Bone Morphogenetic Protein-2 Stimulates Angiogenesis in Developing Tumors

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Abstract

Bone Morphogenetic Protein-2 (BMP-2) is highly overexpressed in the majority of patient-derived lung carcinomas. However, a mechanism revealing its role in cancer has not been established. Here we report that BMP-2 enhances the neovascularization of developing tumors. Recombinant BMP-2 stimulated blood vessel formation in tumors formed from A549 cells injected s.c. into thymic nude mice. Recombinant BMP-2 also enhanced angiogenesis in Matrigel plugs containing A549 cells in nude mice. The BMP-2 antagonist noggin abrogated BMP-2-induced angiogenic response. Furthermore, antisense transfection of BMP-2 cDNA resulted in a decrease in blood vessel formation in the Matrigel assays. BMP-2 induced tube formation in both human aortic endothelial cells (HAEC) and umbilical vein endothelial cells. BMP-2 also stimulated proliferation of HAEC. The ability of BMP-2 to activate endothelial cells was further demonstrated by its ability to phosphorylate Smad 1/5/8 and ERK-1/2 and to increase expression of Id1. This study reveals that BMP-2 enhanced the angiogenic response in developing tumors. Furthermore, these data suggest that BMP-2 stimulation of angiogenesis may involve the activation of endothelial cells.

Introduction

Bone Morphogenetic Protein-2 (BMP-2) was originally described for its ability to induce the entire cascade of endochondral bone formation (1). BMP-2 is now recognized as a multipurpose cytokine that stimulates migration (2–4) and induces differentiation of many different cell types (5, 6). BMP-2/4 are required at very early stages of embryonic development. Animals with functional knockout of BMP-2/4 die between 6.5 and 9.5 days p.c. with little to no mesoderm differentiation (7, 8). BMP-2 is expressed in a variety of carcinoma cell lines (9) and tumors originating from patients with head and neck (10) and pancreatic carcinomas (11).

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Recently, the mature active form of BMP-2 was shown to be highly expressed in the majority of patient-derived non-small cell lung carcinomas (NSCLC), with little to no expression in normal lung tissue (12). Recombinant BMP-2 caused an approximately 50% increase in early tumor growth of A549 cells in athymic nude mice, while BMP-2 antagonists inhibited tumor growth by 50% (12). However, the mechanisms by which BMP-2 stimulates tumor growth have not been established.

BMP-2 is a member of the transforming growth factor (TGF) superfamily. There are 20 isotypes of the BMP family with BMP-2 and BMP-4 sharing 92% homology (13). The BMPs are synthesized as variable length inactive precursors, which are proteolytically cleaved at the COOH-terminal end by a proprotein convertase to produce the mature active protein (14). The mature BMP-2 protein is secreted from the cell, which then can act in a paracrine and/or autocrine manner. Signaling by the BMPs is mediated by serine/threonine kinase called the type IA, IB, and IIB receptors (15, 16). The activated BMP type I receptor then phosphorylates Smad1, Smad 5, and/or Smad 8, inducing its translocation into the nucleus activating the transcription of target genes (17).

Studies have suggested, although indirectly, that the BMPs regulate vasculogenesis during embryonic development (18). Functional deletion of BMP-4 and the BMP I receptor in mice leads to impaired mesoderm precursors required for vascular development (7, 19). Mice with a functional knockout of the BMP transcription factors Smad 1 or Smad 5 died at approximately 9.5–10.5 weeks and had defects in angiogenesis (20, 21). Smad 5 mutant embryos had enlarged blood vessels, a decrease in smooth muscle cells, and contained mesenchymal cells, which were unable to direct angiogenesis (21). Mice lacking TGF receptors also died in midgestation with defects in angiogenesis (22). The role of BMP-2 in the induction of a blood supply in postnatal tissues has not been established.

In this study, we found that BMP-2 greatly enhanced blood vessel formation in tumors formed by A549 cells in nude mice. *In vitro* studies demonstrated that BMP-2 stimulated in endothelial cells Smad 1/5, Erk 1/2, and Id1 expression, which was associated with an increase in tube formation and proliferation. This study provides evidence that BMP-2 promotes tumor growth by stimulating angiogenesis. Furthermore, these data suggest that BMP-2-mediated angiogenic response is regulated at least in part through the activation of endothelial cells.

