A low-M_r chemoattractant for vascular endothelial cells

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The formation of new blood vessels occurs by sprouting from previously existing microvasculature. The process involved directed migration of the vascular endothelial cells towards chemical signals released from the target tissue. We have used the Boyden chemotaxis chamber method to identify chemotactic signals for fetal bovine vascular endothelial cells. Human placenta organ cultures produce a high-M_r chemoattractant for the endothelial cells from which a low-M_r factor can be liberated with trichloroacetic acid treatment and ethanol extraction. This activity was isolated from extracts of human placenta using Sephadex LH-20, Amberlite XAD-2, and silica gel thin-layer chromatography. The M_r of the factor is less than 400, it is lipophilic and resistant to proteolytic enzymes. The factor induces chemotactic migration of both aortic endothelial cells and capillary endothelial cells from the retina, but has no effect on fibroblasts or leukocytes suggesting a specific function of the compound for the vascular endothelial cells.

Neovascularization *Angiogenesis* *Vascular endothelial cell* *Chemotaxis* *Cell motility* *Placenta*

1. INTRODUCTION

In new blood vessel formation, or angiogenesis, the vascular endothelial cells at the tips of the neovascular sprouts actively migrate towards the inciting stimulus [1,4]. It was previously suggested by Seppä et al. [5,6] that chemotaxis assays may be used as an in vitro assay to elucidate angiogenic substances that act directly on the vascular endothelial cells. In the in vivo angiogenesis assays [7–11] it is not possible to differentiate between indirect and direct action of a compound on the final target, the vascular endothelium. We have now used the Boyden chamber chemotaxis assay to characterize a chemotactic activity for vascular endothelial cells present in organ culture medium and extracts of human placenta, a tissue which is highly vascular and is available alive in large amounts for the isolation procedure.

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2. MATERIALS AND METHODS

2.1. *Chemotaxis assays*

Chemotaxis assays were performed using the multiwell version of the modified Boyden chamber (Neuroprobe, Bethesda, MD) with 8 μm pore size polycarbonate filters (Nucleopore, Pleasanton, CA) which were coated with gelatin and fibronectin as described [6,12]. Briefly, the lower plate of the chamber consists of 48 wells, each of which has a surface area of approx. 8 mm^2 and holds 25 μl of chemoattractant. The upper plate consists of 48 holes which correspond to the wells on the bottom plate. Between the upper and lower plates are interposed a gelatin- and fibronectin-coated polycarbonate filter of 10 μm thickness with 8-μm pores and a silicon gasket to make a tight seal. The upper wells hold up to 50 μl of cell suspension. Cell suspensions were incubated in the chemotaxis chambers for 4 h at 37°C in a humidified atmosphere of 95% air/5% CO_2. After incubation, the filter was removed from the chamber, and stained using Diff-Quick. The filter with stained cells was placed on a microscope slide with the

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lower surface (containing the migrated cells) facing the slide. Cells which did not migrate through the filter were wiped off the upper surface with a cotton swab. Migrated cells on the lower surface of the filter were counted in 15 consecutive fields along the diameter of each well at a magnification of 400 ×. The counted area corresponds to approx. 1 mm^2, and the results are expressed as number of cells per mm^2 on the lower surface of the filter. Checkerboard assays were performed using the single well chamber versions (Neuroprobe, Bethesda, MD). Assays for leukocyte chemotaxis were performed using 5 μm pore size filters which were without PVP-coating, as described [13]. Positive controls were retinal extract for endothelial cells, platelet derived growth factor (a kind gift from Dr C.-H. Heldin, University of Uppsala, Sweden) for fibroblasts, and formylmethionylleucylphenylalanine (Sigma) for leukocytes. Samples were diluted into DMEM containing bovine serum albumin at 1 mg/ml (= DMEM-BSA).

