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Isolation of a nonmitogenic angiogenesis factor from wound fluid

(neovascularization/capillary endothelial cells/directed migration/capillary growth/tumor angiogenesis factor)

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ABSTRACT **Angiogenesis, or new capillary growth, is essential to normal growth and wound healing. It is also active in several pathologic states, including the growth of malignant tumors. An extracellular, nonneoplastic angiogenesis factor has been isolated from cell-free rabbit wound fluid by pore-limit dialysis and chromatography on a size-exclusion HPLC column. The isolated angiogenesis factor was purified 9,600-fold with a yield of 81% and has a molecular weight between 2,000 and 14,000. Wound fluid angiogenesis factor was completely separated from the mitogenic activity of wound fluid; it did not increase the number of capillary endothelial cells *in vitro* or stimulate [^{3}H]thymidine uptake by these cells. The isolated angiogenesis factor stimulated endothelial cell migration *in vitro*, and less than 200 ng of the factor stimulated angiogenesis *in vivo* in the corneal implant assay.**

Angiogenesis is required in normal processes such as growth and wound repair. It is also an active component of pathologic states such as tumor growth, atherosclerosis, arthritis, and diabetic retinopathy. In each case the identity of the angiogenic substance either has not been determined or has not been agreed upon.

Angiogenesis can be described as the directed outgrowth of new capillaries toward a specific stimulus. By microscopic analysis of capillary growth (1) endothelial cells can be seen to migrate from the tip of the capillary before any indication of mitosis. Therefore, the ability to stimulate directed capillary endothelial cell migration is an expected prerequisite of an angiogenesis factor. Because capillary growth can be stimulated at a distance from the angiogenic source (2), angiogenesis factor must act extracellularly. To date several purification procedures have been reported for angiogenesis factor extracted from cells but not for an extracellular form. The starting material for these purifications has been lysates of tumor cells (3–5) or an extract of retinal cells (6). Some of these purifications have also relied on endothelial cell mitogenesis *in vitro* as a characterizing assay of the purified material (5, 6), but have not included capillary endothelial cell migration in the characterization.

Angiogenesis is an essential part of wound healing, and some wounds have an easily definable extracellular fluid filling the central space of the wound. This wound fluid is a plasma-like substance that has been conditioned by the inflammatory cells at the wound site. Unlike plasma, wound fluid stimulates new capillary growth (7). In this communication, we report the isolation of an angiogenesis factor from cell-free rabbit wound fluid. Wound fluid angiogenesis factor was characterized for the ability to stimulate new capillary growth *in vivo* and for mitogenic and migratory potential for rabbit brain capillary endothelial cells *in vitro*.

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MATERIALS AND METHODS

Rabbit Wound Fluid. Bacteriologically sterile wound fluid was obtained by pooling fluid withdrawn from stainless steel, wire-mesh wound cylinders implanted 21 days earlier in the flanks of New Zealand White rabbits (8). Any cells present in pooled wound fluid were removed by centrifugation at 1,000 × *g* for 20 min. Each cylinder yielded approximately 10 ml of fluid.

Acidification and Dialysis of Wound Fluid. Pooled wound fluid was clarified by centrifugation at 20,000 × *g* for 30 min at 4°C. The clarified wound fluid was then placed in an M_r 2,000-limit dialysis bag (Spectrapore 6, Spectrum Medical, Los Angeles, CA) and dialyzed against three changes, 24 hr each, of 10 vol of 0.1 M acetic acid at 4°C. Acid-insoluble material was removed from the retentate by centrifugation at 20,000 × *g* for 30 min at 4°C. The clarified retentate was then placed in a standard M_r 14,000-limit dialysis bag and dialyzed against 2 liters of 4°C water for 24 hr. The 2 liters of dialysate was then lyophilized and the retentate was discarded.

Chromatography. The dialyzed and lyophilized wound fluid was reconstituted to 10 mg/ml (wt/vol) (≈7 mg of protein per ml) in 0.01 M acetic acid (Pierce). Any insoluble material was removed by filtration through a 0.45-μm-pore-diameter filter (Millipore). This material was then applied to a pair of Aquapore OH-100 size-exclusion columns (4.6 × 250 mm each) fitted with an Aquapore guard cartridge and an Aquapore presaturation column (Brownlee, Santa Clara, CA). Chromatography was carried out with 0.01 M acetic acid as the mobile phase delivered at 1.0 ml/min at 25°C by a Hewlett–Packard model 1084B liquid chromatograph equipped with a model 79825A fraction collector, a model 79842A automatic sampling system, and a model 7987A variable wavelength detector with stop-flow-scan capability. For this column system the theoretical void and total inclusion volumes for globular proteins that do not bind to the column were determined with blue dextran (M_r 2 × 10^6, retention time 3.67 min) and $K_3Fe(CN)_6$ (M_r 329, retention time 6.11 min). Preparative chromatography experiments involved 20–30 cycles of 100 μl per injection per cycle with pooled fractions collected on ice.

