

Molecular Basis of the Synergistic Antiangiogenic Activity of Bevacizumab and Mithramycin A

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Abstract

The impact of antiangiogenic therapy on the Sp1/vascular endothelial growth factor (VEGF) pathway and that of alteration of Sp1 signaling on the efficacy of antiangiogenic therapy is unclear, yet understanding their interactions has significant clinical implications. Treatment with bevacizumab, a neutralizing antibody against VEGF, suppressed human pancreatic cancer growth in nude mice. Gene expression analyses revealed that this treatment substantially up-regulated the expression of Sp1 and its downstream target genes, including VEGF and epidermal growth factor receptor, in tumor tissues, whereas it did not have this effect on pancreatic cancer cells in culture. Treatment with mithramycin A, an Sp1 inhibitor, suppressed the expression of Sp1 and its downstream target genes in both cell culture and tumors growing in nude mice. Combined treatment with bevacizumab and mithramycin A produced synergistic tumor suppression, which was consistent with suppression of the expression of Sp1 and its downstream target genes. Thus, treatment with bevacizumab may block VEGF function but activate the pathway of its expression via positive feedback. Given the fact that Sp1 is an important regulator of the expression of multiple angiogenic factors, bevacizumab-initiated up-regulation of Sp1 and subsequent overexpression of its downstream target genes may profoundly affect the potential angiogenic phenotype and effectiveness of antiangiogenic strategies for human pancreatic cancer. Therefore, this study is the first to show the significance and clinical implications of alteration of Sp1 signaling in antiangiogenic therapy for pancreatic cancer and other cancers. [Cancer Res 2007;67(10):4878–85]

Introduction

Pancreatic cancer is currently the fourth leading cause of cancer-related deaths. The median survival duration from diagnosis to death is about 4 to 6 months, and the overall 5-year survival rate is <5% (1–3). A full understanding of the cellular and molecular mechanisms of the development and progression of pancreatic cancer is crucial for identifying new targets of effective treatment modalities for this deadly disease. Among the various potential targets are numerous proangiogenic and antiangiogenic factors released by tumor and host cells (4–6). These factors regulate

angiogenesis, which determines the growth and metastasis of pancreatic cancer (6–8). Of the numerous angiogenic factors discovered thus far, studies have identified vascular endothelial growth factor (VEGF) as a key mediator of tumor angiogenesis (9–11). Authors have reported elevated expression of VEGF in human pancreatic cancer specimens (12, 13), that its expression level correlates with microvessel density (MVD; refs. 4, 6, 14–16), and that VEGF-targeted therapy significantly inhibits angiogenesis and growth of pancreatic cancer in animal models (4, 6, 17). Other identified proangiogenic factors that are overexpressed in human pancreatic cancer include epidermal growth factor (EGF) and EGF receptor (EGFR; refs. 4, 18), insulin-like growth factor-I (IGF-I) and IGF-I receptor (IGF-IR; ref. 19), hepatocyte growth factor (HGF) and its receptor Met (20, 21), and fibroblast growth factor (FGF) and FGF receptor (22).

The molecular mechanisms by which these angiogenic molecules are regulated remain unclear, yet understanding these mechanisms is crucial for designing effective antiangiogenic therapies. Our previous studies showed that the transcription factor Sp1 plays an important role in regulating expression of VEGF and angiogenesis of human pancreatic cancer (23, 24). However, whether and, if so, how treatments targeting molecules such as VEGF impact the expression of *Sp1* and its downstream target genes, including *VEGF*, are unclear. We hypothesize that strategies such as neutralization of VEGF by treatment with bevacizumab lead to feedback activation of Sp1 and subsequent up-regulation of VEGF, EGFR, IGF-IR, and other factors, leading to bevacizumab resistance, whereas blockade of Sp1 expression and function sensitizes tumors to bevacizumab and/or reverses bevacizumab resistance. Mechanistic studies of these strategies will have significant clinical implications on the treatment of bevacizumab-resistant tumors, including pancreatic cancer.

Sp1 is a zinc finger transcription factor that is important to the transcription of many cellular and viral genes that contain GC boxes in their promoters. Researchers have cloned additional transcription factors similar to Sp1 in their structural and transcriptional properties (Sp2, Sp3, and Sp4), thus forming the *Sp1* multigene family (25). Although Sp1 has been perceived to be a basal transcription factor since its discovery, increasing evidence suggests that it regulates a variety of biological functions, including cell survival, growth, and differentiation and tumor development and progression (25–29). We previously reported that Sp1 overexpression is directly correlated with the angiogenic potential of and poor prognosis for human gastric and pancreatic cancer (22, 24, 30). Therefore, Sp1 inhibitors such as mithramycin A may have profound antiangiogenic effects.

Mithramycin A, also known as aureolic acid and plicamycin (Mithracin), is an aureolic acid-type polyketide produced by various soil bacteria of the genus *Streptomyces* (31, 32). In the past,

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physicians used mithramycin A to manage hypercalcemia in patients with bone metastases from various malignancies, whereas others used it to treat Paget's disease and several types of cancer, including testicular carcinoma, chronic myeloid leukemia, and acute myeloid leukemia (33–35). Furthermore, researchers have shown that mithramycin A acts as a neuroprotective drug (36). Mithramycin A binds to GC-rich regions in chromatin and interferes with the transcription of genes that bear GC-rich motifs in their promoters (33, 37). Its mechanism of action involves a reversible interaction with double-stranded DNA with GC-base specificity. Mithramycin A is believed to act, in part, by selectively regulating transcription of genes that have GC-rich promoter sequences (38). In addition, recent studies have shown that mithramycin A sensitizes tumor cells to apoptosis induced by tumor necrosis factor and inhibits p53-mediated transcriptional responses (39, 40). Inhibition of Sp1 activity is considered to be a major mechanism of the antitumor activity of mithramycin A. Thus, we did the present study to determine the effect of and molecular basis for the combined use of bevacizumab and mithramycin A as an antiangiogenic therapy for pancreatic cancer.

