A Monoclonal Antibody to Human Angiogenin. Inhibition of Ribonucleolytic and Angiogenic Activities and Localization of the Antigenic Epitope†

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ABSTRACT: A monoclonal antibody (mAb) to human angiogenin, a protein that induces formation of new blood vessels, was produced by somatic cell fusion techniques and designated as 26-2F. It is an IgG1κ whose binding affinity, expressed as an IC_{50}, is $(1.6 \pm 0.1) \times 10^{-9}$ M as determined by a competition radioimmunoassay. mAb 26-2F neutralizes the ribonucleolytic activity of angiogenin as assessed by *in vitro* protein synthesis and tRNA degradation assays. It also effectively inhibits neovascularization induced by angiogenin on the chick chorioallantoic membrane. Epitope mapping indicates that the binding region of angiogenin recognized by mAb 26-2F is discontinuous and involves both Trp-89 and residues in the segment 38–41. This epitope is formed by two surface loops which are juxtaposed in the three-dimensional structure of human angiogenin recently determined by X-ray crystallography. Thus mAb 26-2F, along with similar antibodies under investigation, will facilitate structure/function studies of angiogenin, help define its physiological role, and lead to an understanding of the consequences of its inhibition in pathological situations in which angiogenin may be involved.

Angiogenin is a 14.1-kDa basic protein that elicits neovascularization in the chick chorioallantoic membrane and rabbit cornea and meniscus (Fett et al., 1985; Denèfle et al., 1987; King & Vallee, 1991). It was purified originally from medium conditioned by human colon adenocarcinoma cells, but subsequently has been isolated from normal human serum and bovine milk (Shapiro et al., 1987a; Bond & Vallee, 1988; Maes et al., 1988; Bond et al., 1993). Sequence analysis revealed a 35% identity with pancreatic ribonuclease (RNase)[1] including the principal active-site residues (Strydom et al., 1985; Kurachi et al., 1985), although its enzymatic activity toward various RNA substrates differs substantially (Shapiro et al., 1986a; St. Clair et al., 1987; Rybak & Vallee, 1988). Angiogenin also abolishes cell-free protein synthesis by ribonucleolytic inactivation of RNA (St. Clair et al., 1987, 1988; Saxena et al., 1992). Unlike RNase, which is not angiogenic, angiogenin activates second messenger pathways in vascular endothelial and smooth muscle cells *in vitro* (Bicknell & Vallee, 1988, 1989; Moore & Riordan, 1990), supports endothelial cell adhesion (Soncin, 1992), and undergoes nuclear translocation (Moroianu & Riordan, 1994). These biological activities of angiogenin are presumably induced via interaction with specific cell surface receptors (Badet al., 1989). Indeed, a 42-kDa angiogenin binding protein, identified as a type of muscle actin, has been isolated from surface membranes of cultured endothelial cells and may represent a component of a functional angiogenin receptor (Hu et al., 1991, 1993). A putative receptor binding site encompassing residues 60–68 and Asn-109, which is distinct from the ribonucleolytic active site (Shapiro & Vallee, 1989; Shapiro et al., 1989; Hallahan et al., 1991, 1992) has been identified and shown to be essential for angiogenic activity.

Although extensive chemical and biological characterizations of angiogenin have been reported (see Strydom et al., 1989; Fox and Riordan, 1990, for reviews) and are the subjects of continuing investigations, questions remain regarding the physiological role for this protein, its site(s) of synthesis, and the relationship between ribonucleolytic and biological activity, as well as its possible contribution to pathological conditions characterized by abnormal blood vessel proliferation. In order to provide tools toward the eventual elucidation of answers to the types of question posed above, we have generated mAbs to angiogenin. The present report describes the production and characterization of a mAb that neutralizes both the ribonucleolytic and angiogenic activities of angiogenin and thus provides a useful probe for studies of structure/function and physiological/clinical relevance of this mediator of angiogenesis.

MATERIALS AND METHODS

Materials. Human angiogenin was purified from media conditioned by genetically altered baby hamster kidney cells or from an *Escherichia coli* recombinant system as described previously (Kurachi et al., 1988; Shapiro et al., 1988). Angiogenins from the two sources differ only with respect to their N-terminal sequences: Met-Gln- (kidney cells) vs <Glu- (*E. coli*). This difference does not affect enzymatic or angiogenic activities (Shapiro et al., 1988) or binding to mAb 26-2F. Bovine angiogenin was purified from milk as described (Bond & Vallee, 1988). Porcine and rabbit serum angiogenin (Bond et al., 1993); human RNase IV[2] (Shapiro et al., 1986b;

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[1] Abbreviations: RNase, ribonuclease; BMV, Brome Mosaic Virus; CAM, chorioallantoic membrane; ELISA, enzyme-linked immunosorbant assay; FBS, fetal bovine serum; HAT, hypoxanthine–aminopterin–thymidine; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PRI, placental ribonuclease inhibitor; RIA, radioimmunoassay; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

[2] A RNase derived from HT-29 adenocarcinoma cell conditioned medium and normal human plasma.

