Tumor Angiogenic Factor
PURIFICATION FROM THE WALKER 256 RAT TUMOR*

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We report here the purification of an angiogenic substance from the Walker 256 rat ascites tumor which is mitogenic for fetal bovine aortic endothelial cells in culture and which also stimulates new blood vessel growth in vivo. Purification was monitored in endothelial cell cultures by cell counting or by [3H]thymidine incorporation into DNA. Lyophilized crude tumor cell homogenate was extracted with absolute ethanol, and the extract was further purified by silica gel column chromatography. The most purified material eluted with ethyl acetate:methanol (25:1) and behaved as a single component when analyzed either by thin layer chromatography with both silica gel and polyethyleneimine-cellulose systems or by reversed phase (C18) high pressure liquid chromatography. Bio-Gel P-30 chromatography indicates a M, 400–800 for the active material. The mitogenic activity of the purified material was observed with the vascular endothelial cells cultured in serum-less as well as serum-containing media. The angiogenic activity of this material was revealed in both the chicken choioallantoic membrane and rat corneal micropocket bioassays. Our results demonstrate that a factor with mitogenic and angiogenic activities can be purified from the Walker rat tumor by a novel, facile, and high yield process.

The significance of the interaction between host vasculature and solid malignancies to the survival and growth of tumors has been recognized for over a century. Both Virchow (1) and Thiersch (2) described the altered vascular network about tumors. Ribbert (3), Goldmann (4), and Russell (5), in separate studies, noted the irregular and tortuous vessels about the tumor and in the adjacent tissue, and concluded that such findings were useful as an indicator of the presence of a malignancy. In fact, Goldmann observed further that tumor malignancy. In fact, Goldmann observed further that tumor

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0.10 ml. Replica wells on the same dishes were employed for any given test condition. The actual number of cells present at the start of the test was measured by detaching the cells from several wells with 0.1% trypsin (Type III from bovine pancreas, Sigma) and 0.05% EDTA, and carrying out cell counts. At 5 h attachment efficiencies of 60–75% were obtained; slightly higher cell counts were obtained after an overnight (16 h) incubation in growth medium.

Test cultures were incubated at 37 °C in a humidified 5% CO2 atmosphere for the specified period of time. At the end of this period, the cultures were processed for quantitation by cell counting or incorporating tritiated deoxythymidine into cellular DNA. Cell counting was performed with a Coulter counter on cells detached with a tryptic solution. Incorporation of [3H]deoxythymidine was achieved by the following pulse-labeling technique: 1 h prior to the end of the experiment the test media were removed and replaced with 1.0 ml/well of serum-free minimum essential medium containing 0.625 μCi/ml of [3H]Tdr (methyl-3H)thymidine obtained from Amersham (Arlington Heights, IL) as a sterile, aqueous solution of 20–25 Ci/mmol and used within 2 months. After a 1-h incubation at 37 °C the incorporation was terminated by removing the culture fluid and rinse each well once with cold (4 °C) Ca- and Mg-free phosphate-buffered saline solution. This was followed by a 5-min rinse at room temperature with a solution of 50% CaCl2 and MgCl2, 0.25 ml of cold saline and then thoroughly rinsed with H2O. Solubilization of cellular materials was accomplished by sonication of the contents of a well with 0.2 ml of 0.25 N sodium hydroxide solution for 20 min at room temperature. The contents of each well were transferred to 4-ml glass counting vials using an additional 0.2 ml of H2O for rinsing the well. Each vial then received 3.2 ml of counting fluid (Budget-Solv, Research Products International, Elk Grove Village, IL); the contents were mixed, and the vials were counted in a Beckman LS-100 liquid scintillation counter (with a tritium-counting efficiency of approximately 31%). All data are minimally the average of duplicate samples. Separate autoradiographic studies performed on cells labeled and fixed in an identical manner revealed that the tritium label was localized to the nuclear regions of the cells.

