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Antiangiogenic therapy of transgenic mice impairs *de novo* tumor growth

(tumor angiogenesis/angiogenesis inhibitors/apoptosis/antitumor therapy/interferon/TNP-470)

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ABSTRACT **Angiogenesis is activated during multistage tumorigenesis prior to the emergence of solid tumors. Using a transgenic mouse model, we have tested the proposition that treatment with angiogenesis inhibitors can inhibit the progression of tumorigenesis after the switch to the angiogenic phenotype. In this model, islet cell carcinomas develop from multifocal, hyperproliferative nodules that show the histological hallmarks of human carcinoma *in situ*. Mice were treated with a combination of the angiogenesis inhibitor AGM-1470 (TNP-470), the antibiotic minocycline, and interferon α/β. The treatment regimen markedly attenuated tumor growth but did not prevent tumor formation; tumor volume was reduced to 11% and capillary density to 40% of controls. The proliferation index of tumor cells in treated and control mice was similar, whereas the apoptotic index was doubled in treated tumors. This study shows that *de novo* tumor progression can be restricted solely by antiangiogenic therapy. The results suggest that angiogenesis inhibitors represent a valid component of anticancer strategies aimed at progression from discrete stages of tumorigenesis and demonstrate that transgenic mouse models can be used to evaluate efficacy of candidate antiangiogenic agents.**

It is evident from genetic and histological analyses of biopsies from human cancers that tumor development is a multistage process (1, 2). Transgenic mice carrying dominant oncogenes or tumor suppressor gene knockouts have confirmed the multistage nature of carcinogenesis and provide reproducible access to the different stages for analysis. In one such model (RIP1-Tag2), expression of the simian virus 40 large T antigen (SV40 Tag) oncogene in the pancreatic β cells under control of the insulin gene regulatory region results in the reproducible development of islet cell carcinomas (3). Tumorigenesis proceeds through two distinct preneoplastic stages that can be readily visualized among the ≈400 pancreatic islets, which represent natural focal nodules. The first involves a switch from quiescence to hyperproliferation of the oncogene-expressing pancreatic β cells concomitant with activation of expression of the growth/survival factor insulin-like growth factor II (IGF-II) (4). The second step is induction of angiogenesis, wherein the normally quiescent vasculature is activated to proliferate and form new capillaries (5). The angiogenic switch is discrete, and the three stages are both statistically and temporally separable (Fig. 1*A*).

The hyperproliferative switch (Fig. 1*A*) first occurs in individual islets, beginning at 3–5 weeks of age; eventually >50% of the islets activate IGF-II and become hyperplastic. In fact, these so-called "hyperplastic" or hyperproliferative islets are populated with cells showing the hallmarks of cellular malignancy; as such these numerous nodules are analagous to the multifocal carcinoma-*in-situ* lesions seen in many epithelial cancers. Subsequently, the angiogenic switch occurs in a subset of the hyperproliferative islets, beginning at about 5 weeks; by 9–10 weeks, ≈10% of the islets are angiogenic. Finally, solid tumors appear, beginning at 12 weeks of age; 1–2% of the oncogene-expressing islets progress to this stage. All animals die of tumor-induced hypoglycemia by 14 weeks of age. At the time of death, there is typically no evidence of metastasis. The switch to the angiogenic phenotype has subsequently been described in preneoplastic lesions arising in a transgenic mouse model of dermal fibrosarcoma (6) and in preneoplastic lesions associated with breast and cervical cancer in humans (7, 8). Together, these studies argue that induction of angiogenesis is a discrete and necessary step in tumor development. Thus, interference with angiogenesis presents a target for design of cancer therapeutics that target not the tumor cells themselves but another critical component of the tumorigenesis process.