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Zhou & Strydom, 1993); proteolytic products angiogenin E (Hallahan et al., 1991) and angiogenin K (Harper & Vallee, 1988); and the human angiogenin mutants ARH I (Harper & Vallee, 1989); ARH II (Harper et al., 1990); R5A,[3] R31A, R32A, R33A, R66A, and R70A (Shapiro & Vallee, 1992); H13A and H114A (Shapiro & Vallee, 1989); K40Q (Shapiro et al., 1989); T44A, E108Q, and S118R (Curran et al., 1993); and N109D (Hallahan et al., 1992) were from earlier studies. Mouse angiogenin (Bond & Vallee, 1990) and the human angiogenin mutants S37R, K73A, and W89M were provided by Dr. R. Shapiro. All four were obtained from a recombinant system in *E. coli* by using the expression plasmid pAng3 (Shapiro & Vallee, 1992); primary structures were confirmed by DNA sequencing and amino acid analysis. Protein A-Sepharose was obtained from Pharmacia-LKB, Piscataway, NJ. All tissue culture components, including FBS, were obtained from BioWhittaker, Walkersville, MD, and all plastics and culture vessels were supplied by Costar, Cambridge, MA. Hypoxanthine, thymidine, aminopterin, bovine RNase A, tRNA (Type X from yeast), human serum albumin, ovalbumin, protamine sulfate, *p*-nitrophenyl phosphate, and PMSF were purchased from Sigma, St. Louis, MO, while poly(ethylene glycol) 3350 was from J. T. Baker, Phillipsburg, NJ. RNase-free bovine serum albumin was obtained from Worthington Biochemicals, Freehold, NJ. The nuclease-treated rabbit reticulocyte lysate translation system and PRI were purchased from Promega, Madison, WI. The 5′ DNA terminus labeling system was obtained from Bethesda Research Laboratories, Bethesda, MD. [^{35}S]Methionine (1200 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from DuPont, Boston, MA. The nonsecreting mouse myeloma P3X63-Ag8.653 and the nonspecific IgG1κ secreting plasmacytoma, MOPC 31C, were obtained from the American Type Culture Collection, Rockville, MD. H37RA mycobacterium was purchased from VWR Scientific, Boston, MA. Antibody subtyping reagents, purified nonspecific MOPC 21 IgG1κ, and affinity purified goat anti-mouse immunoglobulin were obtained from Organon Teknika, Kensington, MD. Alkaline phosphatase-labeled goat anti-mouse IgG (H- and L-chain specific) was purchased from Kirkegaard and Perry, Gaithersburg, MD. Anti-human angiogenin and anti-angiogenin peptide polyclonal antibodies were produced in rabbits by standard immunization techniques using Freund's adjuvant. Monoclonal anti-human angiogenin antibodies in addition to 26-2F were generated as described below and will be the subject of a future publication. Synthetic peptides were provided by Dr. D. S. Auld or purchased from Bachem Fine Chemicals, Torrance, CA. Normal Balb/c mice were obtained from Taconic Farms, Germantown, NY, while athymic (Crl:nu/nu) mice were from Charles River Laboratories, Kingston, NY.

Immunization. Eight-week-old Balb/c mice were immunized subcutaneously with 30 μg of human angiogenin emulsified in complete Freund's adjuvant supplemented with 10 mg/mL of mycobacterium. At 10 and 17 days following the initial injection animals were boosted with an additional 30 μg of angiogenin in incomplete Freund's adjuvant. One week later mice were given 30 μg of angiogenin in PBS intraperitoneally, and 4 days later spleens were harvested for fusion.

Generation of Hybridomas. Splenocytes were fused with mouse myeloma cells essentially as described by Kearney (1984). These were plated into Dulbecco's Modified Eagle's Medium/20% heat-inactivated FBS supplemented with HAT along with mouse peritoneal exudate cells as a feeder layer. After 7 days in culture, hybridomas were transferred into HAT-free medium over a 2-week period. Supernatants from wells containing hybridomas were assayed for antibody production by ELISA. Antibody-positive hybridomas were cloned and recloned by limiting dilution using splenocyte feeder layers.

Production and Purification of mAb 26-2F. From one fusion a stable, antibody-producing hybridoma was expanded to provide supernatants for further characterization and antibody purification. Twice-cloned hybridoma cells were transferred to 24-well plates and subsequently into T-flasks. Supernatants thus generated were used for initial evaluations and purification. Additionally, ascites were produced by intraperitoneal injection of 1×10^6 hybridoma cells into athymic mice which had been primed with 0.5 mL of pristane 7 days earlier. mAb 26-2F was precipitated from either conditioned supernatants or ascites by ammonium sulfate (50% saturated). Precipitates were dissolved in PBS containing 3 M NaCl, 1.5 M glycine, pH 8.5, and applied onto a protein A-Sepharose column equilibrated in the same buffer. The IgG fraction was eluted with 0.1 M sodium citrate, pH 3.5, dialyzed versus saline and stored as aliquots at −70 °C for later use. Control MOPC 31C IgG produced as an ascites was purified similarly.