Determination of Protein Concentration. The protein concentration of whole wound fluid and of dialyzed wound fluid was estimated by determining the absorbance at 280 nm in a 1.0-cm light path with a model 250 spectrophotometer (Gilford). An absorbance of 1.0 was considered 1.0 mg/ml. The amount of protein in the HPLC-purified peak was determined by integrating the area under the peak that was detected at 280 nm. The detector was calibrated with bovine insulin B chain (Sigma).

Corneal Implant Assay. Angiogenesis was determined by the corneal implant assay (9). The solution to be tested was mixed with an equal volume of Hydron (Hydron, New Brunswick, NJ), dropped in 20-μl aliquots onto a polyethylene sheet, then dried

Abbreviation: RBCE, rabbit brain capillary endothelial cells.

Table 1. Wound fluid as a source of angiogenesis factor

Source	Activity,* units/ml	No. of preparations
Serum	0	6
Wound fluid	25 ± 31.6	6
Acidified wound fluid†	62 ± 17.7	2

* Mean ± SD.
† Acidified to pH 2.9 with glacial acetic acid; not dialyzed.

under reduced pressure (10). The resulting pellets of polymerized Hydron and test substance were implanted in corneas 2 mm proximal to the superior limbus. Eyes were evaluated at 3 through 14 days after implantation. The sustained growth of well-defined individual capillaries from the limbus toward or into the implant was considered positive for angiogenesis. Angiogenic response was scored blindly on a 0–4 relative scale. A normal or negative eye was scored as 0, whereas a maximal response was scored as 4. Because a reading of 4 was maximal it was not used in quantification. A response of 2 on the relative scale was defined as 1.0 unit of angiogenic activity, and a reading of 1 was considered 0.5 unit; readings of 1 and 2 were the preferred range for quantification. Eyes that demonstrated positive angiogenesis were removed, fixed, and processed for histologic examination to rule out angiogenesis due to the influx of inflammatory cells.

Rabbit Brain Capillary Endothelial Cells (RBCE). RBCE were isolated and cultured by a modification (unpublished data) of a method for isolating rat brain capillary endothelial cells (11). The purification of capillary endothelium from basement membrane and pericytes was modified by employing an enzyme cocktail consisting of DNase at 200 μg/ml (7×10^4 dornase units/mg, B grade, Calbiochem-Behring), Pronase at 200 μg/ml (45 proteolytic units/mg, B grade, Calbiochem), and collagenase at 500 μg/ml (125 units/mg, U.S. Biochemical, Cleveland, OH) in Hanks' balanced salt solution (Ca^{2+},Mg^{2+}-free). RBCE were collected after centrifugation at the interface between 30% and 50% in a discontinuous Percoll gradient. The isolated RBCE were cultured on gelatin-coated plates [the plates were covered with 1% gelatin (type III, Sigma) in distilled water for 1–2 hr, excess fluid was removed, and the plates were dried at 60°C] in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 10% normal rabbit serum, and 2 mM glutamine. RBCE were passaged with the same enzyme cocktail used in the isolation. The endothelial origin of these cells was confirmed by light microscopy, by the presence of Weibel–Palade bodies visible in electron microscopy and by immunofluorescent staining with antibody against factor VIII (Atlantic Antibodies, Scarborough, ME). The cells used in both the migration and mitogenesis assays were nonconfluent RBCE from passages 1–4 that had been in culture less than 4 days from the time of passage. The doubling time of RBCE was 35 hr in the presence of rabbit wound fluid and 50 hr in the presence of normal rabbit serum, but they did not proliferate in rabbit platelet-poor plasma serum.

Table 2. Mitogenic potential of wound fluid and serum

Source	Mitogenic index*	
	Rabbit skin fibroblasts	RBCE
Normal rabbit serum	78.7	27.8
Rabbit wound fluid	32.6	26.9
Acid dialysate†	15.2	1.1

* [^{3}H]Thymidine incorporated; experimental/control = index. Assay volume was 20 μl.
† The acid dialysate of rabbit wound fluid was concentrated 37-fold over whole wound fluid.

