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Isolation and Characterization of Angiogenin, an Angiogenic Protein from Human Carcinoma Cells†

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ABSTRACT: The first human tumor derived protein with in vivo angiogenic activity to be obtained in pure form has been isolated from serum-free supernatants of an established human adenocarcinoma cell line (HT-29) and named angiogenin. It was purified by cation-exchange and reversed-phase high-performance liquid chromatography; the yield was ~0.5 μg/L of medium. Biological activity of angiogenin was monitored throughout purification by using the chick embryo chorioallantoic membrane assay. Statistical evaluation demonstrates that it displays activity in this system with as little as 35 fmol per egg. Moreover, only 3.5 pmol is required to induce extensive blood vessel growth in the rabbit cornea. The amino acid composition of this basic (isoelectric point >9.5), single-chain protein of molecular weight ~14 400 has been determined. The amino terminus is blocked, and the carboxyl-terminal residue is proline.

Angiogenesis, the induction of the formation of blood vessels, is critical to the development, progression, and metastasis of animal and human tumors (Goldman, 1907; Ide et al., 1939; Algire & Chalkey, 1945; Ehrmann & Knoth, 1968; Greenblatt & Shubik, 1968; Folkman & Cotran, 1976). The molecular messengers that arise from tumor cells to mediate this process have long been sought (Folkman et al., 1971; Tuan et al., 1973; Phillips & Kumar, 1979; Weiss et al., 1979; McAuslan, 1980; Fenselau et al., 1981; Kumar et al., 1983; McAuslan et al.,

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1983; Shing et al., 1984). The majority of such studies have examined materials derived from rat Walker 256 carcinoma cells, but to date, none of the angiogenic substances from this source has been characterized chemically [for a review, see Vallee et al. (1985)]. The identification and purification to chemical homogeneity of a human tumor derived angiogenic factor (TAF)[1] have hitherto not been achieved. In one instance, a substance was isolated from human lung tumor cells but it, too, has thus far not been identified (Kumar et al., 1983).

An account of the considerations pertinent to the initial phase of our work in this field, which also employed the rat tumor cell line (Vallee et al., 1985), delineated the theoretical and experimental problems to be encountered in a study of angiogenesis. For reasons detailed therein, we subsequently examined the possibility of isolating TAFs from human tumors (Alderman et al., 1985b; Fett et al., 1985b), employing medium conditioned by HT-29 human colon adenocarcinoma cells, grown in the presence of FBS, as the starting material (Fett et al., 1985b). Although the serum introduced a major complication, a number of angiogenic entities were identified, among which one of molecular weight 12 500–17 500 was partially purified. Purification to homogeneity was hindered by both an inadequate amount of starting material and a lack of separation methods with satisfactory resolving power, problems since overcome.

This report details the isolation of human tumor derived angiogenin and describes its chemical and biological properties. The following paper presents its amino acid sequence (Strydom et al., 1985).

Experimental Procedures

Materials. CM-cellulose (grade CM-52) was a product of Whatman Ltd. All dialyses were performed with 6000–8000 molecular weight cutoff tubing (Spectra/Por). Deionized, sterile water was provided by a Milli RO-20 reverse-osmosis/Milli Q water purification system (Millipore Corp., Bedford, MA). Pepstatin A and hen egg white lysozyme were from Sigma Chemical Co. (St. Louis, MO).

Cell Line and Culture Conditions. Cells from the human colonic adenocarcinoma line HT-29 (Fogh & Trempe, 1975) were routinely grown at 37 °C as monolayer cultures in T flasks (Costar, Cambridge, MA) utilizing Dulbecco's modified Eagle's medium (M.A. Bioproducts, Walkersville, MD) containing 4.5 g/L glucose, 50 mg/L gentamycin, and 500 μg/L fungizone (DME) supplemented with 2 mM L-glutamine and 5% heat-inactivated FBS (DME/5) in a humidified, 7.5% CO_2 (in air) atmosphere. Medium was changed every 2–3 days, and cells were subcultured according to standard trypsinization procedures.

Large-scale, serum-free cultures of HT-29 cells employed 6000-cm^2 Nunc multilevel cell factories (Vangard International, Inc., Neptune, NJ) as described (Alderman et al., 1985a). Briefly, 1×10^8 cells, grown in DME/5 as above, were inoculated into a cell factory containing 1.5 L of DME/5 and allowed to attach and proliferate in a humidified, 7.5% CO_2 in air environment at 37 °C until confluent. The DME/5 was then replaced by 1.5 L of a serum-free maintenance medium consisting of DME without FBS but with an L-glutamine concentration of 5 mM. This maintenance medium was changed at 2–3-day intervals, and all collections made after day 7 were processed as described below.

