Angiogenin Single-Chain Immunofusions: Influence of Peptide Linkers and Spacers between Fusion Protein Domains

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ABSTRACT: The gene for human angiogenin (Ang), a member of the ribonuclease superfamily, was fused to a gene encoding a single-chain antibody (sFv) against the human transferrin receptor. Three Ang single-chain immunofusion proteins (AngsFvs) were constructed with variations in the type of linker connecting the V_L and V_H chain [EGKSSGSGSESKEF, L1 or $(GGGGS)_3$, L2] as well as with or without a spacer (FB) connecting the Ang and sFv (AngFBsFvL1 or L2; AngsFv(L2)]. Although the nature of the linker did not affect the enzymatic activity of the FB-containing fusion proteins, the fusion protein containing the L2 linker was 2.3-fold more effective than the L1 linker in competing with the labeled monoclonal IgG1 antibody for binding to the transferrin receptor. The fusion protein containing the L2 linker without the FB spacer exhibited a 13-fold decrease in binding to the transferrin receptor as well as a decrease in its capacity to degrade tRNA and to inhibit translation in the rabbit reticulocyte lysate compared to its counterpart containing the FB spacer. Binding of placental ribonuclease inhibitor (PRI) to Ang also was affected by the nature of the linker and by the presence or absence of a spacer. PRI bound to Ang and AngFBsFv(L2) and inhibited their ribonuclease activity. A 3-fold greater concentration of PRI, however, did not affect the activity of AngFBsFv(L1) or AngsFv(L2), suggesting that the conformation of these fusion proteins was altered. Binding of monoclonal and polyclonal anti-Ang antibodies to AngsFvs was also used to investigate conformational alterations of the fusion proteins. AngFBsFv(L2) was the least altered while AngFBsFv(L1) exhibited the greatest change in structure. Yet maximal concentrations of all AngsFvs elicited angiogenesis in the chick chorioallantoic membrane assay, demonstrating that Ang in all three fusion proteins remained functionally active. Consistent with all the activities, the fusion protein containing the FB spacer and L2 linker was the most cytotoxic to three different human tumor cell lines. The fusion protein lacking the FB spacer exhibited the least cytotoxicity. These data demonstrate that the linker connecting the V_H-V_L chains can affect the binding and cellular cytotoxicity of Ang immunofusions and that placement of a spacer between the antibody binding domains and Ang is necessary for optimal activity. Thus, a new class of targeted therapeutic agents containing Ang as the toxic moiety can be designed that potentially will be less immunogenic and less toxic than immunotoxins available currently.

Noncytotoxic members of the ribonuclease (RNase)[1] A superfamily have been linked to tumor-associated cell-surface binding ligands by chemical (Rybak et al., 1991; Newton et al., 1992) or recombinant methods (Rybak et al., 1992; Newton et al., 1994) for the purpose of selectively killing tumor cells while exhibiting fewer side effects than current strategies employing plant and bacterial toxins (Rybak & Youle, 1991). Single chain antibodies (sFvs) composed of the variable heavy and light chains of an antibody connected by a peptide linker have been produced in bacteria (Huston et al., 1988; Bird et al., 1988). Immunotoxins have been constructed between these sFvs and bacterially derived toxins (Chaudhary et al., 1989; Brinkmann & Pastan, 1994) and the human eosinophil RNase, EDN (Newton et al., 1994). Problems of stability and aggregation encountered in the expression and characterization of EDNsFv prompted the current investigation into certain components of sFv fusion protein architecture as well as the use of an alternative human RNase, angiogenin (Ang).

Ang was isolated originally from conditioned tumor cell medium by following angiogenic activity in the chicken embryo chorioallantoic membrane (CAM) and rabbit cornea (Fett et al., 1985). It is 65% homologous to EDN and

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[1] Abbreviations: Ang, human angiogenin; mAb, monoclonal antibody; E6, antitransferrin receptor IgG monoclonal antibody; PRI, placental ribonuclease inhibitor; EDN, eosinophil-derived neurotoxin; RNases, ribonucleases; tRNA, transfer ribonucleic acid; PCR, polymerase chain reaction; CAM, chick chorioallantoic membrane assay; PBS, phosphate-buffered saline pH 7.4; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; V_L, light chain variable region; V_H, heavy chain variable region; sFv, single chain antibody; L1, 14 amino acid residue linker composed of EGKSSGSGSESKEF; L2, 15 amino acid residue linker composed of $(GGGGS)_3$; FB, residues 48−60 of fragment B of staphylococcal protein A; AngsFvs, Ang single-chain immunofusion proteins; AngsFv(L2), AngsFvs containing linker L2; AngFBsFv(L1), AngsFvs containing spacer FB and linker L1; AngFBsFv(L2), AngsFvs containing spacer FB and linker L2; IC_{50}, the concentration of the fusion protein which inhibits protein synthesis by 50%.

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exhibits RNase activity although the RNA substrate specificities of the two enzymes differ markedly (Shapiro et al., 1986; Sorrentino et al., 1992). The characteristic ribonucleolytic activity of Ang (Shapiro et al., 1986) is responsible for inhibition of protein synthesis in cell-free systems (St. Clair et al., 1987) and in oocytes after microinjection of the protein (Saxena et al., 1992). Thus we sought to determine the biological properties of AngsFvs as compared to those previously published for the EDNsFv (Newton et al., 1994).

The properties of the peptide used to connect the antibody to the effector protein influences both the folding and aggregation of sFv-fusion proteins (Brinkmann et al., 1992). Additionally, linkers connecting the carboxyl terminus of one variable antibody domain and the amino terminus of the other have been designed to maintain flexibility and solubility of the molecule as well as integrity of the antigen-binding site (Huston et al., 1988; Bird et al., 1988). In the current study, we demonstrate that the type of linker connecting the V_L and V_H domains of the sFv affects binding of the sFv to the human transferrin receptor and that the presence of a spacer connecting the RNase and sFv is necessary for obtaining optimal activity of each half of the fusion protein. Most importantly, each of these modifications is reflected in the overall cytotoxicity of the fusion proteins toward cancer cells. These results indicate that a stable, active RNase-based fusion protein can be constructed with human Ang. The implications with regard to targeted cancer therapy are discussed.