Tumor-derived Materials and Chromatographic Techniques—Walker 256 ascitic tumor cells, carried in male Sprague-Dawley rats, were collected, centrifuged at 800–1000 × g for 10 min to yield a tumor cell pellet, washed twice or three times in 0.87% (w/v) ammonium chloride to lyse the red blood cells, and reconstituted. The nearly white cell pellet was resuspended at 80 A2W cells. The pellet-limbus distance was measured with an ocular micrometer (Carl Zeiss, Inc., New York, NY). Maximum vessel length and the pellet-limbus distance were measured with an ocular micrometer. Vascularization was markedly reduced in all cases the procedures employed with rabbit (18) and mouse corneas (19). Briefly, corneal micropockets were prepared in the eyes of male Sprague-Dawley rats; an Ethicon pellet containing the test material was inserted into the pocket, which was sealed by repinning the temporally displaced corneal epithelium. Every 4 days eyes with these implants were examined using a Zeiss slit lamp stereomicroscope (Carl Zeiss, Inc., New York, NY). Maximum vessel length and the pellet-limbus distance were measured with an ocular micrometer. The number of blood vessels and the presence of any corneal edema were noted. At the end of the test (day 16), pathological studies on enucleated and formalin-fixed eyes were performed using paraffin sections stained with hematoxylin and eosin (17).

RESULTS

In order to simplify and accelerate certain steps in our assay technique, without sacrificing the reliability and flexibility of our previously described procedures (13), we determined conditions for an endothelial cell growth assay based upon the incorporation of radiolabeled thymidine into cellular DNA. As an example of the criteria that were used for judging the acceptability of any experimental condition was the close agreement between cell counts and [3H]Tdr incorporation for parallel test cultures. Two major considerations emerged from these studies: 1) the

Recrphotography on silica gel was performed with active material that eluted with ethyl acetate-methanol (3:1). This fraction (7.5 A250 nm in 1 ml of methanol) was placed on a silica gel column (1.2 × 9 cm). The components were eluted isocratically at a flow rate of 1 ml/min with chloroform: ethyl acetate, and mixtures of ethyl acetate-methanol in the ratios of 50:1, 25:1, 10:1, 5:1, and 2:1, in the order stated. The absorbance at 260 nm was measured with a Gilford spectrophotometer for each of the 1.0-ml fractions collected.

Thin layer chromatography, using both silica gel and polyethyleneimine-cellulose absorbents, was carried out on Brinkmann plates (with fluorescent indicator) in closed glass chambers. Samples of 10 μl were spotted in methanol using glass capillary tubes. Silica gel plates were developed with methanol; ion exchange plates, with 1 M NaCl in 0.1 M Tris-HCl (pH 7.0). The components were routinely visualized using three methods: 1) fluorescence quenching; 2) iodine adsorption; and 3) reaction with ninhydrin (15). Silica gel plates on occasion were treated with Zinazide reagent, bromthymol blue, or charring after spraying with sulfuric acid (15); however, no additional components were detected with these reagents other than those observed with the other three methods.

High pressure liquid chromatography was performed using a Waters system with an analytical Whatman ODS3 reversed-phase column (column volume, 2 μl). The components were eluted isocratically with 5% aqueous methanol at a flow rate of 1 ml/min. Eluents were routinely detected spectrophotometrically by means of absorbance at 260 nm and doped cafully by the alternative means of refractive index differences. Results from these two detection methods were always in excellent agreement.

Vascularization Assays—Neovascularization was examined for the grade choroidal lamellar membrane using the previously described techniques (16). Corneal neovascularization in rats (17) was studied using essentially the procedures employed with rabbit (18) and mouse corneas (19). Briefly, corneal micropockets were prepared in the eyes of male Sprague-Dawley rats; an Ethicon pellet containing the test material was inserted into the pocket, which was sealed by repinning the temporally displaced corneal epithelium. Every 4 days eyes with these implants were examined using a Zeiss slit lamp stereomicroscope (Carl Zeiss, Inc., New York, NY). Maximum vessel length and the pellet-limbus distance were measured with an ocular micrometer. The number of blood vessels and the presence of any corneal edema were noted. At the end of the test (day 16), pathological studies on enucleated and formalin-fixed eyes were performed using paraffin sections stained with hematoxylin and eosin (17).
need to use subconfluent cell cultures, and 2) the need to eliminate artifacts arising from the trypsinization of cells during subculturing. First, the narrow range of cell density (5-50 x 10^3 cells/cm^2) over which [^3H]TdR incorporation into pulse-labeled cells was linear did not prove to be a serious limitation. Even though slightly more than 2 cell doublings are possible during the test period (2-3 days), we normally observe between 1-2 doublings in cell numbers with control cultures grown in Medium 199 containing 2% (or less) FBS. Second, cells for testing must be allowed to remain on the plating medium for a minimum of 5 h before changing to the test medium. The preferred procedure for tests that required 2-3 days in serum-less medium was to use an overnight preincubation in the original plating medium. In order to allow for some growth during the 16- to 20-h period before the start of a test, cells can be plated at a lower cell density (5-10 x 10^3 cells/cm^2). If this protocol was followed, fewer cells from the control wells detached during the test period.