Results

BMP-2 Stimulates Neoangiogenesis in Tumors

To assess the effects of BMP-2 on blood vessel formation in developing tumors, A549 cells were co-injected with Affi-Blue
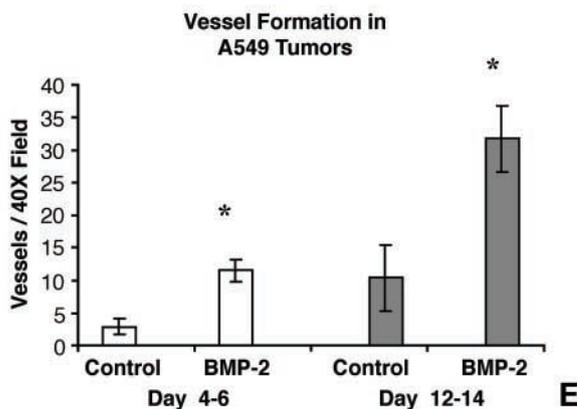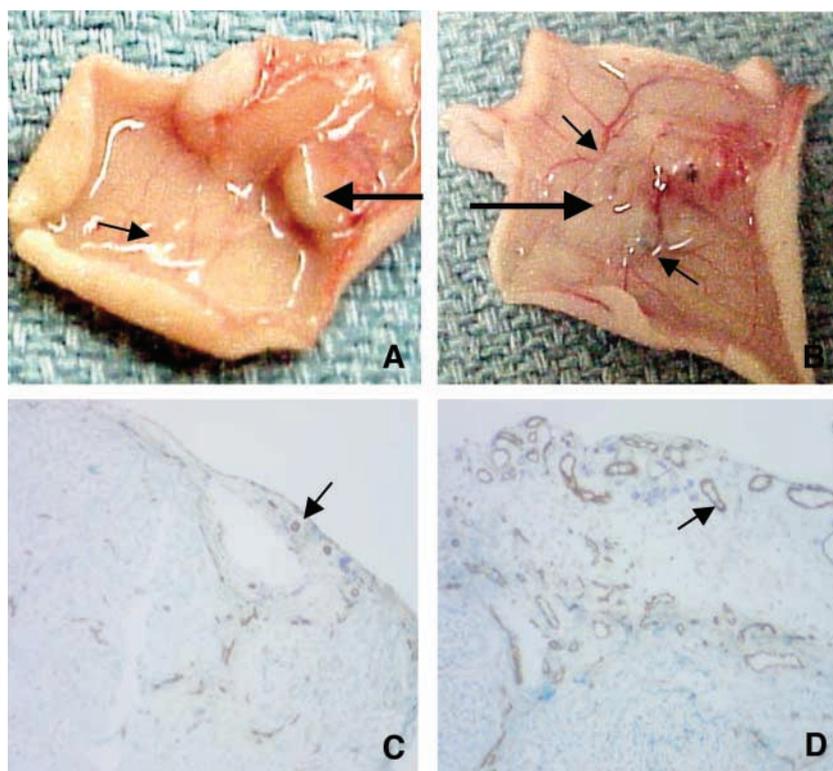
agarose beads coated with recombinant BMP-2 s.c. into athymic nude mice. On visual inspection, compared to controls (Fig. 1A), tumors treated with BMP-2 had larger and more numerous blood vessels (Fig. 1B). Immunohistochemistry studies revealed a 3-fold increase in the number of blood vessels that formed within the tumors treated with BMP-2 (Fig. 1D). The BMP-2-mediated angiogenic response occurred rapidly, demonstrating an increase in blood vessels by day 4 that persisted for at least 12 days (Fig. 1E).

