ACTIBIND, a T₂ RNase, Competes with Angiogenin and Inhibits Human Melanoma Growth, Angiogenesis, and Metastasis

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Abstract

Melanoma is a very aggressive and highly angiogenic tumor in which standard treatments have had only limited success. Patients with advanced disease have a 5-year survival rate of 5%. In search for alternatives, we identified a natural product extracted from the fungus Aspergillus niger, termed ACTIBIND, that inhibits tumor growth and metastasis of melanoma in vivo. ACTIBIND, a T₂ RNase, exerts antitumorigenic and antiangiogenic activities by competing with the angiogenic factor angiogenin (itself an RNase homologue). Thus, there was decreased expression and activity of the matrix metalloproteinase 2 in melanoma and vascular endothelial cells, decreased vascularization, and increased tumor cell apoptosis in vivo. ACTIBIND significantly inhibited angiogenesis in an in vivo angiogenesis assay with sponges containing angiogenin. In vitro, ACTIBIND was internalized by both melanoma and human umbilical vein endothelial cells, reached the cell nuclei, and inhibited the activity of angiogenin response elements in a dose-dependent manner. Collectively, our data indicate that ACTIBIND should be tested for its potential as a new antiangiogenic modality for the treatment of melanoma. [Cancer Res 2007;67(11):5258–66]

Introduction

Identifying novel therapies against metastatic melanoma is crucial in light of its aggressive nature, its position as the leading cause of skin cancer death, and its notorious resistance to currently available cancer therapies. Overall, melanoma accounts for about 3% of all malignant tumors and has dramatically increased in incidence in the last 10 years (1). When diagnosed as a thin lesion, cure with surgical resection is possible in a high percentage of cases, with a 5-year survival rate of >80%. However, once the metastatic phase develops, it is almost always fatal, with an estimated median survival range of 6 to 9 months and a 5-year survival rate of <5% (2). The gold standard for melanoma treatment is with the chemotherapeutic drug dacarbazine (DTIC). Other modalities include the Food and Drug Administration–approved adjuvant therapies with high-dose IFN-α2b for patients with high-risk stage II and III melanoma and treatment with a high-dose interleukin 2 for stage IV melanoma (3, 4). None of these treatments, however, have had a substantial effect on outcomes; thus, we and others have begun to search for alternative modalities. Among the most promising are the RNases.

RNases, defined as 2’ 3’-cycling enzymes, have been the subjects of intensive research in the past two decades. The RNases are classified into three distinct families: RNase A, RNase T₁, and RNase T₂ according to base specificity, structure, function, optimal pH, and origin (5). The specific impetus for recent scientific attention is the possible therapeutic potential of these enzymes shown by the discovery that a number of them exhibit antitumor and antiviral activities both in vitro and in vivo (6). For example, onconase, an amphibian RNase, is now being tested in a phase III cancer therapy trial after it proved effective against a wide range of cancer cells in culture and in animal studies (7). The family of T₂ RNases (EC 3.1.27.1), ubiquitous in nature and present in all organisms thus far examined, are mostly located where RNA is not thought to be readily available (e.g., outside the cell or in the vacuole; ref. 8). Thus, researchers have proposed biological functions for these enzymes other than the processing of cellular RNA. Our group was the first to isolate and characterize a T₂ RNase with antitumorigenic properties (9, 10). We extracted a T₂ RNase from the fungus Aspergillus niger and established that it inhibits the elongation and alters the orientation of pollen tubes in plants by interfering with the intracellular actin network; thus, we named it ACTIBIND (9).

More recently, we reported that ACTIBIND exerted preventive and therapeutic effects in two different models of colorectal cancer (10). Our results indicated that ACTIBIND may function as an antiangiogenic and antivascular drug, targeting tumor-associated blood vessels (10). We postulated that ACTIBIND might exert its antiangiogenic activities through inhibition of angiogenin.

This hypothesized mechanism of action is intriguing. Angiogenin is one of the most potent angiogenic factors in vivo. Although it is an RNase A homologue, its proangiogenic properties make it a tumor-promoting factor and thus a potential cancer therapeutic target rather than a therapeutic agent like some of the other RNases (11). Angiogenin has been implicated in a variety of tumors; increased expression in tumor specimens and in patient sera was observed in breast, colorectal, gastric, pancreatic, kidney, and lung cancers, and the increases correlated with decreased time to tumor progression and a shortened disease-free survival (12–17). Similarly, increased angiogenin levels were found in the serum of melanoma patients (18). Furthermore, Hartmann et al. showed that angiogenin is induced by hypoxia in human melanoma cells, and that enhanced expression correlated with tumor aggressiveness in vitro and in vivo (19). Thus, we chose angiogenin as a target for testing our hypothesized mechanism of action for ACTIBIND. In addition,
we measured the effects of ACTIBIND on human melanoma growth and metastasis using an in vivo nude mouse model.