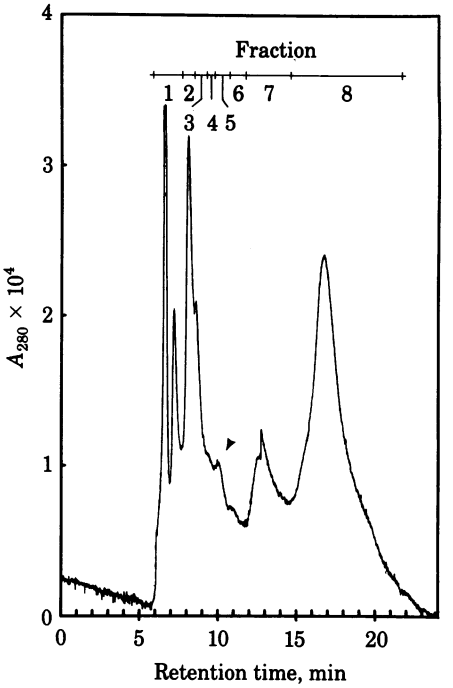

FIG. 1. Chromatography of acid dialysate of rabbit wound fluid. Ten microliters of a 10 mg of solids per ml solution of the acid dialysate was chromatographed on an Aquapore OH-100 column. Mobile phase was 0.01 M acetic acid delivered at 1.0 ml/min. Fraction numbers are indicated at the top. The arrow indicates the peak containing angiogenic activity, fraction 5.

Mitogenesis Assay. Mitogenic activity was determined by measuring the incorporation of [^{3}H]thymidine (12) by confluent serum-starved RBCE or primary rabbit skin fibroblasts. Cells were grown in 24-well plates (10^5 cells per well) (Costar, Microbiological Associates, Los Angeles) for 5 days in 1 ml of Dulbecco's modified Eagle's medium supplemented with 2% heat-inactivated bovine calf serum. On day 6, the medium was replaced with 20–100 μl of test material diluted to 1 ml in the same medium. On day 7, 0.5 μCi of [^{3}H]thymidine (specific activity 11 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was added to each well. After an additional 24 hr the cells were washed twice with saline and lysed, and the radioactivity was determined by liquid scintillation counting. Data are expressed as a mitogenic index ([^{3}H]thymidine incorporated; experimental/control). Under these conditions RBCE had a mitogenic index of 5.1 when exposed to a crude preparation of rabbit platelet-derived growth

Table 3. Biologic activity of chromatographic fractions of rabbit wound fluid

Fraction	Start of fraction, min	RBCE mitogenesis, index*	Angiogenesis, units/ml	RBCE migration,† cells/filter
1	5.79	0.4	0	7
2	7.64	0.7	0	8
3	8.43	1.55	0	7
4	9.21	1.06	0	6
5	9.71	1.03	50	128
6	10.66	1.15	0	45
7	11.69	1.70	0	33
8	14.55	0.62	0	11

* Index of wound fluid = 26.9. Assay volume was 100 μl.
† Area = 12.6 mm^2.

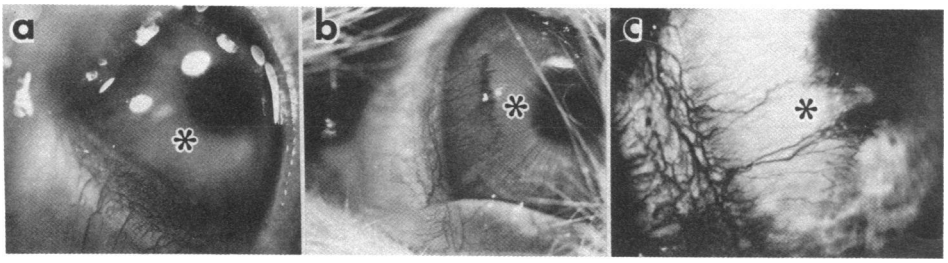

FIG. 2. Corneal implant assay. Each eye was implanted with two 20-μl pellets of 50% Hydron/50% test substance. The pellets contained M_r >14,000 acid retentate (*a*), M_r 2,000–14,000 acid dialysate (*b*), or isolated angiogenesis factor (fraction 5) (*c*). Asterisks indicate the positions of the implants. The relative angiogenic response in *a* is 0, in *b* it is 4, and in *c* it is 2. *c* shows the equivalent of 1.0 unit of angiogenic activity and has been magnified ×1.5 relative to *a* and *b*.

factor and 25.8 when exposed to normal rabbit serum, whereas bovine aortic arch endothelial cells had a mitogenic index of 3.2 when exposed to normal rabbit serum and 1.1 when exposed to rabbit wound fluid.