The dose-dependence plot of Fig. 1 illustrates some of these points. In this experiment, cell growth effects were examined as a function of the medium concentration of impure tumor-derived material (obtained by an ethanol extraction of the lyophilized crude tumor homogenate). Stimulation of endothelial cell growth by this tumor-derived material is evident, regardless of the means for monitoring growth effects, cell counts, or [^3H]TdR incorporation. The results from these two methods are in excellent agreement.

Furthermore, this dose-dependence plot permits a determination of the amount of endothelial cell growth stimulatory activity present in a sample. A unit of activity is defined as the minimal amount of material (on the basis of volume of addition or any other parameter) that is needed to produce a maximal stimulatory effect on endothelial cell growth. From Fig. 1, then, 1 unit corresponds to material with an absorbance of 0.150 at 260 nm (or 10 mllilitres at A_{260} nm). Although there is some degree of variability inherent in measuring activity, it is still possible to employ this definition for gaining an approximation of the recoveries of active material for each step in the purification process.

Deserving of mention at this point are two aspects of the specificity of this in vitro assay. When the ethanol extract of the crude tumor homogenate (as was used in Fig. 1) was tested on two nonendothelial cell types (adult bovine corneal fibroblasts and fetal bovine smooth muscle cells), no growth stimulation was observed under conditions that produced a positive response from the fetal bovine aortic endothelial cells. In addition, if the growth response of these endothelial cells was examined with crude tissue homogenates, or their ethanol extracts, prepared from normal rat liver, kidney, or skeletal muscle, no stimulation of cell growth could be measured, i.e. cell counts (or [^3H]TdR incorporation) were within 20% of control values. These observations, in accord with our previous results obtained with crude tumor homogenates and the vascular endothelial cells (13), demonstrate the selective nature of the in vitro assay.

With the development of this convenient and credible assay we began studies on the purification of the endothelial cell growth factor from the Walker 256 tumor. We observed that dialysis of dilute solutions of crude tumor cell homogenates produced a small but real loss in active materials. Activity could be recovered from the lyophilized dialysates, suggesting that the active materials were small molecules. We then studied the elution of activity from a Bio-Gel P-30 column under neutral and acidic conditions. In crude tumor cell homogenates at pH 7.6, most of the activity was found in the flow through, or void volume, fractions (Fig. 2A). Since the crude homogenate could be treated to pH 3.9 with minimal loss of activity, we applied this acid-treated homogenate to the column, which was then eluted at pH 3.9. Under these conditions most of the active materials eluted from the column with an apparent Mr, 400-800 (Fig. 2B). Additionally, when crude tumor homogenate was adjusted to pH 3.9, then brought back to neutrality and chromatographed at pH 7.0, the active materials also eluted from the same column in the molecular weight range of 400-800.

Purification of the active component was next attempted using extraction of crude tumor homogenates with an organic solvent, instead of acid treatment. When lyophilized crude tumor homogenates were sonicated with ethanol, greater than 95% of the total activity was recovered in the ethanol supernatants. This extract was about five times more active than the crude homogenate in our assay system (based on A_{260}). When this ethanol extract was dissolved in a neutral buffer and applied to the Bio-Gel P-30 column, the molecular weight of the active material was also found to be in the range of 400-800 (as in Fig. 2B).

