Isolation of Angiogenin from Normal Human Plasma†

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ABSTRACT: Angiogenin, a potent blood vessel inducing protein, was previously isolated from medium conditioned by a human adenocarcinoma cell line [Fett, J. W., Strydom, D. J., Lobb, R. R., Alderman, E. M., Bethune, J. L., Riordan, J. F., & Vallee, B. L. (1985) *Biochemistry 24*, 5480–5486]. We now report that a protein which is physically and functionally identical with angiogenin is present in normal human plasma and can be purified to homogeneity by CM 52 and Mono S cation-exchange chromatography. The plasma-derived angiogenin exhibits the same angiogenic and ribonucleolytic activities, amino acid composition, molecular weight, immunoreactivity, and chromatographic behavior as the tumor cell derived protein. Peptide mapping and sequencing studies indicate chemical identity of the two proteins. The present yield of angiogenin from either plasma or serum is 60–150 μg/L. These findings demonstrate that angiogenin is not a tumor-specific product and provide further opportunities for the investigation of the role and mechanism of action of angiogenin and its potential diagnostic or prognostic utility.

Angiogenin, a potent blood vessel inducing protein isolated from medium conditioned by the human colon adenocarcinoma cell line HT-29, is a cationic single-chain polypeptide of M_r 14 124 with 35% sequence identity to human pancreatic ribonuclease (RNase)[1] (Fett et al., 1985; Strydom et al., 1985; Kurachi et al., 1985). Angiogenin indeed exhibits ribonucleolytic activity which is, however, distinctly different from that of the pancreatic RNases (Shapiro et al., 1986a). Its gene and cDNA have been isolated from libraries generated from normal fetal liver, and their chemical structures have been determined (Kurachi et al., 1985).

The presence of angiogenin cDNA in such a normal liver library suggested that the protein might also exist in normal tissue and fluids and prompted the present investigation of human plasma as a readily accessible potential source of angiogenin. We here report the isolation of a protein from normal human plasma which is physically and functionally indistinguishable from the angiogenin obtained previously from HT-29 tumor cell conditioned medium. The yield was 60–150 μg/L. It is the first demonstration of the existence of angiogenin in any normal body fluid or tissue.

Experimental Procedures

Materials. Fresh frozen human plasma was purchased from the American Red Cross. Serum samples were obtained from normal volunteers. CM-cellulose (grade CM-52) was a product of Whatman Ltd. and was precycled according to the manufacturer's instructions before use. Sources of other materials are identical with those listed in Shapiro et al. (1986a,b) and Shapiro and Vallee (1987).

Purification of Angiogenin. Angiogenin was isolated by a modification of the procedure previously described for its purification from HT-29 tumor cell conditioned medium (Fett et al., 1985). Plasma (typically 3–10 L) was brought to pH 6.6 with 3 N HCl. After filtering through Whatman 934-AH glass microfiber paper, it was loaded onto a 300-mL filter cake of CM-52 cation-exchange resin in a 9.4-cm diameter sintered glass funnel. The plasma was drawn through the resin under vacuum at a flow rate of 1–2 L/h. The resin was then washed with several volumes of 0.1 M sodium phosphate, pH 6.6 (starting buffer), and transferred into a 2.5-cm diameter column. Additional starting buffer was passed through the resin until the absorbance at 280 nm decreased to <0.05. The column was then eluted with a solution of 1 M NaCl in starting buffer. Salt-eluted material (designated as CM 2) was dialyzed extensively vs. water and lyophilized. All of these steps were performed at 4 °C.

In one experiment, as indicated, an alternative protocol was followed. Plasma was acidified with glacial acetic acid to a final concentration of 5% (v/v), frozen at −20 °C, thawed, and centrifuged at 23000*g* for 20 min. The supernatant was then dialyzed vs. water, lyophilized, and reconstituted in CM-52 starting buffer. After adjustment of the pH to 6.6, it was then filtered and chromatographed on CM-52 resin as described above.

Lyophilized CM 2 was reconstituted in 10 mM Tris, pH 8.0, clarified by centrifugation at 15600*g* for 15 min, and applied to either a Mono S cation-exchange column (50 × 5 mm; Pharmacia) or a Synchropak RP-P C18 HPLC column (250 × 4.1 mm; Synchrom Inc.). The Mono S column was eluted with a 50-min linear gradient from 0.15 to 0.55 M NaCl in 10 mM Tris, pH 8.0, at a flow rate of 0.8 mL/min at ambient temperature. The C18 column was eluted with a 90-min linear gradient from 20% to 40% solvent B, where solvent A is 0.1% TFA in water (v/v) and solvent B is 2-propanol/acetonitrile/water (3:2:2 v/v) with 0.08% TFA. The flow rate was 1 mL/min. Column effluents were monitored at either 214 or 254 nm. One-minute fractions were collected.

