatant produced by thrombin cleavage of fibrinogen had marked stimulatory effects on fibroblast replication. The maximal stimulation of about 50% above control was obtained at a protein concentration of 7 μg ml^{-1}. This assay was performed in the absence of serum, but to allow comparison of this stimulation with that obtained in an optimal medium, we examined the effect of 10% serum on the same cells. This gave a stimulation of about twice that seen with clot supernatant alone. Thus, in two experiments in 96-well plates the ratio of activity in 10% serum to that in clot supernatant (7 μg ml^{-1}) was 2.6 and 1.86, respectively. Fig. 1B and C also show the effects on fibroblast replication of fibrinogen and thrombin alone. Fibrinogen displayed no mitogenic activity at eight concentrations ranging from 10^{-8} to 10^{-6}. The mean values obtained were all within 5% of medium control, and at no concentration did they significantly differ from controls. Thrombin inhibited fibroblast growth at concentrations above 10^{-9} M. However, below this concentration it displayed mitogenic activity and this was still apparent at a concentration of 10^{-11} M.

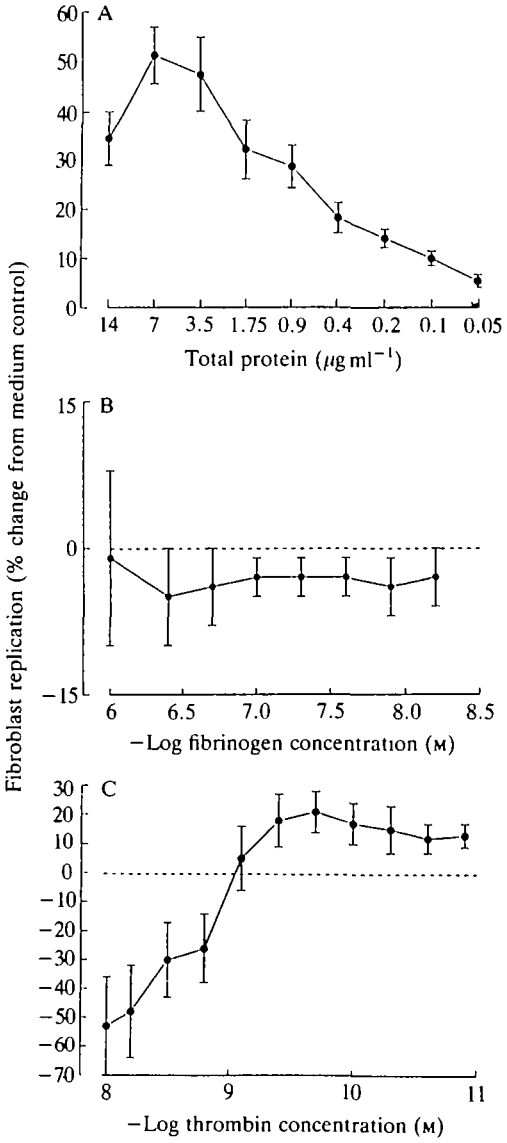

Fig. 1. Fibroblast mitogenic activity in response to: soluble products from the fibronogen – thrombin reaction (A), fibrinogen (B), and thrombin (C). The clot supernatant is expressed as total protein in solution, estimated as s7 μg ml^{-1} (see Results).

Fibroblast replication assays in response to clot supernatant were also performed on individual 96-well plates using other human cell lines. The human fetal lung fibroblast IMR90 showed maximal stimulation of 52% above medium control at a protein concentration of 3.75 μg ml^{-1}. A third human fetal lung line (HFL1) showed a maximal response of 58% above control at a protein concentration of 7 μg ml^{-1}. All these cell lines showed comparable proliferative responses to clot supernatant.