![Fig. 1. Comparison of [^3H]thymidine incorporation and cell density of vascular endothelial cells grown in the presence of varying amounts of Walker tumor-derived materials. Fetal bovine aortic endothelial cells were plated in growth medium at 2.3 x 10^4 cells/cm^2. After an overnight incubation the cells were given fresh Medium 199 containing 1% fetal bovine serum and various amounts of tumor-derived materials. For this study aqueous solutions of the ethanol extract from the crude cell homogenates of the Walker tumor were added. Two days later the cells were counted (Curve B) or pulse-labeled with [^3H]thymidine (Curve A) as described under "Materials and Methods."](image1)

![Fig. 2. Bio-Gel P-30 column chromatography of Walker tumor cell materials. Specific conditions are described under "Materials and Methods." Stippled areas indicate fractions active in stimulating endothelial cell growth. The arrows in A and B indicate the elution position of [^3H]adenosine (M_r = 267). In B are indicated the elution positions of molecular weight markers: I, chymotrypsinogen (M_r = 25,700); II, vitamin B_12 (M_r = 1,300); and III, adenosine. A, crude tumor homogenate; eluting buffer was 50 mm Tris-HCl (pH 7.0). B, acid-treated tumor homogenate; eluting buffer was 50 mm formic acid (pH 3.9).](image2)
The mixture from the ethanol extraction of the tumor cell homogenates could be separated further using silica gel chromatography. By applying the sample in ethanol to a silica gel column packed in chloroform and by carrying out the elution scheme indicated in Fig. 3, seven separate fractions could be resolved. Only those fractions eluted with ethyl acetate:methanol (3:1) displayed activity in the endothelial cell growth assay.

In order to ascertain the composition of the recovered active materials, three analytical systems, two based on the use of thin layer chromatography, and the third on the use of high pressure liquid chromatography, were developed for conveniently but accurately determining the purity of the column fractions. The TLC methods use different adsorbents, silica gel (Fig. 4) and polyethyleneimine-cellulose (Fig. 5), thereby effecting separations on the basis of different molecular properties. In Fig. 4 can be seen results of silica gel TLC of the ethanol extract of the tumor cell homogenates (Lane A), as well as the seven fractions from silica gel column chromatography (Lanes B–H). The means of detection routinely employed and shown in this representation include fluorescence quenching, and iodine- and ninhydrin-staining. Because no one detection method revealed the presence of all of the components in a given sample, only the combination of the routine methods (on a single TLC plate) was considered to provide a fair picture of the state of purity for a sample. Worth noting in the context of the purification procedure, fraction C1 from the silica gel column appeared to be comprised of 3–4 major components by silica gel chromatography (Fig. 4, Lane D). Corroboration of this finding was obtained by polyethyleneimine-cellulose TLC (Fig. 5, Lane B) and by HPLC with a reversed-phase C18 analytic column (Fig. 6A). A similar finding resulted when HPLC detection was accomplished by means of refractive index differences, instead of by ultraviolet absorption.

On the basis of these observations, silica gel chromatography was repeated using a shallower solvent gradient. A methanolic solution of fraction C1 from the first silica gel chromatography was placed on a silica gel column (as before), but elution was carried out in stepwise fashion with ethyl acetate to methanol ratios of 50:1 down to 2:1. Six fractions containing UV-absorbing material could be separated by this procedure, but only material eluting with ethyl acetate:methanol (25:1) contained endothelial cell growth stimulatory activity. When this material was examined by silica gel 'TLC (Fig. 4, Lane J), polyethyleneimine-cellulose (Fig. 5, Lane C), and HPLC (Fig. 6B), it was found to be essentially homogeneous. Alteration of detection methods and elution conditions did not lead to any different conclusion.

**Fig. 3.** Silica gel column chromatography of ethanol extracts of Walker tumor cell homogenates. Specific conditions for applying the ethanol extracts of the tumor cell homogenates are described under "Materials and Methods." Stippled areas indicate fractions active in stimulating endothelial cell growth.