MATERIALS AND METHODS

Materials. Yeast transfer ribonucleic acid (tRNA) was purchased from Sigma (St. Louis, MO), placental ribonuclease inhibitor (PRI) from Promega (Madison, WI). The CM-Sephadex C-50 and Ni^{2+}-NTA-agarose were obtained from Pharmacia Biotech Inc. (Piscataway, NJ) and Qiagen (Chatsworth, CA), respectively. Tris/glycine gradient electrophoresis gels were from Novex (San Diego, CA). Reagents for performing the polymerase chain reaction (PCR) were obtained from Perkin-Elmer Corp. (Norwalk, CT). Recombinant human Ang was purified from *Escherichia coli* as described previously (Saxena et al., 1991; Shapiro et al., 1988). It differed from the native only with respect to the N-terminal sequence, Met-Gln-(*E. coli*), a difference that does not affect its enzymatic or angiogenic properties (Shapiro et al., 1988). Anti-Ang monoclonal antibodies (mAbs) 26-2F and 36u were produced as described (Fett et al., 1994). The rabbit polyclonal anti-Ang antibody R113 was produced by immunization with Freund's adjuvant using classical techniques. Materials for the enzyme-linked immunosorbent assay (ELISA): ELISA plates, Costar (Cambridge, MA); ovalbumin, bovine serum albumin (BSA) and *p*-nitrophenyl phosphate, Sigma (St. Louis, MO); alkaline phosphatase-labeled goat anti-mouse IgG and anti-rabbit IgG reagents, Kirkegaard and Perry (Gaithersburt, MD). All cell culture supplies were from Gibco/BRL (Grand Island, NY). [^{14}C]Leucine (310 mCi/mmol) and [^{35}S]methionine (1134 mCi/mmol) were from Dupont-New England Nuclear (Beverly, MA). MDA-MB-231 human breast and HT-29 human colon carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD). MDA-MB-231 and HT-29 parental cells were infected with a pHamdr1/A retrovirus carrying the human mdr1 complementary DNA (MDA-231^{mdr1} and HT-29^{mdr1}), and clones cross-resistant to colchicine, doxorubicin and vinblastine were isolated as described (Pearson et al., 1991). ACHN renal carcinoma cells were obtained from the Anti-Cancer Drug Screening Program of the National Cancer Institute.

Plasmid Construction. The Ang gene (Rybak et al., 1992; Kurachi et al., 1985) was joined to the 5′ end of the gene encoding a murine single chain antibody (sFv) against the human transferrin receptor as described (Newton et al., 1994). Two of the constructs, AngFBsFv(L1) and AngFBsFv(L2) contained a 13 amino acid residue spacer composed of residues 48−60 of fragment B (FB) of staphylococcal protein A (Tai et al., 1990) that separated the Ang protein from the sFv protein. In the third construct the Ang gene was fused directly to the sFv gene [AngsFv(L2)]. Two different linkers were used to connect the 3′ end of the antibody V_L domain to the 5′ end of the antibody V_H domain; one linker was a modification of that reported by Bird et al. (1988) (EGKSSGSGSESKEF, L1), the other linker, L2, was $(GGGGS)_3$ designed by Huston et al. (1988). Primers were designed that introduced three histidyl residues at the 3′ end of the V_H gene in order to facilite purification of the fusion proteins by metal chelate chromatography. The assembled genes were inserted between the *XbaI* and *BamHI* sites of the bacterial expression vector pET-11d, Novagen (Madison, WI).

Protein Expression and Purification. The plasmids were expressed in BL21(DE3) *E. coli* cells as recommended by the supplier, Novagen (Madison, WI). The fusion proteins were isolated from inclusion bodies, denatured, renatured, and dialyzed as described (Newton et al., 1994) before being applied to a CM-Sephadex C-50 column. Briefly, after induction for 2 h in the presence of IPTG, the inclusion bodies containing the fusion proteins as insoluble aggregates were isolated and purified as described (Newton et al., 1994). The fusion proteins were then denatured in a buffer consisting of 0.1 M Tris-HCl, pH 8, 6 M guanidine HCl, 2 mM EDTA, and 0.3 M DTE and renatured by rapid dilution into 0.1 M Tris-HCl, pH 8, containing 2 mM EDTA, 0.5 M L-arginine, and 4 mM oxidized glutathione. After exhausitve dialysis in 20 mM Tris-HCl, pH 7.5, containing 100 mM urea (Brinkmann et al., 1992), purification to homogeneity was achieved by chromatography on a CM-Sephadex C-50 column followed by Ni^{2+}-NTA-agarose as described (Newton et al., 1994). The majority of contaminating proteins were not retained on the cationic exchanger column. After washing the column with 20 mM Tris-HCl, pH 7.5, containing 10% glycerol, the fusion proteins were eluted with 1 M NaCl. The eluted fractions were adjusted to 0.8 mM imidazole and 1% Triton X-100 and applied to Ni^{2+}-NTA agarose and chromatography was carried out as described (Newton et al., 1994).

Protein Determination. Protein was monitored throughout the purification procedure using the BCA protein assay reagent, Pierce (Rockford, IL) according to the manufacturer's instructions using BSA dissolved in the fusion protein buffer as the standard. SDS−polyacrylamide gel electrophoresis and Western blot analysis were used to monitor protein purification.

Ribonuclease Assay. The RNase activity of Ang and the Ang single-chain immunofusion proteins (AngsFvs) was determined by a modification of a protocol using tRNA as substrate (Shapiro et al., 1987b). The activity was measured in a final volume of 0.3 mL containing 0.33 mg/mL yeast tRNA, 30 mM HEPES, pH 6.5, 30 mM NaCl, 0.17 mg/mL

human serum albumin, Calbiochem (San Diego, CA), and the appropriate concentrations of RNases (dilutions were made in 0.5 mg/mL human serum albumin). The mixtures were incubated for 18 h at 37 °C before termination with 700 μL of 3.4% ice-cold perchloric acid. The remaining steps were performed as described previously (Rybak et al., 1991). Assays were performed in the linear range of the enzyme. Absorbance readings of the appropriate blanks were subtracted from assays containing enzyme.

Transferrin Receptor Binding Assay. Binding of the antibody single-chain portion of AngsFvs was measured as described (Newton et al., 1994).

In Vitro Translation Assay. This was performed using the rabbit reticulocyte lysate as described previously (St. Clair et al., 1987). Briefly, assay mixtures contained Brom mosaic viral RNA, 7 μL of rabbit reticulocyte lysate, 3.5 μCi of [^{35}S]methionine, 1 mM each of the other 19 amino acids, and Ang or AngsFvs diluted in 0.5 mg/mL human serum albumin (final volume, 25 μL). After a 1 h incubation at 30 °C, the amount of protein synthesis was determined by the incorporation of [^{35}S]methionine into products precipitable by 10% trichloroacetic acid.

Angiogenesis Assay. The CAM assay was employed as described previously (Fett et al., 1985). AngsFvs were stored at 4 °C and diluted just prior to use.

Protein Synthesis Assay. Protein synthesis was measured as previously described (Rybak et al., 1991). Briefly, cells were plated at concentrations given in the figure legends into 96-well microtiter plates in Dulbecco's minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum; additions were made in a total volume of 10 μL, and the plates were incubated at 37 °C for the times indicated. Phosphate-buffered saline (PBS) containing 0.1 mCi of [^{14}C]-leucine was added for 2−4 h, and the cells were harvested onto glass fiber filters using a PHD cell harvester, washed with water, dried with ethanol, and counted. The results are expressed as per cent of [^{14}C]leucine incorporation in the mock-treated wells or as total CPM.

