Characterization of a Chemoattractant for Endothelium Induced by Angiogenesis Effectors

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ABSTRACT

The mechanism of neovascularization was further explored by the use of chemically defined angiogenesis effectors. The vascularization of the rabbit cornea was selected as an experimental approach that permits comparison of one cornea treated by the angiogenesis effector with the contralateral cornea of the same subject treated by the same molecule deprived of angiogenic capacity. Under these conditions, we observed that neovascularization was initiated by the appearance of a chemoattractant for the bovine capillary endothelium only in the cornea treated by the angiogenesis effector. The chemoattractant was purified about 150-fold by a single-step procedure, using gelatin-Sepharose affinity chromatography. Chemoattraction resulted from the combined effect of a chemoattractant factor(s) and an activating factor(s). The association of the two enhanced 5- to 8-fold the motility of the capillary endothelium in a concentration-dependent manner with optimum at 0.2 mg/ml. The activating factor(s) do not have chemotactic capacity, but without it, chemotaxis is reduced to about one half. The chemotactic complex was present in the cornea regardless of the nature of the angiogenesis effector used as the triggering device. Heat and proteases eliminated chemotaxis and destroyed the chemotactic complex. Thus, neovascularization may be triggered by effectors able to induce in the cornea proteins, normally not present, that influence angiogenesis via mobilization of capillary endothelium.

INTRODUCTION

Angiogenesis is a morphogenetic event indispensable for the growth of tissues and organs. We observed that the capacity to induce new formation of vessels is a property acquired very early by cells undergoing neoplastic transformation (17, 25, 26). Almost all neoplastic tissues tested have shown strong angiogenic capacity (4, 10, 13). Under appropriate conditions, transplants of neoplastic cells in avascular organs fail to produce a tumor (3, 7, 11), suggesting that angiogenesis is indispensable for neoplastic growth and that interference with the angiogenesis process may alter the ability of a tumor to grow in an apparently unrestrained way (3, 6, 7, 11).

In previous work, we have found that several small molecules, in particular molecules carrying copper ions, can trigger the angiogenesis process and can also lose this property when the molecule is modified; for instance, by removal of the copper ion (22, 23). We also observed that, among the angiogenesis effectors evaluated, only the heparin-copper complex was able to induce both angiogenesis in vitro (rabbit cornea assay) and mobilization of bovine capillary endothelium in vitro (1). All other effectors such as prostaglandin E1, ceruloplasmin or fractions thereof carrying copper, and the G-H-Ly4-copper complex were unable to mobilize capillary endothelium by themselves, but when they acted in vivo on the corneal tissue, a strong chemotactic activity toward the capillary endothelium was detected in the corneal extract. The experiments reported aimed at assessing the nature of this chemotactic activity. The approach was based on the comparison between one cornea treated with the angiogenesis effector and the contralateral cornea of the same rabbit treated with the same or similar molecule deprived of angiogenic capacity.

MATERIALS AND METHODS

Preparation of Angiogenesis Effectors. Native ceruloplasmin, with about 6 copper atoms/molecule, was obtained from commercial preparation of human ceruloplasmin (type III ceruloplasmin; Sigma Chemical Co., St. Louis, MO) by DEAE-cellulose chromatography as described earlier in our laboratory (23). Apoceruloplasmin, containing 0.44 copper atoms/molecule or less, was prepared by the diethyldithiocarbamate method of Morell and Scheinberg (19); M, 11,000 copper-rich fragments and the correspondent apopeptides were prepared from human ceruloplasmin as described by Raju et al. (22, 23).

Copper was bound to porcine heparin (ammonium salt; Sigma) as described by Lages and Stivala (15). Twenty-five mg of heparin and 5 mg of CuSO4 were dissolved in 3 ml of 0.1 M Tris-HCl buffer (pH 7.5), allowed to stand overnight, and chromatographed on a Sephadex G-25 column equilibrated in water. Fractions containing copper-heparin complexes were detected by absorbance at 237 nm (15) or by copper content determination (18).

G-H-Ly4-copper complexes were obtained by the procedure of Pick and Thaler (21). CuSO4 (0.5 mM) and G-H-Ly tripptide (0.5 mM) were dissolved together in PBS at pH 7.4, allowed to stand overnight, and chromatographed on a Sephadex G-10 column equilibrated in water. G-H-Ly4-copper complexes elute before G-H-Ly and were detected by chromatographic profile and by copper content determination.