In this report, we describe a therapeutic trial in which RIP1-Tag2 transgenic mice were treated with a set of angiogenesis inhibitors, beginning during the period of the angiogenic switch at 6 weeks of age and continuing to a time 5.5–7.5 weeks later, when islet cell carcinomas had formed. Although islet cell tumors are themselves rare in humans, we believe this transgenic model presents an important tool for studying development and progression of common human epithelial cancers, such as breast, prostate, and bladder carcinoma. Progression from the multifocal hyperproliferative stage can also be considered to model the situation when a primary tumor is excised, leaving behind multiple carcinoma-*in-situ* lesions with the capability to progress to full malignancy.

An increasing number of angiogenesis inhibitors are candidates for therapeutic studies. These include the following: endogenous endothelial inhibitors, such as thrombospondin (9), the 16-kD fragment of prolactin (10), and angiostatin, a fragment of plasminogen (11); synthetic derivatives of fungal and bacterial compounds, such as AGM-1470 (12) and the antibiotic minocycline (13); and regulatory cytokines, such as interferon α/β (IFN α/β) (14). Based on a series of previous experiments involving treatment of tumor-bearing mice resulting from inoculation of established tumor cell lines, we chose a combination of three known angiogenesis inhibitors for this trial: AGM-1470, minocycline, and IFN α/β. AGM-1470 (TNP-470) is a synthetic analogue of fumagillin, a fungal-derived angiogenesis inhibitor. Minocycline is a locally effective angiogenesis inhibitor which potentiates other angiogenesis inhibitors when administered systemically. The α/β IFNs are angiogenesis inhibitors (14); among their capabilities, the

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Abbreviations: TdT, terminal deoxytransferase; vWF, von Willebrand factor; IFN, interferon.

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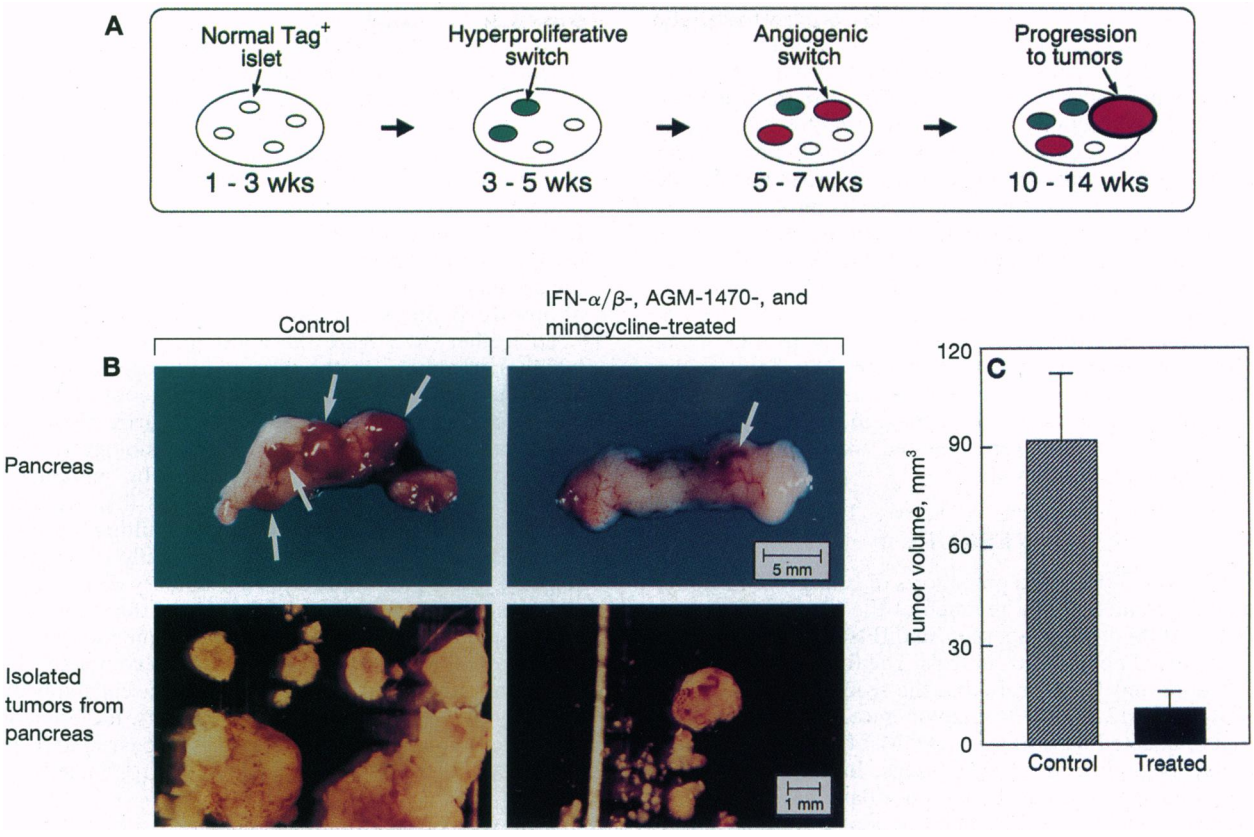