Processing of Conditioned Medium. Cell debris was removed from the conditioned medium by sequential passage through Whatman 40 filter paper and Whatman 934-AH glass microfiber filters. Glacial acetic acid was added to the filtrate to a concentration of 5%. The acidified serum-free conditioned medium was treated with pepstatin A (5 mg/L), frozen, stored at −20 °C, then thawed, and clarified by filtration through Whatman 934-AH microfiber filters. The filtrate was subsequently concentrated 200-fold on a Model DC2 hollow-fiber dialyzer/concentrator unit equipped with HP2 (molecular weight cutoff of 2000) hollow-fiber filters (Amicon Corp., Lexington, MA), dialyzed vs. water, and lyophilized.

CM-cellulose Chromatography and HPLC. The lyophilized material from acidified serum-free conditioned medium was dissolved in 100 mM sodium phosphate, pH 6.6, and chromatographed on CM-52 as described (Fett et al., 1985a). Both the unbound (designated as CM 1) and the bound, salt-eluted fractions (CM 2) were extensively dialyzed vs. water and lyophilized.

Reversed-phase HPLC was performed by utilizing a Waters Associates liquid chromatography system consisting of a Model 440 absorbance detector (254 nm), an LKB 2138 detector (206 nm), two Model 6000 solvent delivery systems, and a WISP 710A automatic sample loader plus a data module and systems controller. Fractionations were performed by using an octadecylsilane Synchropak RP-P column (10-μm particle size, 250 × 4.1 mm) (Synchrom, Inc., Linden, IN) at a flow rate of 1 mL/min at room temperature. Column effluents were monitored at 206 and at 254 nm. Lyophilized preparations (CM 2) to be fractionated were reconstituted in 0.1% (v/v) TFA in water (solvent A) (Mahoney & Hermodson, 1980) and applied to the column through the automatic sample injector. Columns were eluted with linear gradients using final buffers of either 75% acetonitrile and water with 0.09% TFA (solvent B) over 80 min or 2-propanol/acetonitrile/water (5:5:4 v/v) with 0.08% TFA (solvent C) over 120 min. Following elution, pooled fractions were dialyzed against water, lyophilized, and reconstituted for biological analyses. Chemical analyses were performed directly on column eluates.

Gel filtration HPLC was performed on an LKB Ultropac TSK-G3000SW column (300 × 7.5 mm) equilibrated with PBS containing 6 M guanidine hydrochloride at a flow rate of 0.5 mL/min. Column effluents were monitored at 206 nm. BSA (M_r 68 000), ovalbumin (M_r 43 000), chymotrypsinogen (M_r 25 000), lysozyme (M_r 14 400), and insulin (M_r 6000) were used as standards.

Cation-exchange HPLC employed a Synchropak CM-300 column (250 × 4.1 mm; Synchrom, Inc.) equilibrated with 20 mM sodium phosphate, pH 7.0, at a flow rate of 0.8 mL/min. Elutions were performed by using a linear gradient of NaCl in the above buffer. Standards used were ribonuclease A (p*I* 9.5), cytochrome *c* (p*I* 10.2), and lysozyme (p*I* 10.5).

Gel Electrophoretic Procedures. Analytical SDS–PAGE using 15% gels with or without reducing reagent was performed according to Laemmli (1970) except that the stacking gel was omitted. Gels were silver stained by using a commercially available kit (Bio-Rad Laboratories, Richmond, CA). For extraction experiments, SDS–PAGE was performed on unboiled samples. Five micrograms of angiogenin (plus 20 μg of lysozyme as carrier) was run on 15% gels as above.

[1] Abbreviations: TAF(s), tumor-derived angiogenic factor(s); HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; CM, carboxymethyl; IEF, isoelectric focusing; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; FBS, fetal bovine serum; CAM, chorioallantoic membrane; BSA, bovine serum albumin; PBS, Dulbecco's calcium- and magnesium-free phosphate-buffered saline, pH 7.4; EDTA, ethylenediaminetetraacetic acid; DME, Dulbecco's modified Eagle's medium supplemented with 4.5 g/L glucose, 50 mg/L gentamycin, and 500 μg/L fungizone; DME/5, DME supplemented with 5% heat-inactivated FBS and 2 mM L-glutamine.