Characterization of Anti-Ang Antibody Binding to AngsFvs by ELISA. The relative affinities of three different anti-Ang antibodies to the AngsFvs in comparison with Ang were determined by ELISA. Two monoclonal anti-Ang antibodies with different epitope specificities, mAb 26-2F (epitope: residues 38−41 plus 89; Fett et al., 1994) and mAb 36u (epitope: residues 58−73, K.A.O., unpublished results), were tested using a modification of the ELISA described in Fett et al. (1994). Ang and the AngsFvs were coated on the plate (at the molar equivalent of 0.5 mg/mL of Ang, 50 μL/well) overnight at room temperature in borate coating buffer, pH 8.3. After the plates were washed four times with PBS-Tween (PBS containing 0.5% Tween 20) followed by three washes with PBS, the wells were blocked with ovalbumin (0.5% in PBS). Dilutions of the mAb in 0.17% ovalbumin in PBS were then made in the wells and allowed to bind for 2 h. After the plates were washed, alkaline phosphatase-labeled goat anti-mouse IgG was added to the wells (62.5 ng/mL) for 1 h, at which time the plates were washed and the *p*-nitrophenyl phosphate substrate was added (1 mg/mL, 100 μL/well) in diethanolamine buffer, pH 9.8. The plates were read and processed 1 h later at 405 nm with a computer-controlled Dynatech MR 600 ELISA reader using the Immunosoft data analysis program. ELISAs using the polyclonal rabbit anti-Ang were identical except for the use of BSA as the blocker and diluent and the use of alkaline phosphatase-labeled goat anti-rabbit IgG for detection of bound IgG. A double antibody ELISA was also used to characterize the capacity of the anti-Ang antibodies to bind to the AngsFvs. In this assay, the ELISA wells were coated with either mAb 26-2F or mAb 36u in borate coating buffer (10 μg/mL, 100 μL/well, overnight at room temperature). After washing as above, the wells were blocked for at least 6 h with 0.5% BSA in PBS, 200 μL/well. Dilutions of Ang and the AngsFvs in 0.25% BSA in triplicate were then added to each plate (100 μL/well). After incubation overnight at room temperature, the plates were washed, and a 1/4000 dilution of R113 in 0.25% BSA (100 μL/well) was added for 2 h. The plates were then washed again, and bound rabbit IgG was detected with the addition for 1 h of alkaline phosphatase-labeled goat anti-rabbit IgG (31 ng/mL, 100 μL/well) followed by a final washing sequence and the addition of *p*-nitrophenyl phosphate (1 mg/mL, 100 μL/well, 90 min incubation). The plates were read as above. All ELISA data presented are the result of triplicate assays, and controls for nonspecific binding to mouse and rabbit immunoglobulin were included in the data analysis.

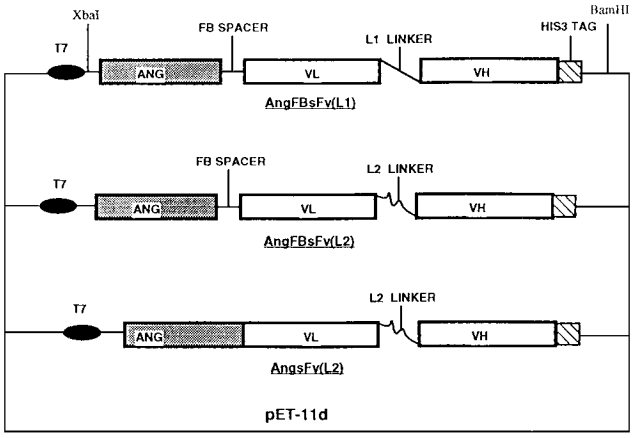

FIGURE 1: Plasmids for expression of Ang single chain fusion proteins. The V_H and V_L domains of the antihuman transferrin receptor monoclonal antibody, E6, are joined by either EGKSSGSG-SESKEF (Bird et al., 1988; L1) or $(GGGGS)_3$ (Huston et al., 1988; L2) peptide linkers. The sFv was then fused with or without a spacer [FB, residues 48−60 of staphylococcal protein A (Tai et al., 1990)], AKKLNDAQAPKSD, to the gene encoding Ang. Three histidyl residues (HIS3) were included at the 3′ end of the assembled gene to facilitate purification by metal chelate chromatography. Primers were designed to incorporate *Xba*I and *Bam*HI restriction sites at the 5′ and 3′ ends of the assembled gene for cloning into the expression vector pET-11d as described (Newton et al., 1994).

RESULTS

Cloning and Expression of AngsFvs. The structures of the three AngsFvs used in this study are shown in Figure 1. The gene encoding the entire 123 amino acids of human Ang was fused with or without a spacer (FB) to the amino terminus of the gene coding for the first 107 amino acid residues of the V_L chain of the antihuman transferrin receptor. The presence of the FB spacer was inserted to provide flexibility between Ang and the sFv and to improve the solubility of the sFv constructs. The gene encoding the V_L chain was connected to the first 115 amino acids of the antitransferrin receptor heavy chain by a modified (amino acid residues Ser 13 and Thr 14 were replaced with Glu and

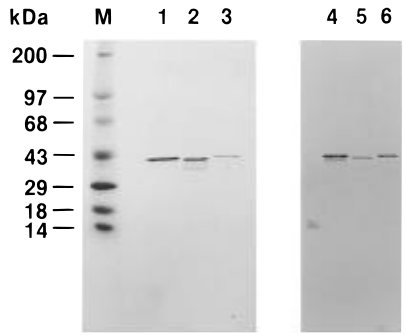

FIGURE 2: SDS−polyacrylamide gel electrophoresis of AngsFvs. Lanes 1−3 are stained with Coomassie blue. Lanes 4−6 are Western blots using polyclonal rabbit anti-Ang. Lane M contains the molecular mass markers; the numbers at the left indicate their sizes in kilodaltons. Lanes 1 and 4, AngFBsFv(L2); lanes 2 and 5, AngsFv(L2); lanes 3 and 6, AngFBsFv(L1).

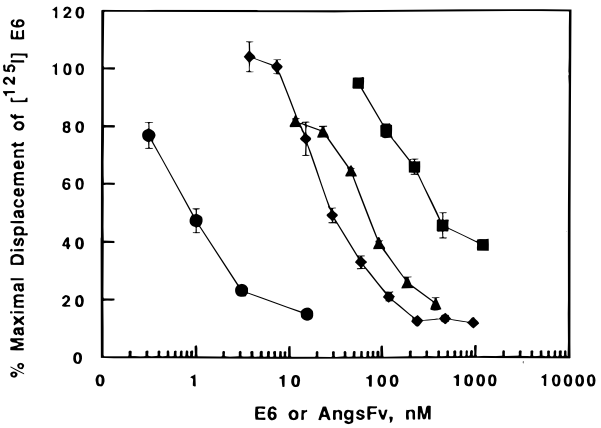

FIGURE 3: Competition of angiogenin single-chain fusion proteins with E6 IgG1 antibody for binding to the transferrin receptor. The binding analyses were conducted on K562 cells as described in Materials and Methods. Data from 2−3 experiments were pooled, averaged, and plotted ±SEM for each point. Fold differences in binding were assessed at 50% displacement. E6, circles; AngFBsFv(L2) diamonds; AngFBsFv(L1), triangles; AngsFv(L2), squares.