Tumor tissue extracts were prepared from mammary carcinomas induced with 7,12-dimethylbenz(a)anthracene. Virgin rat mammary glands were used as controls. The tissue was minced, washed in 0.87% (w/v) ammonium chloride to lyse the RBC, and centrifuged at 1000 x g for 10 min to yield a pellet. The washing and recentrifugation was repeated 3 times. The washed pellet was resuspended at 1 g/ml in 50 mM Tris-HCl (pH 7.3) and homogenized with Polytron. The homogenate was centrifuged at 25,000 x g for 20 min; the supernatant was lyophilized and stored frozen at −20°C.

Angiogenesis Assay in Vivo. The procedure has been described previously (9, 12). Briefly, a New Zealand white rabbit (Dutchland Laboratories, Inc., Denver, PA), 2- to 3-kg body weight, male or female, was anesthetized with Nembutal (25 mg/kg). One pocket, about 2 x 3 mm, was surgically prepared in the lower half of each cornea. Attention must...
be paid to have the bottom of the pocket about 2 mm from the limbus. The material to be treated was introduced into the pocket of one cornea and the control material into the pocket of the opposite cornea of the same animal. Thus, differences in response due to the variability of the host were minimized. Four to 5 days after implantation, capillary buds appeared on the limbal vessels facing the angiogenesis effector. By the seventh to eighth day, a dense network of capillaries had involved the cornea and surrounded the implant (12). The cornea is avascular and transparent, and the newly formed capillaries, clearly visible with a stereomicroscope, were photographed.

Preparation of Corneal Extracts. An elvax pellet containing the appropriate amount of an angiogenesis effector was inserted into one cornea, and a similar pellet containing equal amounts of the same molecule deprived of angiogenic activity was inserted into the contralateral cornea of the same rabbit. The elvax pellet was used as a way of obtaining a slow continuous release of the effector (12). After 80 hr, when the budding of capillaries from the limbal vessels was just beginning but the cornea was free of vessels, the rabbit was anesthetized, both corneas were frozen by a spray of Cryowik (dichlorodifluoromethane; Shield Chemical Co., Needham Heights, MA), and rapidly removed. The segment of cornea underlying the elvax pellet, where the penetration of capillaries occurs as time progresses, was cut with a razor blade and stored at -80°C until use. The extract was prepared by chopping the thawed samples in fragments of about 1 cm mm and incubating them overnight at 37°C in DMEM or PBS. The volume of fluid was adjusted to obtain, after about 1 hr of extraction, a supernatant (2000 x g for 15 min) with 1.5 mg protein/ml.

Cell Populations. Endothelial cells isolated from capillaries of BACE were used for the migration assays. The cells were donated by Dr. J. Feder (Monsanto Co., St. Louis, MO) and used at passages 15 to 22. The endothelial origin of the cell population has been shown (20). BACES were grown in DMEM plus 10% calf serum (Grand Island Biological Co., Grand Island, NY) mixed 1:1 with conditioned medium from mouse sarcoma 180. BACES were kept in T-75 sq cm Falcon flasks (Falcon Labware; Becton-Dickinson Co., Oxnard, CA) coated with 1% (w/v) gelatin made up in PBS (without calcium and magnesium). BACES were split once weekly at a ratio of 1:3. The conditioned medium was obtained from cultures of sarcoma 180 (American Type Culture Collection, Rockville, MD) grown in DMEM plus 10% calf serum in T-75 sq cm Falcon flasks. The conditioned medium was collected as described (8) and was used to maintain the cells, never for the endothelium mobilization assays.

Human foreskin fibroblasts (Meloy Laboratories, Inc., Springfield, VA), fetal bovine aortic endothelium, and adult rabbit cornea fibroblasts were maintained in our laboratory as described earlier (1) and used in the migration assay as control cell populations.