A control lane contained 20 μg of lysozyme alone. After electrophoresis, the gel was washed twice for 15 min each with 20% v/v 2-propanol in PBS to remove SDS (Blank et al., 1982) followed by three 10-min washes with sterile water to remove 2-propanol. Gel slices (2.5 mm) were incubated for 72 h in 200 μL of 5 mM sodium phosphate buffer, pH 7.0, containing 0.02% (w/v) BSA, and supernatants were then assayed directly on the CAM for angiogenic activity. The efficiency of extraction for lysozyme, determined enzymatically, was 20%.

Analytical IEF was carried out on an LKB 2117 multiphor unit using preformed plates (PAG plates, pH range 3.5–9.5; LKB). Gels were stained with Coomassie blue according to the manufacturer's recommendations. Standards used were ribonuclease A, cytochrome *c*, and lysozyme.

Protein Assays. Protein concentrations was determined by the dye binding method of Bradford (1976) using BSA as standard.

Amino Acid and Sequence Analyses. Proteins were reduced with tributylphosphine in 0.25 M sodium bicarbonate in 1-propanol (50% in water) and alkylated with 1,3-propane sultone (Sigma Chemical Co.) according to Ruegg & Rudinger (1974). Alkylated samples were desalted by chromatography on an I-125 HPLC column (Waters Associates) in 17.9% (v/v) acetonitrile, 17.9% (v/v) 2-propanol, and 0.1% (v/v) TFA in water. Performic acid oxidation was done according to Moore (1963). Lyophilized samples were hydrolyzed in vacuo with 6 N HCl and 0.1% phenol for 20 h at 100 °C (Sanger & Thompson, 1963). Hydrolysates were dried under vacuum at 25–35 °C, redissolved in citrate buffer, pH 2.2, and analyzed on a Durrum D-500 amino acid analyzer using ninhydrin as reagent. A Hewlett Packard 3390A integrator was used for quantitation.

Automated Edman degradation was performed on 300–3000 pmol of protein with a Beckman 890C sequencer using 0.1 M quadrol coupling buffer with Beckman program 121078 as described (Fett et al., 1985a). Carboxyl-terminal determinations were performed on 200 pmol of protein by hydrazinolysis with anhydrous hydrazine (Pierce Chemical Co., Rockford, IL) for 18 h at 80 °C in vacuo (Akabori et al., 1952) followed by direct analysis of hydrazinolysates by the "Pico-Tag" method (Waters Associates; Bidlingmeyer et al., 1984).

Biological Assays. Angiogenesis was routinely assessed by using the chick embryo chorioallantoic membrane (CAM) method of Knighton et al. (1977) with modifications. Fertile chick embryos were received from Spafas, Inc. (Norwich, CT), held at 18 °C for ~45 h, placed in a humidified, 37 °C incubator (Leahy Manufacturing Co., Higginsville, MO), and designated as day 0 eggs. Albumin was aspirated from the embryos on day 3, and 1–2-cm "windows" were cut through the shell on day 4. Five-microliter volumes of aqueous, salt-free samples were applied to sterile Thermanox 15-mm disks (Flow Laboratories Inc., Rockville, MD) and allowed to dry under laminar flow conditions. The loaded disks were inverted and applied to the CAM surface of 9-day-old embryos through the windows. Negative or positive [i.e., the appearance of a typical "spokewheel" pattern (Folkman, 1974)] responses were microscopically assessed at 30 ± 2, 54 ± 2, and 78 ± 2 h after implantation (designated as days 1, 2, and 3, respectively) and recorded as the number of positive angiogenic responses per number of eggs surviving per sample dilution. Statistical analysis was performed on the day 2 data as defined above. The definition of the time of reading is important since the number of positive responses increases at a rate of approximately 30% per 24 h. The background for the method of analysis and details of tests for fits to different distributions will be described elsewhere.[2] In brief, since the only designations are as positive or negative, the assays constitute Bernoulli trials and can be analyzed as binomial distributions (Kendall & Stuart, 1969). The frequency of positive responses in a series of 1834 controls is 0.0676 (124 positive and 1710 negative), with a standard deviation of 0.0059 (Kendall & Stuart, 1973), yielding upper and lower 0.1% confidence limits of 0.0857 and 0.0495, respectively. Unless indicated otherwise, we have employed 0.0857, the upper limit, as the probability of obtaining a positive result with a test group of N eggs. While this decision increases the chance that the sample is classed as inactive when it is in fact active, we feel it more important to decrease the chance of an inactive sample being considered active. Tables of the cumulative probabilities of positive results for $N = 2$–30 were prepared by using 0.0857 as the probability of a positive result, and test results are interpreted in terms of these tables. For values of N greater than 30, the cumulative distribution was evaluated by using the incomplete β function (Kendall & Stuart, 1969). Analogue dose–response curves were constructed by plotting the resultant probabilities against the weight of sample applied per egg. However, such plots cannot be interpreted in quantitative terms (i.e., that a 50% response can result from a defined dose) but rather as yielding the range over which and the lower limit at which a positive response is significant. A significance level of ≤5% has to be attained for a sample to be considered active. Since the cumulative probabilities refer to discrete events, they are themselves discrete, and therefore, only inequalities may be specified in general.