**Fig. 4.** Silica gel thin layer chromatography of fractions from the silica gel column chromatography of the Walker tumor cell ethanol extracts. The pooled fractions from the silica gel column chromatography of the ethanol extracts of the Walker tumor cell homogenates, shown in Fig. 3, were rechromatographed using silica gel thin layer chromatography and methanol as the eluant. Specific details are presented under "Materials and Methods." O, fluorescence quenching; @, iodine staining; $\oplus$, ninhydrin reacting. Lane A, ethanol extract of tumor cell homogenate. From chromatogram shown in Fig. 3: Lanes B; fraction A; C; fraction B; D, fraction C1 (active); E, fraction C2; F, fraction; C3; G, fraction D; H, fraction E. From second silica gel chromatography using fraction C1: Lane J, active material eluting with ethyl acetate:methanol, 25:1.

**Fig. 5.** Polyethyleneimine-cellulose thin layer chromatography of active materials separated by silica gel column chromatography. Conditions for carrying out thin layer chromatography on polyethyleneimine-cellulose strips are described under "Materials and Methods." A 1 M NaCl solution in 0.1 M Tris-HCl (pH 7) was used as the eluant; components were visualized by fluorescence quenching. Lane A, ethanol extract of tumor cell homogenate; Lane B, active fraction C1 from silica gel chromatography (Fig. 3); and Lane C, active material eluting from second silica gel chromatography with ethyl acetate:methanol, 25:1.
Thus, a homogeneous preparation of a tumor cell-derived growth factor for endothelial cells can be obtained in reasonable yields by this facile, 3-step process. A summary of the process is provided in Table I. Due to our arbitrary definition of a unit of activity, the yields at the various stages of the purification process must be considered only as estimations. However, that the purified material is the major (if not only) endothelial cell growth stimulator present in the Walker tumor cell homogenates seems a reasonable conclusion.

We next examine two features of this purified material: 1) its ability to stimulate endothelial cell growth in a serum-less medium, and 2) its ability to stimulate vessel growth in two assays for neovascularization, the corneal implant and chicken chorioallantoic membrane assays. The effects of purified tumor factor on aortic endothelial cell growth were studied in Medium 199 containing no serum, 2.5% dialyzed fetal bovine serum, or 1% FBS (Table II). The ability of each of these media to support endothelial cell growth, in the absence of added tumor factor, increases in the order given. The addition of these media of maximal stimulatory amounts of purified tumor factor (20 milliunits at A360) resulted in stimulatory ratios (Table II) that decreased in the order serum-less media > media with 2.5% dialyzed FBS > media with 1% FBS. The mitogenic action of the tumor factor is best demonstrated in the serum-less medium, where the contribution to cell growth by serum factors has essentially been eliminated. The inference from these results is that the tumor factor is capable of stimulating cell growth independently of other serum factors.

Also worth noting is that the same results are obtained by cell counts (shown in the table) and by incorporation of [3H]dThd. Furthermore, at concentrations 10-fold higher than that used in Table II, the purified tumor factor continued to produce a maximal growth stimulatory effect. These results indicate that an inhibitory (or cytotoxic) agent, present in the crude cell homogenates (13), had been removed during the course of the purification. Thus, in summary, these observations reveal that the tumor factor is capable of initiating cellular DNA synthesis and cell replication for cultured fetal bovine aortic endothelial cells, and that it can act directly on the cells without the intervention of other serum factors.

The angiogenic activity of this tumor mitogen for cultured endothelial cells was examined in the two most commonly used neovascularization assays, the chicken chorioallantoic membrane and the corneal implant assays. In both instances, preparations of the tumor factor were encapsulated in a slow release polymer (Elvax), which was then implanted in the test system (20). Typical results on the chicken chorioallantoic membrane are shown in Fig. 7. In this micrograph the vascularizing responses elicited by a control implant of tumor-derived materials inactive in the in vitro assay and an implant containing active tumor-derived materials are being directly compared at day 7 after implantation. A sizable effect, in terms of the number of new vessels loops directed toward the Elvax pellet, is obtained only when material active in stimulating endothelial cell growth is included in the pellet (Fig. 7B). Essentially no effect is seen with the inactive control fractions (Fig. 7A); this vascular pattern is identical with that obtained when only a blank pellet is used.