Boyden Chamber Migration Assay. The method (2) is based on the passage of cells across pores against a gradient of a migration effector. The Boyden chamber used had an upper well of 200 μl in size and a lower well of 40 μl. The 2 wells were separated by a 0.1 mm thick polycarbonate membrane (Nucleopore Corp., Pleasanton, CA), 13 mm in diameter, precoated with type I collagen. A 5 μm micropore-sized filter was used. Both chambers were filled with DMEM added with 1% fetal calf serum. After the cells had attached, the supernatant was removed, and fresh DMEM plus 1% calf serum were placed in the central well. Test and control material in 20 μl DMEM plus 1% calf serum were pipetted into the central well incubated at 37°C in 5% CO2 atmosphere for 3 to 4 hr. After the cells had attached, the supernatant was removed, and fresh DMEM plus 1% calf serum were placed in the central well. Cell migration was followed by the medium with a slow continuous flow of fresh DMEM through the upper well and fresh DMEM with an angiogenesis effector into the lower well. After 80 hr, when the cells had migrated to the lower filter surface were fixed in 10% formalin in buffer phosphates; the layer of agarose was carefully peeled off, and the cells migrated on the gelatin were stained with Wright’s solution. A migration index was determined as the product between the number of cells migrated as a halo around the central well and the distance traveled beyond the background movement (1).

Isolation of Chemotactic Activity Complex. Sepharose-6B gel filtration was used for isolation of the chemotactic activity complex. Sepharose-6B (Pharmacia, Piscataway, NJ) was soaked in 50 mM ammonium acetate buffer, pH 7.0, for at least 24 hr and deaerated before packing in a column of 1.5 cm diameter to a height of 50 cm. A 0.5 cm layer of Sephadex G-25, coarse grade, was placed on top of the Sepharose-6B bed to afford stability to the surface. Gel filtration was carried out at 4°C. Approximately 5 to 6 mg of corneal extract protein in 2 ml of PBS solution were placed on the column and eluted with 50 mM ammonium acetate buffer at a flow rate of about 10 ml/hr. Fractions of 2.2 ml were collected, and absorbance at 280 nm was recorded with an ISCO absorbance monitor.

Protein fractions under a zone were pooled, lyophilized, and tested for chemotactic activity. Zones 3, 4, and 5 of Sepharose-6B contained salts and were desalted on PD-10 columns (Pharmacia) before testing for chemotactic activity.

Purification of Chemotactic Factor(s). From the corneal extracts, the chemotactic factor(s) were purified by gelatin-Sepharose 4B (Pharmacia) affinity chromatography. Gelatin-Sepharose 4B, 2- to 3-ml bed volume, was loaded in a (0.7 x 20 cm) column and equilibrated in 0.02 M sodium phosphate buffer, 0.15 M NaCl, pH 7.4. The corneal extract (G-H-Ly plus copper-implanted corneas) in PBS (8 mg protein in 2.5 ml) was applied to the column and washed with equilibrating buffer. The bound protein was eluted with 0.05 M sodium acetate and 1.0 M sodium bromide, pH 5.0. The bound eluate was dialyzed against 50 mM ammonium acetate buffer to remove salts and was lyophilized.

Purification of Activating Factor(s). The corneal extract (G-H-Ly plus copper-implanted corneas) was passed through a gelatin-Sepharose column to remove the chemotactic factor(s). The gelatin-unbound fraction was dialyzed against 50 mM ammonium acetate buffer and lyophilized. The lyophilized material (5 mg protein) was dissolved in 50 mM ammonium acetate buffer, pH 7.0, and fractionated in Sepharose-6B column (1.5 x 50 cm) equilibrated with 0.05 M ammonium acetate buffer, pH 7.0. The fractions under a peak were pooled, lyophilized, and tested for chemotactic activity individually or in combination with the chemotactic factor(s) (gelatin-bound fraction).

Purification of Rabbit Fibronectin from Serum. All procedures were carried out at room temperature. Rabbit serum (100 ml) was passed through a Sepharose-6B column (2.6 x 40 cm), equilibrated with PBS containing 0.1% NaH2SO4 to remove Sepharose-binding proteins. Fractions having absorbance at 280 nm >1.0 were pooled and passed through
the gelatin:Sepharose column (1.5 cm x 20 cm), equilibrated with PBS containing 0.1% NaN3 to bind fibronectin (5). The column was first washed with PBS containing 0.1% NaN3 and then with 0.05 M Tris-HCl, pH 7.1, in 4 mM NaCl containing 0.1% NaN3 to remove nonspecifically bound proteins. Finally, fibronectin was eluted with 3 M urea in PBS containing NaN3 (24). The fractions obtained by washing with 3 M urea were pooled, dialyzed against PBS, and stored at -20°C.