Materials and Methods

Chemicals and reagents. Mithramycin A (1 mg per vial crystal powder) was purchased from Sigma Chemical Co. and diluted in sterile water. Bevacizumab (25 mg/mL) was purchased from Genentech, Inc. For animal experiments, mithramycin A (0.1–0.4 mg/kg body weight) and bevacizumab (25–100 μ g per mouse) were given by i.p. injection twice a week or as indicated otherwise.

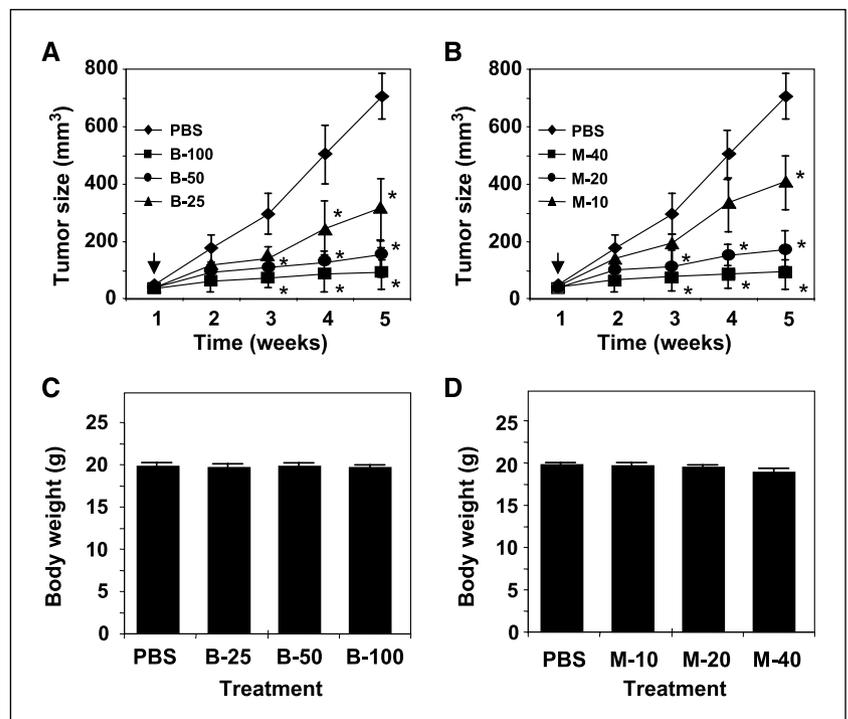
Cell lines and culture conditions. The human pancreatic adenocarcinoma cell lines BxPC3 and PANC-1 were purchased from the American Type Culture Collection. The cell lines were maintained in plastic flasks as adherent monolayers in MEM supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and a vitamin solution (Flow Laboratories).

Animals. Female athymic BALB/c nude mice were purchased from The Jackson Laboratory. The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used when they were 8 weeks old. The animals were maintained in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care in accordance with the current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and NIH.

Western blot analysis. Whole-cell lysates were prepared from human pancreatic cancer cell lines and tissues (23). Standard Western blotting was done using polyclonal rabbit antibodies against human and mouse Sp1, VEGF, and EGFR (Santa Cruz Biotechnology) and the anti-rabbit immunoglobulin G (IgG) antibody, a horseradish peroxidase-linked F(ab)₂ fragment obtained from a donkey (Amersham). Equal protein-sample loading was monitored by probing the same membrane filter with antibodies against anti- β -actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ref. 23). The probe proteins were detected using the Amersham enhanced chemiluminescence system according to the manufacturer's instructions.

Immunohistochemical analysis and quantification of tumor MVD. For CD31 staining, frozen tissue sections (5 μ m thick) were fixed in acetone. Endogenous peroxidase in the specimens was blocked using 3% hydrogen peroxide in PBS for 12 min. The specimens were incubated for 20 min at room temperature in a protein-blocking solution consisting of PBS (pH, 7.5) containing 5% normal horse serum and 1% normal goat serum and then incubated overnight at 4°C in a 1:100 dilution of monoclonal goat anti-CD31 (PECAM1-M20), polyclonal rabbit anti-Sp1, or polyclonal rabbit anti-VEGF antibodies (Santa Cruz Biotechnology). The specimens were then rinsed and incubated with peroxidase-conjugated anti-goat or anti-rabbit IgG for 1 h at room temperature. Next, slides were rinsed with PBS and incubated with diaminobenzidine (Research Genetics) for 5 min. Frozen sections of the specimens were then washed thrice with distilled water, counterstained with Mayer's hematoxylin (Biogenex Laboratories), and washed once each with distilled water and PBS. Sections were mounted on the slides using Universal Mount (Research Genetics), and the slides were examined under a bright-field microscope. A CD31-positive and Sp1-positive reaction was indicated by a reddish-brown precipitate in the cytoplasm or nuclei, respectively. For quantification of tumor MVD, vessels

Figure 1. Dose-dependent antitumor effects of bevacizumab and mithramycin A in xenograft models of human pancreatic cancer. *A* and *B*, BxPC3 cells were injected into the subcutis of nude mice ($n = 5$). When tumors reached around 4 mm in diameter, the animals received different doses of (*A*) bevacizumab [25 (*B-25*), 50 (*B-50*), and 100 (*B-100*) μ g] and (*B*) mithramycin A [0.10 (*M-10*), 0.20 (*M-20*), and 0.40 (*M-40*) mg/kg] via i.p. injection twice a week. Tumors were measured once every week, and at each measurement, the mean \pm SD tumor volume in the five mice in each group was calculated. *C* and *D*, the control mice and mice that received (*C*) bevacizumab and (*D*) mithramycin A were weighed at the time of experiment termination. Columns, mean weights; bars, SD. *, $P < 0.01$ in a comparison between the treated and respective control groups. This was one representative experiment of two with similar results.



