A Monoclonal Antibody to Human Angiogenin Suppresses Tumor Growth inAthymic Mice

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Abstract

Human angiogenin, a potent inducer of neovascularization, is secreted by HT-29 colon adenocarcinoma cells. μg doses of a monoclonal antibody that neutralizes the in vitro and in vivo activities of angiogenin prevent or delay the appearance of s.c. HT-29 tumors in athymic mice in a statistically significant, dose-dependent manner. The antibody is not cytotoxic to tumor cells in vitro, which indicates that inhibition of tumor growth most likely occurs by neutralization of the activity of angiogenin in vivo and further implies a critical role for angiogenin in the early development of HT-29 tumors. The results suggest a therapeutically useful approach to the treatment of angiogenin-dependent malignancy.

Introduction

Angiogenin, a M, 14,124 protein secreted by and first isolated from medium conditioned by HT-29 human colon adenocarcinoma cells (1) and later from normal mammalian plasma and milk (2, 3), elicits new blood vessel formation in the rabbit cornea and meniscus and in the chick chorioallantoic membrane (1, 4). A member of the ribonuclease superfamily of proteins, it exhibits ribonuclease activity, although markedly less than and different from that of pancreatic RNase, which is angiogenically inactive (5). Also, unlike RNase, angiogenin binds to smooth muscle and vascular endothelial cells in vitro to induce second messenger responses (6), presumably mediated by cell surface receptors (7). Chemical modification as well as targeted mutagenesis have defined many of the residues responsible for the enzymatic and angiogenic activities of angiogenin, including a putative receptor-binding domain necessary for function (8). More recently, angiogenin, unlike RNase, was found to support endothelial cell adhesion (9), a property which may contribute to its role in angiogenesis.

Although the physico-chemical properties of angiogenin and studies of its mechanisms of in vitro signal transduction and receptor binding have been described, little is known about its functions in normal or pathological cellular processes, including tumor growth. A mAb, 26-2F, that neutralizes both the in vivo angiogenic as well as the in vitro ribonuclease activities of angiogenin has recently been produced and characterized (10). We here examine the effect of this mAb on the temporal appearance and growth of HT-29 cell xenografts in athymic mice. Inspection of the data and Wilcoxon and t test analyses indicate that all mAb 26-2F treatments significantly affect tumor growth, resulting in subsets of tumor-free mice in some treatment groups. Subsequent survival analysis reveals that, in these latter groups, antibody treatment significantly prevents tumor appearance and that, even in tumors that develop, mAb 26-2F affects their growth by delaying appearance and/or decreasing the early rate of palpable tumor growth. Thus, angiogenin appears to play a critical role in the early development of HT-29 tumors.

Materials and Methods

Materials. Five-week-old outbred male athymic mice (Crl:nu/nu) from the isolator-bred colony of Charles River Laboratories were maintained under specific pathogen-free conditions. HT-29 human colon adenocarcinoma cells (HTB-38; American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (BioWhittaker) supplemented with 2 mM L-glutamine, 5% heat-inactivated fetal bovine serum, and antibiotics. The mAb 26-2F, an IgGk which recognizes human but not mouse angiogenin, was purified from ascites fluid by ammonium sulfate precipitation and protein A-Sepharose chromatography and quantitated by solid-phase enzyme-linked immunosorbent assay (10). F(ab')2 fragments of this mAb were prepared and isolated by standard procedures. MOPC 31C, a nonspecific IgGk-secreting mouse hybridoma (CCL 130; American Type Culture Collection), was propagated, and IgG was purified from ascites as above.