Angiogenic activity was evaluated also in the rabbit cornea by using a modification of established procedures (Langer & Folkman, 1976) that employed methylcellulose instead of Elvax pellets as the implant (Alderman et al., 1985b). Stereomicroscopic observations were made every 5 days to detect infiltrating vessels extending from the corneal limbus toward the sample implant.

Table I: Recovery of Protein on Purification of Angiogenin from Conditioned Medium of Human Colon Adenocarcinoma Cells

purification step	protein recovered (μg)
serum-free conditioned medium (10 L)	16000
acidification, freeze-thaw, filter	6300
CM-cellulose	
CM 1 (unbound)	3200
CM 2 (bound, eluted with 1 M NaCl)	2300
reversed-phase HPLC of CM 2	
acetonitrile elution (pools A and B)	19
acetonitrile/2-propanol elution (pool C)	8
acetonitrile/2-propanol elution (pool D)	5

RESULTS

Isolation Procedures. Table I lists the recovery of protein and steps used for purification of angiogenin from 10 L of pooled, serum-free conditioned medium. Biological activity, monitored by using the chick embryo CAM assay, is retained following acidification of the conditioned medium to 5% v/v with acetic acid, while many contaminating proteins, largely of high molecular weight, are removed as microprecipitates after a freeze–thaw cycle (Fett et al., 1985b).

Lyophilized acidified serum-free conditioned medium was dissolved in and dialyzed overnight vs. 100 mM sodium phosphate buffer, pH 6.6, filtered, and applied to a CM-cellulose column (Fett et al., 1985a). A typical experiment

[2] J. L. Bethune, unpublished experiments.

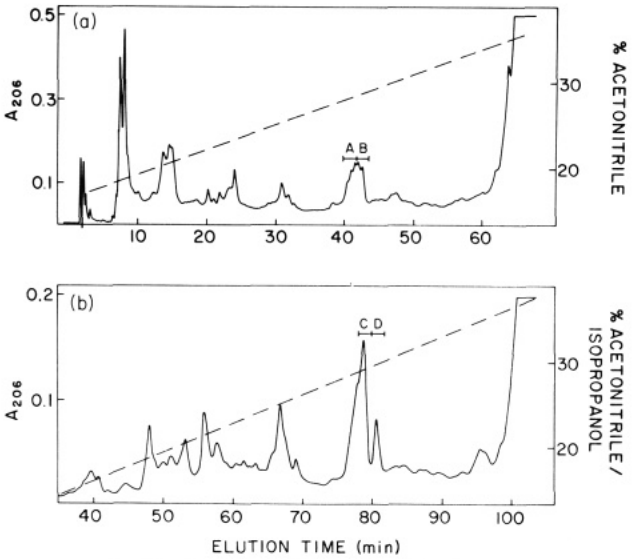

FIGURE 1: Purification of angiogenic activity from CM 2 by reversed-phase HPLC. The HPLC conditions employed are described under Experimental Procedures. (a) Chromatography of 9 mg of CM 2 using acetonitrile (---) as the elution buffer (solvent B) over 80 min. (b) Chromatography of 6.5 mg of CM 2 using acetonitrile/2-propanol (---) as the elution buffer (solvent C) over 120 min. Pools A–D containing angiogenic activity were characterized as described in the text.

(Table I) employed 6.3 mg of starting protein, from which 3.2 mg of CM 1 (unbound) and 2.3 mg of CM 2 (bound and eluted with 1 M NaCl) were obtained. While the activity of CM 2 varies in different preparations, positive results at a significance level of <1% are routinely found at a dose of 20 μg per egg and, with some preparations, at a dose of 10 μg per egg. The CAM activity of the CM 1 fraction, on the other hand, is much lower, i.e., requiring minimally 100 μg per egg to achieve even a 5% significance level.