Screening ELISA. Antibody-producing hybridomas were detected by a solid-phase ELISA in which angiogenin was coated overnight at room temperature onto 96-well plates at 1 μg/mL in 10 mM borate coating buffer, pH 8.3 (50 μL/well). The plates were then washed three times in PBS, pH 7.2, containing 0.05% Tween 20 (PBS-Tween), followed by two washes with PBS. The wells were blocked with 0.5% ovalbumin in PBS (200 μL/well) overnight at 4 °C. After the blocked plates were washed three times with PBS-Tween and twice with PBS, 50 μL of the hybridoma culture supernatant, diluted 1:1 with 0.16% ovalbumin/PBS, was added to each well. The plates were incubated at room temperature for 2 h and washed four times with PBS-Tween and three times with PBS. Alkaline phosphatase-labeled goat anti-mouse IgG (1.25 μg/mL, 50 μL/well) diluted in 0.16% ovalbumin/PBS was then added for 1 h at room temperature. After washing four times with PBS-Tween and three times with PBS, *p*-nitrophenyl phosphate (1 mg/mL, 100 μL/well) diluted in diethanolamine buffer, pH 9.8, was added. The reaction was stopped after 1 h of incubation at room temperature with 3 N NaOH (50 μL/well), and adsorptivities were measured on a Dynatech MR600 ELISA plate reader at 405 nm with a turbidity reference of 630 nm.

Class and Isotype Classification. These were performed by gel double-diffusion analysis using specific antibody typing reagents.

Western Blot. The immunoblot analysis was performed as described previously with minor modifications (Kurachi et al., 1988).

In Vitro Translation Assay. Methods for cell-free protein synthesis have been described in detail elsewhere (St. Clair et al., 1987). The amount of protein synthesis was determined by the incorporation of [^{35}S]methionine into products precipitable by 10% trichloroacetic acid and by autoradiography following separation of proteins by SDS–PAGE (10% polyacrylamide).

For isolation of RNA fragments from angiogenin treated- or angiogenin plus mAb 26-2F treated-lysates, the translation

[3] Single replacement mutants are designated by the single-letter code for the original amino acid followed by its position in the sequence and the single-letter code for the new amino acid.

mixture was diluted with 10 mM Tris-HCl, 10 mM NaCl, 1.5 mM $MgCl_2$, pH 7.5, which contained 0.5% Nonidet P-40. SDS (0.5%) was then added followed by sequential extraction with phenol saturated with the above buffer and with chloroform:isoamyl alcohol (24:1). The RNA was precipitated with ethanol, dissolved in water, and analyzed by labeling the 5′ terminal ends with T4 kinase and [^{32}P]ATP followed by 7 M urea/10% polyacrylamide gel electrophoresis and autoradiography.

tRNA Assay. Formation of perchloric acid soluble fragments from yeast tRNA was measured by a modification of the method of Shapiro et al. (1987b) except that incubations were for 30–45 min. Mixtures of angiogenin with mAb 26-2F or control MOPC 21, containing 4 μg of human angiogenin and 40 μg of immunoglobulin in 30–60 μL of PBS, were preincubated for 60 min at room temperature before addition to the incubation mixture. Samples containing mAb 26-2F or MOPC 21 alone were also assayed, and the absorbances obtained were subtracted from those determined for the corresponding angiogenin–immunoglobulin mixtures. Activities were quantitated by reference to a standard curve obtained with 0, 0.5, 1.0, 2.0, and 4.0 μg of angiogenin.

Angiogenesis Assay. The CAM assay was employed as described previously (Fett et al., 1985; Shapiro & Vallee, 1992). Mixing of angiogenin with IgGs was done just prior to application to the disks.

ELISA for Determination of Cross-Reactivity and Epitope Mapping. A variation of the solid-phase ELISA used for screening hybridoma supernatants was used to test the binding of mAb 26-2F to synthetic peptides, angiogenin derivatives and several other proteins of known sequence homology to human angiogenin. Angiogenins from different species, bovine RNase A, RNase IV, human angiogenin mutants, and proteolytic products were coated onto ELISA plates at 1–3 μg/mL (50 μL/well). For determination of binding to synthetic peptides based on the sequence of human angiogenin, peptides were coated onto ELISA plates at 10 μg/mL (50 μL/well, an approximately 100-fold molar excess over native angiogenin). After blocking with ovalbumin, mAb 26-2F, MOPC 21, or MOPC 31C (100 ng/mL, 50 μL/well) diluted in 0.25% ovalbumin/PBS was added to the washed plate and incubated overnight at room temperature. The ELISA was then performed as above except that incubation with alkaline phosphatase-labeled goat anti-mouse IgG was for 2 h. Under these conditions, the A_{405} for binding of mAb 26-2F to human angiogenin is within the linear portion of the A_{405} versus mAb concentration curve, approaching the absorbance plateau. Thus any decrease in binding of mAb 26-2F to peptides or other proteins is readily detectable. The efficiency of binding of peptides, angiogenin derivatives, and nonhuman angiogenins to the ELISA plate was monitored by performing the same assay using either polyclonal rabbit anti-peptide or anti-human angiogenin antisera or other monoclonal anti-angiogenin antibodies. In all cases wells were shown to be coated satisfactorily.