FIG. 1. Experimental schematic and gross pathology of effects on tumor burden. (*A*) Illustration of the transgenic islet cell carcinoma model in which expression of the simian virus 40 large T oncoprotein (Tag) under control of the rat insulin promoter elicits tumorigenesis. Expression of Tag begins in the β cells of the islets of Langerhans during embryogenesis. Four distinct stages of tumorigenesis are discernible: (*i*) normality, (*ii*) hyperproliferation (hyperplasia), (*iii*) onset of angiogenesis, and (*iv*) formation of solid, encapsulated tumors. Antiangiogenic therapy commenced at 6 weeks of age, coinciding with onset of angiogenesis, and continued to either 11.5 (trial I) or 13.5 weeks of age (trial II). (*B*) (*Upper*) Gross anatomy of pancreata at 13.5 weeks of age from a control mouse and a mouse treated in trial II with the regimen of antiangiogenic therapy is shown. Arrows point out the visible tumors in each pancreas. (*Lower*) All solid tumors were isolated from one control and one treated pancreas by using collagenase digestion and then collectively displayed by using a dissecting microscope. (*C*) Mean tumor volume per pancreas at 13.5 weeks of age in control (90.7 ± 20.3 mm^3) (hatched bar) and treated animals (10.0 ± 5.1 mm^3) (black bar) for trial II is shown. These differences are statistically significant, as revealed by an analysis performed by using Student's *t* test ($P < 0.001$).

α/β IFNs can specifically block basic fibroblast growth factor (bFGF) mRNA and protein in human cancer cells (15). Two of these compounds (AGM-1470 and IFN α/β) previously showed combinatorial efficacy in a transplantation model: when C57BL/6 mice were inoculated subcutaneously with Lewis lung carcinoma cells and treatment commenced, AGM-1470 alone reduced tumor volume to 35% of controls, IFN α/β reduced tumor volume to 75%, and the combination of the two reduced tumor volume to 20% (16). Given both the logistics of producing and treating by daily injections a statistically significant age-matched cohort of transgenic mice and the extremely limited availability of IFN α/β active on murine cells, we chose to use a combination of these three compounds at the optimum values suggested by traditional tumor transplantation studies for our experimental trial in transgenic mice.

MATERIALS AND METHODS

Treatment Groups and Drug Dosages. All treated mice received 30 mg of AGM-1470 (a gift of Takeda Chemical Company, Osaka) per kg s.c. every other day and 10 mg of minocycline (Sigma) per kg i.p. every day. The low-dose murine IFN group received 2×10^5 units i.p. daily, while the high-dose murine IFN group received 1×10^6 units. Murine IFN α/β was a gift from L. Kronenberg (Lee Biomolecular Laboratories, San Diego). The animals were treated starting at 6 weeks of age and euthanized at either 11.5 weeks of age (trial I) or 13.5 weeks (trial II). In the second trial, all treated animals received the above specified regimen of AGM-1470 and minocycline, and 1×10^6 units of a hybrid human IFN, IFN-α B/D (a gift from D. Gangemi, Clemson University, and H. Hochkeppel, CIBA–Geigy) that is active on murine cells. All control mice received i.p. and s.c. saline injections. Earlier initiation of the drug regimen was considered but was technically difficult due to issues such as weaning and typing of transgenic mice.