Purification of angiogenin from the CM 2 material was achieved by HPLC employing a Synchropak RP-P column. One protocol utilized acetonitrile/TFA as the elution buffer. When 9 mg of CM 2 (pooled from several CM-cellulose fractionations) was applied in 0.1% TFA and eluted with a linear gradient of increasing acetonitrile concentration (solvent B) over 80 min, angiogenic activity was found reproducibly only in a single peak (Figure 1a) eluting over a range of 27–30% acetonitrile and containing 85.5 μg of protein. SDS–PAGE of each half of this peak (pools A and B) revealed that HPLC under these conditions did not resolve single components (Figure 2).

When a gradient of 2-propanol/acetonitrile (solvent C) was used as the elution buffer over 120 min, the elution time for angiogenic activity was extended to about 80 min after sample injection (~30% total organic concentration). In a typical experiment, 6.5 mg of CM 2 was applied to the Synchropak RP-P column, followed by linear gradient elution with solvent C. Angiogenic CAM activity (vide infra) was found in pools C and D which eluted at total organic concentrations of 29% and 30%, respectively (Figure 1b). SDS–PAGE demonstrated that pool D (13.5 μg of protein) comprises a single species with an apparent molecular weight of 14000 (Figure 2). This species is designated as tumor-derived angiogenin. Pool C (21.8 μg of protein) contained a major protein component of M_r ~16000 and lesser amounts of the M_r 14000 species (Figure 2). The yield of angiogenin in pool D was ~0.5 μg/L of conditioned medium (Table I).

Biological Characterization. Analogue dose–response analyses were performed on the two active fractions by using

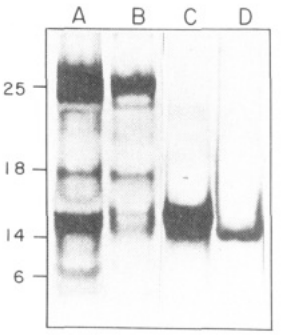

FIGURE 2: SDS–PAGE of pools A (3.5 μg), B (1.6 μg), C (1.3 μg), and D (0.8 μg) from HPLC separations of CM 2 (Figure 1). Conditions were as described under Experimental Procedures. Positions of molecular weight markers (Bethesda Research Laboratories, low molecular weight standards) are at the left (×10^{-3}).

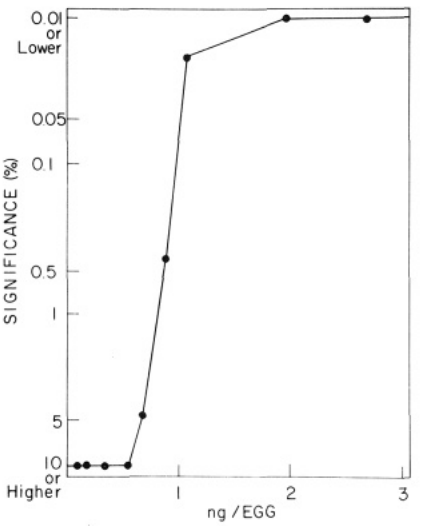

FIGURE 3: Analogue dose–response curve of angiogenin. The control level for the frequency of positives was set at the 0.01% level; i.e., the probability was 0.0925 to allow interpretation of significance levels of the order of 0.01%.

the chick embryo CAM assay. Angiogenin (pool D) was reproducibly active, $0.01\% \leq P \leq 5\%$, at levels ranging from 290 to 0.5 ng per egg (i.e., from 20 pmol to 35 fmol per egg), the major change in the response occurring below 1.4 ng per egg (Figure 3).

Pool C was also active but less so than pool D. Thus, pool C reached a significance level of ≤5% only above 40 ng per egg. These data suggest that the angiogenic activity in pool C is due to the M_r 14000 species detected in this region (Figure 2).

Angiogenin also induces the growth of new blood vessels when implanted in a pocket in the rabbit cornea (Figure 4). Considerable outgrowth of new vessels from the limbus toward and into the area of sample implantation can be seen. No vessel growth was observed in control experiments employing equivalent amounts of lysozyme (data not shown). Positive angiogenic responses are observed reproducibly in the rabbit cornea at a level of ~50 ng (3.5 pmol).

CAM angiogenic activity at a significance level of <0.2% was eluted from gel slices from the region of the electrophoretogram corresponding to M_r 14000 after 15% SDS–PAGE of an angiogenin/lysozyme mixture. No activity (significance level >50%) was eluted from slices from an adjacent control lane containing lysozyme alone.