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[1] Abbreviations: RNase(s), ribonuclease(s); CM, carboxymethyl; HPLC, high-performance liquid chromatography; C18, octadecylsilane; TFA, trifluoroacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; CAM, chorioallantoic membrane; C>p, cytidine cyclic 2′,3′-phosphate; U>p, uridine cyclic 2′,3′-phosphate; poly(C), poly(cytidylic acid); poly(U), poly(uridylic acid); poly(A), poly(adenylic acid); poly(G), poly(guanylic acid); Tris, tris(hydroxymethyl)aminomethane; PBS, phosphate-buffered saline.

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A Waters Associates liquid chromatography system was employed together with a Hewlett-Packard 3390A integrator.

For experiments in which the ribonucleolytic activity of plasma-derived angiogenin was examined, a particularly rigorous purification method was employed that removes all traces of extraneous RNases which are abundant in plasma (Akagi et al., 1976; Blank & Dekker, 1981; Shapiro et al., 1986b). Column fractions eluting from the Mono S column were diluted with an equal volume of 0.1 M sodium phosphate, pH 7.0, containing 2.64 M ammonium sulfate and applied to a Bio-Gel TSK-phenyl-5-PW column (75 × 7.5 mm; Bio-Rad), which had been equilibrated with 0.1 M sodium phosphate, pH 7.0, containing 1.7 M ammonium sulfate (buffer A). Angiogenin was eluted with a linear 3-h gradient of 0–100% buffer B (0.1 M sodium phosphate, pH 7.0) at a flow rate of 0.8 mL/min at ambient temperature. One-minute fractions were collected. Those containing angiogenin were injected directly onto the C18 column and eluted with a 45-min linear gradient from 30% to 40% solvent B. Angiogenin eluting from the C18 column was then rechromatographed on the same column.

Plasma angiogenin employed in CAM assays was purified from CM 2 by chromatography on (1) a Mono S column alone, (2) both Mono S and C18 columns, or (3) both phenyl and Mono S columns, as indicated. For C18 HPLC, Mono S fractions containing angiogenin were injected onto the column and eluted as described above. For phenyl chromatography, CM 2 was dissolved in 0.1 M sodium phosphate, pH 7.0, containing 1.36 M ammonium sulfate and eluted as detailed above, but using a 2-h gradient. Fractions containing angiogenin were dialyzed vs. water, lyophilized, reconstituted in 10 mM Tris, pH 8.0, and chromatographed on the Mono S column as described. Plasma angiogenin prepared by all three of these methods was dialyzed vs. water prior to assay.

Polyacrylamide Gel Electrophoresis. SDS–PAGE was performed in the absence of reducing agents by using 15% gels as described by Laemmli (1970). Protein bands were visualized by silver staining (Rapid-Ag-stain; ICN Radiochemicals).

Protein Assays. Protein concentrations were determined by amino acid analysis.

Enzyme-Linked Immunosorbent Assay (ELISA). Angiogenin (tumor cell or plasma derived) in 10 mM borate buffer, pH 8.3, was coated onto wells of 96-well ELISA plates (Costar) overnight at 4 °C in dilutions ranging from 15.6 ng/mL to 4 μg/mL. The plates were then blocked overnight with 0.2% nonfat dry milk in 0.1 M phosphate-buffered saline, pH 7.2 (milk/PBS). After addition of a 1:50 dilution of rabbit antiserum in milk/PBS, the plates were incubated overnight at 4 °C. The two rabbit antisera used were prepared by immunization with two synthetic peptides representing amino acids 6–21 and 108–121 of tumor cell derived angiogenin. Details of the immunization procedures and antiserum characteristics will be reported elsewhere. Binding of the rabbit antisera to angiogenin was detected by using alkaline phosphatase conjugated goat anti-rabbit immunoglobulin G (heavy and light chain specific) (Kirkegaard and Perry) diluted in milk/PBS and incubated in the wells for 2 h at room temperature. A substrate solution consisting of *p*-nitrophenyl phosphate (0.5 mg/mL) diluted in 10% diethanolamine/5 mM $MgCl_2$, pH 9.6, was subsequently added to the wells and allowed to incubate for 1 h at room temperature before the reaction was stopped with 3 N NaOH. All experiments were performed in duplicate, and the absorbance was read at 410 nm by using a Dynatech MR 600 microplate reader.

