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A MACROPHAGE FACTOR THAT STIMULATES THE PROLIFERATION OF VASCULAR ENDOTHELIAL CELLS

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SUMMARY: Sarcoid macrophage-epithelioid cells have been shown to release a growth factor that stimulates the proliferation of vascular endothelial cells in vitro. In the presence of this factor, cultured endothelial cells can proliferate in a serum-free medium. Gel-chromatography on Sephadex G-75 revealed a single peak of activity on endothelial cells. The molecular weight was estimated at 7,000-10,000. The activity was heat-labile and trypsin-sensitive, and did not adhere to heparin-Sepharose.

It has been known that activated mononuclear phagocytes secrete a growth-promoting activity that can stimulate the directional outgrowth of capillaries in vivo (1). Although there are many reports on the in vivo angiogenesis induced by activated macrophages (2-5), studies on the responsible factors for this phenomenon have been limited (6). No growth-promoting factor(s) stimulating angiogenesis has been isolated from macrophages. Sarcoidosis is a chronic granulomatous disease. The granulomata are compact focal collections of inflammatory cells characterized, primarily, by the presence of cells derived from mononuclear phagocytes, i.e., macrophages, epithelioid cells, and multinucleated giant cells (7). Recently, we have succeeded in the cultivation of macrophage-epithelioid cells of sarcoid granulomata (8,9). These cells have been shown to secrete large amounts of lysosomal enzymes, suggesting that they are significantly activated mononuclear phagocytes. Since activated mononuclear phagocytes have been known to induce neovasculization in vivo, and since prominent neovasculization is found near sarcoid lesions, it may be of great interest to examine whether the sarcoid macrophage-epithelioid cells release a growth

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factor that stimulates proliferation of vascular endothelial cells. This study describes partial purification and characterization of a factor that stimulates proliferation of vascular endothelial cells.

MATERIALS AND METHODS

Source of cells: The materials used were biopsied lymph nodes from 6 patients with active sarcoidosis. The diagnosis of sarcoidosis was made on the basis of clinical findings, chest roentogenograms and pathologic examination of biopsied specimens from scalene lymph nodes. Each lymph node contained non-necrotizing granulomas. Acid-fast bacilli, fungi, or other microbial agents were not demonstrated in any of the specimens by microscopic and culture examinations.

Cell culture and media: The primary culture of sarcoid macrophage-epithelioid cells was carried out as described previously (8). The culture medium used for the primary culture was serum-free HB 101 medium (Hana Media Inc., Berkley, Calif.) which contained only three kinds of proteins, i.e.; bovine serum albumin, transferrin, and insulin. Endothelial cells were collected from a fresh porcine aorta using collagenase digestion as described (9). The endothelial cells were incubated with Ham's F-10 medium containing 10% fetal bovine serum (FBS) and subcultured into 25 cm^2 culture flasks with 0.25% trypsin solution (Gibco) when the cells reached confluency. There was no evidence of transformation or loss of endothelial cell monolayer under these conditions for more than 6 months.

Assay for growth-promoting activity: Growth-promoting activity was estimated as the incorporation of [^{3}H] thymidine into serum-deprived endothelial cells (10). Cells were seeded in 24-well (2 cm^2) culture plates (Falcon) at a density of 10,000 per well. Cells were incubated for 24 hr in 0.5 ml of media containing 10% FBS. At this point, samples were added to the wells and after a further 16 hr incubation, all wells were pulsed for 8 hr with 0.4 µCi of [^{3}H] thymidine (6.7 Ci/mmol, New England Nuclear, Boston) per well. The cells were then washed and extracted with 5% trichloroacetic acid. The resultant precipitates were washed twice with ethanol-ether (ratio of vol. 3:1) and solublized with 1.0 N NaOH. Radioactivity was determined by a scintillation counter.

Gel exclusion chromatography: The serum-free conditioned medium of the macrophage-epithelioid cells was concentrated 20-fold by ultra-filtration with YM-5 membrane. We then, transferred 1.5 ml of sample to a Sephadex G-75 superfine column (1.1 x 40 cm, flow rate 4.0 ml/hr), equilibrated with 10 mM Tris-HCl buffer (pH 7.4), containing 1,000 mM NaCl, and 0.01% polyethyleneglycol. The void volume of this column was 14 ml, determined by blue dextran. The column was calibrated using ovalbumin (Mr, 43,000), ribonuclease A (Mr, 13,700), and insulin (Mr, 5,700).