Chemical Characterization of Angiogenin. The molecular weight of angiogenin was estimated by both SDS–PAGE (Figure 2) and gel filtration HPLC in the presence of guan-

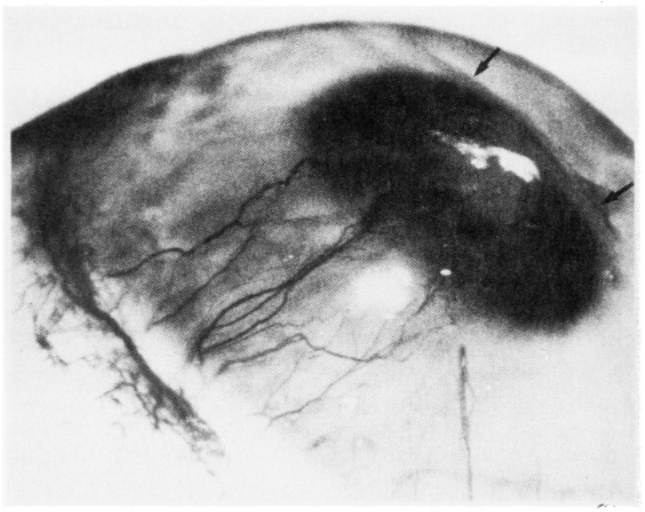

FIGURE 4: Activity of angiogenin in the rabbit corneal assay. 500 ng was implanted as described under Experimental Procedures, and the photograph shown was taken 15 days later. The pocket extends from the corneal vertex (arrows) to 2–3 mm from the limbus (at left). The light areas are caused by photographic reflection.

Table II: Amino Acid Composition of Angiogenin

amino acid	residues/mol ± SD[a]	amino acid	residues/mol ± SD[a]
Cys	6.08 ± 0.64[b]	Met	0.97 ± 0.31
Asp	15.96 ± 1.02	Ile	6.31 ± 1.27
Thr	7.35 ± 0.32	Leu	6.10 ± 0.33
Ser	9.80 ± 0.77	Tyr	3.99 ± 0.43
Glu	11.47 ± 0.43	Phe	4.90 ± 0.39
Pro	7.93 ± 2.38	His	5.72 ± 0.36
Gly	10.41 ± 0.86	Lys	7.49 ± 0.40
Ala	6.05 ± 0.40	Arg	13.00 ± 0.54
Val	4.86 ± 0.58	Trp	1[c]

[a] Calculated for 20-h hydrolysates on 13 different preparations. [b] Determined as *S*-(sulfopropyl)cysteine and cysteic acid. [c] Estimated from the UV absorption spectrum (data not shown).

idine hydrochloride. Both yielded a molecular weight of ~14000. Amino acid analysis gave a value of M_r ~14400 (Table II).

Six cycles of Edman degradation performed on either native or performic acid oxidized protein did not reveal a free end group. Only a single carboxy-terminal amino acid, proline, is found after hydrazinolysis. Furthermore, no lower molecular weight subunits are found when angiogenin is examined by SDS–PAGE in the presence of 2-mercaptoethanol. Taken together, these data indicate that angiogenin is a single-chain polypeptide.

Analytical IEF and cation-exchange HPLC demonstrate that angiogenin is extremely basic. It migrates to the front during IEF, indicating an isoelectric point greater than 9.5. Additionally, angiogenin elutes after lysozyme (p*I* 10.5) in cation-exchange HPLC. This behavior is consistent with its binding to CM-cellulose under the conditions employed and suggests that along with its high content of basic amino acids (Table II) many of the side-chain carboxyl groups are amidated.

Discussion

The angiogenic molecule whose isolation is described here has been designated angiogenin. It is the first pure angiogenic molecule derived from a human tumor to be identified, purified to homogeneity, and characterized. Its isolation and chemical/biological characterization required the solution of fundamental problems inherent in such research, as detailed elsewhere (Fett et al., 1985b; Vallee et al., 1985). In particular, these were the definition of conditions for cell growth in the absence of exogenous proteins and isolation in pure form of extremely small amounts of protein from the resultant conditioned media. Our initial attempts to isolate TAFs from human HT-29 cells utilized, as starting material, cells grown in either serum-containing medium or the serum-containing conditioned medium itself. These efforts resulted in the partial purification of a plasma membrane associated, carbohydrate-containing TAF from the cells (Alderman et al., 1985b), as well as several fractions from the conditioned medium (Fett et al., 1985b). Among these was a low molecular weight species not yet identified. Another was localized by open column cation-exchange and gel filtration chromatography to a molecular weight range of 12500–17500. This fraction, however, contained many proteins when examined by SDS–PAGE and sensitive silver staining techniques. Although hundreds of liters per week of serum-containing, tumor-conditioned medium eventually became available through industrial-scale tissue culture, the final yield of partially purified product from such sources was too low to provide sufficient material for final purification and subsequent characterization.