Table I: Recovery of Protein on Purification of Angiogenin from Plasma

purification step	protein (mg)
plasma (6.0 L)	3.54×10^5
CM-cellulose: CM 2 (bound, eluted with 1 M NaCl)	11.8
Mono S HPLC of CM2: angiogenin peak	0.45

Biological Assays. Angiogenesis was assessed by using the chick embryo chorioallantoic membrane (CAM) method of Knighton et al. (1977) as described (Fett et al., 1985). The number of eggs employed in any individual set of assays of a given concentration ranged from 6 to 20. Average probabilities were calculated by converting the cumulative binomial probability found for the number of positive results obtained in a given set of assays to a standard normal Z value, employing formula 26.2.23 of Zelen and Severo (1972). These Z values were then averaged, and the standard error of the mean (SEM) was computed. The average Z value and the Z values corresponding to minus and plus SEM were then converted to probabilities employing formula 26.2.17 of the above reference. A significance level of ≤5% has to be attained for a sample to be considered active.

Enzymatic Assays. Assays for RNA degrading activity were performed as described (Shapiro et al., 1986a). In brief, activity toward homopolyribonucleotides was measured by the production of perchloric acid soluble fragments using a modification of the method of Zimmerman and Sandeen (1965). Incubations were performed for 3 h at 37 °C. Activity toward wheat germ RNA was measured by the precipitation assay of Blank and Dekker (1981), using 4-h incubations at 37 °C. Cleavage of CpG, C>p, and U>p was monitored by HPLC. Degradation of 28S and 18S rRNA was examined by agarose gel electrophoresis under denaturing conditions. RNA was visualized with ethidium bromide.

Amino Acid and Sequence Analysis. Methods for amino acid analysis, tryptic peptide mapping, Edman degradation, and C-terminal amino acid determination were identical with those described previously (Fett et al., 1985; Strydom et al., 1985).

Metal Determinations. Concentrations of zinc, copper, iron, and manganese were measured by electrothermal atomic absorption spectroscopy using a Perkin-Elmer Model 5000 spectrophotometer together with a Model 500 graphite furnace and an AS-15 automatic sampler.

Results

Isolation Procedures. Tumor-derived angiogenin binds to CM-52 cation-exchange resin and is eluted with 1 M NaCl in the "CM 2" fraction (Fett et al., 1985). It is then purified to homogeneity by C18 HPLC (Fett et al., 1985). When normal human plasma is chromatographed on CM-52, 0.003% of the total protein elutes in the CM 2 fraction (Table I). C18 HPLC of this material reveals a peak with the retention time of angiogenin. SDS–PAGE results in a single major band (>95% of the total) with the mobility of angiogenin. The amino acid composition of this protein (Table II) is indistinguishable from that of angiogenin.

Mono S cation-exchange chromatography of plasma CM 2 also yields a peak eluting at the same NaCl concentration as angiogenin (Shapiro et al., 1986a) (Figure 1A). SDS–PAGE again reveals a band with the same apparent molecular weight as angiogenin, generally comprising at least 98% of the total protein (Figure 1B). The amino acid composition of this material (Table II) is also virtually identical with that of angiogenin. In some preparations, a protein of $M_r \sim 11\,000$ was found as a 5–10% contaminant. This protein was com-

Table II: Amino Acid Composition[a] of Plasma Angiogenin Obtained by C18 and Cation-Exchange HPLC

amino acid	plasma-derived angiogenin[b] C18	plasma-derived angiogenin[b] Mono S	tumor-derived angiogenin[c]
Asx	14.5	15.0	15
Glx	9.2	10.1	10
Ser	8.2	8.4	9
Gly	8.0	8.0	8
His	5.6	5.3	6
Arg	13.9	12.6	13
Thr	6.7	6.8	7
Ala	5.0	5.2	5
Pro	10.3	8.3	8
Tyr	4.0	3.9	4
Val	4.0	4.4	5
Met	0.8	1.0	1
Ile	6.3	7.1	7
Leu	6.6	6.4	6
Phe	5.2	5.2	5
Lys	6.8	7.3	7
Cys	nd[d]	5.4	6

[a] Determined by the Picotag method (Waters Associates; Bidlingmeyer et al., 1984). Compositions are presented as residues per mole. [b] Plasma angiogenin analyzed was obtained by chromatographing CM2 material on a C18 or Mono S HPLC column. In each case, the elution time is indistinguishable from that of tumor angiogenin. Mono S purified protein was dialyzed vs. H_2O prior to analysis. [c] From sequence (Strydom et al., 1985). [d] Not determined.