RIA for Binding Affinity. The binding affinity, expressed as an IC_{50}, of mAb 26-2F for human angiogenin was determined by a competition RIA. RIA plates were coated with affinity-purified goat anti-mouse immunoglobulin (10 μg/mL in borate coating buffer, 50 μL/well) overnight at room temperature. After the plates were washed with PBS-Tween and PBS, wells were blocked overnight at 4 °C with 0.25% ovalbumin/0.25% protamine sulfate diluted in PBS (150 μL/well). mAb 26-2F (300 ng/mL, 50 μL/well) diluted in 0.25% ovalbumin/PBS was added to the washed plates, which then were incubated for 2 h at room temperature. Various dilutions of proteins to be tested were mixed with a fixed amount of iodinated human angiogenin prepared by the chloramine-T method (93 μCi/μg; Greenwood et al., 1963) and transferred in triplicate to the washed RIA plate (50 μL/well, 5.6×10^4 cpm/well). Following incubation for 2 h at room temperature, the plates were washed, cut, and counted in a Micromedic γ counter. The data were analyzed by four parameter logistics using the RIA. Aid program (Robert Maciel, Inc.) The IC_{50} is defined as the concentration of unlabeled protein at which the binding of iodinated angiogenin is decreased by 50%.

RESULTS AND DISCUSSION

Isolation and Characterization of mAb 26-2F. A stable hybridoma was obtained from a fusion of mouse myeloma cells with splenocytes from a mouse immunized with human angiogenin after two rounds of cloning by limiting dilution. Its antibody product, termed mAb 26-2F, is an IgG1κ as determined by Ouchterlony double diffusion analysis. The typical yield of mAb 26-2F from tissue culture is 40 μg/mL while that from ascites fluid is 2 mg/mL. The latter source is used routinely as the starting material for mAb purification. Western blot analysis confirmed that mAb 26-2F recognizes human angiogenin specifically. An IC_{50} of [1.6 ± 0.1 (SEM)] $\times 10^{-9}$ M for the binding of mAb 26-2F to human angiogenin was determined by competition RIA.

Effect of mAb 26-2F on Angiogenin's Ribonucleolytic Activity. Incubation of rabbit reticulocyte lysate with angiogenin abolishes its capacity to support protein synthesis as judged by incorporation of [^{35}S]methionine into acid-precipitable protein (St. Clair et al., 1987, 1988). As depicted in Figure 1A, mAb 26-2F eliminates this inhibition. A 15-min exposure to angiogenin effectively inhibits protein synthesis initiated by BMV RNA as revealed by visualization of newly synthesized [^{35}S]Met-labeled proteins (lane 3). Preincubation of angiogenin with mAb 26-2F (lane 4), however, results in a labeled protein pattern virtually identical to that of the untreated control (lane 2). The nonspecific IgG, MOPC 21, has no effect on angiogenin's inhibitory activity (lane 5).

RNA isolated from treated reticulocyte lysates was 5′ end-labeled with [^{32}P]ATP and analyzed by urea/polyacrylamide gel electrophoresis. Autoradiography reveals that treatment with angiogenin extensively degrades the RNA to a characteristic pattern of low molecular weight products (St. Clair et al., 1987) (Figure 1B, lane 1). Inclusion of mAb 26-2F in the incubation mixture substantially reduces this degradation (lane 2).

mAb 26-2F also inhibits the capacity of angiogenin to degrade tRNA into acid-soluble fragments. In two separate experiments 40 μg of mAb 26-2F inhibited the activity of 4 μg of angiogenin toward tRNA by an average of 96% as compared to <20% by control MOPC 21 under identical conditions.

Inhibition of in Vivo Biological Activity. mAb 26-2F inhibits the biological activity of angiogenin on the CAM (Table 1). Analysis by the χ^2 test indicates that angiogenin (50 ng/egg) over several sets of assays is highly active (group I, $p < 0.000\,01$) when compared to water controls. mAb 26-2F, in approximately equimolar amounts, effectively inhibits this activity (group II, $p = 0.213$) whereas MOPC 21 does not (group III, $p < 0.000\,01$). Since angiogenin is active even at 0.5–1.0 ng/egg (Fett et al., 1985), inhibition by mAb