In an experiment to assess the mitogenic effects of clot

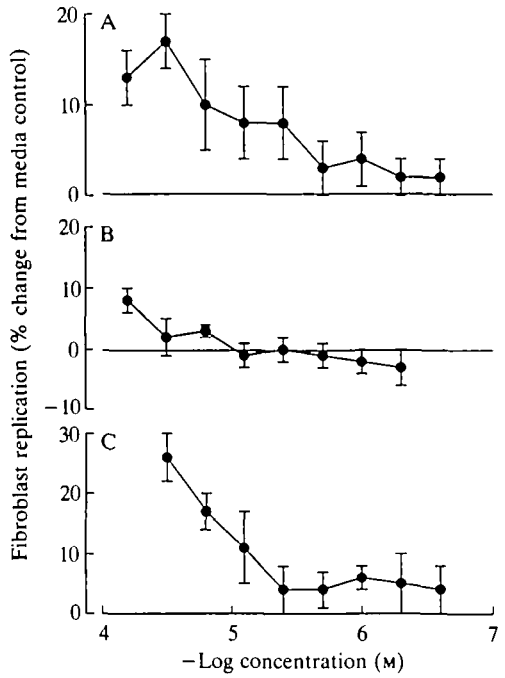

Fig. 2. Fibroblast mitogenic activity of fibrinopeptides A (A), B (B), and A plus B (C).

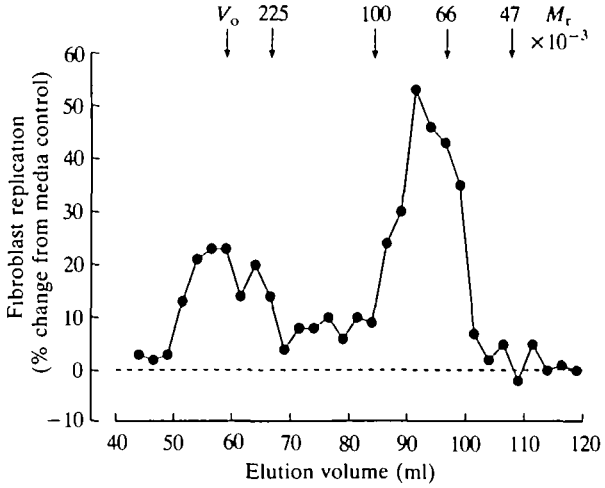

Fig. 3. Fibroblast mitogenic activity of fractions taken from a Sephacryl S-300 gel filtration column. Arrowed along the abscissa are the elution points of selected molecular weight ($\times 10^{-3}$) standards as well as the void volume (V_o).

supernatant by an independent technique, and also to distinguish between cell number and possible change in cell size, we correlated the absorbance obtained in the Methylene Blue assay with numbers of cells assessed by light microscopy. Values obtained by the two methods correlated closely (r=0.91), as observed by Oliver *et al.* 1989) when this assay was applied to other mitogens.

The known products of fibrin clot formation include fibrinopeptides A and B. Fig. 2 shows the effects on fibroblast replication of these peptides alone, and in combination. Fibrinopeptide A gave a maximum stimulation of almost 20% above control at a concentration of 3.2×10^{-5} M. Fibrinopeptide B gave a stimulation of 10% above control at a concentration of 1.3×10^{-4} M and its activity decreased with serial dilution. The combination of fibrinopeptides A and B, each at a concentration of 3.2×10^{-5} M, gave a stimulation of 25% above control.

Fractions collected after gel filtration showed marked mitogenic activity (55% above control) for moieties with an apparent molecular weight of about 70 000 (Fig. 3). There was also mitogenic activity of a smaller magnitude (25% above control) in fractions containing moities with an apparent molecular weight greater than 250 000, eluting at the void volume.

Discussion

This study reports the finding that soluble products of the fibrinogen–thrombin reaction stimulate proliferation of human fibroblasts and this was found consistently for three different cell lines. The stimulation was equivalent to about half that elicited using an optimal concentration (10%) of newborn calf serum. Most experimental analysis was performed using a colormetric technique based on uptake and subsequent elution of Methylene Blue from cells (Oliver *et al.* 1989). This technique is sensitive and convenient for processing large numbers of samples, and is deemed to have some advantages over assays based on thymidine uptake (Elliott *et al.* 1983; Rannels *et al.* 1982). However, because dye uptake may be influenced by factors other than cell number it was important to validate results using an independent approach. In the current study the strong correlation between cell counts and Methylene Blue absorbance demonstrated that the assay was indeed detecting fibroblast mitogenic activity.