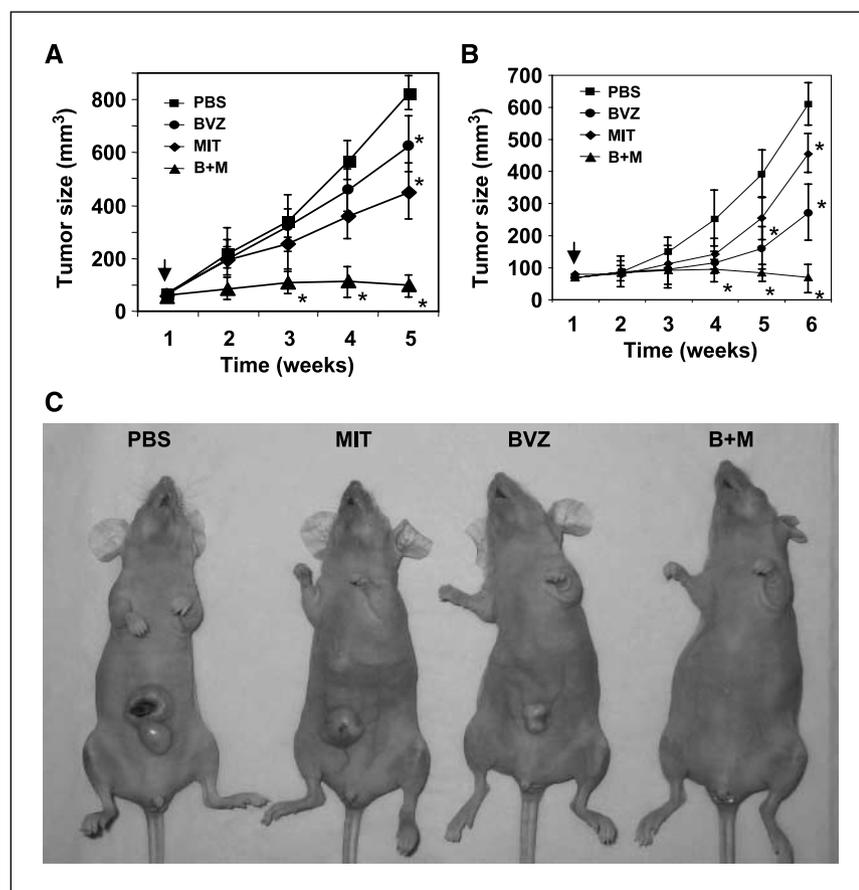


Figure 2. Synergistic antitumor effects of treatment with bevacizumab and mithramycin A in xenograft models of human pancreatic cancer. Both (A) BxPC3 and (B) PANC-1 cells were injected into the subcutis of groups of mice ($n = 5$). Specifically, animals received injections of PBS (controls), bevacizumab (25 μ g), mithramycin A (0.10 mg/kg), or B + M. Tumors were measured once every week, and at each measurement, the mean \pm SD tumor volume in the five mice in each group was calculated. A and B, *, $P < 0.01$ in a comparison between the treated and respective control groups. C, representative tumor sizes in each group of BxPC3 model mice. This was one representative experiment of two with similar results.

on each section were counted in five high-power fields (magnification, $\times 200$ [$\times 20$ objective and $\times 10$ ocular]) as described previously (41).

Sp1 promoter constructs and analysis of Sp1 promoter activity. The minimal Sp1 promoter reporters in pGL3 luciferase constructs were generated and used as described previously (23, 42). To examine the transcriptional regulation of the Sp1 promoters by bevacizumab and mithramycin A, PANC-1 cells were seeded to about 80% confluence in six-well plates (in triplicate) and transiently transfected with 0.6 μ g of minimum Sp1 reporter plasmids and 0.3 μ g of effector expression plasmids as indicated in each experiment using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. The reporter luciferase activity was measured 48 h later using a luciferase assay kit (Promega). Promoter activity was normalized according to the protein concentration as described previously (23, 42).

Chromatin immunoprecipitation. Chromatin was prepared from cells and tumors as described previously (42). Chromatin immunoprecipitation (ChIP) assay was done using the Chromatin Immunoprecipitation Assay Kit (Upstate Cell Signaling Solutions) according to the manufacturer's instructions. Briefly, DNA cross-binding proteins were cross-linked with DNA and lysed in SDS lysis buffer. The lysate was sonicated to shear DNA to 200 to 500 bp. After preclearing with a salmon sperm DNA/protein A agarose-50% slurry for 30 min at 4°C, chromatin samples were immunoprecipitated overnight with no antibody or an anti-Sp1 antibody (PEP2). The region between -224 and -53 bp of the Sp1 promoter was amplified using the following primers: sense, 5'-caggcagcaacttagtc-3', and antisense, 5'-gtaaggaggaggagcag-3'. PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

Statistical analysis. Each experiment was done independently at least twice with similar results; one representative experiment is presented. The significance of the *in vitro* data was determined using Student's *t* test (two-tailed), whereas the significance of the *in vivo* data was determined using the two-tailed Mann-Whitney *U* test. For the *in vivo* experiments, the overall

survival duration was calculated using the Kaplan-Meier method. The log-rank test was used to compare the survival duration between groups. $P \leq 0.05$ was deemed significant.