Tumor Measurements. In both trials, animals were euthanized, and tumor volume (in mm^3) was measured from freshly excised pancreata by using a stereomicroscope, applying the formula [volume = $0.52 \times (\text{width})^2 \times (\text{length})$] for approximating the volume of a spheroid. In addition, to isolate tumors from the pancreas of control and treated individuals, the mice were euthanized and the common bile duct was cannulated and infused with 1.162 mg of collagenase P (Boehringer Mannheim) per ml in Hanks' balanced salt solution without phenol red. Pancreata were collected, and protease digestion continued at 37°C for 10 min. Tumors were then collected by micropipetting and placed in RPMI medium 1640 with 25 mM Hepes for visual examination and measurement. Tumor volumes in mm^3 were measured *in situ* in freshly excised pancreata under a stereomicroscope, as described above.

Tissue Preparation and Histochemistry. To measure the percentage of cells in S phase, mice were injected with 0.25 ml of BrdUrd (5 mg/ml; Amersham) 2 h prior to sacrifice. Pancreata were fixed in either buffered 4% (vol/vol) formaldehyde overnight for terminal deoxynucleotide transferase

(TdT) labeling and BrdUrd staining or in Carnoy's fixative for 4 h for von Willebrand factor (vWF) staining and, in both cases, embedded in paraffin and sectioned with a microtome. For immunoperoxidase visualization of BrdUrd incorporation or vWF staining, sections were pretreated with 0.2 N HCl for 10 min and 2 μg of proteinase K per ml at 37°C for 15 min. Immunohistochemical detection used a monoclonal anti-BrdUrd antibody (Amersham) or a rabbit anti-human vWF antibody (diluted 1:200; Dako) and their reaction products were visualized with a vectastain ABC kit (Vector Labs) by using diaminobenzidine as a chromophore. TdT labeling was performed according to Gavrieli *et al.* (17), except that no proteinase K digestion was performed. Finally, the tissue sections were lightly counterstained with Gills hematoxylin. The number of antibody-positive cells were scored under oil immersion light microscopy at a magnification of 300×. A minimum of 3000 cells were counted from each tumor in control and treated pancreata.

RESULTS

Two trials were conducted on cohorts of transgenic mice that were either treated with a regimen of the three angiogenesis inhibitors AGM-1470, minocyclin, and IFN α/β or maintained as age-matched nontreated controls. The first trial involved 18 treated mice and 11 controls, and the second trial comprised 13 treated and 12 control transgenic mice. In both trials, the mice were injected with 30 mg of AGM-1470 per kg s.c. every other day, beginning at 6 weeks of age. In addition, minocycline was injected (10 mg/kg) i.p. on a daily basis. The trials differed in the source of IFN. The first trial utilized a partially purified preparation of murine IFN-α and IFN-β, supplied at two doses of 200,000 units and 1,000,000 units per day i.p. The second trial utilized a hybrid IFN, derived from a recombinant between the human IFN-α B and D genes (18, 19), which is active on murine cells (20–22). A total of 10^6 units of IFN-α B/D was injected i.p. daily.