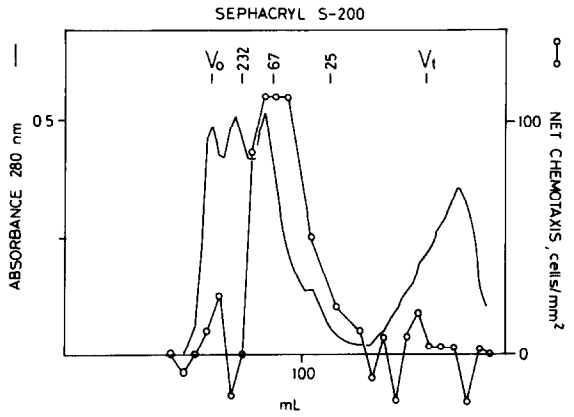

Fig.1. Sephacryl S-200 chromatography. Gel filtration of serum-free tissue culture medium from organ cultures of human placental slices on Sephacryl S-200 equilibrated with 0.3 M NaCl. Molecular mass standards were catalase (232 kDa), serum albumin (67 kDa), chymotrypsinogen (25 kDa). The V_o was determined using Blue Dextran.

2.2. *Cell cultures*

Fetal bovine aortic endothelial cells [14] and bovine retinal capillary endothelial cells [15] were isolated and cultured as described. The identity of the cells was ascertained with immunohistochemical staining for factor VIII antigen [16]. Confluent cultures were used for assays in passages 4–16 of the aortic and passage 4 of the capillary endothelial cells. The human skin fibroblasts were a kind gift from Dr Leena Hämäläinen, Dept of Medical Biochemistry, University of Oulu, and cells from 3 different cultures were used. Leukocytes were produced by injecting thioglycollate intraperitoneally and harvesting on the first day after injection [17]; 50–65% of the cells were neutrophils.

3. RESULTS AND DISCUSSION

3.1. *Live placental slices release a high-M_r endothelial cell chemoattractant*

Tissue culture medium conditioned by pieces of human placenta was found to contain chemoattractant activity towards vascular endothelial cells. A gel filtration on Sephadex G-200 of this conditioned medium is depicted in fig.1, and shows a peak of activity eluting at volumes corresponding to molecular masses of 50–100 kDa. Similar high-M_r attractants have been described previously from cultures of murine sarcoma [6], human meningioma [18], 3T3 adipocytes [19], and in tissue fluid from angiogenesis reaction in the cornea [20].

3.2. *Trichloroacetic acid precipitation and ethanol extraction reveals a low-M_r chemoattractant*

Precipitation of the proteins of the placental conditioned medium with trichloroacetic acid leads to precipitation of the chemoattractant activity. The activity was extractable into ethanol. Pretreatment with trichloroacetic acid seems to be a prerequisite for extractability of the activity in ethanol, possibly through denaturation of carrier protein, since in previous work with endothelial cell attractants from cultures of murine sarcoma [6] and human meningioma [18] no activity was found in ethanol extracts of lyophilized conditioned media. A gel filtration of the placental extract in ethanol on Sephadex LH-20 revealed a peak of activity in the range of 150–400 Da (fig.2).

3.3. *Extraction and purification of the low-M_r chemoattractant*

To prepare larger amounts of the chemoattractant human placentas were minced and treated with trichloroacetic acid and subsequently ex-

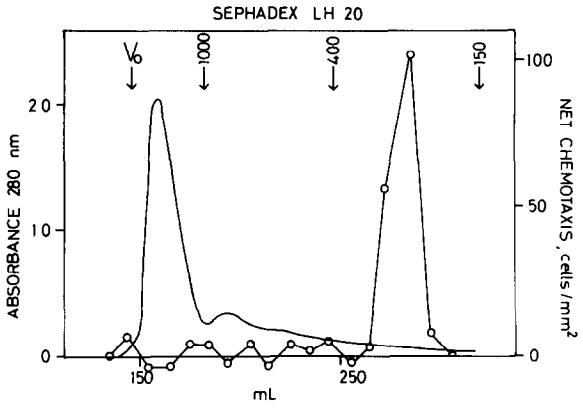

Fig.2. Sephadex LH-20 chromatography. Gel filtration of an ethanol extract of a trichloroacetic acid precipitate of tissue culture medium from organ cultures of human placental slices on a Sephadex LH-20 equilibrated with 96% ethanol. Molecular mass standards were triethylene glycol (150 Da), and polyethylene glycols of 400 Da, 1000 Da, and 20000 Da.

tracted with ethanol. The extract was then purified on Sephadex LH-20. The peak of activity from the LH-20 was diluted with water and adsorbed on an Amberlite XAD-2 resin. The activity was eluted with a gradient of increasing concentration of ethanol (fig.3). The peak fraction was used for further analysis.