Implantation of these materials into the rat cornea produced the same result as that with the chicken chorioallantoic membrane assay (Fig. 8 and 9). The micrograph (Fig. 8) shows considerable outgrowth of limbal vessels only when the corneal implant contained the tumor factor. This response was quantitated for implants containing approximately the same number of milliunits at A360 of inactive tumor material (fraction B from the initial silica gel chromatography), impure but active material (ethanol extracts of the crude tumor homogenate), and the purified tumor factor (Fig. 9). Neovascularization produced by approximately the same number of A360 was greater with the purified preparation than with the impure material; material inactive in the in vitro assay was also inactive in this in vivo assay. The neovascular response observed in this rat corneal assay was not associated with inflammation, as judged by the absence of corneal edema from stereomicroscopic examination. Furthermore, the inflammatory response, apparent from analyzing the histological sections and characterized by the presence of occasional lymphocyte or macrophage and rare polymorphonuclear leu-

### Table II

| Medium | Control (A) | Stimulatory ratio (B/A) |
|--------|-------------|------------------------|
| Serum-less | 7.32 | 12.0 | 1.64 |
| 2.5% Dialyzed FBS | 23.9 | 33.9 | 1.42 |
| 1% FBS | 28.3 | 33.5 | 1.18 |

Effects of purified Walker tumor factor on vascular endothelial cell growth in serumless and serum-containing media

Fetal bovine aortic endothelial cells were plated at an initial cell density of 8.53 x 10^5 cells/cm^2 (uncorrected for attachment efficiency) in minimum essential medium containing 5% FBS. After an overnight incubation, the attached cells were rinsed with Ca- and Mg-free phosphate-buffered saline solution and Medium 199 containing the indicated addition was placed on the cells. After 3 days the cells were counted. The results represent the average values from two tests done in duplicate.

* A unit of activity is defined as the minimal amount of material that is used to produce a maximal effect on endothelial cell growth.

**TABLE I**

| Purification step | Total AZM | Total units | Specific activity | Recovery of activity |
|-------------------|-----------|-------------|------------------|---------------------|
| Crude cell homogenate | 100 | 670 | 6.7 | 100 |
| Ethanol extract | 20 | 660 | 33.0 | 98 |
| Silica gel I | 6.9 | 420 | 63.6 | 63 |
| Silica gel II | 1.7 | 350 | 206 | 52 |

Summary of the purification of the Walker tumor factor from crude cell homogenates

**Fig. 6.** High pressure liquid chromatography of active materials separated by silica gel chromatography. The procedures for effecting HPLC separation of the ultraviolet-absorbing components in the active materials are detailed under "Materials and Methods." A, active fraction C from silica gel chromatography (Fig. 3). B, active material eluting from second silica gel chromatography with ethyl acetatemethanol, 25:1.

**TABLE II**

| Purification step | Total AZM | Total units | Specific activity | Recovery of activity |
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* A unit of activity is defined as the minimal amount of material that is used to produce a maximal effect on endothelial cell growth.
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Fig. 7. Angiogenic activity of purified Walker tumor factor in the chicken choioallantoic membrane assay. The details for assaying test materials on the chicken choioallantoic membrane are given under "Materials and Methods." The micrographs were obtained on day 7 following implantation of an Elvax pellet containing the test materials. A, fraction D (60 milliunits at $A_{290\,\text{nm}}$/pellet) from silica gel chromatography (Fig. 3) (inactive in cell growth assay). B, active purified material (60 milliunits at $A_{290\,\text{nm}}$/pellet).

Fig. 8. Angiogenic activity of purified Walker tumor factor in the rat corneal micropocket assay. The details for assaying test materials in the rat cornea micropocket are given under "Materials and Methods." The micrographs were obtained on day 8 following implantation of an Elvax pellet containing the test materials. A, fraction D (60 milliunits at $A_{290\,\text{nm}}$/pellet) from silica gel chromatography (Fig. 3) (inactive in cell growth assay). B, active purified material (60 milliunits at $A_{290\,\text{nm}}$/pellet).