Protein Estimation and SDS-Polyacrylamide Gel Electrophoresis. Protein was estimated by the method of Lowry et al. (16). Polyacrylamide gel electrophoresis in the presence of SDS was performed according to the method of Laemmli (14) using vertical slab gels. The acrylamide gel electrophoresis in the presence of SDS was performed according to

RESULTS

Angiogenesis in Vivo and Chemotaxis of Capillary Endothelium in Vitro by Corneal Extracts. The capacity to induce corneal angiogenesis in vivo was tested for the effectors listed in Table 1. The first part of Table 1 (in vivo corneal assay) indicates that 4 effectors carrying copper in their molecules are strongly angiogenic, while removal of copper reduces their angiogenic capacity to almost zero. As already shown (27), prostaglandin E2 has a much stronger angiogenic capacity than prostaglandin E1, and normal mammary gland is practically not angiogenic as compared to mammary tumors. The second part of Table 1 (in vitro migration by corneal extracts) shows that a sharp increase of capillary endothelium mobilization was induced in vitro by extracts of cornea implanted for 80 hr with elvax pellets bearing each one of the effectors tested in vivo. Presence of angiogenesis in vivo paralleled the capacity of the corneal extract to mobilize cells in vitro, and absence of angiogenesis in vivo corresponded to absence of cell mobilization in vitro. In previous work (1), we have observed that the angiogenesis effectors listed in Table 1 were unable by themselves to induce mobilization of endothelium in vitro, except the heparin and copper complex. Thus, Table 1 indicates that, under the action of angiogenesis effectors, a new property is acquired by the corneal tissue, the capacity to mobilize capillary endothelium. As observed previously (1), and confirmed in these experiments, the chemotactic effect is exerted on capillary endothelium, not on fibroblasts (data not shown).

Partial Purification of Chemotactic Activity from Corneal Extracts. Rabbit corneas treated with different angiogenesis effectors were extracted, and the extracts were fractionated by gel filtration chromatography on a Sepharose-6B column. The profiles of absorbance at 280 nm reported in Chart 1 showed a remarkable similarity among the different angiogenesis effectors, including mammary tumors. The eluate was first divided into 5 fractions (Chart 1), and the chemotactic capacity of each fraction was tested on bovine capillary endothelium by the Boyden chamber assay (Table 2). Zone 2 (Chart 1) showed the highest chemotactic effect. The response pattern was consistent regardless of the angiogenesis effectors tested in the cornea (Table 2).

The ability of the corneal extract to mobilize capillary endothelium was also tested with the gelatin-agarose assay (1). The results were similar to the Boyden chamber assay, namely Zone 2 (Chart 1), had a chemotactic activity about 3-fold higher than the other fractions (Table 3).

The chemotactic effect of Zone 2 (Chart 1) as well as that of the unfractionated extract increased in a concentration-dependent manner with optimum at 0.2 mg per ml of protein for the first and 1.6 mg per ml of protein for the latter. Chart 2 reports the data for the extracts of corneas treated with G-H-Ly and copper complex.

About 4% of the total corneal proteins were contained in Zone 2 (Chart 1); their molecular weight ranged from 300 to 800 kDa. The unfractionated extract increased in a concentration-dependent manner with optimum at 0.2 mg per ml of protein for the first and 1.6 mg per ml of protein for the latter. Chart 2 reports the data for the extracts of corneas treated with G-H-Ly and copper complex.

In order to improve the purification of the chemotactic factor(s), the corneal extract was passed through a gelatin-Sepharose affinity column. At a concentration of 10 μg/ml, the bound fraction showed a chemotactic effect on endothelium equal to or better than the unbound fraction at a concentration 150-fold higher (Table 4). Thus, a substantial purification of the chemotactic factor(s) was obtained with the gelatin column. When the portion of the corneal extract unbound to the gelatin-Sepharose affinity column was fractionated on Sepharose-6B column, the 280-nm absorbance profile was found to be similar to the profile of the whole corneal extract (Chart 3). However, Zone 2 of the gelatin-unbound extract (Chart 3B) had no chemotactic effect, whereas the same Zone 2 of the whole extract (Chart 3A) induced strong chemotaxis as shown in Table 2 and Chart 2. The gelatin-Sepharose affinity column removed practically all the chemotactic activity of the corneal extract. However, a sharp improvement of endothelial cell mobilization was observed (Table 4) when Zone 2 of the gelatin-unbound extract (Chart 3B), which does not have chemotactic activity, was added to the gelatin-bound fraction of the corneal extract which already has chemotactic activity.
Chart 1. Gel filtration of corneal extract proteins on Sepharose-6B column (1.5 x 50 cm) equilibrated with 0.05 M ammonium acetate buffer (pH 7.0). Flow rate was 10 ml/hr. Fractions (2.2 ml/tube) were collected, and the column effluent was continuously monitored at 280 nm with an ISCO absorbance monitor. Marked zones were pooled and tested for chemotactic activity. This enhancement of the chemotactic effect indicates that, despite a lack of chemotactic activity, the gelatin-bound fraction contains an activator of the endothelial chemotaxis induced by the gelatin-bound fraction of the corneal extract. This suggests that at least 2 components of the corneal extract are necessary for optimal chemotactic effect on capillary endothelium.