Here, we report that ACTIBIND was effective in inhibiting human melanoma growth and metastasis in vivo. We show that ACTIBIND exerts its antitumorigenic and antimetastatic effect by negatively competing with angiogenin at the endothelial cell level, thus inhibiting angiogenesis. Interestingly, the data point to a possible autocrine function of angiogenin affecting melanoma cells directly, which is similarly inhibited by ACTIBIND. Thus, we propose that ACTIBIND could be used as a new modality to treat melanoma patients.

Materials and Methods

Cell lines. The highly tumorigenic and metastatic human melanoma A375/SM cell line was established previously from a pool of lung metastasis produced by i.v. injection of parental A375-P cells (20). Cells were maintained in Eagle's MEM supplemented with 10% fetal bovine serum (FBS), sodium pyruvate, nonessential amino acids, HEPES buffer, and 1% penicillin-streptomycin and incubated at 37°C with 5% CO2. Human umbilical vein endothelial cells (HUVEC) were obtained from the American Type Culture Collection (CRL-1730). HUVECs were plated in 0.5% gelatin-coated flasks and maintained in DMEM supplemented with 15% FBS, 1% glutamine, 1% antibiotic/anti-mycotic solution, and 10 ng/mL basic fibroblast growth factor (bFGF).

ACTIBIND. ACTIBIND was prepared from the A. niger mold as previously described (9). Following purification, ACTIBIND was dissolved in water, sterilized, and applied to cells or animals as described. On a Western blot, ACTIBIND yields 32- and 40-kDa glycoprotein isoforms. All ACTIBIND solutions used herein were tested for the presence of bacterial endotoxin by the Limulus amebocyte lysate detection assay (Associates of Cape Cod, Inc.). The value measured in ACTIBIND solutions was below the detection limit of the assay (0.05 EU/mL) as obtained in standard curves using endotoxin by the asebocyte lysate detection assay (Associates of Cape Cod, Inc.).

Animals. Male athymic BALB/c nude mice were purchased from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used at 7 to 9 weeks of age. Animals were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the U.S. Department of Agriculture, Department of Health and Human Services, and NIH.

In vivo tumor growth and metastasis. Cells in exponential growth phase were harvested by brief exposure to a 0.25% trypsin/0.02% EDTA solution (w/v), washed, and resuspended in Ca2+/Mg2+-free HBSS. Cell viability was determined by trypan blue exclusion, and only single-cell suspensions of >90% viability were used. S.c. tumors were produced by injecting 105 tumor cells per 0.1 mL HBSS over the right scapular region of the mice, and their growth was monitored weekly for 5 weeks (21). Formalin-fixed tumor sections were recovered and processed for immuno-

Zymography. HUVECs or A375/SM cells (5 × 105) were grown in six-well plates and treated with ACTIBIND (1–10 μmol/L) or PBS for 4 days. Treatment for 4 days was found to produce optimal angiogenin effects and MMP-2 release (data not shown). On day 5, CMEM was replaced with serum-free medium. After overnight incubation, the supernatants were collected and analyzed for angiogenin or MMP-2 levels using corresponding quantikine immunoassay kits (R&D Systems). The results were normalized by the cell number.

Immunohistochemistry. For the immunofluorescent staining of CD31/platelet/endothelial cell adhesion molecule 1 in Gelfoam specimens, frozen sections were fixed in acetone/acetone/chloroform/acetone, washed with PBS, blocked with fish gelatin (4% in PBS, 20 min), and incubated for 18 h at 4°C with rat anti-mouse CD31 antibody (1:800; Pharmingen). Samples were then washed with PBS and incubated with goat anti-rat antibody conjugated to Alexa 594 for 1 h (1:200; Molecular Probes, Inc.), counterstained with Hoechst 33342 for visualization of nuclei, washed, and mounted with Vectashield mounting medium (Molecular Probes). Immunofluorescence microscopy was done using a Zeiss Axioplan microscope (Carl Zeiss) equipped with a C5810 Hamamatsu color-chilled CCD camera. For the quantification of microvessel density in Gelfoam specimens, ten 0.2-mm2 fields at ×100 magnification were counted. Microvessel density was expressed as the median number and range of endothelial cells per field.

