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Angiogenin antagonists prevent tumor growth *in vivo*

(angiogenesis/neoplasia/monoclonal antibody/athymic mice)

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ABSTRACT **A noncytotoxic neutralizing monoclonal antibody (mAb), 26-2F, to human angiogenin (Ang), a potent inducer of neovascularization, has been reported to prevent or delay the establishment of HT-29 human tumor xenografts in athymic mice. In the present study the tumor model was modified to increase sensitivity to Ang antagonists to facilitate further investigations and comparisons of their capacity to inhibit tumor growth. An increase in the percentage of tumor-free mice from 10–25% to 65% is observed in this modified model after treatment with mAb 26-2F. An additional neutralizing mAb, 36u, that interacts with a different epitope on Ang similarly prevents the appearance of tumors, both alone and in combination with mAb 26-2F. In those tumors that develop in mice treated with these agents, the number of vascular elements is reduced. Actin, an Ang antagonist that unlike the mAbs binds both human and mouse Ang, also prevents the establishment of tumors while exhibiting no toxic effects at daily doses >50 times the molar amount of circulating mouse Ang. Ang antagonists also inhibit the appearance of tumors derived from two other Ang-secreting human tumor cell lines—i.e., A549 lung adenocarcinoma and HT-1080 fibrosarcoma. These results demonstrate that inhibition of the action of Ang is an effective therapeutic approach for the treatment of malignant disease.**

Angiogenesis is crucial in numerous physiological and pathological processes. In malignancy, both the growth of a primary tumor and its metastatic spread to distant sites require the constant elicitation of host blood vessels by tumor-associated inducer molecules (for review, see refs. 1 and 2). One of these most likely is angiogenin (Ang), a 14.1-kDa protein isolated originally from medium conditioned by HT-29 human colon carcinoma cells (3) and subsequently from normal serum (4) and milk (5). It is a heparin-binding protein (6) whose three-dimensional structure has been determined recently (7). Ang is homologous in amino acid sequence to pancreatic RNase (8)—although differing markedly in enzymatic activity (9)—and contains distinctive catalytic and cell-binding domains that are essential for its angiogenic activity (10, 11). It activates intracellular second-messenger pathways (12), supports tumor and endothelial cell adhesion (6, 13), and undergoes nuclear translocation (14). A 42-kDa Ang-binding protein isolated from the surface of cultured bovine endothelial cells has been identified as a smooth muscle type of α-actin (15–17). It, like commercial actin preparations, binds tightly not only to bovine but also to mouse and human Ang (G.-F. Hu, personal communication). The demonstration that both actin and antiactin antibodies inhibit Ang-induced angiogenesis in the chicken embryo chorioallantoic membrane underscores the functional relevance of this interaction (16).

The inhibition of angiogenesis is an attractive therapeutic target for the treatment of both primary and metastatic cancer. A preliminary study showed that the growth of HT-29 cells in athymic mice is Ang-dependent and that a specific neutralizing monoclonal antibody (mAb) to human Ang is capable of preventing or delaying the appearance of tumors (18). The present study, using a more sensitive model system, demonstrates a dramatic increase in the efficacy of mAb 26-2F in preventing the appearance of HT-29 tumors. Furthermore, two additional Ang antagonists, mAb 36u—which differs in epitope specificity from mAb 26-2F—and actin, are equally effective in preventing the establishment of this tumor. Importantly, the capacity of actin to interfere with human tumor growth in the athymic mouse provides a means to investigate both the potential toxicity and the effectiveness of an Ang antagonist under conditions where the antagonist may bind to endogenous mouse Ang. In addition, the data show that the growth of tumors of two different histological types is inhibited by treatment with Ang-neutralizing mAbs.