Phe, respectively) 14 amino acid residue linker described by (Bird et al., 1988; EGKSSGSGSESKEF, L1) or a 15 amino acid residue linker, $(GGGGS)_3$ (L2), described by Huston et al. (1988). A tail consisting of three histidyl residues was included at the 3′ end of the V_H chain to facilitate purification of the fusion protein by Ni^{2+}-NTA-agarose. The assembled genes were then inserted into the pET-11d vector which has been used for the expression of toxic gene products (Studier et al., 1990). All constructs were sequenced to ensure that no sequence errors due to the PCR technique had occurred. The fusion proteins illustrated in Figure 1 were expressed in *E. coli* BL21 (DE3). SDS−polyacrylamide gel electrophoresis (Figure 2) shows they were >95% pure by both Coomassie blue staining and by Western blot analysis using a specific antibody against human Ang.

Binding of AngsFvs to the Transferrin Receptor. Binding of AngsFvs to tumor cells that overexpress the human transferrin receptor was compared using an ^{125}I-labeled E6 mAb displacement assay (Figure 3). The two fusion proteins containing the FB spacer, AngFBsFv(L1) and AngFBsFv(L2), differing only in regard to the type of linker used, exhibited differences in receptor binding. The fusion protein containing the L2 linker bound approximately 2.3-fold stronger (from 50% displacement values determined directly

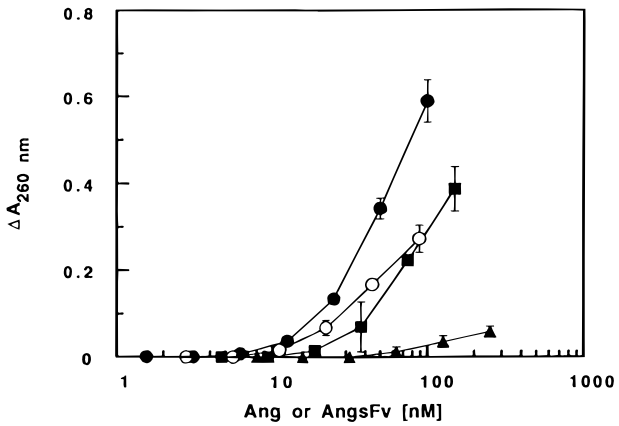

FIGURE 4: Ribonucleolytic activity of Ang and AngsFvs. Acid soluble tRNA fragments were measured as described in Materials and Methods. The data from a representative experiment are plotted ±SEM. Ang, solid circles; AngFBsFv(L2), open circles; AngFBsFv(L1), solid squares; AngsFv(L2), solid triangles.

from the semilogarithmic dose−response curves; 30-fold excess needed compared to E6) than the fusion protein designed with the L1 linker (70-fold excess needed compared to E6). In addition, the presence of the FB spacer also influenced receptor binding. In its absence, approximately 400-fold more AngsFv(L2) was needed to compete for E6 binding compared to 30-fold more for the AngFBsFv(L2) construct containing the spacer. This indicates that the FB spacer most likely allows the V_H and V_L chains to form a receptor binding domain of greater binding affinity, most likely by forming a natural hinge region that provides more flexibility between the sFv and effector protein (Huston et al., 1991).

Ribonuclease Activity of AngsFvs. Ang does not possess degradative ribonucleolytic activity toward most RNase A substrates (Shapiro et al., 1986). However, an assay based on using tRNA as an Ang substrate (Shapiro et al., 1987b) has been used successfully to characterize the enzymatic activity of Ang and Ang mutants (Lee & Vallee, 1989). Therefore, a comparison of the ribonucleolytic activities of AngsFvs with native Ang was made utilizing tRNA as substrate. The two proteins containing the FB spacer were approximately 50% as active as Ang in this assay (Figure 4). To achieve a $\Delta A_{260\ nm}$ of 0.2 required 30 nM angiogenin and 55 nM and 65 nM of AngFBsFv(L2) and AngFBsFv(L1), respectively. AngsFv(L2), without the FB spacer, possessed very little activity at the highest concentration tested (250 nM).

Inhibition of Translation in the Rabbit Reticulocyte Lysate. Ang is a potent inhibitor of the translational capacity of the rabbit reticulocyte lysate by a mechanism that depends upon its ribonucleolytic activity (St. Clair et al., 1987). As depicted in Figure 5, the addition of Ang to a rabbit reticulocyte lysate caused the inhibition of protein synthesis as measured by the incorporation of [^{35}S]methionine into acid-precipitable protein. Whereas Ang inhibited protein synthesis with an IC_{50} of 9 nM, both AngFBsFv(L1) and AngFBsFv(L2) were approximately 6-fold less active. The similarity in activity between these two immunofusion proteins demonstrates that their capacity to inhibit translation is seemingly independent of the type of linker used to connect the V_L chain and the V_H chains. Consistent with the receptor binding and RNase assay data shown in Figures 3 and 4, absence of the FB spacer resulted in a substantial loss of

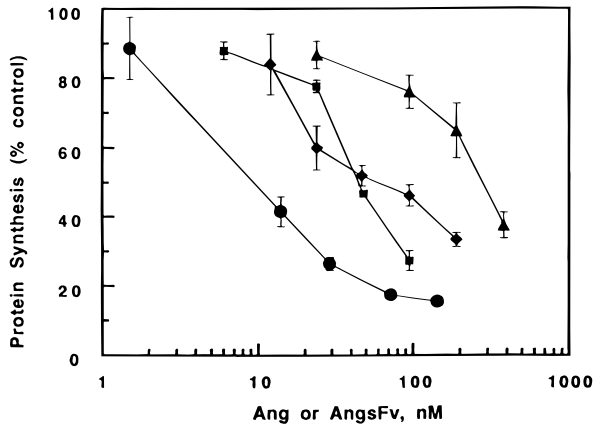

FIGURE 5: Translation of mRNA in the presence of Ang or AngsFvs. Ang or AngsFvs were added to a lysate mixture containing BMV mRNA and [^{35}S]methionine. Protein synthesis was determined by measuring the incorporation of label into newly synthesized proteins as described in Materials and Methods. Data from 2−3 experiments were pooled, averaged, and plotted ±SEM. The results are expressed as percentages of a control reaction. The IC_{50} values, the concentration of Ang or AngsFvs that caused a 50% inhibition of protein synthesis, were determined directly from the semilogarithmic dose response curves. Ang, circles; AngFBsFv(L2), squares; AngFBsFv(L1), diamonds; AngFv(L2), triangles.

activity [AngsFv(L2); IC_{50}, 300 nM]. These results taken together demonstrate that the placement of a spacer between the enzymatic and binding moieties of the fusion protein is critical for increasing the activities of both of these domains in the resultant fusion protein.