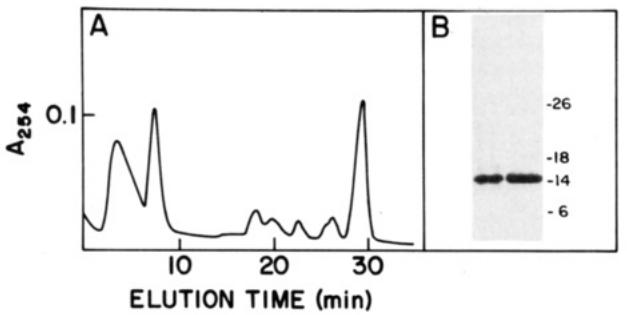

FIGURE 1: (A) Chromatography of CM 2 fraction from normal human plasma on a Mono S cation-exchange column. A lyophilized sample was dissolved in 10 mM Tris, pH 8.0, and eluted with a 50-min linear gradient from 0.15 to 0.55 M NaCl in the same buffer at a flow rate of 0.8 mL/min. (B) SDS–PAGE of tumor cell derived angiogenin (left lane) and of plasma material eluting from the Mono S column at 28 min (right lane). Positions of molecular weight markers (Bethesda Research Laboratories) are at the right ($\times 10^{-3}$).

pletely removed by C18 HPLC.

Although both the C18 and Mono S HPLC procedures yield the same pure angiogenin-like protein from plasma CM 2, the latter method is generally preferable since much greater quantities of CM 2 can be chromatographed at one time without causing overloading or peak broadening. Moreover, repeated use of the C18 column for this purpose is potentially problematic owing to irreversible binding of high molecular weight proteins present in the CM 2 fraction.

The procedure typically yields 60–150 μg/L normal plasma (e.g., see Table I) or serum. Inclusion of an acidification/freeze–thaw step (see Experimental Procedures) used previously in isolating angiogenin from tumor cell conditioned medium (Fett et al., 1985) does not increase the yield from plasma appreciably.

Cochromatography. Equal amounts of the plasma-derived and tumor cell derived proteins were mixed and chromatographed on Mono S and C18 columns. The mixture elutes from the Mono S column as a single sharp symmetrical peak (Figure 2A). When chromatographed on the C18 column individually, both tumor (Strydom et al., 1985) and plasma

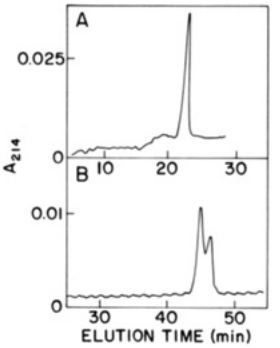

FIGURE 2: Chromatography of a mixture containing 0.6 μg each of tumor cell derived and plasma-derived angiogenin on Mono S (panel A) and C18 HPLC (panel B) columns. Chromatographies were performed as described under Experimental Procedures.

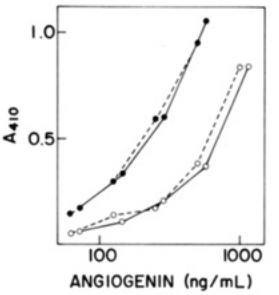

FIGURE 3: Binding of tumor cell derived (—) and plasma-derived (---) angiogenin to rabbit antiserum against synthetic peptides 6–21 (●) and 108–121 (○) of tumor cell derived angiogenin. ELISAs were performed as described under Experimental Procedures. Each point is the average of duplicate determinations.