The gelatin-bound fraction on SDS:polyacrylamide gel electrophoresis appears to be constituted by 5 to 6 protein bands with molecular weights ranging from 35 to 66 x 10^3, a substantial improvement over the number of protein bands of the starting material (Fig. 1). Heating at 70° for 60 min, as well as incubation at 37° with Pronase (Calbiochem-Boehringer Co., La Jolla, CA) for 3 hr (enzyme:protein ratio, 1:30) destroyed the chemotactic effect.

Table 2
Chemoattractant response of capillary endothelial cells to Sepharose-6B fractions of corneal extracts

<table>
<thead>
<tr>
<th>Fractions tested</th>
<th>Protein concentration (mg/ml)</th>
<th>Chemotactic response (cells migrated/field)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ceruloplasmin</td>
<td>11-K fraction</td>
</tr>
<tr>
<td>Sepharose-6B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zone 1</td>
<td>0.2</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>Zone 2</td>
<td>0.2</td>
<td>112 ± 16</td>
</tr>
<tr>
<td>Zone 3</td>
<td>0.2</td>
<td>31 ± 4</td>
</tr>
<tr>
<td>Zone 4</td>
<td>0.2</td>
<td>30 ± 5</td>
</tr>
<tr>
<td>Zone 5</td>
<td>0.2</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>Unfractionated corneal extract</td>
<td>1.5</td>
<td>97 ± 18</td>
</tr>
</tbody>
</table>

* Boyden chambers migration assay.

** Mean ± S.D.

Table 3
Chemoattractant response of capillary endothelial cells to Sepharose-6B fractions by gelatin:agarose assay

<table>
<thead>
<tr>
<th>Migration effector</th>
<th>Protein concentration (mg/ml)</th>
<th>No. of cells migrated</th>
<th>Migration distance (µm)</th>
<th>Migration index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sepharose-6B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zone 1*</td>
<td>0.200</td>
<td>80 ± 13</td>
<td>450 ± 20</td>
<td>330 ± 35</td>
</tr>
<tr>
<td>Zone 2</td>
<td>0.200</td>
<td>321 ± 27</td>
<td>875 ± 25</td>
<td>1028 ± 110</td>
</tr>
<tr>
<td>DMEM + 1% calf serum</td>
<td>0.100</td>
<td>302 ± 21</td>
<td>750 ± 30</td>
<td>800 ± 65</td>
</tr>
</tbody>
</table>

* The results for Zones 3, 4, and 5 were similar to the values for Zone 1 and were omitted.

** Mean ± S.D.
Chart 2. Chemotactic response of capillary endothelial cells to increasing concentrations of chemotaxis-inducing fraction [Sepharose-6B (SE-6B) Zone 2 from G-H-Ly + copper] (O-O) in comparison with unfractionated extract (G-H-Ly + copper) (• •) and control unfractionated extract (G-H-Ly) (A A). Lower wells of Boyden chambers contained test material, and upper wells contained BACE (10⁵ cells/well). Filters were processed, and number of cells migrated was determined as described in "Materials and Methods."