Results

Synergistic antitumor effects of bevacizumab and mithramycin A in a human pancreatic cancer model. Studies have shown that Sp1 is essential for VEGF expression, and that VEGF plays a major role in pancreatic cancer angiogenesis. However, whether combined inhibition of both Sp1 and VEGF signaling has a synergistic antiangiogenic effect is unknown. To determine whether this takes place, we treated pancreatic cancer in nude mice with bevacizumab, mithramycin A, or a combination of the two (B + M). Specifically, we first did dose-response experiments. We injected BxPC3 cells s.c. into nude mice. When their tumors reached 4 mm in diameter, we gave the animals different doses of bevacizumab (25, 50, and 100 μ g; Fig. 1A) and mithramycin A (0.1, 0.2, and 0.4 mg/kg; Fig. 1B) via i.p. injection twice a week. We also gave PBS injections to animals as controls. We found that treatment with B + M produced dose-dependent antitumor activity. Bevacizumab (at a dose of 25 μ g) and mithramycin A (at a dose of 0.10 mg/kg) had slight antitumor effects ($P =$ not significant). We observed no discernible effect on body weight in the mice that received bevacizumab alone (Fig. 1C), suggesting that at the doses given, bevacizumab did not have any systemic side effects. However, at the highest dose given (0.4 mg/kg), mithramycin A slightly reduced body weight (Fig. 1D).

Next, we did experiments using both BxPC3 and PANC-1 tumor cell models in which we gave PBS, bevacizumab (25 μ g),

mithramycin A (0.10 mg/kg), or B + M to a group of nude mice. Consistently, administration of bevacizumab or mithramycin A alone produced marginal antitumor activity. In contrast, administration of B + M produced synergistic antitumor activity without any systemic side effects (Fig. 2). Additionally, the mice that received B + M had body weights similar to those of the control mice that received PBS (data not shown). Therefore, the use of B + M resulted in higher antitumor activity than the use of bevacizumab or mithramycin A alone did without an increase in toxicity, suggesting that treatment of pancreatic cancer with B + M has a significant therapeutic benefit.

Prolonged survival of xenograft models of human pancreatic cancer that received treatment with bevacizumab and mithramycin A. We injected PANC-1 cells into the pancreas of nude mice and then gave treatment to them as described in Fig. 2. We monitored animal survival daily until the termination of the experiment 160 days after tumor-cell injection. We found that the mice that received bevacizumab or mithramycin A alone had a slightly increased survival duration when compared with those that received PBS only, whereas the mice that received B + M had a significantly longer survival duration when compared with the other three groups of mice (Fig. 3A). Furthermore, the incidence of tumor growth in the pancreas and of metastasis in the liver and/or other organs was lowest by far in the mice that received B + M (Fig. 3B).

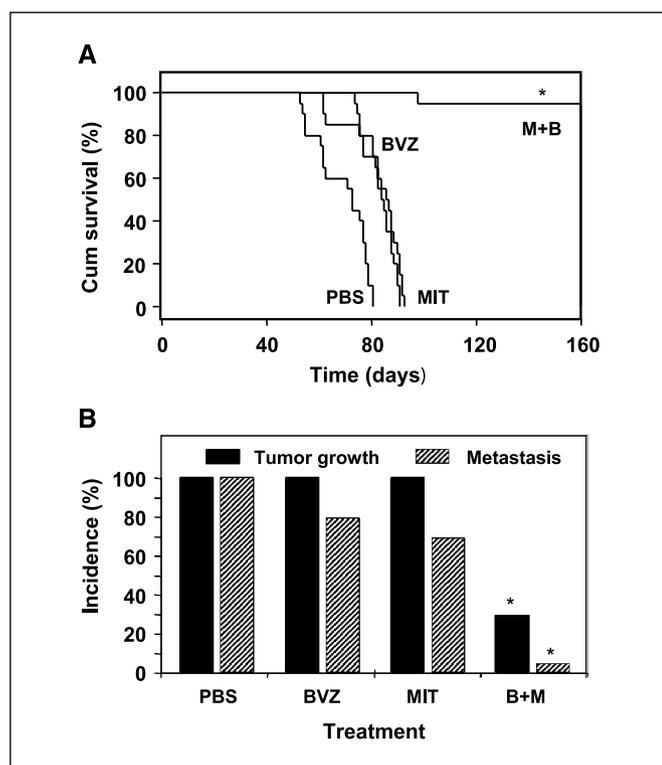


Figure 3. Prolonged survival of mice that received treatment with bevacizumab and mithramycin A in xenograft models of human pancreatic cancer. PANC-1 cells were injected into the pancreas of nude mice (17–20 mice per group). The mice received treatment as described in Fig. 2. The entire experiment was terminated 120 days after tumor-cell injection. *Cum*, cumulative. *B*, tumor growth in the pancreas and metastasis in the liver and/or other organs were evaluated and expressed as the incidence (%). *, $P < 0.01$ in a comparison between the treated and respective control groups. This was one representative experiment of two with similar results.

Up-regulation of Sp1 and VEGF expression by treatment with bevacizumab and its reversal by treatment with mithramycin A. To determine the molecular basis for the synergistic effect of treatment with B + M, we did Western blot analysis using total protein lysates extracted from the PANC-1 tumor tissue specimens collected from mice that received treatment with PBS, bevacizumab, mithramycin A, or B + M. As shown in Fig. 4A and B, treatment with bevacizumab alone increased expression of Sp1 and its downstream molecule VEGF. However, treatment with mithramycin A alone suppressed Sp1 and VEGF expression, which was consistent with reduced MVD, whereas treatment with bevacizumab at a low dose alone did not significantly reduce MVD, which was consistent with increased Sp1 expression (Fig. 4C). These data suggested that the neutralization of VEGF function by bevacizumab may up-regulate the expression of Sp1 via a positive feedback loop and lead to increased VEGF expression. However, because of this neutralization of VEGF function, the sustained MVD levels may be caused by other mechanisms. Indeed, our Western blot analysis showed that the treatment with bevacizumab also up-regulated the expression of other proangiogenic molecules, such as platelet-derived growth factor, IGF-IR, and EGFR (data not shown). Therefore, bevacizumab resistance may result from overexpression not only of VEGF but also of other Sp1 downstream molecules.