To further assess the effects of BMP-2 on tumor neoangiogenesis, A549 cells were mixed in Matrigel supplemented with recombinant BMP-2 or BSA and injected s.c. into athymic nude mice. We also examined blood vessel formation induced from A549 cells engineered to secrete less BMP-2 by antisense transfection (A549-AS; Ref. 12). Grossly, compared to controls (Fig. 2A-1), Matrigel plugs containing

BMP-2 (Fig. 2A-2) had more blood vessels. A549-AS cells showed little evidence of a neovasculature after 6 days (Fig. 2A-3). Immunohistochemistry also demonstrated significantly more blood vessels within the Matrigel plugs treated with recombinant BMP-2 (Fig. 2, B-2 and C). Matrigel plugs containing A549-AS cells (Fig. 2, B-3 and C) had less blood vessels than that of A549 cells treated with BSA (Fig. 2, B-1 and C) or A549 cells transfected with vector alone (vector) (Fig. 2C). Inhibiting the activity of BMP-2 with its antagonist noggin (23) abrogated the angiogenic response induced by recombinant BMP-2 (Fig. 2C).

BMP-2 Activates Smad 1, Id1, and ERK-1/2

We next examined whether BMP-2 stimulated human endothelial cells. BMP type I and II receptors were previously

FIGURE 1. BMP-2 stimulates tumor neovasculature. A549 cells were co-injected s.c. into nude mice with Affi-Blue agarose beads coated with bovine serum albumin (BSA) (**A**) or recombinant human BMP-2 (**B**). *Bold arrow* highlights the tumor and *smaller arrow* a blood vessel feeding the tumor. Endothelial cells within the tumors were measured by immunohistochemistry. Photograph of representative immunohistochemical study showing blood vessels in tumors treated with BSA (**C**) or recombinant BMP-2 (**D**). *Arrow* demonstrates blood vessels that are staining *brown*. **E.** Data showing the number of blood vessels per 40× field in tumors treated for 4–6 or 12–14 days. *Columns,* average of at least three experiments per time point. * $P < 0.05$.
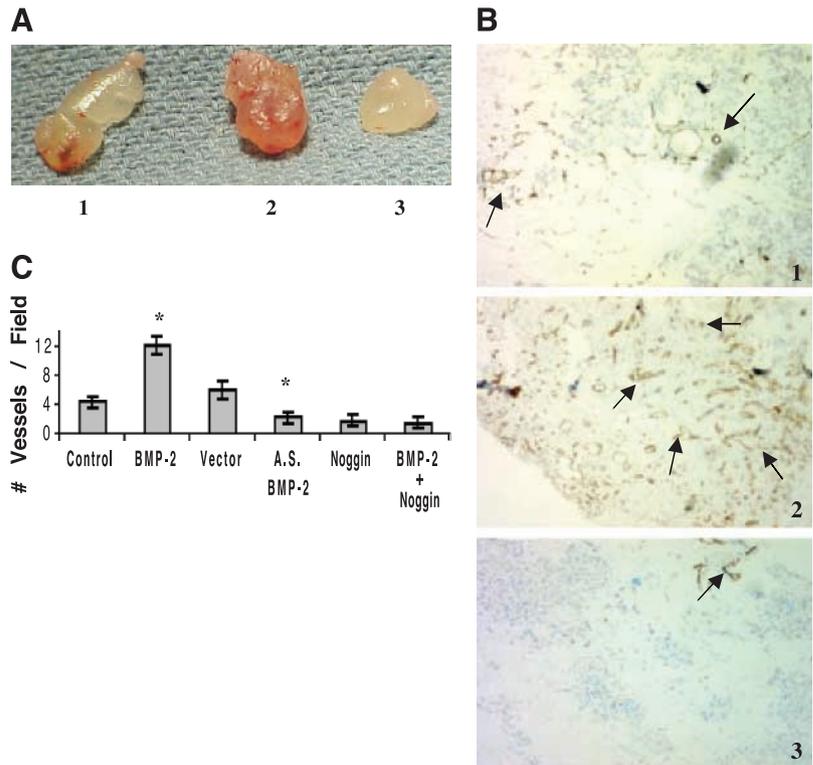
FIGURE 2. Matrigel angiogenesis assay. **A.** Photograph of Matrigel plugs containing A549 cells supplemented with BSA (*1*) or recombinant BMP-2 (*2*) or Matrigel plug containing A549-AS cells alone (AS) (*3*). **B.** Representative photograph of an immunohistochemical study demonstrating endothelial cells within the Matrigel plugs. Blood vessels are staining *brown*. Matrigel plugs containing A549 cells supplemented with BSA (*1*), recombinant BMP-2 (*2*), or A549-AS alone (*3*). Experiments were repeated 6 times. **C.** Graphic representation of the number of blood vessels per high-powered field. Also shown is the number of blood vessels in Matrigel plugs containing BMP-2 preincubated with recombinant noggin and A549 cells transfected with pcDNA3 (*Vector*). * $P < 0.05$ compared to control.