Table III: Angiogenic Activity of Plasma Angiogenin in CAM Assay[a]

ng	sets of assays	average significance (%)	−SEM	+SEM
100	1	<0.1	<0.1	≪0.1
50	7	≪0.1	<0.1	≪0.1
35	2	<0.1	0.7	0.1
25	9	0.2	0.5	0.1
18	6	<0.1	<0.1	<0.1
10	6	0.8	2.1	0.3
7	3	0.4	3.1	<0.1
5	5	3.0	4.9	1.8
2	3	2.9	4.8	1.7
1	4	15.7	30.5	6.6
0.5	6	6.6	13.3	2.9
0.1	4	7.3	29.0	0.9

[a] Average percent significance and SEM values were calculated as described under Experimental Procedures using 0.0925 as the probability of obtaining positive results with a control group of eggs (Fett et al., 1985). Angiogenesis was assessed 68 ± 2 h after sample implantation.

proteins elute as a double peak,[2] and the relative sizes of the two peak components are the same for both. Their mixture elutes as a double peak of identical shape (Figure 2B). These results conclusively demonstrate that the chromatographic behavior of the two proteins is identical in these systems.

Immunoreactivity. The Mono S purified plasma protein was tested by ELISA for reactivity with two antisera raised against peptides from the N- and C-terminal regions of angiogenin (see Experimental Procedures). With both antisera, their reactivity with the plasma protein was closely similar to

[2] This double peak appears to reflect a reversible conformational change in the protein (D. J. Strydom, unpublished results).

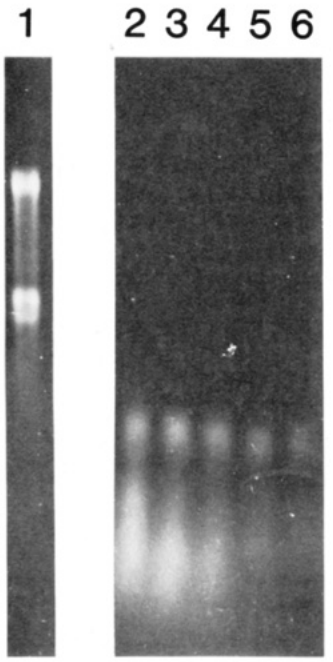

FIGURE 4: Effect of plasma angiogenin on 28S and 18S rRNA. RNA (12 μg) was incubated with or without protein at 37 °C for 30 min. Samples were run on a 1.1% agarose gel under denaturing conditions. The gel was stained with ethidium bromide and photographed under ultraviolet illumination. Lane 1, control RNA sample; lanes 2–6, samples containing 0.8, 1.6, 3.2, 6.4, and 13 μM angiogenin, respectively.

that measured with angiogenin (Figure 3).

Biological Characterization. Statistical analysis of the CAM assay data for the Mono S purified plasma protein (Table III) shows that its activity is comparable to that of angiogenin isolated from the HT-29 adenocarcinoma cell line (Fett et al., 1985). Further chromatography of this material on phenyl or C18 HPLC columns has no detectable effect on activity.

Ribonucleolytic Activity. Angiogenin catalyzes the limited cleavage of 28S and 18S rRNA (Shapiro et al., 1986a). It is apparently devoid of pancreatic RNase-like activity: e.g., it does not produce detectable quantities of acid-soluble fragments from poly(C), poly(U), or high molecular weight wheat germ RNA. The plasma-derived protein displays the same characteristic activity. Thus, it cleaves 28S and 18S rRNA, yielding products 100–500 nucleotides in length which are relatively resistant to further degradation; the apparent stability of the ~500-nucleotide band is particularly striking (Figure 4). The specific activities of the tumor cell derived and plasma-derived proteins are indistinguishable in this assay system. The plasma protein is inactive toward wheat germ RNA, poly(C), poly(U), poly(A), poly(G), C>p, and U>p when assayed at concentrations comparable to those used previously for HT-29 cell angiogenin.[3]

Metal Content. Atomic absorption spectrometry performed on the Mono S purified protein (70 μM) shows that less than 0.01 mol/mol of copper, iron, manganese, or zinc is present.

Peptide Mapping and Sequencing. Automated Edman degradation of 200 pmol of the plasma protein revealed no end group for 4 cycles, indicating a blocked amino terminus. Proline was detected by hydrazinolysis as the carboxyl-terminal amino acid, together with small amounts of serine and glycine.

[3] When much higher concentrations of plasma angiogenin are employed ($\geq$1 μM), an extremely small amount of activity toward wheat germ RNA, poly(U), poly(C), and CpG is observed.