Cell Migration Assay. The potential of purified material to stimulate the directed migration of RBCE was determined by a modified migration assay previously used for fibroblasts (13, 14). Solutions to be tested were diluted 1:10 to a final volume of 300 μl in Dulbecco's modified Eagle's medium supplemented with 10% rabbit platelet-poor plasma serum and placed in the bottom of Boyden blind-well chambers. Gelatin-coated 10-μm-pore-diameter polycarbonate filters (Nuclepore) were placed over the test solution and 2.5×10^4 RBCE suspended in Dulbecco's modified Eagle's medium plus 10% rabbit platelet-poor plasma serum were added to the top compartment. Chambers were incubated for 7 hr at 37°C. At the end of the incubation, the tops of the filters were wiped clean. The filters were then fixed, stained, and evaluated by counting the number of cells that had migrated to the bottom side of the filter.

RESULTS

Angiogenic and Mitogenic Activity of Whole Wound Fluid. The angiogenic potential of whole untreated wound fluid varied from no detectable activity to as high as 75 units/ml. Serum consistently had no angiogenic activity (Table 1). Acidification of the wound fluid to pH 2.9 by the addition of glacial acetic acid to 0.1 M enhanced angiogenic activity 2- to 6-fold (Table 1) and also removed nonangiogenic acid-insoluble material.

When whole wound fluid was dialyzed at a neutral pH in a M_r 14,000-limit dialysis bag, the angiogenic activity was found in both the retentate and the dialysate. However, when dialysis was carried out against 0.1 M acetic acid, all of the angiogenic activity was recovered in the dialysate. When acid dialysis was carried out in a M_r 2,000-limit dialysis bag, the retentate contained all of the angiogenic activity and little or no mitogenic activity toward RBCE or fibroblasts (Table 2).

Chromatographic Isolation and Biologic Activity of Wound Fluid Angiogenesis Factor. The acid-dialyzed wound fluid that was retained in the M_r 2,000-limit bag and passed through the M_r 14,000-limit bag (acid dialysate) was lyophilized and reconstituted in 0.01 M acetic acid, then fractionated on a size-exclusion HPLC column (Fig. 1; Table 3). Angiogenic activity was found only in fraction 5 (Table 3). A corneal implant containing 150 ng of material from fraction 5 had 1.0 unit of angiogenic activity (Fig. 2). By histologic examination, the angiogenesis due to the material in fraction 5 was not the result of the infiltration of inflammatory cells.

These fractions were also assayed for mitogenic activity toward RBCE by measuring [^{3}H]thymidine incorporation (Table 3). The fractions neither had significant mitogenic activity (index ≥ 2.0) when compared to whole wound fluid (index = 26.9) nor increased endothelial cell number. Therefore, most of the mitogenic activity remained in the M_r >14,000 retentate after acid dialysis of the wound fluid. These data show that wound fluid angiogenesis factor is not a mitogen (index of the angiogenic fraction 5 was 1.03). Occasionally, some mitogenic activity was recovered in the acid dialysate. In the two of nine isolation experiments in which this was observed, the mitogenic activity eluted in fraction 1, whereas the angiogenic fraction remained free of mitogenic activity.

Because angiogenesis *in vivo* requires the migration of capillary endothelial cells toward the angiogenic stimulus, all of the fractions were tested for their ability to stimulate directed migration of RBCE through a 10-μm-pore-diameter polycarbonate filter. Whole wound fluid and the acid dialysate stimulated RBCE migration. The migratory activity of the acid dialysate was recovered in the angiogenic fraction 5, and to about one-third this extent in fraction 6 (Table 3). Therefore, only angiogenic fraction 5 at 735 ng/ml stimulated migration of RBCE. The concentration that maximally stimulated migration was 7.4 ng/ml.

Partial Characterization of Wound Fluid Angiogenesis Factor. We made the assumption that the material with an absorbance peak at 280 nm is likely to be protein or polypeptide. As estimated on the basis of this assumption, our isolation scheme produced an angiogenic fraction that was purified 9,600-fold with a recovery of 81% (Table 4). When fraction 5 was rechromatographed (Fig. 3) it ran true to its original retention time of 10.0 min. Although not homogeneous, fraction 5 consisted

Table 4. Purification of angiogenesis factor from rabbit wound fluid

Purification stage	Volume, ml	Total angiogenesis, units	Total protein,* mg	Specific activity, units/mg	Purification, fold	Recovery, %
Wound fluid	67	1,675	2,379	0.7	1	100
Acid dialysate	1.8	†	13	—	—	—
Fraction 5	27	1,350	0.2	6,750	9,643	81

* Material absorbing at 280 nm.