identified by immunohistochemistry to be expressed in both mouse and human endothelial cells (24). By Western blot analysis, we found that human aortic endothelial cells (HAEC) expressed BMP receptors (Fig. 3A). As expected, we also found HUVEC to express all the BMP receptors (data not shown). To determine whether BMP-2 activates BMP-specific Smads, we used an antibody that recognized the phosphorylated form of Smad1. BMP-2 induced the phosphorylation of Smad1 within 10 min in both the HAEC (Fig. 3B) and human umbilical vein endothelial cells (HUVEC; Fig. 3C). BMP-2-mediated Smad phosphorylation persisted for approximately 40 min in both endothelial cell lines. To further assess BMP-2-induced Smad phosphorylation, cell lysate from HAEC treated with and without BMP-2 was immunoprecipitated with an

antibody recognizing Smads 1, 5, and/or 8 and immunoblots probed with an antibody recognizing phosphorylated serines (Fig. 3D). This study also demonstrated that BMP-2 induced the phosphorylation of BMP-2-specific Smads in endothelial cells. An isotype control antibody did not immunoprecipitate Smads 1, 5, and/or 8 (data not shown).

Id is a negative regulator of basic helix loop transcription factors and was shown to regulate endothelial migration and possibly tube formation (24). We found that when compared to untreated controls, BMP-2 induced a transient increase in the expression of Id1 in both the HAEC (Fig. 3B) and HUVEC (Fig. 3C). Proangiogenic proteins vascular endothelial growth factor (VEGF), fibroblastic growth factor (FGF), epidermal growth factor (EGF), and angiogenin are known to mediate a