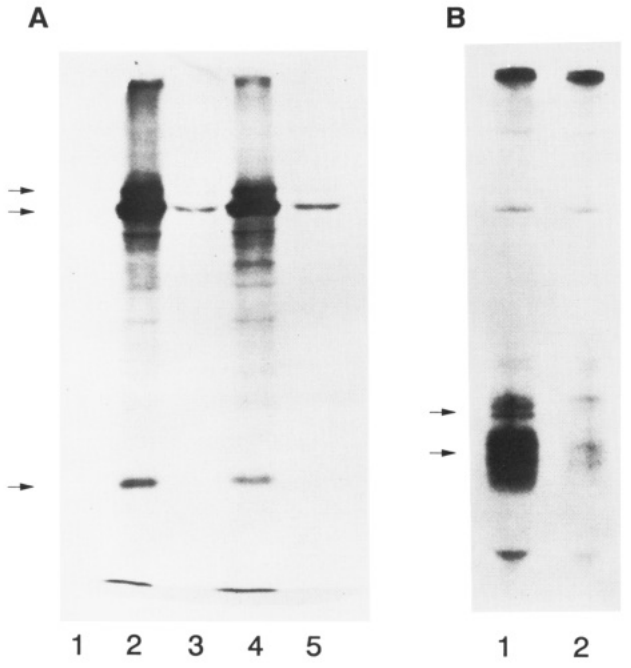

FIGURE 1: (A) The inhibition of cell-free protein synthesis by angiogenin is blocked by mAb 26-2F. Human angiogenin (50 ng) was preincubated with 1.5 μg of mAb 26-2F or MOPC 21 in H_2O for 1 h at 37 °C. Further effects of angiogenin were blocked by the addition of PRI. The mixture was then added to the reticulocyte lysate and incubated for 15 min at 37 °C, and protein synthesis was initiated by the addition of BMV RNA in the presence of [^{35}S]-methionine. After 60 min the [^{35}S]Met-labeled proteins were analyzed by 10% polyacrylamide gel electrophoresis and autoradiography. Lane 1, control translation without exogenous BMV RNA; lane 2, the same as 1 but with 0.2 mg of BMV RNA; lane 3, the same as 2 but with angiogenin; lane 4, the same as 2 but with angiogenin preincubated with mAb 26-2F; lane 5, the same as 2 but with angiogenin preincubated with control MOPC 21. The upper to lower arrows denote the molecular weight positions of major BMV RNA translation products (i.e., 110-, 97-, and 20-kDa, respectively). (B) Angiogenin cleavage of reticulocyte RNA is blocked by mAb 26-2F. RNA was isolated as described in Materials and Methods from reticulocyte lysates which were treated with angiogenin or with angiogenin plus mAb 26-2F as described above. ^{32}P-Labeled RNA was electrophoresed on a 7 M urea/10% polyacrylamide gel and subjected to autoradiography. Lane 1, RNA from lysates incubated with BMV RNA and treated with angiogenin; lane 2, the same as 1 but treated with angiogenin preincubated with mAb 26-2F. The characteristic doublet and group of five to seven prominent cleavage products generated by angiogenin are indicated by arrows.

26-2F must be >98%. The immunoglobulins alone are inactive on the CAM (groups IV and V, p's > 0.05).

Cross-Reactivity of mAb 26-2F. The specificity of binding of mAb 26-2F to several proteins homologous to human angiogenin was investigated using both solid-phase ELISA and competition RIA. The proteins tested were rabbit, pig, bovine, and mouse angiogenins as well as bovine RNase A and human RNase IV, whose sequence identities relative to human angiogenin are 73, 66, 64, 75, 33, and 39%, respectively (Bond et al., 1993; Zhou and Strydom, 1993). As shown in Table 2, rabbit angiogenin is the only one of these proteins recognized by mAb 26-2F in the ELISA, although its binding is less than that observed with human angiogenin. The attenuated reactivity of rabbit angiogenin with mAb 26-2F was examined further by a competition RIA which gave an IC_{50} of 1.3×10^{-7} M as compared with 1.6×10^{-9} M for human angiogenin. No inhibition of binding could be detected with up to a 1×10^{-5} M concentration of the other nonhuman angiogenins or bovine RNase A.

Epitope Determination for mAb 26-2F. Since mAb 26-2F neutralizes the ribonucleolytic and angiogenic activities of angiogenin, it was of interest to determine the structural domains recognized by the antibody. Epitope mapping was therefore initiated by investigating the binding of mAb 26-2F to synthetic peptides representing portions of the primary sequence of human angiogenin as well as to various derivatives of the protein. Fourteen peptides were available which encompass most of the sequence and correspond to residues 1–9, 6–21, 17–26, 30–41, 36–46, 41–50, 41–51, 48–57, 48–61, 59–68, 81–91, 105–114, 108–121, and 115–123. Recognition by mAb 26-2F was evaluated by solid-phase ELISA, which revealed no detectable binding to any of these peptides.