Because the low dose of bevacizumab that activated Sp1 expression (25 μg) did not have significant antitumor activity, we determined the levels of Sp1 expression in residual tumors in mice that received a high dose of bevacizumab (100 μg) that had a significant antitumor activity. As shown in Fig. 4D, we observed significant inhibition of Sp1 expression in the residual tumors of mice that received 100 μg of bevacizumab, whereas we consistently observed up-regulation of Sp1 expression in the residual tumors of mice that received 25 μg of bevacizumab. The alteration of Sp1 expression was consistent with that of VEGF expression. These data suggested that administration of a low, ineffective dose of bevacizumab up-regulated Sp1 expression and contributed to bevacizumab resistance.

Effects of treatment with bevacizumab and mithramycin A on the growth of and gene expression in human pancreatic cancer cells. To further determine whether treatment with bevacizumab directly impacts gene expression in pancreatic cancer cells, we incubated PANC-1 and BxPC3 cells in medium alone or a medium containing 100 $\mu\text{g}/\text{mL}$ bevacizumab for 6 to 48 h. As expected, bevacizumab did not affect the growth of PANC-1 cells (Fig. 5A) or BxPC3 cells (Fig. 5B) *in vitro*. As shown in Fig. 5C, neutralization of VEGF did not affect the expression of Sp1 or its major downstream molecules, VEGF and EGFR. This result was consistent with previous findings showing that bevacizumab primarily blocks the autocrine effect of VEGF on tumor angiogenesis (43–47). In contrast, treatment with mithramycin A produced dose-dependent cytotoxic effects in both PANC-1 cells (Fig. 5D) and BxPC3 cells (data not shown). The calculated IC_{50} s for mithramycin A were $>1.5 \mu\text{mol}/\text{L}$ (24-h assay) and 0.15 $\mu\text{mol}/\text{L}$ (48-h assay; Fig. 5D). Therefore, for 24 and 48 h, we treated PANC-1 cells with mithramycin A at concentrations of 0.05 and 0.10 $\mu\text{mol}/\text{L}$, respectively, which were much lower than the IC_{50} and effectively inhibited the expression of Sp1 and its downstream molecules EGFR and VEGF (Fig. 5E). These data suggested that bevacizumab does not have a direct effect on tumor cells or on the expression of Sp1 and its downstream molecules, but that mithramycin A does.

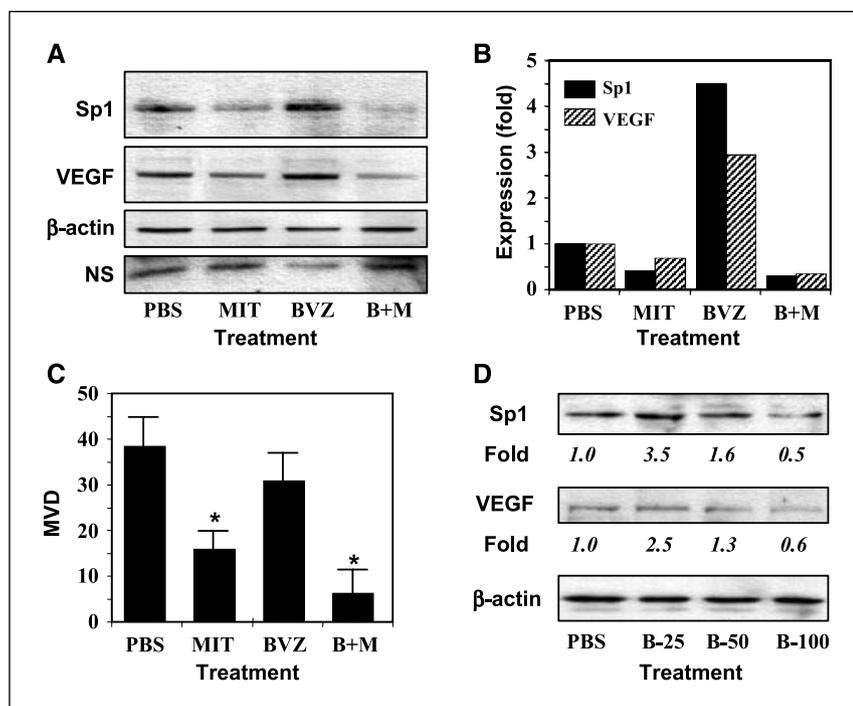


Figure 4. Analysis of gene expression and microvessel formation in tumor tissues. The tumor tissues described in Fig. 2A were collected and processed as described in Materials and Methods. *A*, total protein lysates were harvested from tumor tissues, and the level of protein expression in them was determined using Western blot analysis. Equal protein-sample loading was monitored by probing the same membrane filter with an anti- β -actin antibody. *NS*, nonspecific. *B*, the levels of Sp1 and VEGF expression were quantitated and expressed as fold change. *C*, MVD was quantitated according to CD31 staining. *D*, Western blot analysis was done using total protein lysates harvested from the tumor tissues described in Fig. 1 A and specific antibodies against Sp1, VEGF, and β -actin.

Effects of treatment with bevacizumab and mithramycin A on Sp1 recruitment into the *Sp1* promoter *in vitro* and *in vivo* in human pancreatic cancer cells.