FIGURE 3. BMP-2 activates endothelial cells. **A.** Western blot analysis was performed on cell lysate from HAEC and probed with antibodies specific for BMP receptors IA, IB, and II. HAEC (**B**) and HUVEC (**C**) were treated with recombinant BMP-2 and cell lysates were subjected to Western blot analysis using antibodies specific for phosphorylated Smad1, ID1, and phosphorylated ERK-1/2. An anti-actin antibody was used as a loading control. Experiments were repeated at least 3 times. **D.** HAEC protein was precipitated with an anti-Smad 1/5/8 antibody and Western blot probed with an anti-phosphoserine antibody. HAEC were treated with recombinant BMP-2 (*1*) or vehicle control (*2*).
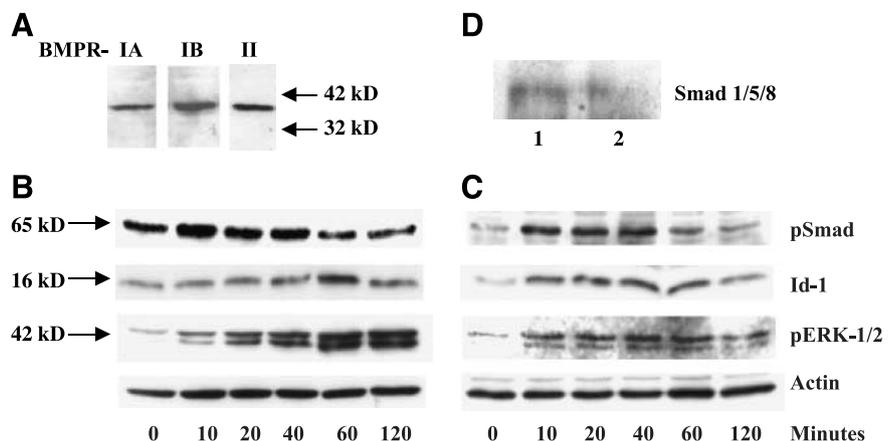
cellular response in endothelial cells through the activation of extracellular signal-regulated kinase (ERK-1/2; Refs. 25–27). To examine whether BMP-2 also activates ERK-1/2, an antibody which specifically recognizes phosphorylated ERK-1/2 was used in Western blot analysis. BMP-2 induced a rapid increase in the phosphorylation of ERK-1/2 in both the HAEC and HUVEC. A robust increase was seen in the HAEC that persisted for at least 2 h (Fig. 3B). Phoshorylation of ERK-1/2 in the HUVEC was not as robust and persisted for only 60 min (Fig. 3C).
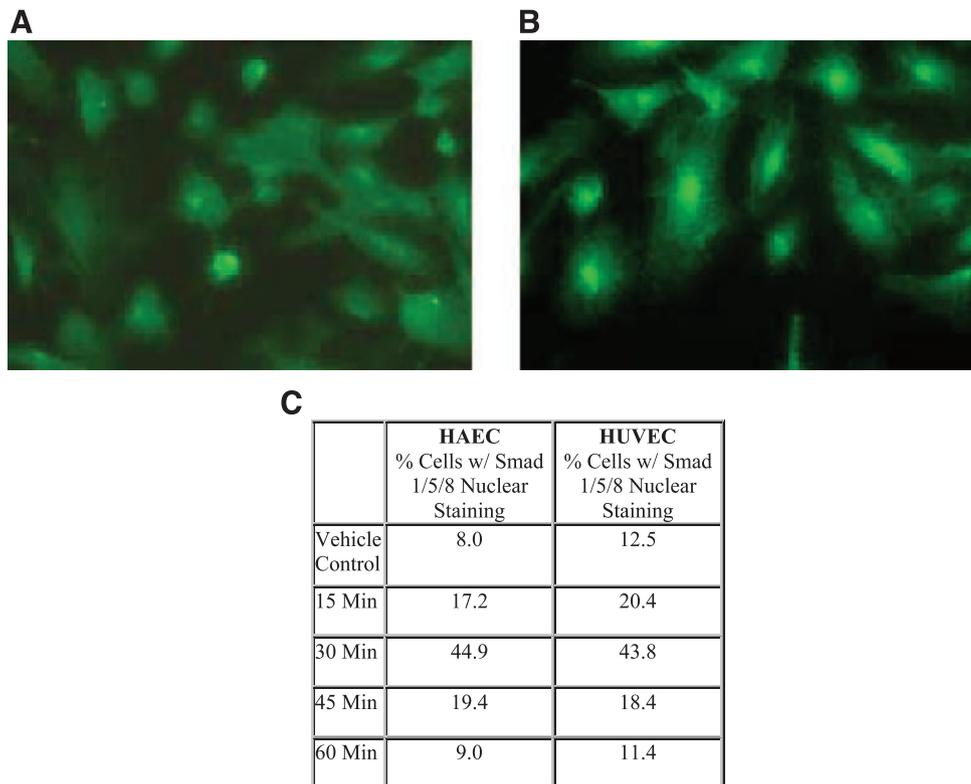
BMP-2 Induced Nuclear Translocation of Smad 1, 5, and/or 8

Phosphorylation of Smads 1, 5, and/or 8 stimulated its translocation from the cytoplasm into the nuclease which then induces transcription of target genes (28). Using immunofluorescence, we examined whether BMP-2 stimulated the translocation of Smads 1/5/8 into the nucleus of human endothelial cells. We found that BMP-2 increased nuclear expression of Smads 1, 5, and/or 8 in both HAEC and HUVEC (Fig. 4). Nuclear staining of Smad 1/5/8 corresponded to the BMP-2-induced phosphorylation of Smad1 found by Western blot analysis. BMP-2-mediated influx of Smads 1/5/8 into the nucleus was greatest after 30 min and returned to baseline levels within 60 min (Fig. 4C). These results are consistent with a previous report demonstrating that BMP-2 mediates a rapid influx of Smads 1/5/8 from the cytoplasm into the nuclease in osteoblasts, which are then actively transported back into the cytoplasm (29). Noggin completely antagonized recombinant BMP-2-induced nuclear accumulation of Smad 1/5/8 (data not shown). Preincubating the anti-Smad 1, 5, and/or 8 antibody with a peptide recognized by this antibody abrogated all fluorescent signals (data not shown).