† The activity of the concentrated acid dialysate was not quantitatively determined, but it gave a maximal, nonlinear response in the corneal implant assay.

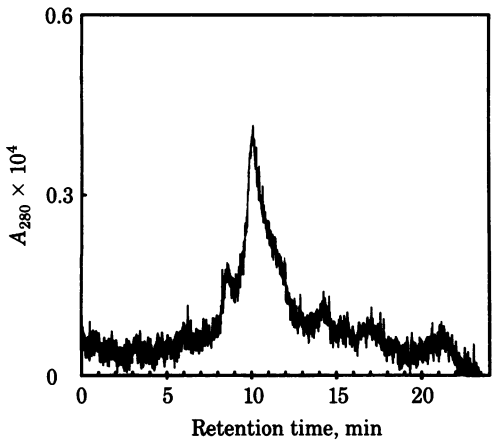

FIG. 3. Rechromatography of isolated wound fluid angiogenesis factor. Chromatogram of 200 μl (1.5 μg) of fraction 5 under conditions identical to those described in the legend to Fig. 1.

primarily of the 10.0-min peak itself with two or three contaminants that were probably components of adjacent fractions (see Fig. 1), which were not angiogenic. The 10.0-min peak may be composed of more than one component and will require further purification steps to establish homogeneity.

The chromatography of the acid dialysate was monitored at 206 nm, 260 nm, and 280 nm to establish optimal fractionation time. At these wavelengths the angiogenic peak fraction absorbed maximally at 206 nm and minimally at 260 nm. When the rechromatography of the angiogenic fraction was interrupted with the 10.0-min peak in the detector window and the peak was scanned from 190 to 540 nm, the resulting absorbance spectrum had a maximum at 206 nm and a prominent peak at 276 nm without a peak or shoulder at 260 nm (Fig. 4). These data are consistent with the interpretation that the angiogenic peak has the absorbance spectrum of a polypeptide or protein and not of a nucleic acid or polysaccharide.

Although angiogenesis factor eluted from a size-exclusion column system, we were not able to assign it a molecular weight. In this system, a void volume marker (blue dextran, M_r 2×10^6) eluted with a retention time of 3.67 min and a total inclusion marker [$K_3Fe(CN)_6$, M_r 329] eluted at 6.11 min. The retention time of the internal standard (insulin B chain, M_r 3,400) used to quantify the angiogenic fraction was 17.0 min. Angiogenesis factor eluted at 10.0 min, outside of the theoretical range of this column for size-exclusion chromatography. It is apparent, therefore, that chromatography was due to chemical interaction with the column rather than size exclusion alone. However, because angiogenic activity was retained by an M_r 2,000-limit dialysis membrane and passed by an M_r 14,000-limit membrane, we can say that its size is approximately between these two limits.

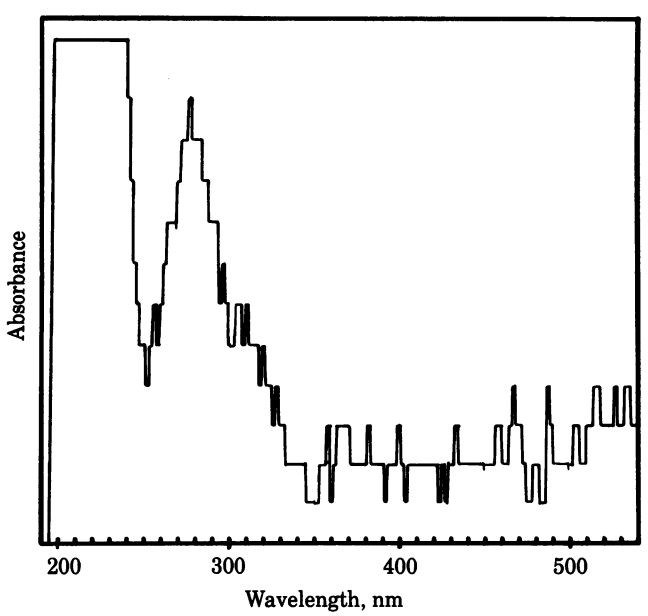

FIG. 4. Ultraviolet absorbance spectrum of isolated wound fluid angiogenesis factor. The chromatography of fraction 5 (see Fig. 3) was interrupted while the material was in the detector window, and it was scanned from 190 to 540 nm in 2-nm increments. Wavelength maxima were 206 and 276 nm.