MATERIALS AND METHODS

Materials. Male outbred athymic mice (Crl:*nu/nu*) were obtained at 5 weeks of age from the isolator bred colony of Charles River Laboratories at Wilmington, MA, and maintained under specific pathogen-free conditions in a temperature- and humidity-controlled environment. The human tumor cell lines HTB-38 colon adenocarcinoma (HT-29), CCL-121 fibrosarcoma (HT-1080), and CCL-185 lung carcinoma (A549) were obtained from the American Type Culture Collection and cultured as described for HT-29 cells (18). mAbs 26-2F and 36u, both IgG1κ mAbs that bind to human Ang with similar affinity, were obtained as described (19). mAb 26-2F, which recognizes a discontinuous epitope on Ang that encompasses residues 38–41 and 89, neutralizes both the ribonucleolytic and angiogenic activities of Ang (19). mAb 36u also inhibits these activities of Ang and interacts with a different region of the molecule that includes residues within the segment 58–73 (K.A.O., unpublished results). MOPC 31C, a nonspecific IgG1κ-secreting mouse hybridoma, was obtained from the American Type Culture Collection (no. CCL-130). All three immunoglobulins were purified from ascites fluid and quantified by ELISA as described (19). Bovine muscle α-skeletal actin was purchased from Sigma.

Anti-Tumor Activity *in Vivo*. This was determined by modifying a described protocol (18). Viable tumor cells (1.25×10^5 cells per mouse) were mixed with antagonist(s), MOPC 31C, or phosphate-buffered saline (PBS) diluent control and injected (150 μl) s.c. behind the left shoulder of age-matched mice (6–8 weeks old; median number of mice per group = 10). Daily injections (100 μl) were then administered for 35 days s.c. in the area of the initial injection. Each experiment included a PBS control group that was compared by survival analysis to the values of the pooled PBS controls to ensure that tumor growth was consistent with the accumulated control data. In 2 out of 15 cases experiments were not included in a

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Abbreviations: Ang, angiogenin; mAb, monoclonal antibody.

particular data set due to a significant difference in PBS-treated tumor growth. Mice were examined daily by palpation for the first sign of tumor appearance at which time caliper measurements were begun (three times per week) and reported as tumor volume (length × width × depth). Tumor-free mice were observed minimally until day 56 and in some cases for as long as day 153. After sacrifice, the area of injection was examined extensively histologically to determine whether previously undetected tumors were present.

Histopathology. Tumor tissues were fixed in buffered formalin, paraffin-embedded, incubated with either a primary rabbit anti-human factor VIII antibody (1:125 dilution; Dako) or nonimmune rabbit IgG, and stained by using a streptavidin/biotin/immunoperoxidase kit (Dako) according to the manufacturer's instructions. Immunostained slides were counterstained with hematoxylin.

Anti-Tumor Activity *in Vitro*. Direct cytotoxicity of HT-29 cells by Ang antagonists was evaluated by using a described [^{3}H]thymidine incorporation assay (18).

Statistical Analysis. The capacity of agents to prevent and/or delay tumor appearance was assessed by using the Kaplan–Meier product-limit method to estimate survivor functions. Mantel–Cox tests of equality of survivor functions (P significant at 0.05) were computed with BMDP program 1L (20). Survival analysis measures the time-to-response, which, in this case, is the time-to-appearance of a palpable tumor. One-tailed Wilcoxon matched-pair tests (Simstat 2.1b; Provalis Research, Montreal) were used to analyze differences in vascular density between size-matched tumors from mAb- and PBS-treated groups (P significant at 0.05).

RESULTS AND DISCUSSION

The earlier study demonstrated that the establishment of HT-29 tumors in athymic mice was delayed significantly and in some cases prevented completely by the administration of the Ang-neutralizing mAb 26-2F (18). These effects were observed above a threshold dose of mAb dependent on the number of tumor cells injected (i.e., 6 μg for 1×10^6 and 3 μg for 5×10^5 tumor cells injected, Table 1). Increasing the mAb dose substantially above this level, however, did not significantly improve the therapeutic outcome. Thus, while inhibition of tumor growth by Ang antagonism was demonstrated, modification of the model used was deemed necessary to increase sensitivity for further analysis and comparison of mAb 26-2F and additional Ang antagonists.