BMP-2 Stimulates Endothelial Cell Proliferation and Tube Formation

We examined whether BMP-2 stimulated proliferation, tube formation, and/or migration of human endothelial cells. One nanogram per milliliter of BMP-2 did not enhance the migration of HAEC when examined at 24 h (Fig. 5A). We were also not able to induce migration of HAEC with 10 or 100 ng/ml when examined at 6 or 24 h (data not shown). VEGF did stimulate the migration of HAEC, demonstrating that cells were cytokine responsive (Fig. 5A). To study the effects of BMP2 on proliferation, the HAEC and HUVEC were treated with recombinant BMP-2 for 24 h and pulsed with [3H]thymidine for 1 h. The effects of BMP-2 on proliferation varied between the HAEC and HUVEC. BMP-2 stimulated DNA synthesis in HAEC (Fig. 5B). However, relatively little change in DNA synthesis was seen in the HUVEC following treatment with BMP-2 (Fig. 5C). We were also unable to find a consistent change in proliferation when [3H]thymidine incorporation

	HAEC % Cells w/ Smad 1/5/8 Nuclear Staining	**HUVEC** % Cells w/ Smad 1/5/8 Nuclear Staining
Vehicle Control	8.0	12.5
15 Min	17.2	20.4
30 Min	44.9	43.8
45 Min	19.4	18.4
60 Min	9.0	11.4

FIGURE 4. BMP-2 induces nuclear accumulation of Smad 1/5/8. Localization of Smad 1, 5, and 8 was determined by immunofluorescence microscopy. HAEC or HUVEC were treated with vehicle control or 10 ng/ml of recombinant BMP-2 for 15, 30, 45, or 60 min. Depicted is a representative image of control (**A**) and HAEC treated with BMP-2 for 30 min (**B**). **C.** Graphic demonstration of the percentage of HAEC and HUVEC having nuclear staining after BMP-2 treatment. A minimum of 100 cells was counted. Each study was performed at least 3 times.
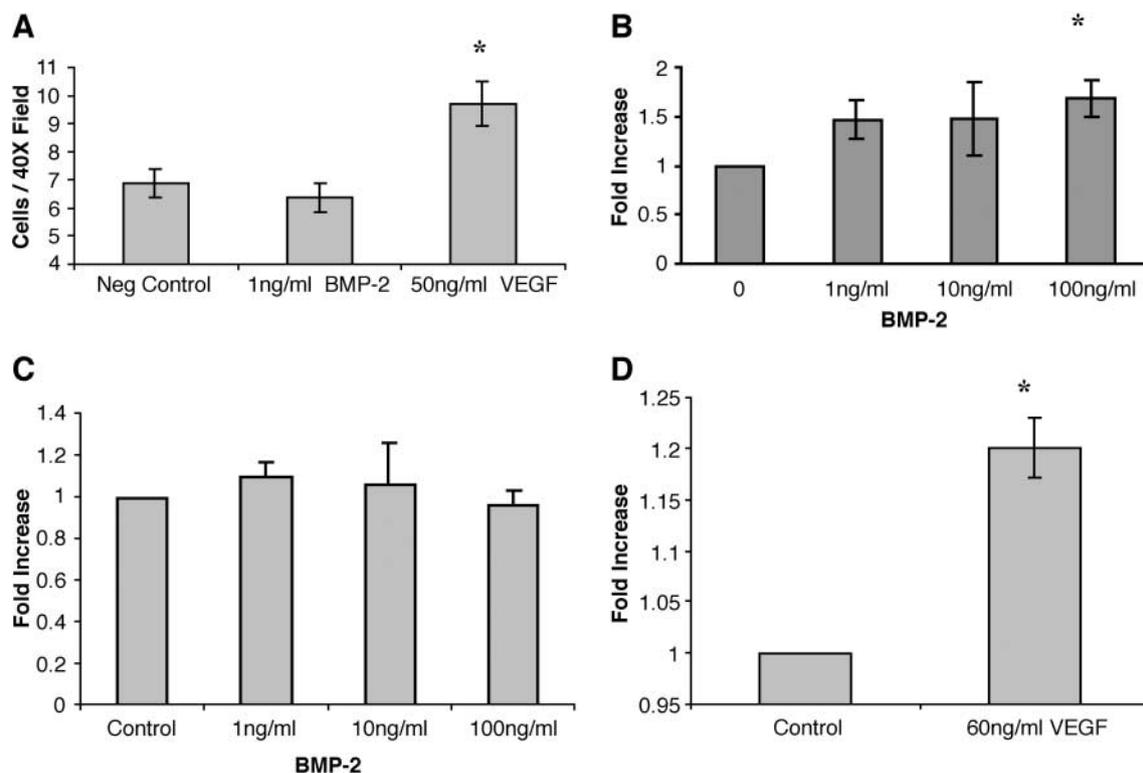
FIGURE 5. BMP-2 stimulates proliferation but not migration of endothelial cells. **A.** Migration of HAEC treated with vehicle control, BMP-2, or VEGF. The rate of DNA synthesis was determined by measuring incorporated [³H]thymidine. Data reported as relative to control. **B.** The rate of DNA synthesis of HAEC treated with recombinant BMP-2 (**C**). The rate of DNA synthesis of HUVEC treated with recombinant BMP-2. **D.** DNA synthesis of HAEC treated with recombinant VEGF. *Columns,* average of three independent experiments performed in duplicate. * $P < 0.05$.