Inhibition of Ang Fusion Proteins by Placental Ribonuclease Inhibitor. Placental ribonuclease inhibitor (PRI), an RNase inhibitor, binds tightly to Ang and abolishes both its angiogenic and ribonucleolytic activities (Shapiro & Vallee, 1987) as well as its protein synthesis inhibitory activity in the rabbit reticulocyte lysate (St. Clair et al., 1987). Therefore, the capacity of PRI to inhibit the activity of AngsFvs was examined in the lysate system. As shown in Figure 6, the activity of Ang and only one of the fusion proteins, AngFBsFv(L2), was decreased by 40 units of PRI. Whereas there was a difference in IC_{50}s of Ang (90 vs 10 nM in the presence or absence of PRI, respectively) and AngFBsFv(L2) (250 vs 45 nM in the presence or absence of PRI, respectively), the other two proteins were unaffected by the RNase inhibitor. Furthermore, increasing the concentration of PRI still did not inhibit the activities of the latter two fusion proteins. The effects of Ang (143 nM) on protein synthesis was almost completely reversed by 40 units of PRI, while up to 120 units of PRI did not affect the activity of either AngFBsFV(L1) (95 nM) or AngsFv(L2) (190 nM, Figure 6D).

Binding of Anti-Ang Antibodies to Ang Fusion Proteins. Two anti-Ang mAbs (26-2F and 36u) with different epitope specificities as well as a polyclonal anti-Ang antibody were tested for their capacity to bind to AngsFvs. Both mAbs displayed a similar pattern of binding to the AngsFvs relative to Ang, with the largest decreases in binding observed for AngFBsFv(L1) (18.7% and 22.6% of the binding to Ang for mAbs 26-2F and 36u, respectively; Table 1). The binding of mAbs 26-2F and 36u to AngsFv(L2) (73.9% and 83.4%, respectively) and AngFBsFv(L2) (78% and 81.5%, respectively) demonstrates that the epitopes (or access thereof) were less altered when the L2 linker was used and that the presence or absence of the spacer had little effect. Polyclonal antibody, R113, binding was again the most diminished toward AngFBsFv(L1) (14.8%, Table 1). Unlike the mAbs, binding of R113 was moderately affected by the spacer, as shown by the improvement in the relative binding of R113 to AngFBsFv(L2) in comparison to AngsFv(L2) (67.8% vs 48.7%). To determine if the decreased binding of the anti-Ang antibodies resulted from insufficient coating of the AngsFvs to the ELISA wells, a second type of ELISA was employed. In this assay, the wells were coated with either mAb 26-2F or mAb 36u. Ang or AngsFvs in solution was then incubated in the wells, and their presence was detected by the addition of R113. As shown in Table 1, the pattern of binding of antibodies to AngsFvs was consistent with the previous ELISA results. Thus, both the choice of linker and the presence of a spacer contribute to the extent of conformational change of the AngsFvs, with AngFBsFv(L2) exhibiting the least alteration as detected by anti-Ang antibodies.

Angiogenic Activity of the AngsFvs. Angiogenic activity was assessed in the CAM. Since multiple mutagenesis studies have explored the relationship between enzymatic and angiogenic activities of Ang (Hallahan et al., 1991, and references therein), this study only addressed one question: Does Ang retain angiogenic activity when expressed as a fusion protein? For this reason, experiments were performed using maximal concentrations of Ang (Fett et al., 1985) and the molar equivalent of each fusion protein. Analysis by the χ^2 test indicated that Ang (10 ng/egg) was active when compared to water controls (Table 2, 47% positive responses, $p = 0.0219$). Molar equivalents of all three fusion proteins displayed angiogenic activity as well: AngFBsFv(L2), 55%; AngFBsFv(L1), 62%; AngsFv(L2), 50% positive responses, $p < 0.003$. These results demonstrate that Ang still retains its biological activity following fusion to a single-chain antibody.

AngsFvs Inhibit Protein Synthesis in Three Human Tumor Cell Lines. The cytotoxic effect of AngsFvs on human tumor cells was assessed by measuring [^{14}C]leucine incorporation into newly synthesized proteins. As depicted in Figure 7, the FB-containing protein designed with the L2 linker was consistently more cytotoxic than that with the L1 linker [IC_{50s} of 10, 7, and 4 nM (for L2 containing) vs 30, >100, and 20 nM (for L1 containing)] in MDA-231^{mdr1}, HT-29^{mdr1}, and ACHN cells respectively. AngsFv(L2) without the FB spacer was the least cytotoxic (IC_{50s} of, 40, >100, and >100 for the same cell lines, respectively). In contrast to the fusion proteins, free Ang was not cytotoxic. No inhibition of protein synthesis was observed on MDA-231^{mdr1} cells at the highest concentration of Ang tested (0.85 μM, data not shown), consistent with the biological actions of this protein (Riordan & Vallee, 1988). In addition, no activity of AngFBsFv(L2) was observed on an antigen negative cell line (mouse NIH3T3 cells, data not shown). To facilitate a comparison of the different activities of the AngsFv fusion proteins, functional data presented in Figures 3, 5, and 7 are compared in Table 3.

DISCUSSION

Ang, a member of the human RNase A superfamily, is a component of normal human plasma (Shapiro et al., 1987a), is not itself cytotoxic to human cultured cells, yet is a potent