Antitumor Activity in Vivo. Age-matched athymic mice, 6- to 8-weeks old, were randomly assigned to subgroups (n = 7–19) for each experiment. Tumor cells were harvested by trypsinization; washed with Hanks' buffered salt solution; mixed with mAb 26-2F, its F(ab')2 fragment, MOPC 31C or PBS as a diluent control; and the mixtures (150 μl) were injected s.c. behind the left shoulder. Subsequent injections (100 μl) were given s.c. daily in the immediate vicinity of the initial injection for 14 days, unless otherwise indicated. Mice were examined for tumor growth daily by palpation until the first appearance of a tumor, at which time caliper measurements commenced and continued on a schedule of 3 times/week. Tumor volume was computed as the product of length, width, and depth. Tumor-bearing mice were sacrificed on days 25–27, by which time all control mice had developed tumors. Tumor-free mice were kept for at least an additional 3 weeks and were examined at autopsy for the presence of tumor. In some cases where no tumor was observed, the area of injection was excised, fixed in buffered formalin, serially sectioned, stained with hematoxylin and eosin, and examined microscopically for tumor cells.

Statistical Analyses. Differences in tumor volumes between control and treatment groups were analyzed with the Wilcoxon rank sum test with correction for ties, a distribution-free test of equality of medians (11). Two-group t tests for differences between mean volumes were performed with BMDP program 3D (12) after applying a 2/3 power transformation. Pooled controls transformed in this manner were normally distributed. Estimates of survivor functions, obtained by the Kaplan-Meier product-limit method, and Mantel-Cox tests of equality of survivor functions (P significant <0.05) were computed with BMDP program 1L (13). Survivor functions are measures of time to-response and, therefore, are measures of the time to appearance of a tumor in this study. The time courses for first appearance of tumors in the controls of individual experiments are reproducible; hence, the results of individual experiments were pooled for survival analysis.

Antitumor Activity in Vitro. HT-29 cells were plated in triplicate into 96-well microtiter plates (1 × 10⁴ cells/ml, 200 μl/well). After 2 days in culture, mAb 26-2F (15 μl, 0.15 μg/ml) and 15 μl of [3H]thymidine (1 μCi/ml, 6.7 Ci/mmol) were added. Cells were detached with trypsin 48 h later, and DNA, harvested onto filters using a Mini-Mash II (BioWhittaker), was counted in a Beckman LS 1801 scintillation counter. In additional experiments,
Results and Discussion

All Doses of mAb 26–2F Used Decrease HT-29 Tumor Growth in Athymic Mice. Table 1 lists the results of individual experiments investigating the effect of mAb 26–2F on tumor growth. Injection protocols included treatment with PBS, mAb 26–2F (3, 6, 30, or 300 μg), or control MOPC 31C (30 μg). Earlier studies demonstrated that the time between tumor cell injection and appearance of palpable tumor depends on the number of cells injected. Therefore, to determine whether sensitivity to angiogenin antagonism is also related to this parameter, the subsequent studies were carried out with either 10^6 or 5 × 10^5 cells/mouse.

In these mice, all doses of mAb 26–2F used effectively decreased tumor volume when individual treatment groups were compared with PBS controls for each number of injected cells (Table 1). Of the 19 experiments performed, 15 were successful (i.e., median α < 0.05). Moreover, the overall outcome of these 19 experiments is highly significant, as indicated by the median of all individual median α values (0.009). At the lower cell number, prolonged treatment with an increased amount of mAb 26–2F (300 μg on day 0; 90 μg daily for days 1–26; median α = 0.015) is not more effective than administration of 30 μg for 15 days (median α of the 8 experiments, 0.034). The nonspecific IgG, MOPC 31C, does not affect tumor growth either at the higher or lower number of cells (median α at both cell numbers > 0.27).

Treatment with the High Doses of mAb 26–2F Delays and, in Some Cases, Completely Prevents HT-29 Tumor Appearance. The most striking effect of treatment with mAb 26–2F is the prevention of the appearance of palpable tumors in a subset of mice over a 7- to 9-week time course in several experiments (Table 2). This observation is supported by histological analysis of the area of cell injection. In contrast, all PBS-treated mice developed tumors rapidly. Survival analysis reveals that treatment with the two higher doses of mAb 26–2F at each of the tumor cell numbers results in a significant difference between survivor functions when compared with PBS controls (P ≤ 0.0001). This indicates that mAb 26–2F can both delay and prevent tumor appearance as illustrated in Fig. 1 for treatment with PBS or 30 μg of mAb 26–2F at the lower cell number. On day 26, 25% of the mAb-treated mice had not developed tumors, whereas the higher or lower number of cells (median α at both cell numbers > 0.27).