Modified Protocol for Examination of the Effect of Ang Antagonists on HT-29 Tumor Growth. As previously, athymic mice are injected s.c. on day 0 with a mixture of HT-29 human adenocarcinoma cells and either control or antagonist solutions. Subsequently daily treatments are administered at the site of initial tumor cell injection. The previous study indicated that the period of sensitivity to Ang antagonism occurred between the time of initial tumor cell injection and the appearance of a palpable tumor; hence, this period of time was increased by reducing the number of HT-29 cells administered to potentially render the tumors more sensitive to the Ang antagonists. Initially, several different numbers of HT-29 tumor cells were injected s.c. to determine the lowest number that would produce tumors in essentially all of the mice. Because injection of 1.25×10^5 cells per mouse consistently produced tumors in all but 3% of the PBS-treated mice, this number of tumor cells was used for all further experiments with HT-29 cells. Additionally, an increased period of treatment through day 35 was adopted because 9% of all mAb 26-2F-treated tumors that developed in the initial study appeared subsequent to the last mAb injection (day 14) and to compensate for the increased mean time for tumors to appear (12.6 days) with 1.25×10^5 cells as compared to 5.4 days with 5×10^5 cells. Because previously the primary therapeutic effect of mAb 26-2F was on the temporal appearance of HT-29 tumors rather than on their growth after establishment, survival analysis is used here to focus exclusively on the capacity of mAb 26-2F and other Ang antagonists tested to affect tumor appearance.

Effect of mAb 26-2F Treatment on Tumor Growth Is Increased in the Modified Tumor Model. In the previous study (18) 10–25% of the mice in the mAb 26-2F-treated groups that exhibited successful prevention and delay of tumor growth ($P \leq 0.0001$) were tumor-free after treatment, with no significant difference in the percentages of tumor-free mice among these groups (Table 1). With the modified protocol, 30 μg of mAb 26-2F was administered daily for 35 days at the 1.25×10^5 injected cell number. While this regimen is successful in preventing tumors, as indicated by the significant difference in the survivor function as compared with that of the PBS controls ($P = 0.0003$), the percentage of mice remaining tumor-free, 14%, is well within the range of that obtained previously. However, when mice are treated with 60 μg of mAb 26-2F, not only is the treatment successful as determined by survival analysis ($P < 0.0001$), but the percentage of tumor-free mice increases markedly. In this group, in which the ratio of mAb to tumor cells is now increased by 60% above the highest ratio used in the previous protocol, 65% of the mice are tumor-free at the termination of treatment (Table 1). Comparison of the results obtained with the two different doses of mAb 26-2F in the modified protocol appears to indicate that the increased percentage of tumor-free mice in the 60-μg dose group is attributable primarily to the increase in mAb/cell ratio and not to other alterations in the protocols—i.e., treatment duration, lower number of tumor cells injected, increased time to tumor appearance. An investigation using several other doses of mAb 26-2F in the two protocols would be required to demonstrate that the increased mAb/cell ratio is both necessary and sufficient to increase the percentage of tumor-free mice. However, the results indicate that the modification of the protocol is successful in providing a more sensitive system in which to test and compare Ang antagonists.

Table 1. Efficacy of mAb 26-2F in preventing growth of HT-29 tumor xenografts in athymic mice as determined by survival analysis

Tumor cells, no.	Treatment* (μg)	Mice, no.	mAb/cells ratio†	P‡	Tumor-free mice,§ %
1×10^6¶	PBS	50	—	—	0
	26-2F (300)	20	30	<0.0001	10
	26-2F (30)	20	3	<0.0001	15
	26-2F (6)	50	0.6	0.1323	0
5×10^5¶	PBS	110	—	—	0
	26-2F (30)	67	6	<0.0001	25
	26-2F (6)	10	1.2	0.0001	20
	26-2F (3)	10	0.6	0.0774	0
1.25×10^5	PBS	145	—	—	3
	26-2F (60)	20	48	<0.0001	65
	26-2F (30)	14	24	0.0030	14

PBS, diluent control.

*Daily dose of mAb 26-2F was given on day 0–14 (1×10^6 or 5×10^5 tumor cell number) or on day 0–35 (at 1.25×10^5 tumor cell number).

†Dose of mAb 26-2F (μg) divided by number of tumor cells injected ($\times 10^{-5}$).

‡Mantel–Cox P, the probability that the survivor functions of the PBS control and mAb 26-2F-treated groups are identical by chance for all mice treated with the indicated regimen. $P < 0.05$ is significant.