Isoelectric focusing: A concentrate (2 ml) of the conditioned medium (40 ml) was dialysed against distilled water and applied to a LKB flat bed unit (Ultrophor, LKB-Produkter AB, Sweden) using a granular bed composed of Ampholyte gel and focused for 18 hr at 4°C. The gel was divided into slices and soluble content of each gel slice was eluted from the gel with 0.15 M NaCl. All isoelectric focusing reagents were obtained from LKB-Produkter and electrophoresis procedure was performed as instructed by the manufacturer.

Characterization of the growth-promoting activity on endothelial cells: The major active fractions (No. 17-19) from Sephadex G-75 chromatography were mixed prior to testing. An aliquot was incubated with insoluble trypsin (100 units/ml gel; Sigma), or insoluble RNase (20 units/ml

gel; Sigma), or immobilized DNase I (2,000 units/ml; Worthington Diagnostic System Inc.) for 2 hr at 37°C. The reaction was terminated by removing the insoluble enzyme by centrifugation. Heat stability was tested by separate treatment of aliquots of the active fractions at 56°C or 80°C for 30 min. Samples were also exposed to dithiothreitol (60 mM, Sigma) for 60 min at 37°C to examine the sensitivity to reducing agents.

RESULTS AND DISCUSSION

When trypsinized endothelial cells were placed in a serum-free medium, most cells enter into a resting state of G_1 phase of cell cycle, with a substantial reduction of cell number. Exposing the endothelial cells to supernates from sarcoid macrophage-epithelioid cells resulted in a marked increase in endothelial cell proliferation rate (Fig. 1a). In the example shown, whereas cell number increased approximately 500% with supernates from the macrophage-epithelioid cell cultures, endothelial cells exposed to control HB 101 medium alone decreased

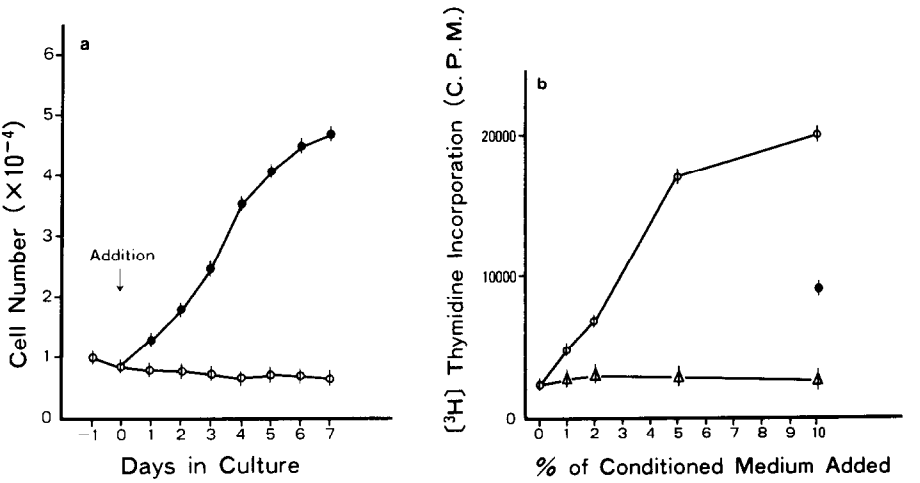

Figure 1a Stimulation of proliferation in vascular endothelial cell cultures by macrophage-epithelioid cell conditioned medium. Endothelial cells were replicate-plated in F-10 medium with 10% FBS. After 24 hr incubations, the dishes were washed with F-10 medium, and incubated with F-10 without FBS in the presence of the serum-free macrophage-epithelioid cell conditioned medium (3%) (●) or control HB 101 medium (3%) (o). Each point represents cell number (mean ± SD) measured in duplicate.

Figure 1b Stimulation of DNA synthesis in vascular endothelial cell cultures by macrophage-epithelioid cell conditioned medium. Growth-promoting activity was estimated as the incorporation of [^{3}H] thymidine into serum-deprived endothelial cells as described in Materials and Methods. The macrophage-epithelioid cell conditioned medium (0-10%) (o), control HB 101 medium (0-10%) (Δ), or 10% FBS (●) was added to the serum-deprived endothelial cells. Each point represents the mean value from two wells. Bars, S.D.