Table 1 Efficacy of mAb 26-2F in reducing the volume of HT-29 tumor xenografts in athymic mice as determined by Wilcoxon rank sum test analysis

<table>
<thead>
<tr>
<th>IgG</th>
<th>Dose (μg)</th>
<th>Mice treated</th>
<th>Median α (of all mice)</th>
<th>IgG</th>
<th>Dose (μg)</th>
<th>Mice treated</th>
<th>Median α</th>
</tr>
</thead>
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<tr>
<td>26-2F PBS</td>
<td>300</td>
<td>10</td>
<td>0.004</td>
<td>26-2F</td>
<td>30</td>
<td>10</td>
<td>0.001</td>
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<td>10</td>
<td>0.091</td>
<td>26-2F</td>
<td>30</td>
<td>8</td>
<td>0.128</td>
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<td>0.001</td>
<td>26-2F</td>
<td>30</td>
<td>7</td>
<td>0.000</td>
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<td>10</td>
<td>0.013</td>
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<td>8</td>
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<td>10</td>
<td>0.000</td>
<td>26-2F</td>
<td>3</td>
<td>10</td>
<td>0.015</td>
</tr>
<tr>
<td>MOPC 31C</td>
<td>30</td>
<td>19</td>
<td>0.500</td>
<td>MOPC 31C</td>
<td>30</td>
<td>19</td>
<td>0.274</td>
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* Mantel-Cox P, the probability that the survivor functions of the PBS control and IgG-treated group 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 days 25-27 divided by the total number of mice in the group (percentage).**

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

<table>
<thead>
<tr>
<th>Control or IgG</th>
<th>Dose (μg)</th>
<th>Total no. of mice</th>
<th>P* (all mice)</th>
<th>P* (tumor-bearing mice)</th>
<th>Tumor-free (%)</th>
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</thead>
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<tr>
<td>1 × 10^6 tumor cell dose</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PBS</td>
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<td>50</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0</td>
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<tr>
<td>26-2F</td>
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<td>20</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>10</td>
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<tr>
<td>26-2F</td>
<td>30</td>
<td>20</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>15</td>
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<tr>
<td>26-2F</td>
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<td>50</td>
<td>0.1323</td>
<td>0</td>
<td>0</td>
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<tr>
<td>MOPC 31C</td>
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<td>19</td>
<td>0.7538</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5 × 10^5 tumor cell dose</td>
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<td></td>
<td></td>
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<tr>
<td>PBS</td>
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<td>110</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0</td>
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<td>67</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>25</td>
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<td>10</td>
<td>0.0001</td>
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<td>6</td>
<td>10</td>
<td>0.0774</td>
<td>0</td>
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<tr>
<td>MOPC 31C</td>
<td>30</td>
<td>19</td>
<td>0.5637</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Mantel-Cox P, the probability that the survivor functions of the PBS control and IgG-treated group 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 days 25-27 divided by the total number of mice in the group (percentage).**

* These values are identical to those calculated for their corresponding groups containing all mice, since no tumor-free animals were observed.

 athymic mouse serum (6% v/v) was added as a source of complement, and growth medium was replaced with serum-free medium immediately prior to the addition of mAb, [3H]thymidine, or serum.