In view of these problems, it was necessary to devise tissue culture and separation techniques to achieve the objectives of high yields of this material and specific purification protocols. Thus, the present system, while based on these preliminary observations, differs fundamentally in approach. It involves a procedure to culture and maintain cells of the HT-29 adenocarcinoma colon line in a serum-free and exogenous protein-free, defined growth medium (Alderman et al., 1985a). When maintained under these conditions, the cells do not proliferate and yet actively secrete angiogenin. Moreover, this protocol eliminates manipulation by extensive subculturing while providing a conditioned medium containing only tumor-derived products. In addition, the last step in the isolation of angiogenin employed HPLC, which during this period of investigation emerged as a powerful means of separating biologically active proteins and peptides, allowing development of gradients optimized for the purification of angiogenin.

The application of statistical techniques allowed quantitation of in vivo assay results. Several approaches which had previously been employed for this purpose were considered. In one, used to test supernatants of cultured central nervous system tumor cells, the number of vessels crossing two concentric circles—one that closely enclosed the site of implantation and one at twice its diameter—were counted, and the differences was analyzed by the Student's *t* test for paired differences (Matsuno, 1981). While this is probably the most rigorous approach to analyzing such data, since each sample serves as its own control, it is extremely time consuming and only allows quantitation of a limited number of such tests. Moreover, if the implant itself causes a localization proliferation of capillaries, through agents other than pure angiogenin, it will be considered as positive. In another, Fisher's exact test for 2 × 2 tables was employed, again for a limited number of samples, analyzing counts of the number of vessel loops growing toward the implant (Glaser et al., 1980; D'Amore et al., 1981). However, definition of the significance of the statistic (Pearson, 1947) and the choice of controls are not immediately evident. In yet other studies, the response was scored, and the scores were subjected to conventional analyses (Tsukamoto & Sugino, 1979; Form & Auerbach, 1983). In this approach, the assignment of scores to the response involved an arbitrary, subjective element the variance of which is difficult to assess. In another case, the sign test was employed,

but details of its use were not delineated (Pliskin et al., 1980).

We have chosen to use a simple positive or negative response as our measure of attribution in a Bernoulli trial. Since we have accumulated 1834 controls, we can calculate with a defined precision the frequency of false positives obtained in the assay (the size of the control groups ranged from 3 to 108 eggs) and can interpret the assay results on the basis of binomial distributions. We have chosen as an upper limit for the probability the mean frequency plus three standard deviations to remove bias resulting from any uncertainty in the control value. While this undoubtedly scores a larger proportion of samples as negative when they are in fact positive, we have accepted this loss of power in the assay test to avoid possible incorrect conclusions into which false positives might lead us. This technique allows rapid, precise quantitation of assay results involving large numbers of samples and defines the in vivo biological activity of angiogenin as occurring down to 35 fmol in the chick embryo CAM assay.

Angiogenin is a single-chain polypeptide of $M_r \sim 14\,400$ as determined by SDS–PAGE, gel filtration HPLC, and amino acid analysis, and by these criteria is quite distinctly different from recently characterized heparin binding growth factors isolated from both normal (Lobb & Fett, 1984) and tumor (Shing et al., 1984) tissue. It contains one tryptophan, one methionine, six cysteines (either as free sulfhydryls or as half-cystines), and a high percentage of basic amino acids which contribute to its very high isoelectric point ($pI > 9.5$). The ultraviolet absorption spectrum is typical of tryptophan and tyrosine residues (data not shown). No unusual amino acids other than the blocked amino terminus have been found so far, and no species characteristic of amino sugars have been observed during amino acid analysis. The carboxyl-terminal residue is proline.

The yield of angiogenin from HT-29 cells is approximately 0.5 μg/L of serum-free conditioned medium harvested. It is exceedingly potent, exhibiting biological activity with as little as 35 fmol in the chick embryo CAM assay and with 3.5 pmol in the rabbit cornea assay. Should angiogenin require a diffusible cofactor, or if the biological activity were to reside in a moiety carried by angiogenin, its binding affinity and specificity would have to be quite extraordinary. To date, we have been unable to detect or recognize the presence of any such essential cofactor in angiogenin preparations.