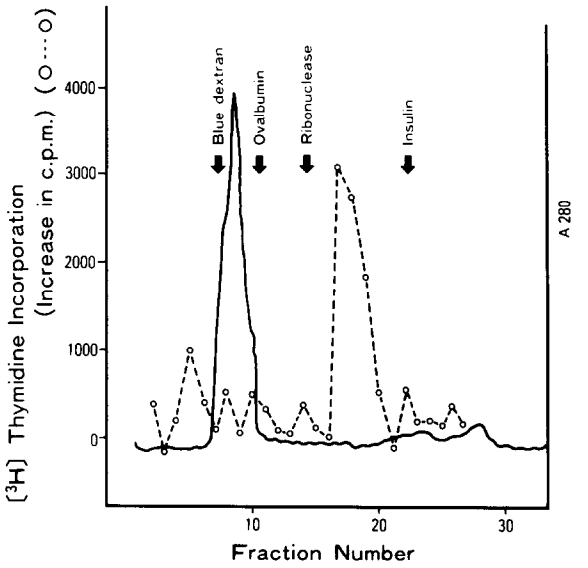

Figure 2 Gel exclusion chromatography of macrophage-epithelioid cell supernates. A concentrate (1.5 ml) of the supernates of the macrophage-epithelioid cell conditioned medium was chromatographed on Sephadex G-75 and fractions were evaluated for vascular endothelial cell growth-promoting activity (o---o). The column was calibrated using ovalbumin (Mr, 43,000), ribonuclease (Mr, 13,700), and insulin (Mr, 5,700).

in cell number by 30% in the same time period. Marked increase in the incorporation of [^{3}H] thymidine into DNA was also observed (Fig. 1b). To determine the approximate size of the molecule(s) responsible for the growth-promoting activity, a concentrate of the supernates was loaded on a Sephadex G-75 chromatography. As shown in Fig. 2, the activity was detected as a single peak at a molecular weight of 7,000-10,000. When a concentrate of the supernates was electrofocused on a flat bed apparatus using granulated gel, a large peak of endothelial cell proliferation activity was observed at pI of 4.5-6.0 (Fig. 3). The activity was destroyed following incubation at 56°C for 30 min, incubation at 80°C for 30 min, or treatment with trypsin (Table 1). Loss of the activity occurred with dithiothreitol treatment. No biological activity was lost by treatment with DNase or RNase.

Macrophages secrete a wide variety of growth-promoting factors in the host defense system. These include such diverse activities as interferon, interleukin I, endogenous pyrogen, acute phase protein synthesis factor, angiogenesis factor, fibroblast proliferation factor

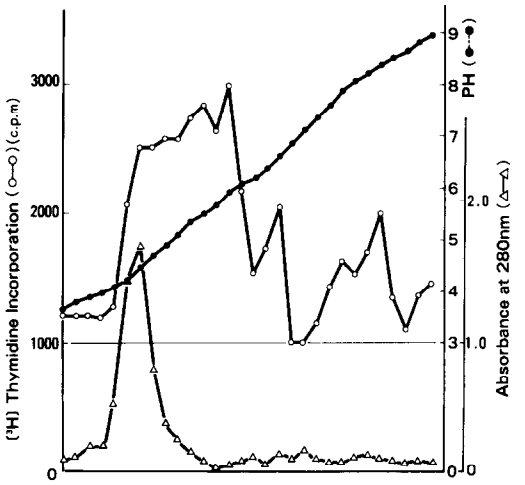

Figure 3 Isoelectric focusing of macrophage-epithelioid cell supernates . A concentrate (2.0 ml) of the supernates was applied to a LKB flat bed unit using a granular bed composed of Ampholyte gel and electro-focused for 18 hr. Each fraction was assayed for endothelial cell growth-promoting activity (o—o). ●—●, pH. Δ—Δ, absorbance at 280nm.