The Wilcoxon rank sum test results are supported by two-group t tests performed after sufficient data were accumulated to determine the appropriate transformation of tumor volumes that would yield normal distributions and to ensure that results from the individual experiments could be pooled. Median one-tailed Ps over all observation days at all doses of mAb 26–2F and at both levels of tumor cell inoculation shown in Table 1 were highly significant (median Ps < 0.003), whereas the Ps for treatment with MOPC 31C were not significant. Since the data for some mAb-treated groups revealed a bimodal distribution of tumor volumes due to pronounced delay and prevention of tumor appearance, the t test was not used further.
none of the PBS control mice were tumor-free. Of these tumor-free mice, 88% remained so until the termination of the experiment on day 49. Results with the lowest dose of mAb 26–2F used for each of the tumor cell amounts, however, indicated that there is a threshold for efficacy (Table 2), i.e., survivor functions differ from the PBS controls only above 6 µg of mAb 26–2F at the higher cell number and 3 µg at the lower cell number. Treatment with 30 µg of MOPC 31C at both tumor cell numbers also does not differ from treatment with PBS (P > 0.5). In terms of prevention of tumor appearance, treatment with the two higher doses of mAb 26–2F at each cell number (Table 2), as well as with the more vigorous regimen at the lower cell number (300 µg on day 0 and 90 µg daily for days 1–26; 15% tumor-free mice), results in similar percentages of tumor-free mice on days 25–27 within the range of SEs. Moreover, 92% of all tumor-free mice remain so 5 weeks after the cessation of treatment. No tumor-free mice, however, were present in the groups treated with either the low dose of mAb 26–2F or 30 µg of MOPC 31C at each tumor cell number. The two higher doses of mAb 26–2F also delayed the growth of those tumors that appear at either tumor cell number, as seen by comparison of the survivor functions of these subsets of the experimental groups with the appropriate PBS controls (P ≤ 0.0065; Table 2). However, treatment with the lowest dose of mAb 26–2F at either cell number failed to delay tumor appearance (P > 0.077; Table 2).

All Doses of mAb 26–2F Used Affect the Early Growth of Developing HT-29 Tumors and Thus Decrease Tumor Size. Although the lowest dose of mAb 26–2F at either cell number failed to delay or prevent tumor growth (Table 2), the tumor volumes in these two low dose groups were, nevertheless, significantly affected by antibody treatment (Table 1). Comparison of their tumor growth curves with the PBS controls (Fig. 2) revealed that, after approximately day 10, the growth rates were quite similar (a left lateral displacement superimposes each treatment curve on its control curve). This implies that, once the treated tumors reach a certain size, their growth is no longer affected by mAb 26–2F. The difference in volumes between the control and treated curves for these low-dose groups must, therefore, reflect events occurring between the time of injection of the tumor cells and approximately day 10. Since measurements made during this time period are relatively imprecise, it is not possible to determine whether this volume difference results from an effect of mAb 26–2F on the growth rate of the small, palpable tumor or from an undetectable effect on the time of appearance, or both.

Further examination of the tumor growth curves for PBS controls and for those mice treated with the higher doses of mAb 26–2F that eventually did develop tumors also revealed that treatment caused a decrease in tumor volumes. Fig. 2 shows examples for a high-dose treatment (30 µg) at each of the cell numbers. The shapes of the treatment growth curves after day 10 are again very similar to the appropriate PBS control curves, indicating that the decreased volume must result from mAb effects before this time. In contrast with the low-dose treatment groups, however, there was also a delay in the appearance of these tumors, as indicated by the survivor functions of these mice compared with PBS controls (P ≤ 0.0065; Table 2). Therefore, in these cases, the decrease in tumor volumes can result from the observed delay in the tumor appearance, either alone or in combination with an effect on the growth of the small but palpable tumor. For each tumor cell number given, however, there was no significant difference in the reduction of tumor volume by the different doses of mAb 26–2F. Thus, in cases where tumor growth is not prevented entirely, there appears to be an early effect of mAb 26–2F on the initial growth of tumors that results in a decreased size compared with time-matched PBS controls; but once the tumor diameters exceed a few mm, it no longer affects growth rate.