While angiogenin is the first human tumor derived angiogenic molecule to be purified and characterized, other evidence (vide supra) points to a variety of soluble tumor-derived mediators that might be involved in the induction of angiogenesis. As indicated above, angiogenic activity has been detected both in the CM 1 fraction and also the plasma membrane fraction obtained from HT-29 cell lysates. The relationships, if any, among these and the angiogenin described in the present report must obviously remain conjectural. It is clear that major efforts are still needed to critically examine, confirm, and correlate other entities that might be involved in tumor angiogenesis.

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Amino Acid Sequence of Human Tumor Derived Angiogenin†

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ABSTRACT: The amino acid sequence and disulfide bond pairing of human tumor derived angiogenin, the first tumor angiogenesis factor to be isolated in pure form from human sources, have been determined by conventional sequencing techniques adapted and applied to nanomole and subnanomole levels of material. Angiogenin, obtained from conditioned media of a human colonic adenocarcinoma cell line, is a single-chain protein consisting of 123 amino acids with the following sequence: <Glu^{1}-Asp-Asn-Ser-Arg-Tyr-Thr-His-Phe-Leu-Thr-Gln-His-Tyr-Asp^{15}-Ala-Lys-Pro-Gln-Gly-Arg-Asp-Asp-Arg-Tyr-Cys-Glu-Ser-Ile-Met^{30}-Arg-Arg-Arg-Gly-Leu-Thr-Ser-Pro-Cys-Lys-Asp-Ile-Asn-Thr-Phe^{45}-Ile-His-Gly-Asn-Lys-Arg-Ser-Ile-Lys-Ala-Ile-Cys-Glu-Asn-Lys^{60}-Asn-Gly-Asn-Pro-His-Arg-Glu-Asn-Leu-Arg-Ile-Ser-Lys-Ser-Ser^{75}-Phe-Gln-Val-Thr-Thr-Cys-Lys-Leu-His-Gly-Gly-Ser-Pro-Trp-Pro^{90}-Pro-Cys-Gln-Tyr-Arg-Ala-Thr-Ala-Gly-Phe-Arg-Asn-Val-Val-Val^{105}-Ala-Cys-Glu-Asn-Gly-Leu-Pro-Val-His-Leu-Asp-Gln-Ser-Ile-Phe^{120}-Arg-Arg-Pro^{123}-OH. Three disulfide bonds link the half-cystinyl residues 26–81, 39–92, and 57–107. The sequence is homologous to that of the pancreatic ribonucleases with 35% identity and many of the remaining residues conservatively replaced. Similarities are especially apparent around the major active-site residues His-12, Lys-41, and His-119 of ribonuclease which are conserved as are three of the four disulfide bonds. The complete chemical characterization of a unique human organogenic messenger molecule, i.e., one that can induce organ formation, accomplishes the first major objective of this long-term investigation of organogenesis in general and angiogenesis in particular. The unexpected homology to ribonuclease suggests novel approaches to the investigation of the biological process of angiogenesis.

The preceding paper describes the isolation and purification of an angiogenic protein from human tumor cells (Fett et al., 1985b). We have designated this protein human tumor *angiogenin* to differentiate it from other angiogenic factors encountered in normal or pathological sources. It is capable of inducing blood vessel growth in both the chick embryo chorioallantoic membrane and the rabbit cornea. It is secreted by HT-29 human adenocarcinoma cells into the extracellular medium from which it can be isolated in yields of about 0.5 μg/L. Detailed physical and chemical characterization has been hampered owing to this limited availability. However, recent experimental advances have facilitated both isolation of angiogenin and examination of its structural properties. It is a single polypeptide of M_r 14400 with a blocked amino terminus and a proline carboxyl terminus (Fett et al., 1985b).

The present paper describes the results of amino acid sequence studies carried out by Edman degradation chemistry and other procedures adapted to nanomolar and subnanomolar amounts of material. The sequence of four polypeptide segments accounts for all 123 amino acid residues. The amino- and carboxyl-terminal segments are identified on the basis of the unique nature of their respective terminal residues. The alignment of the remaining two segments to give the overall primary structure is based on a surprising homology to the family of pancreatic ribonucleases, and on the corresponding cDNA sequence determined concurrently.

Angiogenin is the first human tumor derived angiogenic factor to be isolated in pure form and sequenced. Other mediators of blood vessel growth have also been described but are as yet uncharacterized, and hence, their relationship to angiogenin is still unknown. We expect, however, that angiogenin will be a member of what will prove to be a new and important class of proteins that can induce organ formation, i.e., organogenic messenger molecules.

Experimental Procedures

Materials. Angiogenin was isolated from the serum-free conditioned medium of an established human colonic adeno-

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