and others (11). In this study, we have reported a growth factor that stimulates the proliferation of vascular endothelial cells. Biochemical nature of the endothelial cell proliferation factor produced by macrophages has not been fully elucidated (6). Only recently, Baird et al reported that macrophage-derived growth factor (MDGF) which is a potent mitogen for nonlymphoid mesenchymal cells including fibroblasts, smooth muscle and endothelial cells, would be structurally related to pituitary fibroblast growth factor (FGF)(12). They have demonstrated

Table 1. Stability of vascular endothelial cell proliferation factor

Treatment	% residual activity
None	100%
Heat	
56°C 30 min	-14%
80°C 30 min	-17%
Enzyme	
Trypsin	4%
RNase	89%
DNase	84%
Dithiothreitol (60 mM)	24%

Residual activity was calculated as follows:

$$\% \text{ residual activity} = \frac{\text{c.p.m. in treated VEPF - c.p.m. in controls}}{\text{c.p.m. in untreated VEPF - c.p.m. in controls}} \times 100\%$$

The value of 100% in the controls (100%) was 3,672 c.p.m.

that MDGF is a potent stimulator of vascular endothelial cells in vitro and crossreacts with sequence specific antisera to bovine FGF. The MDGF was retained on heparin Sepharose affinity columns and eluted at more than 2 M NaCl. The MDGF had similar retention behavior or reverse-phase high pressure liquid chromatography as native pituitary or brain FGF. Both MDGF and FGF have been shown to be stable at 56°C and have molecular weights of 18,000 (MDGF)(13) and 16,000 (FGF) with pI of 9.6 (14). Our vascular endothelial cell proliferation factor (VEPF) was heat-labile and the apparent molecular weight was 7,000-10,000. Isoelectric focusing revealed that the pI of VEPF was 4.5-6.0. Thus, apparent molecular size and isoelctric point of VEPF is clearly distinct from those of the MDGF and FGF. In addition, the MDGF and FGF were reported to bind to immobilized heparin and were eluted at more than 2.0 M NaCl (12,14), whereas VEPF did not have an affinity for heparin (Fig. 4). When a concentrate of the supernates was applied to a heparin Sepharose column, most of the VEPF activity was eluted in the void volume and nearly all the remainder was eluted with 0.15-0.5 M NaCl (Fig. 4). VEPF was found to be inactive against

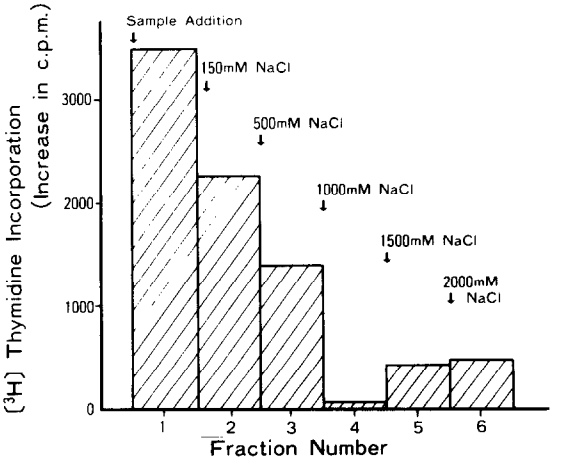

Figure 4 Chromatography of macrophage-epithelioid cell supernates on heparin-Sepharose. A concentrate (0.5 ml) was applied to a column (1.2 x 10. cm, 1.1 ml) of heparin-Sepharose (flow rate, 10 ml/hr, at 4°C). The column was eluted by increasing the NaCl concentration stepwise (150 mM, 500 mM, 1000 mM, 1500 mM and 2000 mM). Fractions were collected and measured for vascular endothelial cell growth-promoting activity (▨).

normal rat kidney fibroblasts (data not shown), whereas the MDGF and FGF were potent stimulators of mesenchymal cells including fibroblasts (12). These observations suggest that VEPF is functionally as well as biochemically distinct from the MDGF and FGF.

Alveolar macrophages from normal volunteers did not secrete VEPF activities (data not shown). The detection and demonstration that VEPF is secreted by sarcoid macrophage-epithelioid cells has some implications as to its possible function in vivo. It is possible to consider that VEPF locally released from sarcoid macrophage-epithelioid cells is transferred to the adjacent vascular vessels to stimulate the endothelial cells to proliferate and mediates the neovasculization process in sarcoidosis patients.

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