For CD31 and matrix metalloproteinase 2 (MMP-2) staining in frozen tumor tissues, sections were washed, treated with 3% hydrogen peroxide to block activity of the endogenous peroxidase, washed again, blocked with 5% normal horse serum/1% normal goat serum, and incubated overnight with mouse monoclonal anti-CD31 (1:800), or rabbit polyclonal anti-MMP-2 antibody (1:200; Pharmingen). After being washed, samples were incubated for 1 h with peroxidase-conjugated anti-mouse IgG1 or anti-rabbit IgG, rinsed with PBS, and incubated with diaminobenzidine (Research Genetics). The sections were then counterstained with Gill's hematoxalin. For the quantification of microvessel density, 10 fields of the CD31 stained samples were counted at ×100 magnification.

Confocal microscopy. For ACTIBIND and CD31 co-staining in HUVECs, cells were cultured on chamber slides, fixed with acetone, blocked with 4% fish gelatin, and incubated overnight at 4°C with rabbit polyclonal anti-CD31 antibody (1:800 dilution; prepared at Anilab) and mouse anti-human CD31 antibody (DAKO Corp.). After washing, the slides were incubated for 1 h with FITC-conjugated anti-rabbit antibody and Hoechst 33342-conjugated anti-mouse antibody, washed, and mounted with Vectashield mounting medium (Molecular Probes). The slides were viewed on Zeiss LSM510 laser scanning confocal microscope. Z-sections and XZ-sections were obtained from three-dimensional scanning by using LSM510 software.

In situ terminal deoxynucleotid transferase–mediated nick-end labeling assay. Thin sections (4 μm) from frozen tissue were prepared, and the terminal deoxynucleotid transferase–mediated nick-end labeling (TUNEL) assay was done using a commercial kit according to the manufacturer's protocol (Promega). The results were determined as mean percentage ± SD of apoptotic cells from the total number of cells counted in eight fields per slide.

ELISA. A375/SM cells (5 × 105) and HUVEC (5 × 103) were plated in six-well plates and treated with ACTIBIND (1–10 μmol/L) or PBS for 4 days. Treatment for 4 days was found to produce optimal angiogenin effects and MMP-2 release (data not shown). On day 5, CMEM was replaced with serum-free medium. After overnight incubation, the supernatants were collected and analyzed for angiogenin or MMP-2 levels using corresponding quantikine immunoassay kits (R&D Systems). The results were normalized by the cell number.
Invasion assay. A375SM cells grown in six-well plates were treated with 1 or 10 μmol/L ACTIBIND or with CMEM for 4 days, released from the plates by a brief exposure to trypsin-EDTA (Life Technologies, Inc.), washed, and resuspended in serum-free medium at 5 × 10⁶ per mL. Cell invasion was tested using BioCoat Matrigel invasion chambers (Becton Dickinson) as described earlier (22). The data were expressed as average number of cells from 10 fields in each of three experiments done ± SD.

Luciferase reporter assay. Angiogenin-binding DNA element (ABE, 5’-CTCTCTCTCTCTGTTCCTCTC-3’) sequence was cloned into the pGL3-E luciferase expression vector (24). A total of 25 × 10⁵ cells per well in a 24-well plate were transfected with 0.5 μg of the basic pGL3-E vector with no promoter or enhancer sequence or with 0.5 μg of the pGL3-E/ABE construct using LipofectAMINE 2000 (Life Technologies). After 6 h, the transfection medium was replaced with serum-containing growth medium. Ten-micromolar angiogenin was added to the transfected cells for 48 h in the presence or absence of ACTIBIND. For each transfection, 30 ng Renilla luciferase reporter pRL-Bactin (Promega) was included to normalize for differences in transfection efficiency. Luciferase activity was assayed using a dual luciferase reporter assay system (Promega).

Angiogenin small interfering RNA transfection. To knock down the angiogenin expression, A375SM cells were transfected with 10 nmol/L angiogenin-specific Dicer-substrate small interfering RNA (siRNA) oligonucleotide duplexes (Integrated DNA Technologies) using LipofectAMINE 2000 (25). Specific RNA interference duplexes selected and probed for efficacy for knockdown angiogenin secretion were as follows: sense, 5’-rCrGrArArCrArArGrArArCrArCrUrUrCrUrUrCrUrUrGrUrUrGrUrUrGrUrUrGrUrUrU; antisense, 5’-rCrGrCrArGrArGrArGrArGrUrArGrArGrUrUrGrUrUrGrUrUrGrUrUrGrUrU. Cells were treated with siRNA at a final concentration of 200 nmol/L. The control sequence was a non-targeting sequence of the same length with no homology to any known human gene. Control cells were A375SM cells incubated with transfection reagent only. After 24 h of transfection, medium was replaced with fresh medium devoid of serum. This final medium was harvested after 48 h for angiogenin or MMP secretion analysis by ELISA or MMP in-gel activity analysis as detailed above.