Mutant and proteolytic derivatives of angiogenin, many utilized previously for structure/function studies, were then investigated for their capacity to bind to mAb 26-2F. Initially two angiogenin/RNase A hybrid proteins, ARH-I and ARH-II, were used. In ARH-I the angiogenin segment 58–70, containing part of the putative receptor binding site, is replaced by the corresponding residues 59–73 of RNase. The sequence of the RNase segment is quite dissimilar from that of angiogenin and contains two additional residues and a disulfide bond which is lacking in angiogenin. In ARH-II, the angiogenin segment 38–41 (Pro-Cys-Lys-Asp), containing the catalytic residue Lys-40, is replaced by the corresponding RNase residues (Asp-Arg-Cys-Lys-Pro). Although the Cys-

Table 1: Effect of mAb 26-2F on the Activity of Human Angiogenin in the CAM Assay

group	angio-genin	mAb 26-2F	MOPC 21	sets of assays	assay results[b]	p[c]	status
I	+	–	–	8	49/88 (56)	<0.000 01	active
II	+	+	–	5	14/72 (19)	0.213	inactive
III	+	–	+	5	36/70 (51)	<0.000 01	active
IV	–	+	–	5	15/65 (23)	0.080	inactive
V	–	–	+	3	9/50 (18)	0.379	inactive

[a] Each individual assay employed between 8 and 30 eggs. [b] These are expressed as the ratio of positive to total surviving eggs. The percentage of positive eggs is given in parentheses. [c] Significance was calculated from χ^2 values of data recorded at 68 ± 2 h based on comparison with water controls tested simultaneously (9 positive eggs/82 total surviving eggs, 11% positive). To be designated active samples must have a value of $p < 0.05$. Amount applied per egg is 500 ng of either IgG and 50 ng of angiogenin.

Table 2: Cross-Reactivity of mAb 26-2F with Angiogenins of Different Species, Human Angiogenin Mutants, and Homologous RNases[a]

protein	ELISA concentration (μg/mL)[b]	ELISA A_{405}[c]	RIA IC_{50} [M (×10^9)[d]]
angiogenin, human	1.0	1.072	1.6
angiogenin, bovine	3.0	–0.003	NI
angiogenin, murine	1.0	–0.001	NI
angiogenin, porcine	3.0	–0.008	–
angiogenin, rabbit	1.0	0.570	130
bovine RNase A	3.0	0.001	NI
RNase IV	3.0	–0.007	–
ARH-II	1.0	0.058	400
K40Q	1.0	0.845	2.4
W89M	1.0	0.084	5.3

[a] Analysis of binding of mAb 26-2F to the proteins listed was performed by ELISA and RIA as described in Materials and Methods. [b] Concentration of protein coated onto ELISA wells. [c] The ELISA results are corrected for the absorbance due to binding of the nonspecific IgGs, MOPC 21, or MOPC 31C (typically ~0.030) to the same coated protein. Additional angiogenin derivatives tested (ARH-I, R5A, H13A, R31A, R32A, R33A, S37R, T44A, R66A, R70A, K73A, E108Q, N109D, H114A, S118R, angiogenin E, and angiogenin K) all were within 20% of the A_{405} value observed for native angiogenin in the ELISA. [d] Determined by competition RIA. NI = no inhibition at concentrations up to 1×10^{-5} M, i.e., $IC_{50} > 1 \times 10^{-4}$ M. – = not measured.

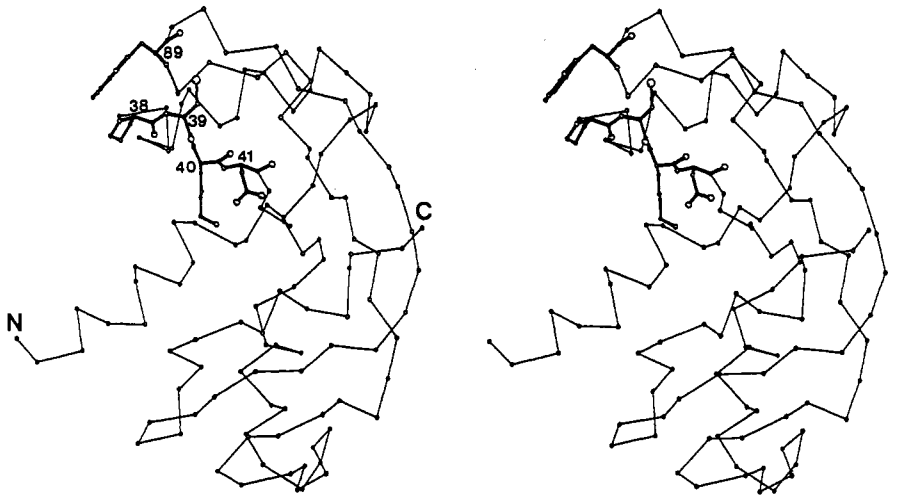

FIGURE 2: Stereodrawing of the three-dimensional structure of human angiogenin determined by X-ray diffraction at 2.4-Å resolution (Acharya et al., 1994). The α-carbon backbone is shown along with all non-hydrogen atoms of residues implicated in the binding to mAb 26-2F (i.e., Pro-38, Cys-39, Lys-40, Asp-41, and Trp-89). The proposed cell-binding domain of angiogenin (residues 60–68 and 109) are contained within loop structures seen at the bottom of the molecule (Hallahan et al., 1991, 1992).

39–Lys-40 sequence is retained in ARH-II, the Asp and Pro on either side are transposed and an additional residue is inserted; the local secondary structures of angiogenin and ARH-II are therefore predicted to differ considerably.