§Number of mice that are tumor-free on day 25–27 (1×10^6 or 5×10^5 tumor cell number) or on day 35 (1.25×10^5 tumor cell number) divided by total number of mice in the group × 100 to yield percentage.

¶Data from groups injected with 1×10^6 or 5×10^5 tumor cell number are from Olson *et al.* (18).

Another Ang-Neutralizing mAb, 36u, Also Prevents HT-29 Tumor Appearance. Treatment with 60 μg of mAb 36u is successful in preventing tumors ($P < 0.0001$, Table 2) and is strikingly similar to treatment with 60 μg of mAb 26-2F, as illustrated by comparison of their survivor functions ($P = 0.732$, Fig. 1*A*). In contrast to the results obtained with mAb 26-2F, however, there is no difference in the percentage of tumor-free mice resulting from the 30- and 60-μg doses of mAb 36u (64 and 56%, respectively). Treatment with the control IgG, MOPC 31C, at the 60-μg dose fails to result in a survivor function that differs from that of the PBS controls ($P = 0.4580$, Table 2), and there are no tumor-free mice at the termination of treatment on day 35.

Treatment with a Combination of mAbs 26-2F and 36u Is Not More Efficacious Than That with the Individual mAbs. To investigate whether a combination of these two mAbs, both of which neutralize the ribonucleolytic and angiogenic activity of Ang but bind to distinctly different epitopes, will function synergistically to prevent tumor appearance, mice were treated with a combined dose of 30 μg each of mAb 26-2F and 36u. Although this treatment results in a significant difference in the survivor function (Fig. 1*A*) in comparison with that of the PBS-treated controls ($P < 0.0001$, Table 2), the results do not differ from those obtained after treatment with 60 μg of either mAb 26-2F ($P = 0.793$) or mAb 36u ($P = 0.996$), nor was there an increase in the percentage of mice remaining tumor-free (Table 2, 60% tumor-free mice). However, because there is no detectable difference between the 30- and 60-μg dose of mAb 36u, it cannot be concluded as yet whether the two mAbs can have an additive therapeutic effect.

Vascular Density Is Decreased in Tumors of Mice Treated with mAb 26-2F or 36u. Histological examination reveals marked differences between 11 pairs of size-matched tumors of mAb- and PBS-treated mice sacrificed within a 3-day period in a representative experiment, as illustrated in Fig. 2 for treatment with mAb 26-2F. The number of factor VIII-positive endothelial elements is decreased substantially in tumors of mice treated with anti-Ang mAbs (60 μg of mAb 26-2F or 36u or a combined treatment of 30 μg of each of these mAbs) compared with tumors of PBS control mice ($P = 0.0038$). These data support the proposition that the antitumor effect of treatment with mAbs 26-2F and 36u is due to inhibition of tumor-induced angiogenesis. Interestingly, a decrease in vascular density is accompanied by a concomitant decrease in the amount of tumor necrosis, suggesting a positive correlation between these two variables. Although the exact nature of this relationship has not been established as yet, an increase in necrotic area could result from blood vessel collapse, owing to the higher proliferative state of tumor cells in untreated versus mAb-treated animals (21).

Table 2. Efficacy in the modified model of Ang antagonists in preventing growth of HT-29 tumor xenografts as determined by survival analysis

Control or antagonist	Dose,* μg	Mice, no.	P†	Tumor free,‡ %
PBS		145		3
26-2F	30	14	0.0030	14
26-2F	60	20	<0.0001	65
36u	30	14	<0.0001	64
36u	60	41	<0.0001	58
26-2F + 36u	60§	47	<0.0001	60
Actin	18	28	<0.0001	63
MOPC 31C	60	30	0.4580	0

PBS, diluent control.
*Daily dose of Ang antagonist was given on day 0–35 at the 1.25×10^5 tumor cell number.
†Mantel–Cox P, the probability that the survivor functions of the PBS control and Ang antagonist or IgG control are identical by chance for all mice treated with the indicated regimen. $P < 0.05$ is significant.
‡Number of mice that are tumor-free on day 35 divided by total number of mice in the group × 100 to yield percentage.
§Thirty micrograms of each mAb was administered daily.