In the two trials, the mice were euthanized after 5.5 or 7.5 weeks of treatment, and pancreata were collected for analysis. There was no indication of toxicity or other side effects resulting from this regimen; body weight (Table 1) and other physiological features—e.g., general activity and tactile responsiveness—were comparable in treated and control mice. In both trials, the treated mice still developed tumors; however, the gross pathology indicated a substantial reduction in tumor size. Cumulative tumor volume was determined for each of the treated and control transgenic mice. Table 1 summarizes the trials, the inhibitors supplied, and the effects on tumor volume. It should be noted that the mice in trial II were allowed to live an additional 2 weeks before concluding the experiment; thus, the tumor volumes were greater than in trial I. Fig. 1 *B* and *C* presents the results for the second trial. It is evident that in both trials tumor burden was substantially reduced, up to 11% of controls in trial II. Moreover, a dose dependence for the IFN can be seen in trial I, indicating that the IFN is contributing to the therapeutic outcome and further suggesting that the optimal dose might be even higher. (The limited quantities of both IFNs precluded testing a higher dose regimen.)

To further investigate the consequences of angiogenesis-inhibitor treatment on the tumors, histological analyses were performed on tumors that developed in control and treated mice from trial II. Fig. 2 and 3 summarize these analyses statistically and by representative histopathology, respectively. The capillary density was determined by immunostaining tissue sections of tumors with antibodies that recognize the endothelial-cell protein vWF and then counting the number of vWF-positive capillaries in grids, essentially as described (7) (Fig. 2*A* and Fig. 3 *A* and *B*). The capillary density in tumors of treated mice was reduced to ≈40% of that seen in control tumors, indicating that this treatment regimen was attenuating the rate of neovascularization, but had not completely blocked the initial activation of angiogenesis nor the capability of every capillary to grow. Indeed, angiogenic islets, the preneoplastic stage preceeding tumor formation (5), appeared to be present at similar numbers in treated and control mice (data not shown).

Next, we assessed tumor cell characteristics by histopathology. The tumor cells in treated animals were more disorganized, lacking the focal clusters of cells surrounded by capillaries apparent in the untreated controls (in Fig. 3, compare *A* with *B* and *E* with *F*). The tumor cell proliferation index, as assessed by the incidence of cells in S phase determined by using BrdUrd labeling, was similar in treated and control mice (Fig. 2*B*). In contrast, the incidence of apoptotic cells, determined either histologically as pycnotic cell bodies in plastic embedded sections (Fig. 3 *E* and *F*) or by the TdT assay (17) (Fig. 3 *C* and *D*), was about 2-fold higher in treated tumors (Fig. 2*C*). One explanation is that these compounds are directly cytotoxic for the tumor cells, despite our expectation of selectivity for endothelium. To address this possibility, we collected serum from inhibitor-treated nontransgenic mice to approximate the effective dose at which these compounds are

Table 1. Trials of antiangiogenesis therapy during islet-cell carcinogenesis

Treatment	No. of animals	Length of therapy, wk	No. of tumors	Mean tumor volume, mm^3	Range, mm^3	Ratio of mean tumor volumes, treated to control	Body weight, g
Trial I							
Saline	11	5.5	27	27.4 ± 7.1	0.52–57	1.0	ND
AGM-1470 + Minocycline + Low-dose mIFN-α/β	13	5.5	22	8.0 ± 2.8	0.01–32	0.30	ND
AGM-1470 + Minocycline + High-dose mIFN-α/β	5	5.5	8	4.5 ± 1.9	0.03–5.5	0.16	ND
Trial II							
Saline	12	7.5	29	90 ± 20	6.8–212	1.0	23.2 ± 0.7
AGM-1470 + Minocycline + High-dose hIFN-α B/D	13	7.5	23	10 ± 5.1	0.06–40	0.11	21.5 ± 1.2

All treated animals received the same dosages of AGM-1470 and minocycline. In addition, animals in trial I received either a low (1×10^5 units) or high (2×10^6 units) dose of partially purified murine IFNs (mIFN-α/β), whereas animals in trial II received a high dose (1×10^6 units) of a recombinant, hybrid human IFN [hIFN-α B/D, (18)]. Control animals received saline injections. The number of animals in each treatment group, the length of therapy, the tumor volume per pancreas, the ratio of tumor volumes in treated animals to control animals, and the body weights at end of treatment regimen were tabulated for both trials. ND, not done.