Statistical analysis. The in vitro data were analyzed for significance by the Student’s t-test (two tailed), and the in vivo data were analyzed by the Mann-Whitney U test.

Results

ACTIBIND inhibits human melanoma growth in vivo. Because angiogenin is thought to play a role in human melanoma progression (18), we first investigated whether ACTIBIND can suppress melanoma growth in vivo. To that end, A375SM melanoma cells (5 × 10⁵) were injected s.c. into nude mice (n = 5). Beginning 3 days later, the mice were treated with 1 mg ACTIBIND or control PBS i.p. every other day for 30 days. The latency period was significantly prolonged in the ACTIBIND-treated group: all animals in the control group developed a palpable tumor within 5 days of A375SM cells injection, whereas mice in the ACTIBIND-treated group did not have any evidence of tumor (Fig. 1). Sixteen days into the study, tumors in control mice had a mean volume of 100 mm³, whereas tumor volume in treated mice reached <10 mm³. Still later, tumors in control animals grew progressively and produced large tumors reaching 800 mm³ mean volume (Fig. 1), as opposed to 100 mm³ mean volume for treated mice. Body weights measured at regular intervals were not significantly different in the treated group in comparison with the control group, indicating that toxicity was low.

ACTIBIND inhibits human melanoma metastasis in vivo. To determine the effect of ACTIBIND on human melanoma metastasis development, we have used an experimental melanoma lung metastasis mouse model established by i.v. injection of A375SM cells into the tail vein of nude mice. The mice were treated with either ACTIBIND or PBS delivered i.p. every other day for 60 days, starting at day 5 after tumor injection. As shown in Table 1, both the incidence and number of lung metastasis of A375SM cells were reduced in the ACTIBIND-treated mice in comparison with the control group. In control mice, A375SM cells produced numerous lung metastases (median, 65; range, 16–200), whereas treatment with ACTIBIND significantly reduced the number (median, 10; range, 0–75; P < 0.05). Collectively, these data show that ACTIBIND treatment suppressed melanoma growth and metastasis. We next set out to identify the molecular mechanisms responsible for the antitumorigenic and antimetastatic effects of ACTIBIND.

ACTIBIND inhibits angiogenin-induced angiogenesis in vivo. Our previous data suggested that ACTIBIND possesses antiangiogenic properties (10). To confirm and further establish its antiangiogenic effects, we did an in vivo Gelofoam sponge angiogenic assay as previously described (23) using either angiogenin or bFGF as angiogenic stimulators. Each nude mouse was implanted with Gelofoam sponges treated with agarose containing PBS, bFGF, or angiogenin into one flank and Gelofoams containing PBS + ACTIBIND, bFGF + ACTIBIND, or angiogenin + ACTIBIND implanted into the other flank. Two weeks after

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<th>Table 1. Experimental lung metastasis of A375SM cells in nude mice</th>
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NOTE: A375SM cells were injected i.v. into groups of nude mice. Mice were treated with ACTIBIND or with PBS. Experimental lung metastasis was determined 60 d after cancer cell injection. *P < 0.05 as determined by Mann-Whitney U test.

Figure 1. ACTIBIND inhibits tumor growth of A375SM human melanoma cells in nude mice. A375SM cells were implanted into the flanks of nude mice, and after 3 d, the mice were treated either with PBS or ACTIBIND every other day for 30 d.
implantation, the mice were sacrificed, and Gelfoams were retrieved for further evaluation. Both angiogenin- and bFGF-induced neovascularization was visible within the Gelfoam, and the number of vessels was significantly greater than in implanted Gelfoams impregnated with PBS alone. Immunohistochemical staining of the Gelfoams for CD31 showed a median number of 30 (range, 3–45), 110 (range, 60–132), and 75 (range, 42–105) CD31-positive cells per × 100 field in the PBS-, angiogenin-, and bFGF-impregnated Gelfoams, respectively. ACTIBIND somewhat inhibited neovascularization in PBS- and bFGF-treated Gelfoams and almost completely abolished it in angiogenin-treated Gelfoams. The median number of CD31-positive cells per × 100 field were 12 (range, 0–33), 11 (range, 0–25), and 21 (range, 2–33) in Gelfoams impregnated with PBS + ACTIBIND, angiogenin + ACTIBIND, and bFGF + ACTIBIND, respectively. The degree of ACTIBIND inhibition of angiogenin-induced angiogenesis was statistically significant (P < 0.001). The angiogenesis observed in the PBS-implanted sponges was likely the result of a wound healing.