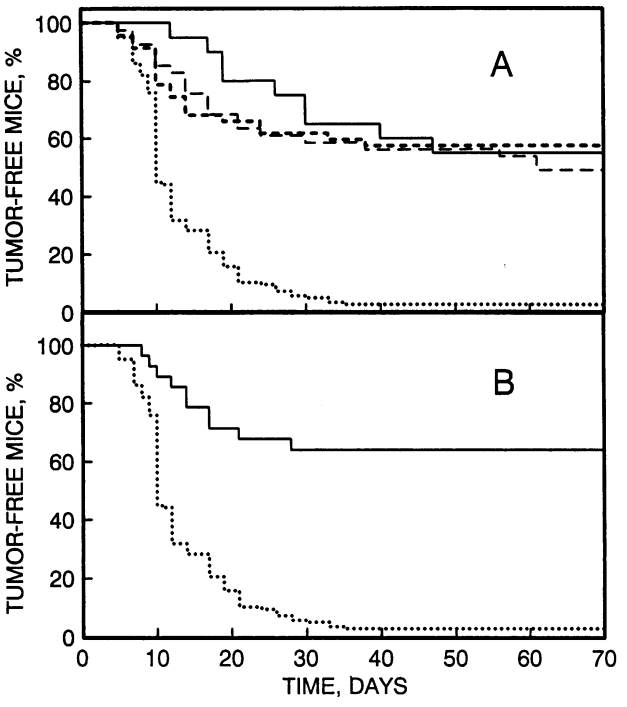

FIG. 1. Survivor functions showing prevention of HT-29 tumor growth in athymic mice by treatment with Ang antagonists. On day 0 HT-29 cells (1.25×10^5 per mouse) are mixed with PBS or the Ang antagonist and injected s.c. Daily injections of PBS or the Ang antagonist are then given for the next 35 days. (*A*) · · ·, PBS ($n = 145$); —, mAb 26-2F (60 μg; $n = 20$); – –, mAb 36u (60 μg; $n = 41$); - - -, mAb 26-2F + mAb 36u (30 μg of each; $n = 47$). (*B*) · · ·, PBS ($n = 145$); —, bovine actin (18 μg; $n = 28$).

Actin, a non-mAb Ang Antagonist, Is as Effective as mAbs 26-2F and 36u. Actin, an additional Ang antagonist, was tested for its capacity to delay or prevent tumor growth. It resembles the above anti-Ang mAbs in binding to and neutralizing the angiogenic activity of human Ang (16); indeed, its binding epitope overlaps that of mAb 36u (15). However, unlike the above mAbs, it also binds to mouse Ang. The combined data from three separate experiments indicate that treatment with actin at a dose of 18 μg (the molar equivalent of 60 μg of mAb) results in a survivor function that differs significantly from that of the PBS-treated mice ($P < 0.0001$, Table 2), with 63% of the actin-treated mice remaining tumor-free at the end of treatment. There is no evidence of toxic side effects, despite the capacity of this antagonist to interact with and potentially neutralize the endogenous Ang of the mice. Treatment with the actin diluent buffer (2 mM Tris·HCl, pH 8.0/0.2 mM ATP/0.2 mM $CaCl_2$) does not result in any tumor-free mice ($n = 20$; data not shown). The similarity between the survivor functions for mice treated with actin (Fig. 1*B*) and for mice treated with anti-Ang mAbs ($P > 0.23$, Fig. 1*A*) is most striking. Tumor-free mice treated with these Ang antagonists were observed for up to 22 weeks after tumor cell injection (median observation time for each experiment = 8 weeks). During this period 93% of these mice remained tumor-free.