No significant difference was found in the binding of mAb 26-2F to ARH-I as compared with human angiogenin by ELISA. However, binding to ARH-II was reduced substantially, as determined by both ELISA and competition RIA (Table 2). The IC_{50} for ARH-II was 4.0×10^{-7} M, 250 times greater than that obtained with the unmodified protein. These data suggested that amino acids in the segment 38–41 are involved in the epitope and hence three additional mutants—S37R, K40Q, and T44A—with single replacements within or flanking this region were examined. ELISA revealed no significant difference in recognition by mAb 26-2F of either S37R or T44A in comparison with human angiogenin, indicating that the epitope does not include the side chains of either Ser-37 or Thr-44. Binding to K40Q in the ELISA was somewhat reduced, however, as also reflected in its slightly increased IC_{50} (2.4×10^{-9} M) in the RIA (Table 2). Thus Lys-40 may play a minor role, perhaps indirect, in antibody binding.

To explore the involvement of additional regions of the molecule, 13 other single-site mutants and two proteolytic products of angiogenin were examined. These derivatives included some modified within or near the enzymatic active site (R5A, H13A, R33A, E108Q, H114A, and S118R), others altered at the putative receptor binding site [R66A, N109D, angiogenin E (angiogenin cleaved between Glu-67 and Asn-68)[4] and angiogenin K (angiogenin cleaved between Lys-60 and Asn-61)[4]], and several modified outside these two sites (R31A, R32A, R70A, K73A, and W89M). W89M was the only one of these proteins to show any appreciable decrease in binding to mAb 26-2F as detected by ELISA (Table 2). Competition RIA with W89M yielded an IC_{50} of 5.3×10^{-9} M, more than 3 times greater than that obtained with the native protein. The magnitude of this change is smaller than that observed by ELISA, suggesting that epitope conformation may be perturbed to a greater extent in the solid phase than in solution. The RIA, in any case, should provide a more reliable quantitative indication of the actual decrease in binding strength.

The preceding results suggest that the epitope recognized by mAb 26-2F contains, minimally, Trp-89 and residues in the segment 38–41. The nature of this epitope can now be considered in light of the recently determined 2.4-Å resolution crystal structure of human angiogenin (Acharya et al., 1994). A stereoview of the α-carbon backbone of the molecule plus all non-hydrogen atoms of residues 38–41 and 89 is presented in Figure 2. Angiogenin consists of seven segments of β structure, including a central core of antiparallel twisted β strands, together with four helices and nine loops. The proposed epitope is formed by the two largest loop structures (loops 2 and 7), which contain residues 34–40 and 85–92, respectively. These loops are juxtaposed in the 3D structure; e.g., the α-carbon of Trp-89 is only 5.5 and 5.9 Å from those of Pro-38 and Cys-39, respectively (the distances from Trp-89 to Lys-40 and Asp-41 are 9.5 and 10.6 Å, respectively). Moreover, they are connected by a disulfide bridge between cysteine residues 39 and 92 as well as through a hydrogen bond between the Cys-39 main-chain N and the carbonyl O of Pro-88. Three H bonds also link amino acids on these loops with residues flanking the opposite loop: the carbonyl O of Asp-41 interacts with the main-chain N of Leu-83 and the phenolic OH of Tyr-94 bonds to both the main-chain N and carbonyl O of Lys-40.

All residues in the putative epitope, and indeed in the two loops encompassing them, with the exception of Cys-39, are exposed to solvent and are potentially available for direct interaction with the antibody. The surface location of Trp-89 in the crystal structure is consistent with earlier findings which showed that the fluorescence of this residue in solution is low and is readily quenched by acrylamide (Lee et al., 1989). Trp-89 is also thought to interact with placental RNase inhibitor (PRI), a tight-binding inhibitor of all known activities of angiogenin. Although PRI, a 50-kDa protein, shares with mAb 26-2F at least one additional contact point, Lys-40, the recognition sites are in large part distinct since Arg-5, Arg-32, and His-114 contribute to binding of PRI (Lee & Vallee, 1989; Shapiro & Vallee, 1989; Shapiro & Vallee, 1992) but not to that of mAb 26-2F.

The characteristics of the mAb 26-2F epitope on angiogenin as now delineated are consistent with the results of X-ray diffraction studies on complexes of monoclonal antibody Fab fragments with protein antigens [reviewed in Davies et al.

[4] The two peptide chains in both angiogenin E and angiogenin K remain connected via three disulfide bonds.