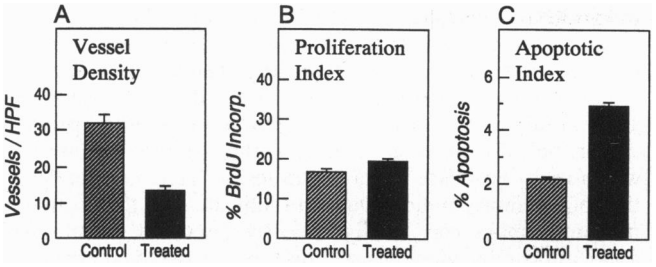

FIG. 2. Statistical analysis of blood vessel density, proliferation index, and apoptotic index in histological sections of pancreatic tumors from control animals (hatched bars) and animals treated with anti-angiogenic therapy in trial II (black bars). (*A*) The number of blood vessels per high-power field (HPF) was determined by immunoperoxidase staining with an antibody to the endothelial cell marker vWF ($P < 0.001$). (*B*) The β tumor cell proliferation index was assessed as the percent of cells in S phase, using BrdUrd incorporation during a 2-h labeling period ($P > 0.05$). (*C*) Apoptotic index was measured by using TdT labeling of fragmented DNA in apoptotic cells using digoxigenin-labeled dUTP ($P < 0.001$). In every case, the differences between treated and control are statistically significant, as assessed by Student's *t* test; the *P* values are indicated for each characteristic analyzed. For reference, both the β tumor cell density and the capillary density in untreated tumors are about 1.5 times greater than the densities of β cells and vessels in normal pancreatic islets.

delivered to the target tumors by the circulation. Cultured β tumor cells (βTC3) (23) were incubated with "activated" serum collected from nontransgenic mice treated with the regimen of compounds used in trial II, in comparison to serum from sham-treated mice. Neither control or activated serum had an effect on tumor cell proliferation *in vitro*, as assessed by increased cell numbers during subconfluent growth (Table 2). However, the activated serum did contain inhibitory compounds for endothelial cells. Bovine capillary endothelial cells were incubated with the same control and activated mouse serum, and the latter inhibited cell proliferation by 50%, compared to the control mouse serum (data not shown).

DISCUSSION

This study demonstrates that tumor progression can be impaired by treatment solely with a set of angiogenesis inhibitors in a regimen that begins prior to the emergence of solid tumors. Capillary density was more than halved, and cumulative tumor volume was about 1/10th that of untreated controls. The tumor cell proliferation rate, as assessed by cells in S phase, was not affected. Interestingly, the apoptotic cell incidence was doubled in treated tumors, despite data indicating that these compounds are not directly cytotoxic to the tumor cells. We infer that inadequate access to serum factors or oxygen, or indeed to the endothelium itself, increased the rate of tumor cell apoptosis, thereby retarding rapid expansion of the tumor mass. The observed effect of angiogenesis inhibitors on apoptosis is consistent with a recent study involving transplantation of Lewis lung carcinoma into syngeneic mice followed by treatment with AGM-1470, which also resulted in increased apoptotic rates (24).

The data from these trials indicate that the initial switch to the angiogenic phenotype during tumorigenesis was not completely blocked; rather, neovascularization was significantly attenuated and could not support rapid tumor growth. It is increasingly evident that multiple positive and negative regu-