In this final set of experiments, we sought to determine whether treatment with B + M regulated Sp1 expression at the transcriptional level. We transfected *Sp1* promoter reporter constructs into PANC-1 cells and then incubated them in medium alone or a medium containing 100 μ g/mL bevacizumab or 0.1 μ mol/L mithramycin A. *In vitro*, treatment with bevacizumab did not suppress *Sp1* promoter activity, whereas treatment with mithramycin A did. However, further deletion of Sp1-binding sites eliminated the ability of mithramycin A to suppress *Sp1* promoter activity (Fig. 6A and B). Consistently, treatment with mithramycin A significantly reduced the recruitment of Sp1 protein to its own promoter as shown in a ChIP assay (Fig. 6C), whereas treatment with bevacizumab did not reduce the recruitment of Sp1 protein to its promoter at either high (100 μ g/mL; Fig. 6C) or low (12.5, 25.0, or 50.0 μ g/mL; data not shown) concentrations. Finally, we did a ChIP assay using tumors formed by PANC-1 cells in nude mice that received treatment as described in Fig. 2A. Treatment with bevacizumab at a high dose inhibited Sp1 recruitment to its own promoter, whereas treatment with bevacizumab at a low dose significantly increased Sp1 recruitment to its own promoter (Fig. 6D). In contrast, both treatment with mithramycin A alone and treatment with B + M suppressed Sp1 protein recruitment to its own promoter (Fig. 6E). These data suggested that treatment with ineffective doses of bevacizumab up-regulates Sp1 expression *in vivo* via positive feedback activation of the *Sp1* gene (positive autoregulation) and then of its downstream target molecules, such as VEGF, whereas mithramycin A disrupts this regulatory loop.

Discussion

In this study, we found that treatment with bevacizumab up-regulated the expression of Sp1, which is a key positive regulator of

the expression of various proangiogenic factors, including VEGF. Inhibition of Sp1 using mithramycin A repressed the expression of VEGF and tumor angiogenesis. Interestingly, treatment with B + M at low doses had a synergistic antiangiogenic effect. This effect was consistent with suppression of Sp1 activity and down-regulation of multiple downstream target molecules of Sp1. Our experimental findings indicate that inhibition of VEGF signaling *in vivo* leads to feedback up-regulation of Sp1 expression, which, in turn, leads to increased expression of multiple proangiogenic factors, including VEGF. Thus, treatment with the anti-VEGF monoclonal antibody bevacizumab can lead to compensatory up-regulation of pathways that, in turn, lead to resistance to bevacizumab. Conversely, inhibition of Sp1 blocked this feedback mechanism. For the first time, our findings not only provide researchers with a novel paradigm of synergism between antiangiogenic agents and inhibitors of transcription factors such as Sp1, but also will help clinicians design rational drug combinations that can improve antiangiogenic activity and/or reverse antiangiogenic resistance.

Angiogenesis plays critical roles in sustained growth and metastasis of most solid tumors, including pancreatic cancer. Previous studies showed that antiangiogenic therapies suppress tumor growth in animal models of pancreatic cancer. For example, several strategies inhibit the angiogenesis, growth, and metastasis of human pancreatic cancer and improve survival in nude mouse models via targeting of VEGF signaling and function, including the use of VEGF antisense oligonucleotides, VEGF-directed ribozymes, VEGF fused to a diphtheria toxin, anti-VEGF antibodies, various types of interference with VEGF receptor-1 and VEGF receptor-2, a dominant-negative flk-1, and the VEGF receptor tyrosine kinase inhibitor PTK-787 (48, 49). Additionally, celecoxib (a cyclooxygenase-2 inhibitor) and genistein (a tyrosine kinase inhibitor) suppress pancreatic cancer growth and metastasis at least in part by inhibiting VEGF expression and angiogenesis (24). Furthermore, the level of VEGF expression correlates with MVD and disease

progression (4, 6). These findings from various experimental and clinical studies using various approaches substantiate the importance of the angiogenic process in pancreatic cancer and support the hypothesis that VEGF plays a crucial role in this process. Moreover, TNP-470 and endostatin reduce the rate of pancreatic cancer angiogenesis, growth, and metastasis (43).

Although studies have shown that a number of VEGF inhibitors have significant antitumor activity in a wide variety of xenograft models, in human studies, the clinical activity of VEGF inhibitors when used as single agents has been limited. Outside of use for renal cell carcinoma, neuroendocrine carcinoma, and some sarcomas, VEGF inhibitors are generally developed in combination

with other therapies. Researchers have shown that antiangiogenic approaches are more effective when combined with chemotherapy or radiation therapy. For example, gemcitabine potentiated antiangiogenic therapies using anti-VEGF, anti-VEGF receptor-2, and anti-EGFR antibodies (44). The limited effectiveness of antiangiogenic therapies that target single molecules also highlights the important issue of diverse angiogenic signals and effector factors (6). Targeting these individual molecules individually may be ineffective and/or result in eventual resistance to bevacizumab. In pancreatic cancer, early clinical trials of bevacizumab, in combination with gemcitabine for advanced disease and in combination with radiotherapy for local-regional disease, had promising results. Investigators have proposed various mechanisms of the synergy between antiangiogenic therapy and chemotherapy. Some have hypothesized that therapy targeting VEGF may normalize tumor vasculature, decrease interstitial fluid pressure, and enhance chemotherapy delivery. Alternatively, others have proposed that neuropilin receptors mediate resistance to apoptosis, which can be reversed by agents such as bevacizumab (45).

Some have recognized that elevated angiogenesis in human pancreatic cancer involves a perturbed local imbalance of proangiogenic and antiangiogenic factors, i.e., proangiogenic factors predominate over antiangiogenic factors (46, 47). Among a growing list of proangiogenic factors, various isoforms of VEGF contribute to the growth and metastasis of pancreatic cancer through a variety of mechanisms (4, 6). Moreover, pancreatic cancer cells overexpress several other mitogenic growth factors that are also angiogenic, such as EGF, transforming growth factor- α , HGF, FGFs (e.g., FGF-1, FGF-2, FGF-5), and platelet-derived growth factor- β (4, 6). Researchers have shown that many of these and other factors correlate with increased vessel formation and poor prognosis in patients with pancreatic cancer; this includes expression of FGF-2 and platelet-derived endothelial cell growth factor, mutation of *K-ras*, and overexpression of hypoxia-inducible factor-1 α , thymidine phosphorylase, thrombospondin-1, and cathepsin B and L (4-6). Therefore, whereas VEGF is crucial for promoting the growth and metastasis of pancreatic cancer, other factors are likely involved in this process as well. Together, these factors may produce mitogenic activity in an autocrine and paracrine fashion, promoting pancreatic tumor-cell growth and angiogenesis and eventually enhancing pancreatic tumor invasion and metastasis (4-6). This notion is supported by the fact that interference with the expression and function of these factors influences the angiogenesis and growth of human pancreatic cancer in animal models. For example, antiangiogenesis can be achieved using treatment with dominant-negative IGF-IR; antithrombin III and vitamin D-binding protein-macrophage activating factor; NK4, a four-kringle fragment of HGF; and the tyrosine kinase inhibitor of EGFR (4, 6). However, simultaneously interfering with multiple target molecules is clinically challenging.