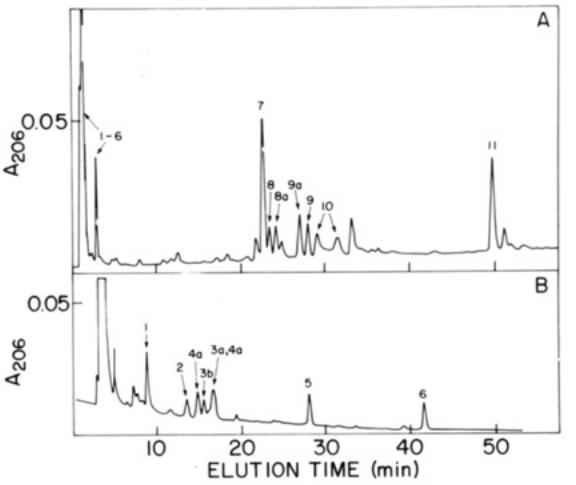

FIGURE 5: (A) Fractionation of a tryptic digest of plasma angiogenin by HPLC on a Beckman Ultrapore (C3) column as described previously (Strydom et al., 1985). (B) Fractionation of breakthrough fractions 1–6 from the C3 column on a Beckman Ultrasphere (C18) column. Elution was achieved with a 2-h linear gradient from 3% to 50% solvent B, where solvent A was 0.1 M sodium perchlorate and 0.1% orthophosphoric acid, pH 2.5, and solvent B was 75% acetonitrile in 25% solvent A. The flow rate was 1 mL/min.

These results are identical with those obtained from tumor cell derived angiogenin (Fett et al., 1985; Strydom et al., 1985).

A tryptic digest of 2.5 nmol of the plasma protein was fractionated by HPLC on a C3 column (Figure 5A), and the breakthrough fractions were rechromatographed on a C18 column (Figure 5B) as described for HT-29 cell-derived angiogenin (Strydom et al., 1985). The resultant chromatographic patterns are essentially identical with those reported in that study, except for the presence of the additional peptides T8a and T9a, and the absence of peptide T12. The amino acid compositions (Table IV) of T8a and T9a are consistent with those expected for amino acids 41–50 and for the disulfide-linked 25–31 plus 74–82 of angiogenin, respectively. These peptides are apparently formed by tryptic cleavages not observed in the earlier work, i.e., peptide T8a from a cleavage between the penultimate lysine and C-terminal arginine of T8 and peptide T9a by removal of the Asp-Asp-Arg tripeptide from T9. The sequence of peptide T9 is, however, identical with that of the tumor cell derived product (Table V).

Amino acids 102–121 of tumor cell derived angiogenin were found previously in two different tryptic peptides, T11 and T12. In the former, this sequence is disulfide linked to peptide T4b, while in the latter it is the free peptide (Strydom et al., 1985). Thus, the absence of a peptide T12 probably indicates that the disulfide bridge joining this peptide to T4b has remained intact during the isolation and digestion procedures.[4] On the basis of its amino acid composition and sequence (Tables IV and V), peptide T11 of the plasma-derived protein indeed appears to contain both the T4b and the T12 portions of the sequence. The amino acid compositions of all remaining peptides (Table IV) are indistinguishable from those found for tumor cell derived angiogenin. Edman degradations performed on two additional large peptides, T8 and T10 (Table V), reveal sequences identical with those of angiogenin.

DISCUSSION

Angiogenesis, the formation of new blood vessels, is critical to a variety of normal and pathological processes, including

[4] The extent to which peptide T12 is present as a separate entity in tryptic digests of tumor cell derived angiogenin is variable.

Table IV: Amino Acid Compositions of Tryptic Peptides[a]