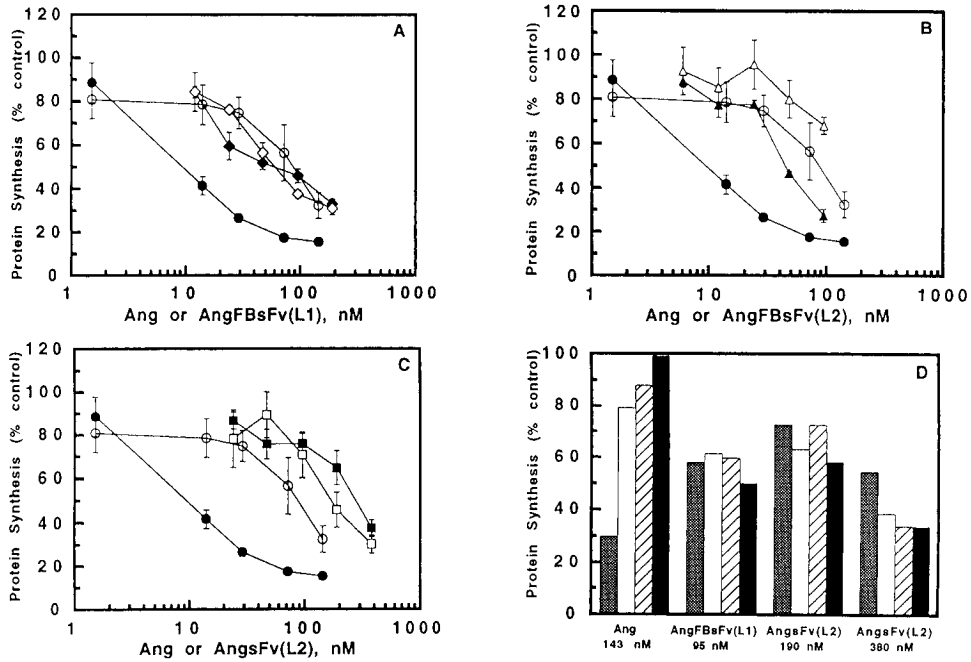

FIGURE 6: Effect of PRI on the inhibition of *in vitro* translation by Ang and AngsFvs. *In vitro* translation was performed as described in the legend to Figure 5 and Materials and Methods. (Panels A−C) Solid symbols, assays performed in the absence of PRI; open symbols, assays performed in the presence of 40 units of PRI. Data from 2−3 experiments were pooled, averaged, and plotted ±SEM. Ang, circles; AngFBsFv(L1), diamonds; AngFBsFv(L2), triangles; AngsFv(L2), squares. The IC_{50} values, the concentration of Ang or AngsFvs that caused a 50% inhibition of protein synthesis, were determined directly from the semi-logarithmic dose response curves. (Panel D) Assays using 143 nM Ang, 95 nM AngFBsFv(L1), and 190 or 380 nM AngsFv(L2) were performed with increasing concentrations of PRI. Stippled bars, no PRI; open bars, 40 units PRI; striped bars, 80 units PRI; solid bars, 120 units PRI. Data from 2−3 experiments were averaged.

Table 1: Binding of Anti-Ang Antibodies to AngsFvs

antibody	AngFBsFv(L1)	AngsFv(L2)	AngFBsFv(L2)
mAb 26−2F[a]	18.7 ± 1.3[b]	73.9 ± 2.7	78.0 ± 2.4
mAb 36u[a]	22.6 ± 0.8	83.4 ± 0.6	81.5 ± 1.4
R113[a]	14.8 ± 0.5	48.7 ± 0.9	67.8 ± 2.0
mAb26-2F/R113[c]	5.7 ± 0.1[d]	9.1 ± 1.4	19.1 ± 0.3
mAb36u/R113[c]	4.3 ± 0.5	7.4 ± 0.1	12.9 ± 0.5

[a] Binding ELISA in which AngsFvs are coated on the wells followed by incubation with the mediated antibody. [b] Binding of the indicated antibody to the sFv, as a percentage of binding of that antibody to Ang ± SEM. [c] Double antibody ELISA using indicated combination of antibodies. [d] Binding of the sFv indicated to the combination of antibodies as a percentage of the binding of Ang to the antibody combination ± SEM.

Table 2: Activity of AngsFv Fusion Proteins in the CAM Assay

protein	ng/egg	assay results[a]	p^b	status
Ang	10	9/19 (47)	0.0219	active
AngFBsFv(L1)	30[c]	25/40 (62)	<0.0001	active
AngFBsFv(L2)	30[c]	22/40 (55)	0.0008	active
AngsFv(L2)	30[c]	20/40 (50)	0.0034	active
imidazole	−[d]	3/16 (19)	0.9773	inactive

[a] Percent positive in parentheses. [b] Chi-squares results tested versus water (7 positive/38 surviving, 18% positive). [c] Molar equivalent of 10 ng of angiogenin. [d] Vehicle control (diluted 1:15 with water).

inhibitor of translation in the cell-free rabbit reticulocyte lysate (St. Clair et al., 1987) and when injected into *Xenopus* oocytes (Saxena et al., 1991). These studies prompted the development of methods to specifically internalize human RNases for the purpose of selective cell killing. In this regard, Ang genetically fused to the C_H2 domain of an antitransferrin receptor antibody and expressed in a myeloma cell engineered to secrete the light chain of the same antibody was selectively cytotoxic to human leukemia cells at concentrations as low as 1×10^{-11} M (Rybak et al., 1992). Unfortunately, low levels of expression prohibited purification to homogeneity. To obtain sufficient quantities of RNase fusion proteins for further evaluation, Ang was expressed in *E. coli* as part of a single-chain antibody immunofusion. The gene for Ang was fused to a single-chain antibody that recognized the human transferrin receptor. Three different configurations of this fusion protein were designed and the most effective identified. These chimeric proteins (1) bound to the transferrin receptor, (2) retained RNase activity as shown by two different assays (degradative RNase assay using tRNA as the substrate and protein synthesis inhibition in the rabbit reticulocyte lysate assay), and (3) retained angiogenic activity in the CAM assay. In addition, the immunofusion proteins were cytotoxic to human tumor cells that overexpressed the human transferrin receptor.

Recent reviews describe sFvs that have been fused to effector proteins such as toxins (Brinkmann & Pastan, 1994) or enzymes (Haber, 1994) to yield bifunctional proteins. The design of these fusion proteins has to accommodate the interaction of the sFv with its receptor as well as the activity of the effector molecule. The L1 linker (Bird et al., 1988) (EGKSSGSGSESKEF) used in these studies was modified by replacing residues 13 and 14, a serine and threonine, respectively, with glutamic acid and phenylalanine (Nicholls et al., 1993). This linker was used previously to construct a fusion protein containing the anti-human transferrin receptor sFv and EDN, a human eosinophil RNase (Newton et al., 1994). Although EDNFBsFv(L1) specifically targeted tumor cells, it was unstable and aggregation limited the concentrations achievable. Linker related sFv aggregation has been described by others suggesting that linkers may need to be varied for different applications. For this reason, we designed AngsFvs with two different peptide linkers (L1