Recent studies have suggested that there is a potential underlying mechanism for overexpression of various proangiogenic factors that collectively regulate pancreatic cancer angiogenesis (6, 9). For example, in previous studies, we showed that Sp1 is constitutively activated in human pancreatic cancer cells, and that constitutive Sp1 activity is essential for constitutive and inducible VEGF expression (6, 9, 23, 27, 28), strongly supporting the initial description of the Sp1/VEGF signaling axis (27, 50). In our previous studies, we also found that overexpression of Sp1 is correlated with

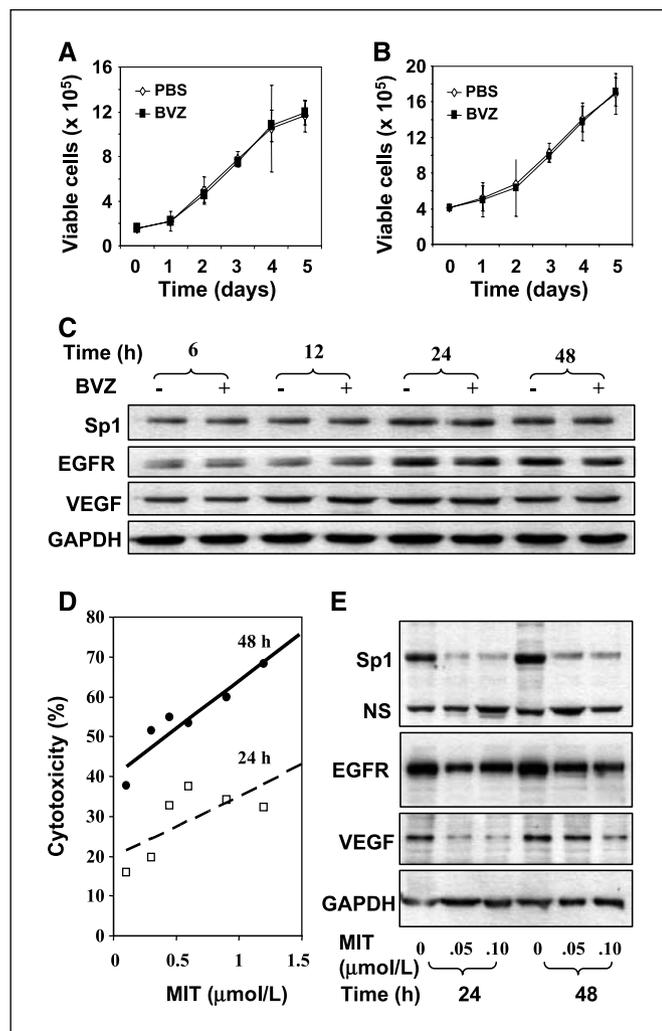


Figure 5. Effects of treatment with bevacizumab and mithramycin A on the growth of and gene expression in human pancreatic cancer cells *in vitro*. BxPC3 (A) and PANC-1 cells (B) were incubated for 1 to 5 d in medium alone or a medium containing 100 $\mu\text{g/mL}$ bevacizumab. The viable cells were counted every 24 h. C, PANC-1 cells were incubated for 6 to 48 h in medium alone or a medium containing 100 $\mu\text{g/mL}$ bevacizumab. Total protein lysates were harvested from the cell cultures, and the level of Sp1, EGFR, and VEGF protein expression was determined using Western blot analysis. Equal protein-sample loading was monitored by probing the same membrane filter with an anti-GAPDH antibody. D and E, PANC-1 cells were treated with mithramycin A at concentrations ranging from 0.1 to 1.2 $\mu\text{mol/L}$ for 24 and 48 h. D, cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. E, the level of gene expression was determined using Western blot analysis. NS, nonspecific. This was one representative experiment of two with similar results.