amino acid	peptide 1	2	4a	3a, 4a[b]	3b	5	6	7	8a	9a	9	10[c]	11
Asx	1.88 (2)		1.67 (2)	1.86		0.98 (1)		1.03 (1)	2.90 (3)	0.18	2.04 (2)	0.25	3.80 (4)
Glx	1.01 (1)					1.00 (1)		1.84 (2)	0.36	1.88 (2)	2.31 (2)	1.00 (1)	2.70 (3)
Ser	1.02 (1)		0.28	2.02	0.88 (1)	0.10		0.54	0.86	3.00 (3)	3.64 (3)	1.93 (2)	1.68 (1)
Gly	0.15		1.21 (1)	1.30		0.13	1.12 (1)	1.17 (1)	1.86 (1)	0.83	1.71	2.81 (3)	1.83 (1)
His			1.20 (1)	1.20				1.69 (2)	0.94 (1)	0.50	0.21	0.80 (1)	0.92 (1)
Arg	1.09 (1)	0.94 (1)	0.95 (1)	1.01		1.00 (1)	1.02 (1)	0.93 (1)	0.25	0.98 (1)	2.05 (2)	1.07 (1)	1.03 (1)
Thr							0.96 (1)	2.02 (2)	1.20 (1)	2.25 (2)	1.94 (2)	1.05 (1)	0.31
Ala							2.00 (2)	1.16 (1)	0.47	0.50	0.81	0.21	2.02 (2)
Pro		1.06 (1)	1.18 (1)	1.28				1.01 (1)	0.54	0.39	0.48	3.58 (4)	1.50 (1)
Tyr								1.88 (2)	0.28	1.38 (1)	1.05 (1)	1.27 (1)	
Val										0.90 (1)	1.29 (1)	0.16	3.03 (4)
Met								0.21	0.22	0.91 (1)	0.99 (1)		
Ile					1.06 (1)				2.03 (2)	0.78 (1)	0.98 (1)	0.21	2.00 (2)
Leu				2.05		1.23 (1)		1.03 (1)	0.25	0.54	0.43	1.88 (2)	2.37 (2)
Phe							1.04 (1)	1.00 (1)	1.10 (1)	1.17 (1)	0.99 (1)	0.16	1.01 (1)
Trp												(+)[d] (1)	
Lys				1.97	1.00 (1)			0.92 (1)	0.72 (1)	1.04 (1)	0.88 (1)	1.00 (1)	1.08 (1)
sequence position[e]	1–5	122–123	61–66	52–54; 61–66	71–73	67–70	96–101	6–21	41–50	(25–31) + (74–82)[f]	(22–31) + (74–82)[f]	(34–40) + (83–95)[f]	(55–60) + (102–121)[f]

[a] Relative molar amounts of amino acids are given, based on the amino acids present in the corresponding peptides obtained for tumor cell derived angiogenin (in parentheses). Between 16 and 170 pmol of peptide was analyzed. [b] Amino acid composition for this peak is consistent with a 2:1 mixture of peptides T3a and T4a of angiogenin. The appearance of peptide T4a at two positions may reflect deamidation of Asn-61. [c] The composition shown is for the second T10 peak. [d] From the absorbance ratio for this peptide in the peptide maps (254 vs. 206 nm). [e] Based on the sequence of tumor cell derived angiogenin (Strydom et al., 1985). [f] Peptides linked by disulfide bonds.

Table V: Edman Degradation of Plasma Angiogenin Peptides

peptide	sequencer results[a]
T8	Asp-Ile-Asn-Thr-Phe-Ile-His-Gly-Asn-Lys 827 817 226 (+) 235 222 92 108 104 14
T9	(Asp+Ser)-(Asp+Ser)-(Arg+Phe)-(Tyr+Gln)-(Cys+Val)- 160 76 101 34 56 91 92 62 19 113 (Glu+Thr)-(Ser+Thr)-(Ile+Cys)-(Met+Lys) 72 21 32 5 58 15 29 23
T10[b]	(Gly+Leu)-(Leu+His)-(Thr+Gly)-(Ser+Gly)-(Pro+Ser)- 294 450 408 200 166 294 139 269 118 152 (Cys-Pro)-(Lys+Trp)-Pro-Pro-Cys-Gln-Tyr-Arg Trace 115 91 175 134 162 45 111 193 10
T11	(Asn+Ala)-(Val+Ile)-Val-(Glu+Val)-(Ala+Asn)-Lys- 627 780 593 520 614 423 444 514 308 73 Glu-Asn-Gly-Leu-Pro 190 143 49 107 Trace

[a] The established sequence is given, with the yield of amino acid residues at each cycle given below the residue, in picomoles. The parentheses indicate that two residues were found after a given degradation cycle, presumably reflecting the linkage of two peptides by a disulfide bridge. Thr and Ser were identified as PTH-Thr or -Ser and their characteristic degradation products. Cys was presumptively identified as a characteristic derivative of cysteine and its degradation products (Harper et al., 1986). [b] This peptide eluted as a doublet (see Figure 5A) just as peptide T10 of tumor-derived angiogenin (Strydom et al., 1985). The material sequenced represents pooled fractions from both peaks. It also contains a contaminating sequence Ala (72 pmol)-Lys (72)-Pro (39)-Gln (43) apparently starting at Ala-16.

embryonic growth, wound healing, diabetic retinopathy, and tumor development (Folkman & Cotran, 1976; Vallee et al., 1985). A protein capable of inducing new blood vessel formation—designated "angiogenin"—was isolated from medium conditioned by the human colon adenocarcinoma cell line HT-29, and its chemical and gene structures were determined (Fett et al., 1985; Strydom et al., 1985; Kurachi et al., 1985).