	38								41																89									
angiogenin, human[a]	R	R	G	L	T	S	-	**P**	**C**	**K**	**D**	I	N	.	.	.	L	H	G	G	S	P	**W**	P	P	C	Q	Y
angiogenin, rabbit[b]	R	R	D	L	T	S	-	P	C	K	D	T	N	.	.	.	H	V	G	G	S	P	W	P	P	C	R	Y
angiogenin, bovine[c]	N	R	R	L	T	R	-	P	C	K	D	R	N	.	.	.	H	K	G	G	S	S	R	P	P	C	R	Y
angiogenin, pig[b]	Q	R	G	L	T	R	-	P	C	K	E	V	N	.	.	.	H	K	G	G	S	N	R	P	P	C	G	Y
angiogenin, mouse[d]	R	R	S	L	T	S	-	P	C	K	D	V	N	.	.	.	H	T	G	G	S	P	R	P	P	C	Q	Y
RNase A[e]	S	R	N	L	T	L	D	R	C	K	P	V	N	.	.	.	A	T	G	S	S	K	Y	P	N	C	A	Y
RNase IV[f]	R	R	K	M	T	L	D	Y	C	K	R	F	N	.	.	.	D	T	G	S	S	R	A	P	N	C	R	Y

FIGURE 3: Amino acid sequences (in single-letter code) for human angiogenin and six homologues in the region of the putative epitope. The human angiogenin residues implicated in antibody binding are in bold-face. The two loops forming this portion of the epitope extend from amino acids 34–40 and 85–92 (Acharya et al., 1994). Residues are designated according to the numbering system for human angiogenin (Strydom et al., 1985). Sequences are from [a]Strydom et al. (1985), [b]Bond et al. (1993), [c]Maes et al. (1988), [d]Bond and Vallee (1990), [e]Smyth et al. (1963), and [f]Zhou and Strydom (1993).

(1990) and Laver et al. (1990)]. Thus antigenic determinants on proteins have generally been found to be discontinuous, containing two to five separate portions of primary structure, and are formed largely by surface loops. At the same time, epitopes usually include between 15 and 22 residues on the antigen, with a buried surface area of about 650–900 Å^2 in the complex. It is therefore likely that numerous additional angiogenin residues beyond 38–41 and 89 form important contacts with mAb 26-2F. At least some of these residues would presumably lie within the same two loops. Adjacent structural elements—helix 2 and β strand 1 for loop 2 and β strands 4 and 5 for loop 7—may also be involved, as well as noncontiguous regions (e.g., helix 1, containing residues 3–13) that are relatively close in the 3D structure. The strong binding of mAb 26-2F to R31A, R32A, and R33A (helix 2), S37R (loop 2), T44A (strand 1), and R5A and H13A (helix 1) rule out interactions with the side chains of these individual residues, although H bonds with the main-chain atoms remain possible.

Given the relatively small sizes reported for antibody–antigen contact regions determined crystallographically thus far, it is not surprising that the mAb 26-2F epitope on angiogenin does not extend to the putative receptor binding site, as indicated by the undiminished recognition of R66A, N109D, and angiogenins E and K. This site, located at the bottom of the structure depicted in Figure 2, is 25–35 Å away from Trp-89. Consequently, the capacity of mAb 26-2F to abolish the angiogenic activity of angiogenin probably derives from its interaction with the ribonucleolytic active site (e.g., Lys-40) rather than from interference with receptor binding.

In conjunction with the angiogenin 3D structure, the present epitope analysis results may also help to explain the weak or undetectable binding of mAb 26-2F to RNase A, RNase IV, and nonhuman angiogenins. The sequences of these proteins in the regions of the proposed epitope are shown in Figure 3. RNase A and RNase IV differ from human angiogenin at three of the five positions corresponding to amino acids 38–41 and 89 and at several positions in the flanking segments that may also form part of the epitope. It is therefore understandable that mAb 26-2F fails to interact with either protein. With the nonhuman angiogenins, the structural basis for decreased binding to mAb 26-2F must lie primarily or exclusively outside residues 38–41 and 89. At all of these positions rabbit angiogenin is identical to human, and yet its interaction with mAb 26-2F is 80-fold weaker. The other angiogenins do not exhibit detectable binding although they differ from the rabbit and human proteins only by the substitution of an arginine for Trp-89 (plus the conservative replacement of Asp-41 by Glu in pig angiogenin). It is unlikely that this substitution by itself can account for such large decreases in binding since mutation of Trp-89 in human angiogenin to Met affects the IC_{50} value by only a factor of 3. Instead, the lack of recognition of bovine, pig, and mouse angiogenins, as well as the reduced binding of the rabbit protein, may reflect the differences from human angiogenin in the segments adjacent to residues 38–41 and 89 (Figure 3). These nonconserved residues themselves may be part of the epitope. It is also possible that replacements of these amino acids affect the conformation of other epitope components, including residues 38–41 and 89. In this regard, the results of preliminary X-ray diffraction analysis of bovine angiogenin (K. R. Acharya, personal communication) suggest that the 3D structure of the Trp-89 loop may be quite sensitive to relatively small changes in sequence. Although six of the eight residues in this loop are the same as their human counterparts, the root mean square deviation in the positions of the α-carbons is 2.2 Å (range 1.5–3.2 Å).

Thus the available crystal structure of human angiogenin has been invaluable for interpretating the binding characteristics of mAb 26-2F. Similar analyses with the panel of neutralizing mAbs currently under development promises to provide additional information in regard to structural domains in this protein responsible for function. Such antibody reagents will also be useful for tissue localization, immunoassays, and affinity purification, as well as for definition of the physiological role(s) of angiogenin. Importantly, evaluation of their therapeutic effects in pathological situations characterized by abnormal blood vessel proliferation is being pursued.

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