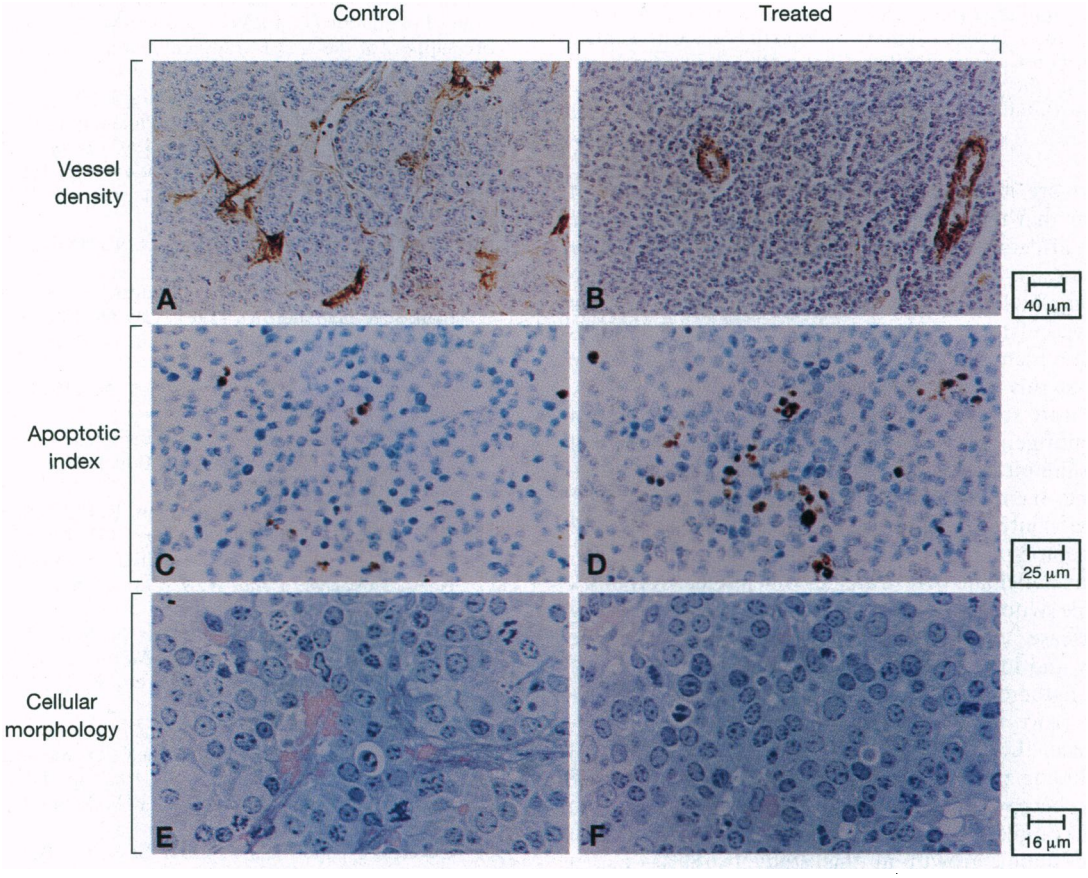

FIG. 3. Histological comparison of tumor vasculature, apoptosis, and morphology in treated and control pancreas. (*A* and *B*) Histologic sections from control and treated paraffin-embedded pancreata were stained with antibodies against vWF to determine vessel density. (*C* and *D*) Apoptotic index was measured by using TdT to label fragmented DNA in apoptotic cells with digoxigenin-labeled dUTP. (*E* and *F*) Cellular morphology of control and treated pancreata embedded in JB4 plastic and stained with hematoxylin/eosin.

Table 2. Sera of mice treated with the antiangiogenesis regimen are not directly cytotoxic to the β tumor cells

Murine serum	Concentration of murine serum per well, %	No. of wells treated	Mean no. of tumor cells per well, $\times 10^{-4}$
None	0	5	32.2 ± 5.20
Saline-treated	2	6	31.0 ± 3.20
Regimen II-treated	2	6	37.2 ± 3.68
Saline-treated	5	5	40.4 ± 3.08
Regimen II-treated	5	5	53.2 ± 2.23
Saline-treated	10	4	40.9 ± 4.01
Regimen II-treated	10	5	59.5 ± 4.21