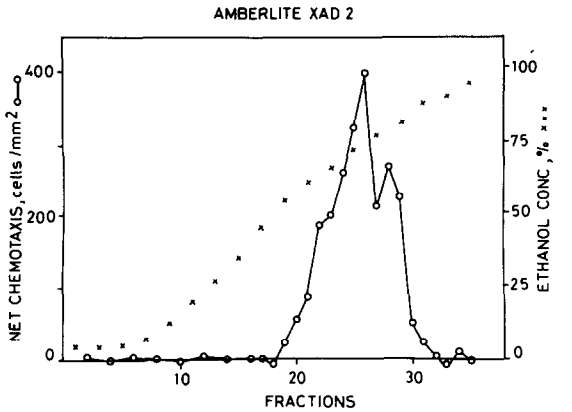

Fig.3. Amberlite XAD-2 chromatography. The peak fractions from the Sephadex LH-20 chromatography were mixed into 10 vols of 25 mM Na-acetate buffer, pH 4.6. The diluted activity was applied to Amberlite XAD-2 resin (Sigma), washed with buffer. The resin was developed with a linear gradient of ethanol in water.

The dry weight of the material in the placental extract was 30 g, the weight of the LH-20 material was approx. 1 g, and the dry weight of the XAD-2 peak was approx. 10 mg. The yield cannot be calculated because of the presence of inhibitors, which does not allow for estimation of the concentrations inducing half maximal responses.

3.4. *Analysis of the purity of the chemoattractant on TLC*

A sample of the peak fraction from the XAD-2 chromatography was analyzed in silica gel TLC (fig.4). The activity migrated with an R_f of 0.35, but no corresponding spot could be visualized by iodine vapor or charring with sulfuric acid. The major impurity present was phosphatidylcholine migrating at an R_f of 0.72. The chemotactic activity was rendered labile after the TLC.

3.5. *Characterization of the chemical properties of the chemoattractant*

To analyze whether a peptide moiety is responsible for the chemoattractant activity, the effect of several proteolytic enzymes on the activity was studied. None of the enzymes totally abolished the

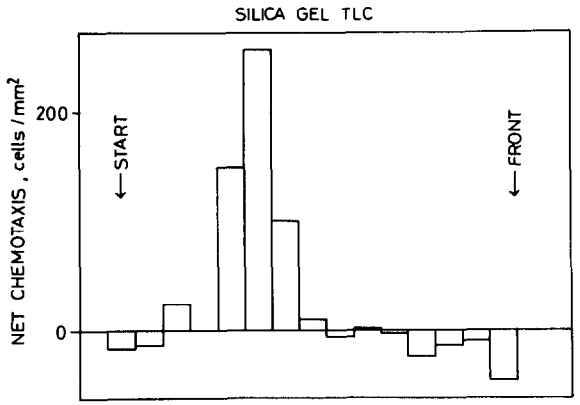

Fig.4. Thin-layer chromatography on silica gel. 20-μl samples were spotted on silica gel 60 TLC plates (Merck), and eluted with chloroform:methanol:water (65:35:6). Chromatograms were dried and split into two halves. The first half was developed with iodine vapor and H_2SO_4, and the second half was cut into 1 cm pieces, each of which was eluted with 0.2 ml methanol. The methanol was evaporated, the residue taken up into 0.005 ml ethanol, and diluted with DMEM-BSA for chemotaxis assay.

activity in 24 h at 37°C, suggesting that no peptide bond susceptible to these enzymes is involved in the chemotactic property of this compound. The activity was resistant to boiling at acidic and neutral pH values, but some loss of activity took place at pH 10.7. Mild oxidation with periodate only led to a slight diminution of the activity, suggesting that a carbohydrate moiety essential for the activity is not present. Because of previous reports of the effect of copper ions on the motility of vascular endothelial cells [21], the effect of a chelating resin, Chelex 100, on the activity was tested. Treatment of the activity with Chelex did not reduce the activity suggesting that no copper ions essential for the activity were present in the preparation. Addition of copper to the attractant did not have any effect on its activity either. Acetylation of the XAD peak fraction with acetic anhydride leads to an essential reduction of the chemotactic activity suggesting the presence of hydroxyl group(s) essential for the activity. The active fractions of the purest samples, the fraction from the TLC, had no absorbance at 254 or 340 nm. It is possible that in spite of the high biological activity of the sample, the molar concentration of the active compound is too low for detection of absorbance. Chemoattractants are known to be active at very low concentrations, e.g., the leukocyte attractant formylmethionylleucylphenylalanine is active at 10^{-12} M [22].