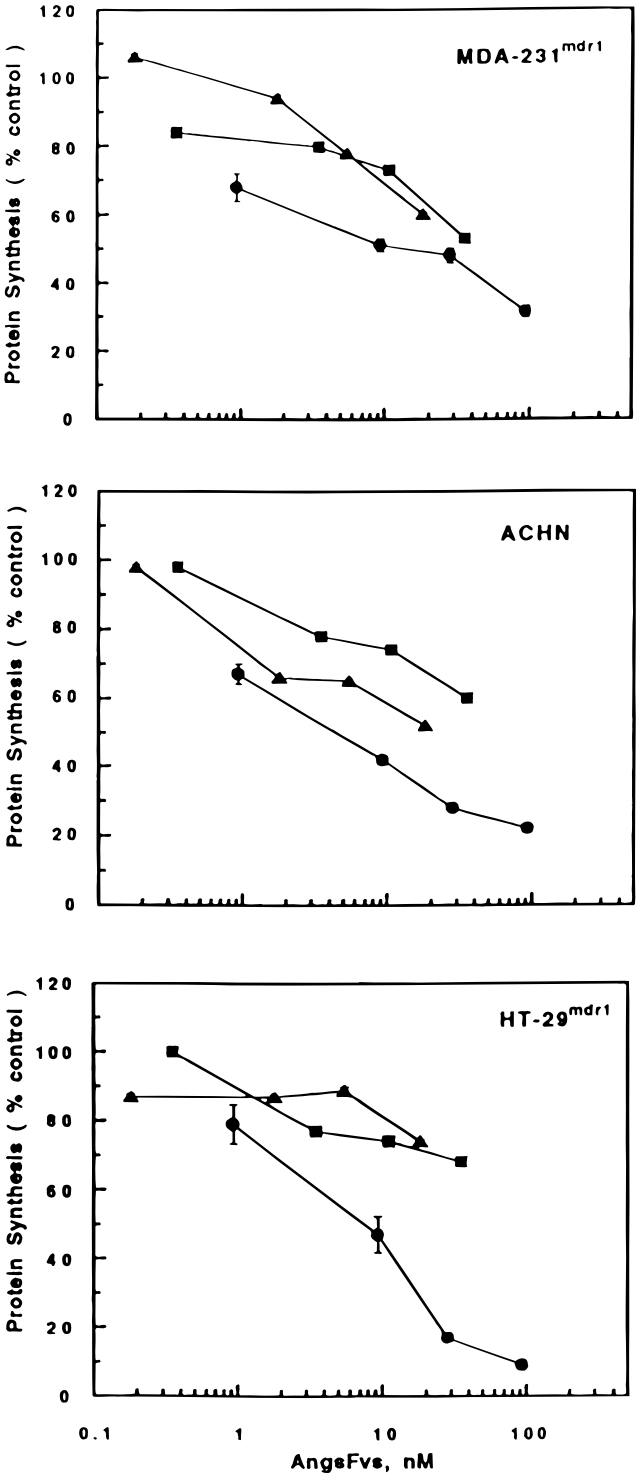

FIGURE 7: *In vitro* toxicity of AngsFvs to three human tumor cell lines as assessed by protein synthesis inhibition. Cytotoxicity assays were performed by measuring the incorporation of [^{14}C]leucine into cell proteins as described in Materials and Methods. The assays were conducted in the presence of serum and changed to leucine- and serum-free medium prior to pulsing with [^{14}C]leucine. The IC_{50} values, the concentration of AngsFvs that caused a 50% inhibition of protein synthesis after a 60−70 h incubation, were determined directly from the semilogarithmic dose−response curves. (Top panel) MDA-231^{mdr1} human breast cancer cells. (Middle panel) ACHN renal carcinoma cells. (Bottom panel) HT-29^{mdr1} colon carcinoma cells. AngFBsFv(L2), circles; AngFBsFv-(L1), triangles; AngsFv(L2), squares. The SEM is shown when it is larger than the symbol.

and L2). Linker length and sequence control characteristics such as charge and conformation; thus, their design has been governed by the structural constraints of the Fv region (Huston et al., 1991). Accordingly, the three GGGGS units that constitute the L2 linker were designed to span the distance between the amino and carboxyl terminals of the variable domains without distorting the conformation of the Fv thus providing a flexible connection (Huston et al., 1988).

Table 3: Comparison of the Properties of the AngsFv Fusion Proteins

	binding EC_{50}[a] (nM)	protein synthesis inhibition IC_{50}[b] (nM)	antitumor activity IC_{50}[c] (nM)		
			ACHN	MDA-231^{mdr1}	HT-29^{mdr1}
AngFBsFv (L2)	30	45	4	10	7
AngFBsFv (L1)	70	60	20	30	>100
AngsFv (L2)	400	300	>100	40	>100
Ang		9		>850	
E6IgG	1				

[a] EC_{50} is the concentration of protein necessary to displace 50% of the [^{125}I]E6IgG monoclonal antibody from the human transferrin receptor. The values are calculated from Figure 3. [b] IC_{50} is the concentration of protein necessary to inhibit protein synthesis by 50% in the rabbit reticulocyte lysate. The values are calculated from Figure 5. [c] IC_{50} is the concentration of protein necessary to inhibit protein synthesis by 50% in three different human tumor cell lines. The values are calculated from Figure 7.

EDNFBsFv(L1) (Newton et al., 1994) competed with E6, the parent IgG monoclonal antibody described by (Hoogenboom et al., 1990) for binding to the human transferrin receptor. As shown for AngFBsFv(L1), approximately 70-fold more of EDNFBsFv(L1), compared to the parent E6 antibody, was required to compete for labeled E6 binding to the transferrin receptor. However, in the current study, only 30-fold excess of the Ang immunofusion protein designed with the L2, $(GGGGS)_3$, linker was required for competition. Thus, binding affinities of the two fusion proteins designed with L1 were not affected by the nature of the RNase (Ang or EDN), yet changing the L1 to the L2 linker increased binding 2.3-fold when the constructs were designed with the same RNase. Additionally, higher concentrations of stable, properly folded material were obtained (400−600 μg/mL with L2 vs 100−200 μg/mL with the L1 linker).

The influence of a spacer connecting the enzymatic and binding sites of RNase immunofusions also was investigated. Residues 48−60 of fragment B of staphylococcal protein A (FB) was inserted between the two domains since it may represent a natural hinge region in the FB-sFv$^{26\text{-}10}$ described by (Tai et al., 1990). In the present study, the FB-connecting region was shown to enhance the activity of both domains of the fusion protein. Expression of the AngsFvs without a spacer resulted in a 13-fold decrease in receptor binding compared to constructions with the same L2 linker but containing the FB spacer. The enzymatic activity of Ang was similarly decreased when assessed by two enzymatic assays. Since this sensitive site can also influence folding kinetics, aggregation, and the yield of correctly folded molecules (Brinkmann et al., 1992), the optimal length and structure of the spacer are being studied currently.

Overall differences in conformation of AngsFvs were detected with PRI, a ribonuclease inhibitor that binds and inactivates angiogenin (Shapiro & Vallee, 1987), as well as with anti-Ang monoclonal and polyclonal antibodies (Fett et al., 1994). The activity of Ang or Ang as part of AngFBsFV(L2) was inhibited to a similar extent by PRI.