To assess the possibility of direct tumor cell cytotoxicity resulting from the antiangiogenesis regimen (which is presumed to be delivered to the tumors via the circulation), three 6-week-old C57BL/6 male mice were treated with the regimen of trial II and three mice were similarly treated with saline injections for 3 days. On the fourth day, the three compounds (or the saline controls) were injected, after 1.5 h the mice were lethally anesthesized, and blood was collected by heart puncture. Serum was prepared and added in increasing amounts to ≈40% semiconfluent monolayer cultures of βTC3 cells (23) in 24-well plates, maintained in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) heat-inactivated bovine calf serum. A total of 1×10^4 cells were seeded in each well, and the cell number had increased to 3.6×10^4 at the beginning of the incubation with control and drug-treated mouse serum. After 48 h, the wells were washed with phosphate-buffered saline and trypsinized, and cell number was determined in a Coulter Counter. The cellular morphology was similar in treated and control cell cultures, and no increase in dead cells was evident by visual inspection. Since the S-phase incidence is similar *in vivo*, and the overall proliferation rate is comparable in treated and control cultures *in vitro*, we infer that these compounds are not directly impairing growth of the tumor cells themselves. In contrast, similar assays performed on bovine capillary endothelial cells with control and activated mouse serum demonstrated a 50% reduction in cell accumulation by the activated serum from the drug-treated mice (data not shown), indicative of inhibitory activity against endothelium.

latory factors are involved in the control of angiogenesis; it would appear that the three inhibitors used in our trials are only partially affecting those signals. Thus, there remains clear potential to design second generation antiangiogenesis regimens that completely block the angiogenic switch, maintaining a quiescent vasculature. Given the number of new inhibitors that have been identified recently (25), there is reason to be optimistic that this is an attainable goal. As such, it will be possible in future studies to compare in transgenic models of *de novo* tumorigenesis the effects of different classes of angiogenesis inhibitors, including the increasing list of proteolytic fragments of endogenous proteins, and inhibitors directed at specific attributes of the endothelial cell, including cell adhesion receptors and intracellular signaling networks. Certainly, one can anticipate that strategies which selectively block the angiogenic switch may only restrict tumor growth, resulting in "stable disease." Nevertheless, this study demonstrates the high efficacy and low toxicity associated with antiangiogenic therapy, strengthening the proposition that angiogenesis inhibitors will prove to be effective in combination with conventional cancer therapeutic strategies that target tumor cells directly, including traditional cytotoxic agents, immunotherapy, or radiotherapy, thereby increasing efficacy, perhaps to the point of complete remission (26). Yet, as it stands, the inhibition of tumor growth in this study by 89%—i.e., a treated/control volume of 0.11—in the absence of direct tumor cell cytotoxicity by a combination of antiangiogenic inhibitors is already as good or better than those of candidate anticancer compounds selected to be tested in clinical trials.

The trials presented herein demonstrate that transgenic mice can serve as a valuable assay system for testing strategies that seek to interfere with tumor angiogenesis. The RIP-Tag 2 model of multifocal islet cell carcinogenesis serves not only as a paradigm for blocking development of an initial primary tumor, but also more generally for the common situation in which multifocal carcinoma-*in-situ* lesions remain after resection of a primary tumor. Our data indicate that the growth of nascent tumors can be significantly impaired by the sole application of angiogenesis inhibitors. In the near term, attenuation of angiogenesis will be most clearly beneficial for cancer patients in whom the primary tumor has been removed but a high likelihood remains for regrowth, progression of carcinoma-in-situ lesions, or expansion of occult metastases. Nevertheless, given its low toxicity, long-term antiangiogenic therapy has potential applications in restricting development and growth of certain primary tumors, especially as methods of early detection improve. Looking ahead, the availability of transgenic mouse models of de novo tumor progression should allow more expedient tests of combinatoric therapies that target discrete aspects of the tumor phenotype.

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