Next, we implanted nude mice with Gelfoams containing only the angiogenic molecules (angiogenin or bFGF, 1 μg/mL; 100 ng per sponge) or PBS and randomized them to receive either ACTIBIND (1 mg/100 μL starting 2 days after the implantation) or PBS i.p., every other day. After 2 weeks of treatment, the Gelfoam sponges from all animals were harvested, washed, and frozen for sectioning. CD31 counts after immunofluorescent staining with CD31 antibody show that i.p. administration of ACTIBIND significantly decreased the number of endothelial cells (CD31+ in sponges containing angiogenin [from a median of 90 (range, 56–123) to a median of 5 (range, 5–20); P < 0.001]. The effect of ACTIBIND on bFGF-impregnated gel foams was less pronounced than for angiogenin but was also significant [from a median of 65 (range, 43–95) to a median of 15 (range, 3–33); P < 0.01]. In the control PBS-impregnated gel foams, ACTIBIND had no significant effect on microvessels density number [median of 26 (range, 2–46) in PBS group compared with median of 17 (range, 0–41) in ACTIBIND-treated group]. Cumulatively, these results, although they do not exclude other mechanisms of action, indicate that ACTIBIND preferentially inhibits angiogenic processes mediated by angiogenin.

**Angiogenin directly regulates rRNA transcription and MMP-2 expression in melanoma cells.** Angiogenin has previously been suggested to play an important role in melanoma progression (18). Although the mechanisms of action of angiogenin are still to be elucidated, most studies focus on the effect on endothelial cell growth and the contribution to the development of neovasculature as the major contributors to angiogenin-induced tumor progression (26). Little is known about the direct role of angiogenin in tumor cells per se. Recently, it was reported that down-regulating angiogenin expression in HeLa cells not only reduced tumor angiogenesis but also diminished tumor cell proliferation, possibly through inhibition of the constitutive translocation of angiogenin to the nucleus where it plays a role in rRNA transcription (27).

Inasmuch as the above results suggest that ACTIBIND acts via inhibition of angiogenin, we first assessed whether our melanoma cells and/or endothelial cells express angiogenin. ELISA showed that A375SM cells and HUVECs released 580 and 650 pg angiogenin per million cells per day, respectively. Next, we set out to confirm that angiogenin is functional, and that it plays a role in melanoma cell growth. We focused on two possible functions of angiogenin: regulation of rRNA transcription and MMP-2 secretion and activity. Crucial for the angiogenic function of angiogenin in endothelial cells is binding to DNA in the nucleolus and stimulating rRNA transcription (27, 28). An ABE has been previously identified from the non-transcribed region of the rRNA gene and characterized as possessing angiogenin-dependent promoter activity that can drive the expression of a luciferase reporter gene (28). We first investigated whether angiogenin can activate the ABE in melanoma cells: pGL3ABE-luciferase reporter construct was transfected into A375SM cells, which were then exposed to increasing concentrations of angiogenin; the luciferase readout was compared with that of control A375SM cells transfected with a pGL3-luciferase vector (Fig. 2A). Basal ABE-driven luciferase activity was observed, suggesting activity of the autocrine angiogenin produced by A375SM cells. Moreover, luciferase expression increased with increasing angiogenin dose and reached a 9-fold increase with 10 μmol/L angiogenin (P < 0.001). These results suggest that angiogenin can induce ABE-driven transcription in A375SM cells and support a possible additional role for angiogenin in melanoma other than the induction of angiogenesis.

One way that angiogenin may induce angiogenesis is through binding to cytoplasmic actin, leading to activation of protease cascades such as MMPs (11, 26). MMP-2 is known to play a crucial role in melanoma angiogenesis and metastasis as it contributes to