On the basis of the present data it is not possible to deduce the structure of the compound. We now know that the attractant is a small lipophilic molecule (soluble in chloroform) with an acetic anhydride acetylatable hydroxyl group essential for the activity. The activity is not leukotriene B_4, prostaglandin E_1 or E_2, or FMLP, as these have previously been tested (Seppä, unpublished) and it is not the platelet activating factor, which we found inactive towards endothelial cells in our assays (not shown). For further biochemical analysis and identification of the compound larger amounts of pure material are required. To achieve this a considerable scaling up of the isolation is needed.

3.6. *Characterization of the biological properties of the chemoattractant*

3.6.1. Analysis of the chemotactic vs chemokinetic property of the compound

The migratory responses of vascular endothelial cells to various concentration gradients between the upper and lower compartment in the Boyden chamber were tested to find out whether the responses were due to increased random migration, or due to directed migration of the cells towards a higher concentration of the compound on the other side of the filter (table 1). The responses to 'negative gradients' (first line of the table) are less than the responses in the control (i.e., in the absence of attractant). The responses to increasing concentrations in the absence of a gradient (diagonal of the table) are equal to the control, showing that the attractant does not act through increasing the random motility of the cells, but acts by inducing increased migration of cells in 'positive gradients' towards increasing concentrations of the placental factor (columns of the table).

3.6.2. Cell specificity

The fetal bovine aortic endothelial cells were used as 'indicator cells' in the isolation of the chemoattractant. As angiogenesis is a phenomenon of the microvasculature, we tested whether capillary endothelial cells would be responsive to the factor. As depicted in fig.5 the bovine retinal capillary cells respond at a concentration nearly 2 orders of magnitude smaller than that giving a maximal response with the aortic endothelial cells, suggesting that the capillary cells are even more sensitive to the compound than the aortic cells. The chemoattractant is not active for human fibroblasts (fig.5) or rat leukocytes (not shown) at

Table 1

Migratory responses of vascular endothelial cells to varied concentration gradients of placental factor

Concentration below filter	Concentration above filter		
	0	1 ×	10 ×
0	351 ± 5	308 ± 15	199 ± 15
1 ×	760 ± 28	438 ± 65	279 ± 28
10 ×	689 ± 42	631 ± 39	339 ± 30

Results expressed as numbers of cells per mm^2 on the lower surface of the filter. The numbers give the mean from triplicates ± SE

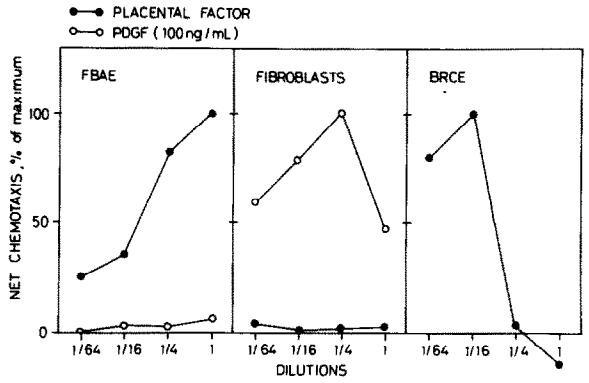

Fig.5. A comparison of chemotactic responses of fetal bovine aortic endothelial cells (= FBAE), human fibroblasts, and bovine retinal capillary endothelial cells (= BRCE) to the chemoattractant from placenta. Platelet-derived growth factor (= PDGF) was used as a positive control for the fibroblasts [12].

the concentrations studied, suggesting that the activity is specific for the endothelial cells. Whether this compound is related to the low-M_r angiogenesis factors that have been described previously [23–26] cannot be decided on the basis of the available information. Our preliminary results from angiogenesis assays using the chick chorionallantois membrane model would, however, suggest that the compound is angiogenic.

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