Analysis of Delay in the Appearance of Those Tumors That Do Develop in Mice Treated with Ang Antagonists. Previously, doses of mAb 26-2F that resulted in the complete prevention of tumor formation in some mice also delayed tumor appearance in the remaining mice that did develop tumors (18). In the current study, the small number of tumor-bearing mice in some of the treated groups precludes statistical analysis of possible

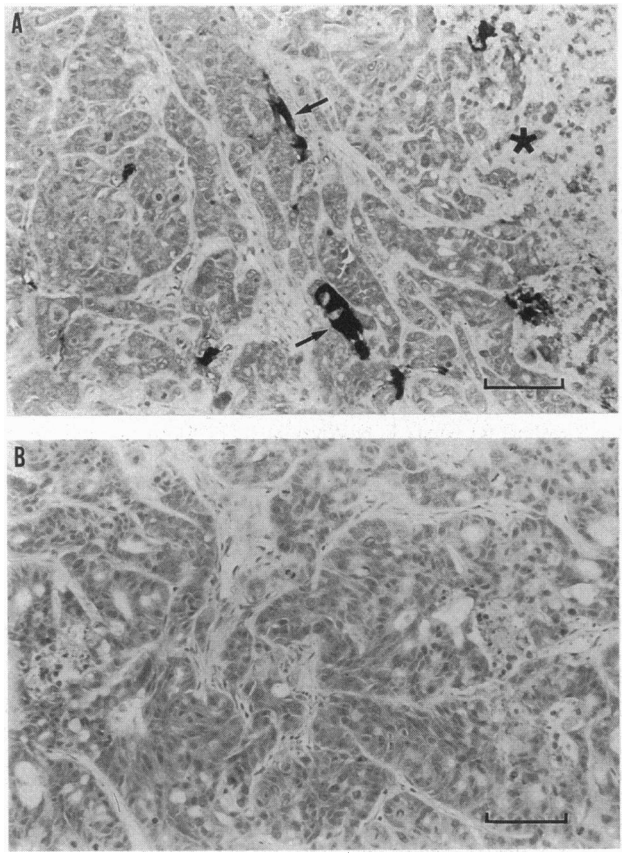

FIG. 2. Histological examination of tumors from PBS control- (*A*) and mAb 26-2F- (*B*) treated mice. Prominent factor VIII-positive blood vessels (arrows) and area of necrosis (star) in tumor from control mouse are indicated. (Bars = 62 μm.)

delay [mAb 26-2F (30- and 60-μg dose groups), mAb 36u (30-μg dose group), actin (18-μg dose group)]. In those groups in which the number of tumor-bearing mice is sufficient for statistical analyses [mAb 36u (60-μg group), mAb 26-2F + 36u (30 μg of each mAb)], there is no significant delay ($P > 0.05$).

Two Additional Human Tumor Cell Lines Are Also Sensitive *in Vivo* to Ang Antagonism. Initial investigations into the effects of treatment with the anti-Ang antagonist mAbs on the appearance of tumors from two other human tumor cell lines known to secrete Ang show a pattern of effectiveness similar to that for HT-29 tumor cells. Both A549 lung adenocarcinoma and HT-1080 fibrosarcoma cells, at a dose of 5×10^5, produce tumors in 100% of the PBS-treated control mice. Treatment with 60 μg of either mAb 26-2F (tested on both tumor cell lines) or 36u (tested only on the A549 cell line) results in 12% of the mice remaining tumor-free (data not shown). Tumor formation is prevented completely in groups treated with 60 μg of either mAb 26-2F or mAb 36u when the number of A549 tumor cells is decreased 4-fold, whereas the percentage of tumor-free mice in the PBS group increases to only 25% (data not shown). Thus, Ang antagonists can prevent growth of tumors of different histological types.