These findings raised the question whether angiogenin is tumor specific or, as seemed more likely, is present in normal tissue and fluids where it would participate in physiologic homeostasis. The presence of cDNAs for angiogenin in a library prepared from normal human liver mRNA (Kurachi et al., 1985) already suggested that the protein is expressed in this and possibly other normal tissues. The relative abundance of this cDNA, however, appeared to be low since only 7 out of the 350 000 clones screened were positive.

Normal human plasma was examined for its content of angiogenin owing to its ready availability and the obvious diagnostic or prognostic relevance of any positive finding. Additionally, it seemed of particular importance to examine plasma since it could serve as a convenient alternative source of the protein if suitable concentrations were detected. The amounts of angiogenin secreted by HT-29 cells—the original source—are extremely small, necessitating large-scale growth and maintenance of these cells (Alderman et al., 1985) to obtain even minimal amounts of protein.

The present results demonstrate that normal plasma contains a protein that exhibits the same biological and enzymatic activities, amino acid composition, molecular weight, chromatographic behavior, and immunoreactivity as does angiogenin. These characteristics clearly identify the plasma protein as angiogenin. Nevertheless, more detailed structural studies were performed in order to rule out the possibility that it might differ from the tumor cell derived molecule in some respect. The N-terminus is blocked and the C-terminus is proline, as for HT-29 cell angiogenin. The tryptic peptide map obtained is closely similar to that reported for the tumor protein, and amino acid compositions and sequences of the peptides generated are in complete agreement with those ex-

pected. These studies encompass almost the entire protein; only residues 32, 33, and 51 are not found in any tryptic peptide, and all three could have been released as single amino acids during proteolytic digestion. Jointly, these data strongly indicate that the plasma protein is chemically identical with the angiogenin isolated previously from tumor cell conditioned medium.

The method described for purification of angiogenin from plasma and serum is simple and rapid. It allows the routine isolation of 0.4–1.0 mg of protein from 5–10 L in 3 days. Preliminary measurements employing an angiogenin radioimmunoassay (K. A. Olson, unpublished results) suggest that the actual content of this protein in plasma is higher than the amount isolated. It is therefore likely that the purification procedure presented can be optimized further to give higher yields. The observed yield could also reflect modification of some of the plasma angiogenin in such a manner as to alter its chromatographic behavior, e.g., by glycosylation or complex formation.

The discovery of angiogenin in normal human plasma raises a number of important questions. What biological role, if any, does this protein play in plasma? Is its activity in blood subject to regulation, and, if so, how is this accomplished? In what manner does its concentration in plasma relate to that which is effective physiologically? Where is plasma angiogenin synthesized? Angiogenin might be a functionally important component of blood, e.g., aiding in normal wound healing. Alternatively, the presence of this protein in plasma could reflect clearance of material released during cell turnover. Any biological and/or enzymatic activities of angiogenin in plasma could be modulated either by activators or by inhibitors.

In this regard, we have recently reported that human placental RNase inhibitor is a potent antagonist of angiogenin (Shapiro & Vallee, 1987). The blood content and state of this and related inhibitors are not yet known nor has it been determined whether angiogenin–inhibitor complexes are present. Even if angiogenin circulates in an active form, its activity might not be expressed unless it were to contact critical substrate(s) or target cells.

At the present time, little can be said about the origin of plasma angiogenin. It does not appear to derive primarily from platelets, since serum and plasma contain similar concentrations. However, substances that induce angiogenesis are reported to be secreted by lymphocytes and macrophages that have been activated by a variety of stimuli (Martin et al., 1981; Knighton et al., 1983). These cell types and others are being examined for their capacity to synthesize this protein. Whether angiogenin is produced by other organs, blood cells, or vascular endothelial cells remains to be determined.

The presence of significant amounts of angiogenin in plasma provides further opportunities for examination of the biological role and mechanism of action of this protein, as well as its potential as a diagnostic index. Thus, the variation of plasma or serum angiogenin levels with various normal and pathological states, particularly those involving blood vessel proliferation, can now be studied. In addition, the present findings suggest that angiogenins may be present in the plasma of other species, thus making them available for investigation. Knowledge of the primary sequences of these proteins should shed light on the evolution of angiogenin and help elucidate those regions and specific amino acid residues that are critical for its activity.

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