Table 4

<table>
<thead>
<tr>
<th>Corneal extract fractions</th>
<th>Protein concentration (µg/ml)</th>
<th>Chemotactic response² (cells migrated/field)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin bound</td>
<td>10</td>
<td>49 ± 15²</td>
</tr>
<tr>
<td>Gelatin unbound</td>
<td>1500</td>
<td>37 ± 15²</td>
</tr>
<tr>
<td>Gelatin bound + unbound</td>
<td>10 + 1500</td>
<td>61 ± 14²</td>
</tr>
<tr>
<td>Gelatin bound + Zone 2 of gelatin unbound</td>
<td>10 + 200</td>
<td>123 ± 18²</td>
</tr>
<tr>
<td>Unfractionated</td>
<td>1500</td>
<td>64 ± 8²</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elvax-40</td>
<td>1500</td>
<td>18 ± 3²</td>
</tr>
<tr>
<td>Elvax + G-H-Ly</td>
<td>1500</td>
<td>16 ± 2²</td>
</tr>
<tr>
<td>DMEM + 1% calf serum</td>
<td></td>
<td>21 ± 3²</td>
</tr>
</tbody>
</table>

²Protein concentration of the fraction added to the lower section of the Boyden chamber.

DISCUSSION

In previous work, we observed that several small molecules could trigger new formation of capillaries (23). The experimental approach used the rabbit cornea assay in which capillaries originating from the limbal vessels were observed as they penetrated into the avascular cornea under the stimulation of an angiogenesis effector slowly released by an elvax pellet. In the experiments reported here, we analyzed the influence of these angiogenesis effectors on the cornea immediately before the penetration of the capillaries had started. The experimental approach had 2 major features: (a) the angiogenic effect was evaluated by quantitating the ability of the corneal extract to mobilize capillary endothelium in vitro; and (b) the action of the angiogenesis effector on one cornea was compared in vivo with the action of a similar molecule lacking angiogenic capacity on the contralateral cornea of the same rabbit.

Regardless of the angiogenesis effector used, the cornea acquired the ability to mobilize capillary endothelium within 75 to 80 hr from treatment, i.e., immediately before the capillaries budded from the limbal vessels initiated the penetration of the cornea. The chemotactic effect was localized in a fraction of the extract and was purified about 150-fold. By SDS-polyacrylamide gel electrophoresis, the molecular weight ranged between 35 and 66 x 10³. Since the best purification was obtained with a
treated with various angiogenesis effectors are able to mobilize capillary endothelium in vivo (4, 10, 13). Extracts of corneas are strongly angiogenic in vivo (4, 10, 13). K. S. Raju et al.

Fig. 2. Polyacrylamide gel electrophoresis of proteins in the presence of SDS. The proteins were analyzed under reducing conditions in a 5% separating gel; Lane 1, purified rabbit plasma fibronectin; 30 to 40 µg of protein sample were loaded; Lane 2, Zone 2 of corneal extract (Sepharose-6B active fraction); 40 µg of protein sample was loaded. Note the absence of fibronectin band; Lane 3, molecular weight markers.

gelatin-Sepharose affinity column that also binds fibronectin, rabbit fibronectin (Fig. 2) was compared in SDS-polyacrylamide gel electrophoresis with the active fraction of the corneal extract. No band at M, 220,000 was observed that could indicate presence of fibronectin in the active corneal fraction. Human (Sigma) and rabbit fibronectins were also tested for chemotactic activity in the gelatin:agarose and Boyden chamber assays. In the first test, a chemotactic activity of fibronectin was not detected; in the second assay, fibronectin showed a moderate chemokinetic effect but a minor chemotactic action on the endothelium. We conclude that fibronectin is not the endothelial chemotactic factor(s) of the corneal extract, although fragments derived from matrix proteins, including fibronectin, may be responsible for the effect.

The mobilization of the endothelium was optimal when a nonchemotactic component of the corneal extract was added to the purified fraction with chemotactic activity. We have no data concerning the nature of this activating effect. Activation was associated with high-molecular-weight material and was inseparable by gel filtration. Heparin and copper were not detected in the purified fraction.

The physiological significance of the data reported is based on the following observations. (a) Angiogenic capacity appears in the course of neoplastic transformation of cells that are normally not angiogenic (17, 25, 26). (b) Most neoplastic tissues are strongly angiogenic in vivo (4, 10, 13). Extracts of corneas treated with various angiogenesis effectors are able to mobilize capillary endothelium in vivo (Table 1). (c) Without neovascularization, a neoplastic cell population does not form a solid tumor (3, 7, 11). (d) Several effectors can trigger angiogenesis (23). In the corneal system, the triggering effect manifests itself through the appearance of proteins normally absent in the unstimulated cornea. Partial purification of these proteins indicates that the triggering event controls their appearance, but not their nature. These proteins influence angiogenesis via mobilization of capillary endothelium. Characterization of these proteins should offer a possibility to interfere with angiogenesis and possibly tumor growth.

REFERENCES

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