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**Figure 2.** A, angiogenin stimulates ABE-driven luciferase activity. A375SM cells were transfected with the pGL3E-ABE vector and pRL-TK control vector and exposed to angiogenin at different concentrations. Luciferase activity was assayed using a dual luciferase reporter assay system. Columns, means from triplicate samples; bars, SD. * P < 0.01; ** P < 0.001. B, angiogenin stimulates secretion of MMP-2 by A375SM cells as determined in the cultured media by ELISA. * P < 0.05. C, effect of angiogenin on MMP-2 activity in A375SM cells as determined by gelatin zymography.
modification of cell surface molecules, proangiogenic factor liberation or activation, pro-MMP activation, extracellular matrix degradation, and integrin modification/interaction (29). To determine whether angiogenin can induce MMP-2 in melanoma cells directly, we stimulated A375SM melanoma cells with angiogenin; the treatment increased MMP-2 secretion and activity in a dose-dependent manner (Fig. 2B and C). The secretion of MMP-2 was significantly increased only at the highest angiogenin concentration, but increased MMP-2 activity was already evident at 1 nmol/L, suggesting that angiogenin regulates MMP-2 both transcriptionally and post-translationally. Our zymography gel (Fig. 2C) did not detect the active form of MMP-2, which is usually the case in melanoma cells. Transfecting A375SM cells with an anti-angiogenin siRNA (10 nmol/L) significantly reduced (~96%) angiogenin secretion (Fig. 3A), which in turn led to reduced MMP-2 secretion observed in an ELISA assay (Fig. 3B) and reduced activity according to zymography (Fig. 3C). Control transfection with non-targeting siRNA did not lead to any change in angiogenin levels nor to any decrease in MMP-2 expression or activity. To the best of our knowledge, these data are the first to suggest that angiogenin plays a role in regulating MMP-2 secretion and activity in melanoma cells. Interestingly, blasting of the MMP-2 promoter revealed no ABE. The regulatory mechanism by which angiogenin leads to increased MMP-2 expression is the subject of future studies.

**ACTIBIND does not affect angiogenin expression level in HUVECs and melanoma cells.** The animal experiments presented above suggested that the anti-melanoma effects of ACTIBIND were at least partially mediated through the inhibition of the function of angiogenin. To examine how ACTIBIND inhibits angiogenin function in human melanoma, we first assessed whether ACTIBIND affects angiogenin secretion by melanoma cells and/or HUVECs. Four days of treatment with 10 nmol/L ACTIBIND did not significantly affect total angiogenin release by A375SM cells or HUVECs (550 and 610 pg angiogenin per 10⁶ cells per day, respectively) as determined via ELISA. These results indicated that ACTIBIND affects angiogenin-related activities without a direct effect on angiogenin production but possibly by competing with it. The exact molecular mechanisms controlled by angiogenin leading to induction of angiogenesis are not well understood, but it has been shown that angiogenin has multiple functions at different cellular levels, including the basement membrane, cytoplasm, and nucleus (11, 28). Based on our hypothesis that ACTIBIND counteracts the effects of angiogenin on endothelial cells, we next studied the localization of ACTIBIND in HUVECs after an 8-h exposure to the drug using immunofluorescent staining analyzed by confocal microscopy (Supplementary Fig. S1). Serial images of a representative HUVEC (see arrow in A) from the upper membrane side of a cell (A) to the lower membrane side of a cell (T) were obtained after double immunofluorescent staining for ACTIBIND (green) and CD31 (red). Combined immunostaining (overlapping, indicated in yellow) for CD31 and ACTIBIND occurred on the cell membrane, but whereas CD31, as expected, was exclusively expressed on the membrane of the endothelial cells, ACTIBIND was also localized to the cytosol and nucleus. These results suggest that ACTIBIND was internalized into endothelial cells and possibly exerted its effects in the same cellular compartments as angiogenin. Next, we asked whether ACTIBIND was also internalized by melanoma cells. Few A375SM cells were stained positive for ACTIBIND following a 1-h exposure to it (data not shown). After 2 h of exposure, ACTIBIND appeared mainly in the cytosol, whereas after an 8-h exposure, ACTIBIND also appeared in the nucleus of the melanoma cells (data not shown). These results show that similar to endothelial cells, melanoma cells retained ACTIBIND, suggesting a possible direct function of ACTIBIND in melanoma cells.

**ACTIBIND inhibits MMP-2 expression in HUVECs and melanoma cells.** Because the role of MMP-2 in melanoma progression and angiogenesis is well established, and based on the above data showing that angiogenin regulates the expression and activity of MMP-2 and our hypothesis that ACTIBIND competes with angiogenin, we next evaluated whether ACTIBIND inhibits MMP-2 expression and/or activity. Figure 4A indicates that ACTIBIND inhibited total MMP-2 release by A375SM cells in a dose-dependent manner. The effect of ACTIBIND on de novo activity of MMP-2 was verified by gelatin zymography. Figure 4B shows that pro-MMP-2 collagenase activity of A375SM cells was significantly decreased by ACTIBIND therapy compared with PBS-treated controls or CMEM-containing sera. Similarly, as shown in Fig. 4C, ACTIBIND treatment decreased pro-MMP-2 and MMP-2 activity in HUVECs in a dose-dependent manner. We next determined whether this reduction in MMP-2 activity was reflected in the ability of melanoma cells to invade through extracellular matrix, an important step in the process of tumor progression and
metastasis. As shown in Fig. 4D, A375SM cells treated with ACTIBIND had a significantly lower ability to invade through Matrigel-coated filters than did control cells (1216 ± 68 versus 725 ± 59 for A375SM cells treated with 1 μmol/L ACTIBIND (P < 0.01) and 211 ± 14 for A375SM cells treated with 10 μmol/L ACTIBIND (P < 0.001)). These results indicate that ACTIBIND inhibited the ability of melanoma cells to penetrate the basement membrane. Collectively, our data indicate that ACTIBIND may exert its antitumorigenic and antimetastatic effects by inhibiting the invasion properties in both melanoma cells and tumor-associated endothelial cells.