Conclusions and Perspective. Ang plays an important role in the development of HT-29 tumors *in vivo* because experiments with the first Ang antagonist tested, mAb 26-2F, demonstrated that such antagonism of Ang can delay, and even prevent, tumor growth (18). In the current study, modification of the model increases the sensitivity of the tumors to Ang antagonism, as indicated by the dramatic increase in the percentage of tumor-free mice in response to treatment with mAb 26-2F. The capacities of actin and another neutralizing mAb, 36u, both of which bind to an epitope distinct from that of mAb 26-2F, to prevent tumor growth are similar and indicate the generality of this effect. mAb 26-2F, which recognizes human but not mouse Ang, affects the appearance of tumors not by cytotoxic or Fc-dependent mechanisms but presumably through the specific extracellular inactivation of the human Ang produced by the tumor cells *in vivo* (18). Further, mAb 36u and actin do not kill HT-29 tumor cells *in vitro* (data not shown). Therefore, while these antagonists are probably incapable of eliminating tumor cells completely *in vivo* by themselves, they most likely interfere with tumor-induced angiogenesis and thereby retard the appearance of palpable tumors by arresting them in a small, avascular stage. In those cases where tumor growth is prevented completely, treatment with the Ang antagonists may render them susceptible to destruction by host factors such as natural killer cells known to be present in athymic mice (22). Moreover, because we have demonstrated recently that Ang supports tumor cell adhesion (6), neutralization of Ang, in addition to an effect on angiogenesis itself, may also interfere with tumor cell attachment and, hence, establishment onto extracellular matrix components. Those cases in which Ang antagonism seemingly fails to affect tumor growth may result from decreased bioavailability of the antagonist, heterogeneity of HT-29 cells (23), or substitution of other HT-29-derived angiogenic factors [e.g., vascular permeability factor (24)].

The effectiveness of both mAbs, which do not bind mouse Ang, in preventing human tumor growth in athymic mice demonstrates that the requirement of the tumor cells for Ang is not met by endogenous mouse Ang. This result could reflect either a species-specific Ang requirement of the tumor cells or an absence of a sufficient amount or gradient of mouse Ang in the environment of the tumor. Thus the tumor cells are vulnerable to neutralization of human Ang, which they secrete. Human Ang is detected in the sera of mice with established HT-29 xenografts (data not shown), confirming that these cells are capable of synthesizing and secreting this protein *in vivo*. Although these murine mAbs have been useful in demonstrating these principles, their utility could be limited, of course, by problems known to occur with the use of murine immunoglobulins for human therapy (25). Humanization of these murine mAbs, however, may circumvent these problems. Unlike mAbs 26-2F and 36u, actin also binds to mouse Ang; thus, the mechanism of action in this case may involve neutralization of available host as well as human Ang.

The present results with actin illustrate the feasibility of tumor prevention in humans with Ang antagonists by demonstrating the apparent lack of toxicity of treatment with these agents. Actin can potentially block the function of the mouse's own Ang, but it does not induce side effects at daily doses >50 times the amount of circulating mouse Ang (≈7 pmol; K.A.O., unpublished results). This lack of toxicity may reflect the infrequent induction of angiogenesis in a normal adult male animal because nonpathologic angiogenesis is associated primarily with embryogenesis, wound healing, and the female reproductive system (26).

Angiogenesis is critical not only for the growth of solid tumors but also for cell shedding from the primary tumor and development of metastases at distant sites (27, 28). Thus, the identification, development, and testing of antagonists of tumor-associated angiogenesis are needed urgently; this need is underscored by convincing evidence reported recently showing that the degree of angiogenesis in an initial primary tumor correlates with metastatic spread and survival in patients (29, 30). A limited number of compounds shown experimentally to inhibit angiogenesis, including platelet factor 4 (31) and synthetic analogues of the antibiotic fumagillin (32), are currently being evaluated clinically for the treatment of malignancy. However, the specific inhibition of tumor-derived angiogenic mediators remains an attractive therapeutic target that has yet to be exploited fully. Although attempts to control experi-

mental tumor growth with antibodies to basic fibroblast growth factor have not been successful reproducibly (33, 34), a mAb directed against vascular endothelial growth factor was shown recently to decrease growth in athymic mice of tumors that secrete this protein, although complete prevention of tumors was not reported (35). Our studies not only further the proposition that neutralization of angiogenic mediators will be therapeutically effective, but also support the idea that complete prevention can be achieved by targeting Ang in particular. In addition to mAbs and actin, other Ang antagonists exist, which include competitive-site-directed mutants (36), inhibitory synthetic peptides (37), and a ribonuclease inhibitor (38) used successfully in preliminary antitumor studies (39). These Ang antagonists, along with other structurally based Ang antagonists under development based on x-ray crystallography, are the subject of ongoing investigations into the design of clinically effective anti-Ang therapeutics.

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