Efficacy of mAb 26–2F in Delaying or Preventing HT-29 Tumor Appearance Is Dose Dependent. Analysis of the relationship between dose and efficacy of mAb 26–2F indicates that, for each tumor cell number given, there is a certain amount of antibody (6 µg
at the high cell number and 3 µg at the low cell number) that will significantly decrease tumor volume (Table 1) without delaying or preventing tumor appearance (Table 2). Thus, a decrease in tumor cell number results in a corresponding decrease in the effective amount of mAb 26–2F. The dose of mAb 26–2F required to neutralize angiogenin sufficiently to prevent tumor appearance depends on the number of tumor cells injected, and, therefore, presumably, on the amount of angiogenin secreted by these cells. Once the particular threshold amount of mAb 26–2F is exceeded, the survivor functions become significant, although any further increase in the dose tested did not increase the prevention or delay of tumor appearance (Table 2).

Comparison of growth curves for the PBS control mice at the two cell number (Fig. 2) demonstrated that, as previously noted, halving the number of tumor cells produces a significant increase in the time between tumor cell injection and tumor appearance (P < 0.0001). This alteration of the growth curve, however, did not lead to increased effectiveness of mAb 26–2F other than that attributable to dose effects.

mAb 26–2F Exerts Its Antitumor Effect not by Cytotoxic or Fc-dependent Mechanisms but Presumably by Specific Extracellular Neutralization of Angiogenin. mAb 26–2F did not inhibit DNA synthesis by HT-29 tumor cells cultured in vitro over a 48-h period in the presence or absence of complement (data not shown), thereby indicating that the antitumor effect is not caused by direct cytotoxicity of tumor cells. Additionally, Fc-dependent effector mechanisms of antibody-mediated killing did not seem to be required for the antitumor activity observed, since treatment with 20 µg of the F(ab′2)2 fragment of mAb 26–2F (equivalent to 30 µg of mAb 26–2F) daily for days 0–14 at the low cell number successfully prevented and delayed tumors (P < 0.0001 for both effects) to an extent that was not significantly different from that of the intact antibody.

From the above observations, plus the fact that human angiogenin can be detected in the serum of mice bearing HT-29 xenografts (data not shown), it is most likely that the antitumor effects exerted by mAb 26–2F (which does not recognize mouse angiogenin) result from the specific extracellular inactivation of tumor-derived human angiogenin and a consequent disruption of the angiogenic process. Since tumors up to 2 mm in diameter can exist in a diffusion-controlled avascular state (14), we postulate that host factors, e.g., NK cells known to be present in athymic mice (15), although unable to prevent tumor establishment and growth without mAb 26–2F treatment, can eradicate tumor cells held avascularly, resulting in tumor-free mice. In other cases, although treatment delayed tumor appearance and/or reduced size, tumors eventually appeared and then grew apparently unaffected by subsequent administration of antibody. This escape from anti-angiogenin susceptibility is unexplained but may be attributed to several variables including bioavailability of antibody, demonstrated heterogeneity of HT-29 cells (16), and use of other angiogenic factors known to be synthesized by this cell type, e.g., transforming growth factor α (17) and vascular permeability factor (18).

Angiogenesis, known to be critical for solid tumor growth (19), is also a prerequisite for the development of metastasis. It permits the transport of cells shed from the primary tumor and the subsequent growth of micrometastases at distant sites (20). Interference with the process of tumor-induced angiogenesis should, therefore, have therapeutic efficacy also in the treatment of metastatic disease. The results presented here suggest that the growth of HT-29 tumors can be delayed and even prevented by the inhibition of angiogenin at an early stage of tumor development. Although this study investigates the effects of a mAb to angiogenin, we have demonstrated that angiogenin mRNA is expressed (21) and the protein is secreted in vitro by several other established human tumor cell lines of different histological types. Thus, angiogenin may be a common mediator of tumor angiogenesis and, as such, may serve as a useful target for antitumor therapy.

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References


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