ACTIBIND competes with angiogenin to block ABE-driven luciferase activity and inhibits clonogenicity in A375SM melanoma cells. In the next set of experiments, we determined whether ACTIBIND competed with angiogenin to block ABE-driven transcription as evaluated by luciferase activity. pGL3ABE-luciferase transfected A375SM melanoma cells were treated with angiogenin (10 μmol/L) and increasing concentrations of ACTIBIND. ACTIBIND inhibited the transcription stimulating activity of angiogenin on pGL3ABE-transfected A375SM cells in a dose-dependent manner (Fig. 5A). These data further support our hypothesis that ACTIBIND competes with angiogenin in melanoma cells.

Tsuji et al. have previously shown that angiogenin-driven rRNA transcription can enhance proliferation of HeLa cells (27). Based on our finding that ACTIBIND competes with angiogenin in melanoma cells, we next sought to determine whether ACTIBIND inhibits melanoma cell proliferation. The long-term viability of A375SM cells, as measured by a colony-formation assay, was significantly and dose-dependently decreased after exposure of the cells to ACTIBIND; ACTIBIND added in a concentration of 10 μmol/L reduced melanoma cells colony formation by >5-fold (Fig. 5B). This effect could not be attributed to a direct induction of apoptosis by melanoma cells in vitro (data not shown).

Taken together, the above data provide evidence that ACTIBIND competes with angiogenin and is active against both melanoma cells and melanoma-associated endothelial cells. Our previous data showed that the antitumor activities of ACTIBIND seem to be independent of its RNase activity (10). However, ACTIBIND was found to directly compete with the binding of angiogenin to actin (data not shown).

ACTIBIND therapy results in decreased angiogenesis, reduced MMP-2 expression, and increased apoptosis in vivo. Lastly, to verify our in vitro data, we evaluated whether the in vivo effects of ACTIBIND, evident from its suppression of melanoma growth and metastasis, could be at least partially attributed to its antiangiogenic and anti-MMP-2 properties. Tumor-associated neovascularization as indicated by microvessel density was determined by immunohistochemistry using an anti-CD31 antibody. As shown in Fig. 5C, microvessel density per field was significantly reduced in the tumors described in Fig. 1 after treatment with ACTIBIND compared with control tumors. The mean microvessel density was 12 ± 5 per field in the ACTIBIND-treated A375SM tumors. In contrast, microvessel density was 43 ± 7 per field for control A375SM tumors. Immunohistochemistry using an anti-MMP-2 antibody (Fig. 5C) showed significantly decreased MMP-2 staining in the ACTIBIND-treated tumors in comparison with control-PBS treated A375SM tumors.

TUNEL assay showed that the number of tumor cells undergoing apoptosis was higher in the ACTIBIND-treated animals than in tumors in control mice. The number of TUNEL-positive tumor cells was inversely correlated with microvessel density in the studied tumors (Fig. 5C). The percentage of apoptotic cells was 31.2 ± 7.3% in the ACTIBIND-treated A375SM tumors versus 2.2 ± 1.1% for control A375SM tumors. The above data indicate that ACTIBIND treatment significantly decreased tumor-associated
neovascularization, possibly as a consequence of increases in the number of melanoma cells undergoing apoptosis.

Discussion

In this study, we examined the antitumorigenic and antimetastatic effects of a novel therapeutic agent, ACTIBIND, a T2 RNase, on human melanoma. ACTIBIND therapy in nude mice injected s.c. with an aggressive human melanoma cell line, A375SM, prolonged the tumor latency period, and the tumors that eventually developed were significantly smaller and less vascularized. Similarly, in an experimental animal model of melanoma metastasis, ACTIBIND effectively prevented the development of lung colonies. We showed that the inhibitory effects of ACTIBIND are directed both against the melanoma cells and the tumor-associated endothelial cells, decreasing tumor cell proliferation and invasion (through significant inhibition of MMP-2) and blocking angiogenesis. Furthermore, we showed that the effect of ACTIBIND on both tumor and endothelial cells was mediated, at least partially, through competition with angiogenin.