DISCUSSION

Because of the nature of wound fluid, it is likely that wound fluid angiogenesis factor is from inflammatory cells, such as macrophages, which have been shown to secrete angiogenesis factor (7, 15, 16), or from platelets (17). Ninety percent of the cells in wound fluid 21 days after implantation of wound cylinders are macrophages (18). Although tumor angiogenesis factor is active extracellularly (2), the purifications of that factor (3–5) use cell lysates as starting material and thus may not have yielded an extracellular form of the factor. Therefore, we chose cell-free wound fluid as a starting material.

At this stage we can only speculate whether tumor angiogenesis factor, retinal angiogenesis factor, and wound fluid angiogenesis factor are the same compound. Our functional characterization of wound fluid angiogenesis factor differed from the characterization of either Walker 256 carcinoma angiogenesis factor (3–5) or retinal angiogenesis factor (6) in that wound fluid angiogenesis factor is not an endothelial cell mitogen. It is clear that a mitogen alone cannot cause directed capillary growth, whereas a capillary cell migratory stimulant can. Therefore, it appears unnecessary to attribute mitogenic capability to an angiogenic factor that stimulates directed migration of capillary endothelial cells. A mitogen is, however, necessary to increase endothelial cell number during capillary growth. The abundant mitogenic activity of wound fluid, of plasma already in the capillaries, and of activated platelets in open capillaries could sustain endothelial cell growth, but cannot by itself give direction to that growth. Wound fluid angiogenesis factor eluted with a retention time of 10.0–10.5 min in nine isolation experiments, and it was never associated with mitogenic activity. In five of the nine experiments, migration potential was evaluated, and the stimulation of endothelial cell migration was always associated with the angiogenic fraction.

The biochemical nature of tumor angiogenesis factor has remained controversial (3–5). Retinal angiogenesis factor has been inactivated by soluble Pronase and is thus considered a protein (6). Although the precise nature of wound fluid angiogenesis factor has yet to be determined, the ultraviolet absorbance data presented here suggest that nucleic acids or polysaccharides are not major components of our most highly purified preparations. Because the absorbance spectrum of this factor is like that of a protein, we have assumed it to be a polypeptide. However, the data presented here do not prove that it is a protein. Because proteinases and their inhibitors cause severe irritation to the cornea, preliminary inactivation studies have been uninformative.

As estimated by pore-limit dialysis, the molecular size range of wound fluid angiogenesis factor (M_r 2,000–14,000) is smaller than the M_r 50,000–100,000 of retinal angiogenesis factor (6) but an order of magnitude larger than the M_r 200–800 assigned to tumor angiogenesis factor (4, 5). If wound fluid angiogenesis

factor is a polypeptide, its size range would predict approximately 20 to 90 amino acid residues. The disparity in the size range for angiogenesis factors may indicate the existence of carrier or modifier compounds associated with the factor or anomalous behavior on gel filtration. Alternatively, angiogenesis factor from inflammatory cells may have nothing in common with other angiogenesis factors. The apparent shift in size of wound fluid angiogenesis factor as a result of acid treatment is similar to the change in size associated with the acidification of tumor angiogenesis factor (5). The shift in the molecular size of both wound fluid angiogenesis factor and tumor angiogenesis factor suggests either proteolysis or the association with a larger carrier molecule, which has been demonstrated for small peptides such as epidermal growth factor (19, 20).

Although the preparation of wound fluid angiogenesis factor was not homogeneous, the amount that stimulated angiogenesis in the corneal implant assay, 150 ng per Hydron pellet, approaches biologically active doses of peptide hormones. This is especially true when the radial diffusion during the corneal implant assay is considered. The Hydron pellet slowly releases its contents to establish a gradient that diffuses through 360°, but the capillary bed at the limbus might respond to a 20°–40° dose of angiogenesis factor during a 10-day period. This would imply that the capillary bed would respond to no more than 5–15% or 1–2 ng per day of partially purified angiogenesis factor; the initiation of angiogenesis may have resulted from an order of magnitude less material than that.

More investigation with a homogeneous preparation of wound fluid angiogenesis factor is needed to determine if there are any molecular similarities among the various angiogenesis factors and to probe the possibility that they share a common mechanism of action upon capillary endothelial cells.

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