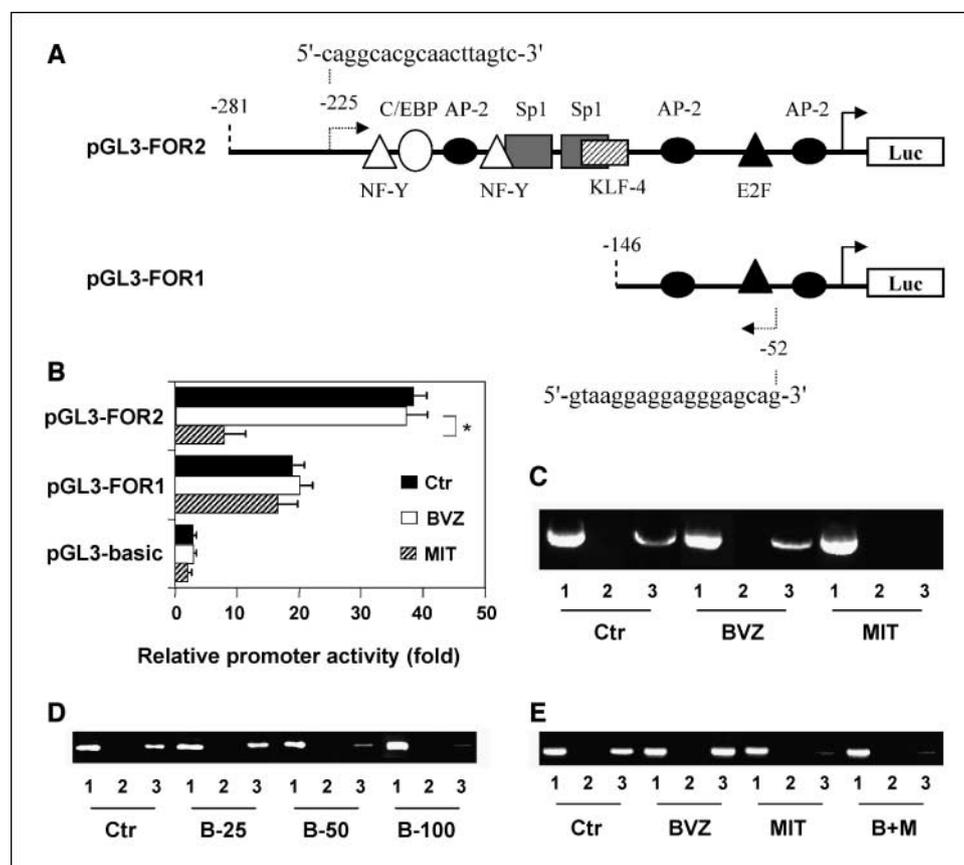


Figure 6. Effects of treatment with bevacizumab and mithramycin A on *Sp1* recruitment into the *Sp1* promoter in human pancreatic cancer cells *in vitro* and *in vivo*. **A**, schematic structures of the minimal *Sp1* promoters. The nucleotide positions and sequences of *Sp1*-binding sites and PCR forward and reverse primers flanking those sites for ChIP assay are shown. **B**, *Sp1* promoter reporter constructs were transfected into PANC-1 cells in triplicate and incubated for 12 h. The cells were then incubated for another 24 h in medium alone or a medium containing 100 $\mu\text{g}/\text{mL}$ bevacizumab or 0.1 $\mu\text{mol}/\text{L}$ mithramycin A. Total protein lysates were harvested from the cell cultures for measurement of *Sp1* promoter activity using a luciferase assay kit. The relative *Sp1* promoter activities were assessed, and the activity in treated groups was expressed as the fold change of that in their respective control groups. **C**, chromatin was extracted from PANC-1 cells that were incubated *in vitro* for 2 d in medium alone or a medium containing 100 $\mu\text{g}/\text{mL}$ bevacizumab or 0.1 $\mu\text{mol}/\text{L}$ mithramycin A. **D**, chromatin was extracted from tumors formed by PANC-1 cells in nude mice that received treatment as described in Fig. 1A. **E**, chromatin was extracted from tumors formed by PANC-1 cells in nude mice that received treatment as described in Fig. 2A. The ChIP assay was done using a specific anti-*Sp1* antibody and oligonucleotides flanking the *Sp1* promoter regions containing *Sp1*-binding sites. Lane 1, input chromatin DNA; lane 2, chromatin DNA with control IgG; lane 3, chromatin DNA with anti-*Sp1* antibody. Ctr, control. This was one representative experiment of two with similar results.

MVD in human gastric cancer tissue, and that manipulating the *Sp1* expression level using a small interfering RNA approach significantly inhibits the tumor angiogenic phenotype (6, 9). This antiangiogenic effect of knocking down *Sp1* expression is consistent with reduced expression of several signaling molecules in the signaling pathways that play important roles in the regulation of tumor angiogenesis (28–30). In fact, one of our recent studies showed that inhibition of VEGF expression and the resulting antitumor effect of celecoxib are mediated at least in part by suppression of *Sp1* activity (24). All of these lines of evidence indicate that *Sp1* plays an important role in the regulation of angiogenesis in human pancreatic cancer. Furthermore, as shown in the present study, blockade of *Sp1* activity by mithramycin A suppresses tumor angiogenesis.

Finally, treatment with a low dose of mithramycin A did not significantly inhibit tumor angiogenesis in the present study, although the overall level of VEGF protein expression in tumors was reduced. This finding suggests that the quantity of extracellular matrix-associated VEGF in the tumor bed may be sufficient to initiate and maintain the tumor angiogenesis phenotype without continuing production of VEGF in the tumor bed. Removal of this angiogenic signal produced by the existing VEGF is necessary,

which may explain why a single treatment with a low dose of mithramycin A is insufficient to eliminate angiogenesis in pancreatic tumors. However, the functional removal of VEGF by treatment with bevacizumab up-regulates the expression of many angiogenic molecules other than VEGF, which may explain why a single treatment with bevacizumab was insufficient to produce sustained antiangiogenesis. The combined use of bevacizumab and mithramycin A should produce sustained antiangiogenesis in pancreatic cancer. This is clearly supported by the present study, in which combined use of low doses of bevacizumab and mithramycin A had a synergistic antiangiogenic effect in human pancreatic cancer models. Therefore, our present findings underscore the important roles of *Sp1*/VEGF signaling in pancreatic cancer angiogenesis.

Collectively, our study suggests that bevacizumab-based treatment targeting VEGF may lead to resistance of pancreatic cancer to bevacizumab in part via positive feedback activation of the transcription factor *Sp1* and subsequent overexpression of the downstream target genes of *Sp1*. The use of mithramycin A in combination with bevacizumab is an important novel strategy of targeting angiogenesis by sequentially interfering with upstream transcriptional regulation by transcription factors such as *Sp1* and

neutralizing downstream effector molecules such as VEGF. Combining bevacizumab and mithramycin A in human clinical studies represents a rationale step forward in the development of effective antiangiogenic therapy for pancreatic cancer and other cancers.

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