The stimulation elicited by clot supernatant was greater than that observed when cells were treated with purified growth factors (ie PDGF and FGF) in the absence of serum (data not shown). This suggests that multiple factors providing competence and progression activity may be present.

Fibrinogen alone had no mitogenic effect. This result is supported by Pohl *et al.* (1979), who found no stimulation of fibroblast proliferation by fibrinogen at concentrations similar to those used in the present study. The absence of serum in both of these studies is probably critical because it may contain trace amounts of thrombin, which would cleave fibrinogen. Indeed we observed clot formation and marked fibroblast proliferation when small amounts of serum were added to fibrinogen, and this stimulation was greater than that caused by serum controls (data not shown).

Thrombin inhibited fibroblast replication at concentrations above 10^{-9} M, but stimulated replication at lower concentrations. Thrombin has been reported to bind to mammalian fibroblasts (Glenn *et al.* 1980), and to stimulate fibroblast proliferation at concentrations of 10^{-8} M to 10^{-7} M (Chen and Buchanan, 1975). However, we have found no reports of proliferative effects at the lower concentrations used in this study.

Fibrinogen consists of three pairs of chains (Aα Bβ γ) that are disulphide bonded. Fibrinopeptides A and B, cleavage products of the Aα and Bβ chains, respectively, have both been reported to cause vasoconstriction (Bayley *et al.* 1967), and fibrinopeptide B is a chemoattractant for neutrophils and fibroblasts at concentrations of 10^{-11} M to 10^{-6} M (Senior *et al.* 1986). Potentiation of fibroblast proliferation by fibrinopeptides A and B has been suggested (Senior *et al.* 1986), but not formally examined. Whilst the present study shows that both peptides possess mitogenic activity, for the concentrations of fibrinogen used in

the present study the mitogenic effect of the fibrinopeptides is calculated to be minimal. Assuming that two moles each of fibrinopeptides A and B are released from one mole of fibrinogen, a fibrinogen concentration of 7.4×10^{-7} M would give a maximum concentration of about 1.5×10^{-6} M for each of the peptides. At this concentration mitogenic activity was only slightly above the medium control (Fig. 2C). These data do not discount a possible role for fibrinopeptides in synergy with other products. Clotting at circulating blood concentrations of fibrinogen could release sufficient fibrinopeptides to stimulate fibroblast proliferation. For example, assuming local tissue concentrations equivalent to a high-normal blood concentration of fibrinogen (4 mg ml^{-1}), it would be possible to generate concentrations of at least 2.3×10^{-5} M for each of fibrinopeptides A and B. In combination these peptides could have significant proliferative effects.

The concentration of thrombin used to generate a clot in our experiments was theoretically sufficient to induce a proliferative response of about 20% above control (see Fig. 1C). However, this assumes a thrombin concentration in the clot supernatant equivalent to that in the initial reaction solution. Since thrombin has been reported to bind polymerised fibrin avidly (Eagle, 1970), the contribution of thrombin to clot supernatant mitogenic activity is likely to be much less than 20%. The results of the gel filtration experiments (Fig. 3) also suggest that thrombin (M_r 37 500) and the fibrinopeptides (M_r 1500 each) contribute minimally to the total proliferative activity, and that the bulk of activity was in moieties, with higher molecular weights, yet to be fully characterised.

In summary, this study reports that elements remaining in solution following the thrombin-catalysed cleavage of fibrinogen are mitogenic for fibroblasts. This finding has potential importance both in normal wound healing and in disease states. If low levels of thrombin are present at the sites of vascular leak and injury, then the reaction of thrombin with fibrinogen could stimulate fibroblast replication *in vivo*. Fibrosis is known to occur in response to chronic vascular injury, and it is possible that the soluble products from clot formation facilitate this.

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