Fig. 9. Vessel growth in the rat cornea in response to Walker 256 tumor-derived materials. See "Materials and Methods" for details. ○, crude ethanol extract of Walker 256 rat tumor cell homogenates (160 milliunits at $A_{290\,\text{nm}}$/Elvax pellet). □, active purified tumor factor (110 milliunits at $A_{290\,\text{nm}}$/Elvax pellet).

kocyte, was comparable in the three experimental groups (in Fig. 9), and could not be correlated with the amount of vascularization. The results from both the chicken choioallantoic membrane and cornea assays unambiguously demonstrate that the endothelial cell growth stimulatory factor is also an angiogenic factor.

DISCUSSION

The purification to homogeneity of a low molecular weight angiogenesis factor from the Walker 256 rat carcinoma has been achieved with good recoveries of activity and with only a few facile steps. In fact, preliminary results is this laboratory indicate that the reported 2-step silica gel chromatography may be simplified to just one step by employing a shallower gradient of ethyl acetate/methanol eluants. The purity of this tumorderived material has been established using two independent TLC and HPLC systems. These convenient analytic techniques, along with the growth assays using endothelial cell cultures, permit a relatively rapid analysis of chromatographic separations of angiogenic agents from the Walker tumor, as well as from other sources. Furthermore, these techniques offer great promise as diagnostic tools in the detection of neovascular disorders, of which tumor vascularization is but one example. For example, the detection of angiogenic activity in various sera, which we have recently accomplished using these techniques, may prove useful in correlating altered serum levels of angiogenic materials with certain neovascular disease states.

The effect of this tumor factor on endothelial cell growth in vitro compared to other growth factors in other cell systems is deserving of further discussion. The cultures of fetal bovine aortic endothelial cells display a direct, selective, and significant growth response to the tumor factor. This observation is in contradistinction to that made with bovine brain capillary endothelial cells (21). In order to observe any growth effects with tumor-derived materials, the bovine cells appear to have stringent requirements for a collagen substratum and added platelets. We have found that cells from fetal bovine aortic endothelium on collagen-coated surfaces respond to the tumor factor as well as do cells on the usual tissue culture plastic surface. Addition of platelets produces in our assays slight growth inhibitory effects. We tend to attribute these effects to the platelet-derived growth factor (22), which when assayed as the purified substance in our system is an inhibitor of fetal bovine aortic endothelial cell growth. Since our assay is straightforward and correlates well with the angiogenic assays, it seems ideally suited for carrying out studies on the detection of certain angiogenic agents (in particular, those agents with endothelial cell mitogenic activity), and perhaps on their mechanism(s) of action.

However, a word of caution must be sounded regarding the general use of these techniques in angiogenesis studies. The process of new vessel growth is comprised of at least two separate processes, and possibly a third. For capillary growth to take place, endothelial cell migration and replication are required. In addition, matrix modification, as in basal membrane degradation, may also be needed. Thus, an angiogenic factor may have for capillary endothelial cells a mitogenic activity or a chemotactic activity or both. Azizkhan et al. (23) have recently demonstrated that heparin affects only the movement of cultured bovine capillary endothelial cells and not their rate of growth. The angiogenic activity of heparin, however, was not determined. A low molecular weight angiogenic factor from the Walker tumor has been claimed to be a nonmitogenic, chemotactic factor; however, no supporting experimental evidence was provided (24). The tumor factor purified by us has been examined in our in vitro assay system,
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which measures only cell growth using endothelial cells from a major vessel, albeit from a fetal source. These same cells have recently been employed in a cell migration assay that has demonstrated the coincidence of mitogenic and chemotactic activities in an angiogenic preparation from bovine retinas (25). Therefore, the relationship between mitogenic and chemotactic activities for the tumor factor should be clarified shortly.

Finally, with the exception of the approximate size of the pure material (<800 daltons) and the ultraviolet absorption at 260 nm, which was associated with the active material through all the steps of its purification, no other physical characteristics of this substance have been reported here. The methods used for isolating and purifying this material do not permit any unequivocal statements regarding molecular structure. In light of the apparent small size and purity of our angiogenic material, it should be amenable to the usual spectroscopic methods of structure determination. The results of these studies will be considered in a future communication.

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