Furthermore, AngFBsFv(L2) was recognized by two different mAbs almost as well as Ang. Conversely, high concentrations of PRI did not affect the activity of AngFBsFv(L1) nor was this fusion protein recognized appreciably by anti-Ang antibodies. AngFBsFv(L2) and AngFBsFv(L1) that differed only in the nature of the linker (L1 vs L2) between the antibody V_H and V_L domains exhibited similar activity in two separate enzymatic assays. It thus appears that the integrity of the catalytic site is maintained similarly in both of these fusion proteins. However, as previously discussed, the AngsFv constructed with the L1 linker bound more weakly to the transfer receptor implying that the antibody portion was not correctly folded. These data coupled with those from the PRI-and anti-Ang antibody binding studies suggest that (1) an altered sFv conformation sterically hindered PRI and antibody binding to Ang in AngFBsFv(L1), (2) L1 altered the conformation of Ang in the immunofusion such that neither the antibody epitopes nor PRI contact sites were accessible, or (3) a combination thereof.

The construct lacking the FB region, AngsFv(L2), was the least active of the fusion proteins with respect to enzymatic activity and binding to the transferrin receptor. Additionally, it no longer interacted with PRI although, both anti-Ang mAbs recognized Ang in this fusion protein. Thus, amino acid residues 38−41 plus 89 (Fett et al., 1994) as well as 58−73 (K.A.O., unpublished results) were available for direct interaction with the antibodies. Previously, mAb 26-2F was shown to inhibit both ribonucleolytic and angiogenic activities of Ang (Fett et al., 1994) while interacting with an epitope (38−41 plus 89) most likely by affecting Lys 40 in the RNase active site. Therefore, the change in Ang conformation caused by the constraints imposed by lack of a spacer could have affected the accessibility of the RNA substrate rather than altered the catalytic site. Irrespective of the exact nature of the conformational defect, the results demonstrate that a spacer between the antibody and effector RNase is essential for optimal activity of both domains.

As discussed above, the nature of the linker separating the V_H and V_L chains of the antibody and the presence of a spacer affected the receptor binding and enzymatic activities as well as the conformation of the immunofusion proteins. Differences in cellular cytotoxicity also were observed. As predicted by binding and enzymatic activities, the AngsFv containing the L2 linker and FB spacer was the most cytotoxic. In another study, a series of anti-Tac(Fv)-PE40 fusion proteins designed with these and other linkers did not exhibit differences in immunotoxin activity (Batra et al., 1990), indicating that smaller differences in cytotoxicity may be masked by variable amounts of improperly folded protein in different constructs. Also, it is possible that the effects of linker may be amplified in certain Fv regions, and that the impact of spacer design will vary for different toxin and RNase fusion partners.

In this study we have identified variations in the design and construction of a single-chain RNase immunofusion that yield bifunctional proteins with markedly different binding and enzymatic activities. Expression of the human RNase, EDN, as a fusion protein with the L2 linker similarly improved the binding, activity, and stability of the molecule (D.L.N., unpublished results) compared to a previous design (Newton et al., 1994). Thus, RNase fusion proteins can be constructed with at least two human RNases, both of which have been reported to have additional interesting biological properties. For example, EDN possesses antiparasitic activities and may also be involved in host defense actions (Youle et al., 1993, and references therein) while Ang promotes neovascularization in the CAM and rabbit corneal (Fett et al., 1985) and meniscus assays (King & Vallee, 1991). Importantly, the potency of the RNase immunofusions is markedly improved compared to RNase chemical conjugates. Bovine pancreatic RNase A conjugated to 454A12, an antihuman transferrin receptor monoclonal antibody, inhibited protein synthesis in human leukemia cells with an IC_{50} of 600 nM (Newton et al., 1992) compared to IC_{50s} between 4 and 10 nM for the most potent AngsFv. These concentrations compared favorably to concentrations needed to achieve 50% protein synthesis inhibition of antitransferrin receptor conjugates made with plant or bacterial toxins such as gelonin (2−5 nM) (Yazdi et al., 1995) or fusion proteins with the same E6sFv and either PE40 or CRM107 (IC_{50}, 0.1 and 1 nM, respectively) (Nicholls et al., 1993). Problems of toxicity and immunogenicity have hampered the clinical development of immunotoxins containing plant and bacterial toxins (Rybak & Youle, 1991); thus, it is expected that comparably potent immunofusions made with a human plasma protein such as Ang could alleviate these problems.

The basis for selective cell killing of immunotoxins is the overexpression of tumor associated receptors (Pastan et al., 1986; Vitetta et al., 1987). Thus, it is expected that the overexpression of the transferrin receptor on tumor cells, relative to normal tissue, will target AngsFvs to the tumor whereby internalization via the transferrin receptor will result in specific antitumor effects. Possibly, the biological properties of Ang may promote enhanced selective tumor cell killing *in vivo*. In the current study, we show that angiogenic activity is maintained even when Ang is expressed as a fusion protein. The interaction of Ang with its putative endothelial binding site (Badet et al,. 1989) is, therefore, preserved. In addition to endothelical cells, Ang has been reported to bind a cell-surface proteoglycan on colon carcinoma cells (Soncin et al., 1994). Thus, an AngsFv could potentially function as a bispecific molecule; the Ang domain enhancing retention in the tumor through its tumor and endothelial associated binding sites while the sFv portion directs the fusion protein to and into a tumor cell. This could be significant since sFvs, due to their small size, exhibit better tumor-penetrating properties than Fab's or IgG's but also are cleared more rapidly from the blood stream (Sutherland et al., 1987; Yokota et al., 1992; Covell et al., 1986; Shealy et al., 1990; Milenic et al., 1991; Colcher et al., 1990).

Importantly, there is precedence for using an angiogenic factor, basic fibroblast growth factor (bFGF; Lobb et al., 1985, and references therein) in the construction of immunotoxins. It has been linked chemically and recombinantly to saporin (SAP), a plant-derived ribosome-inactivating protein (Beitz et al., 1992). This FGF-SAP fusion protein was cytotoxic specifically to tumor cells *in vitro* and *in vivo*. This indicates that FGF-SAP functions as an immunotoxin in tumor-bearing animals rather than as an angiogenic factor, presumably because of the overexpression of FGF receptors on tumor cells relative to normal tissue.

Similarly, if AngsFvs are administered to animals bearing human tumors or human cancer patients, it is expected that the dynamics of the receptor−ligand interaction will be governed by the antitransferrin receptor sFv relative to the Ang binding sites on endothelial cells. In this regard, Ang-

driven angiogenesis in humans must be very tightly controlled process since Ang is present in human plasma at concentrations of approximately 0.4 μg/mL, is continually in contact with the luminal surface of endothelial cells, does not appear to be tightly associated with any carrier or inhibitory molecules (Shapiro et al., 1987a), yet obviously does not promote rampant angiogenesis. These observations, taken together, suggest that an AngsFv should not promote angiogenesis *in vivo*. This hypothesis is being tested currently in a murine model of angiogenesis using a murine homolog of AngsFv.

In summary, Ang can be expressed as a targeted immunofusion protein retaining both its biological and enzymatic activities. Sufficient quantities of stable material can be purified to homogeneity enabling preclinical evaluation of AngsFvs for their potential as a new class of antitumor agents.

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