The utilization of RNases as therapeutic anticancer agents has been the focus of much research in the last two decades (6). Most RNases exhibit cytotoxic effects, and ACTIBIND is no exception. The RNase-based mechanism thought to drive their cytotoxic effect is their ability to adsorb specifically to certain cells, enter their cytosol, degrade the RNA and thereby inhibit protein synthesis, and ultimately cause cell death. In this report, we have established that the RNase ACTIBIND has a different mechanism of action. It is intriguing that the cytotoxic effects of ACTIBIND may be mediated via negative competition with another RNase, angiogenin, the only RNase known to exert tumorigenic and angiogenic effects.

Human angiogenin was first identified as a potent angiogenic factor; thus, subsequent studies had mainly focused on how it induces angiogenesis and how its angiogenic activity can be modulated (30). Following direct interaction with endothelial and smooth muscle cells, angiogenin induces cell proliferation and stimulates cultured endothelial cells to form tubular structures (26). It also mediates cell adhesion, activates proteases, and induces cell invasion (26). The exact cell membrane angiogenin receptors on the cell surface have not yet been clearly determined; various cell surface molecules remain candidates. There are data showing that in confluent cultures, angiogenin binds to the 42-kDa α-smooth muscle type actin and induces cell invasion and migration via activation of tissue plasminogen activator and
plasmin (31). When cells are under sparse culture, angiogenin binds to a 170-kDa putative receptor to induce phosphorylation of mitogen-activated protein kinases (MAPK) and cell proliferation (27). Indeed, MAPK activation was reported to be an early event in melanoma progression (32, 33). Another study found that angiogenin might also bind and activate a member of the FGF receptor family in endothelial cells (26). Following binding to the endothelial cell surface, angiogenin rapidly undergoes endocytosis and is translocated to the nucleus, where it stimulates the synthesis of rRNA (27, 28). This process involves receptor-mediated endocytosis, microtubule- and lysosome-independent transport across the cytoplasm, and nuclear localization sequence-assisted nuclear import. rRNA transcription is the rate-limiting step in ribosome biogenesis and is essential for protein translation and cell growth. Therefore, angiogenin-stimulated rRNA synthesis might be the key process by which angiogenin induces endothelial cell proliferation and new blood vessel formation. Inhibition of nuclear translocation of angiogenin (34) or mutagenesis at its nuclear localization sequence (35) abolishes its angiogenic activity.

To better understand the importance of angiogenin in tumor angiogenesis, several animal experiments have been carried out to block the effects of human angiogenin using monoclonal antibodies (36). These antibodies blocked the growth of human HT-29 colon cancer, lung adenocarcinoma, and fibrosarcoma transplanted into nude mice. Unfortunately, tumor cells escaping the action of antibodies grew into defined tumors and lost sensitivity to the antibodies. To overcome the known limitations of anticancer antibody therapy, numerous nucleoside and nucleotide compounds have been tested as competitive inhibitors for angiogenin (37). Thus far however, no high-affinity (subnanomolar) inhibitors have been found most probably because the full binding potential of the targeted active site is not exploited. Our findings that ACTIBIND is a highly effective angiogenin competitive inhibitor are thus encouraging.

Although the commonly held assumption is that angiogenin is a tumor angiogenic protein, the results presented in our study indicate that melanoma cell themselves can respond to angiogenin in an autocrine fashion. To the best of our knowledge, only one other previous publication investigated a role for angiogenin in cancer cells. Tsuji et al. reported that angiogenin nuclear translocation and rRNA-induced transcription are not exclusive to endothelial cells and also occur constitutively in HeLa cells (27). Down-regulating angiogenin in these cells resulted in inhibition of rRNA transcription, ribosome biogenesis, cell proliferation, and tumorigenesis (27). Their results suggested that angiogenin deregulation of rRNA transcription may be an important determinant in neoplastic transformation. Indeed, they found that inhibiting angiogenin expression reduced tumorigenicity and reversed the malignant phenotype of HeLa cells. Similarly, we observed that angiogenin induces ABE-driven transcription in human melanoma cells, and that inhibition of angiogenin leads to reduced melanoma cell clonogenicity. Interestingly, we also showed that angiogenin regulates MMP-2 secretion and activity with 18 U.S.C. Section 1734 solely to indicate this fact.

In summary, in the present study, we show that the T2 RNase isolated from the fungi A. niger, ACTIBIND, exerts significant antitumor and antimetastatic activities. We further show that this T2 RNase significantly affects the clonogenicity of A375SM human melanoma cells and competes with angiogenin in both melanoma and endothelial cells. Thus, we hope that targeting angiogenin in melanoma using ACTIBIND will be superior to other therapies designed to inhibit either angiogenesis or cancer cell proliferation alone.

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