was measured 6 h after adding BMP-2 to HUVEC (data not shown). In comparison to VEGF, we found BMP-2 to induce an approximately 67% increase in proliferation of the HAEC while VEGF caused a 20% increase (Fig. 5D), suggesting that BMP-2 is at least as strong of a mitogen of endothelial cells as VEGF.

To study whether BMP-2 induced tube formation, the HAEC or HUVEC were plated on Matrigel-coated plates and treated with and without recombinant BMP-2 (Fig. 6, A–F). BMP-2 increased the formation of tube-like structures in both the HAEC (Fig. 6E) and HUVEC (Fig. 6F). The greatest effect on tube formation occurred at 500 pg/ml of BMP-2 with a lesser effect at higher concentrations. Noggin inhibited BMP-2-induced tube formation in both the HAEC (Fig. 6E) and HUVEC (Fig. 6F).

Discussion

The role of BMP family members on vascular development has not been extensively studied. Studies have implicated the BMPs in vasculogenesis, the formation of blood vessels *de novo* from precursor cells during embryonic development. BMPER has been shown to be a BMP-2/4 antagonist secreted from embryonic endothelial cells which inhibits BMP-4-dependent differentiation of endothelial cells in embryoid body differentiation assay (18). Functional deletion of BMP-4 and the BMP I receptor in mice leads to impaired mesoderm

formation, which is required for blood and endothelial development (7, 19). Targeted disruption of the BMP 2/4 transcription factor Smad 5 resulted in the disorganization of yolk sac vasculature and heart development (30). However, a role for the BMPs on postnatal vascular development has not been established.

Using two *in vivo* models, we show that recombinant BMP-2 produces a large increase in the size and number of blood vessels in tumors formed from A549 cells in nude mice. BMP-2 significantly enhanced the tumor's blood supply within 4 days following injection, suggesting that BMP-2 acts early in the establishment of a tumor's blood supply. The finding that inhibition of BMP-2 by either noggin or antisense transfection decreases the tumor vasculature, supports that BMP-2 does not enhance angiogenesis only at high concentrations. BMP-2 is expressed highly in the majority of NSLC when compared to normal lung tissue (12). However, the exact physiological concentration of BMP-2 in tumors is not known. We estimate by Western blot analysis that the concentration of BMP-2 in tumors is between 700 and 1300 ng/ml.[3] This would make the amount of BMP-2 used in our *in vivo* experiments near physiological concentrations. BMP-2 is secreted from cells and is stored in the extracellular matrix (31). Therefore,

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[3]Unpublished data.
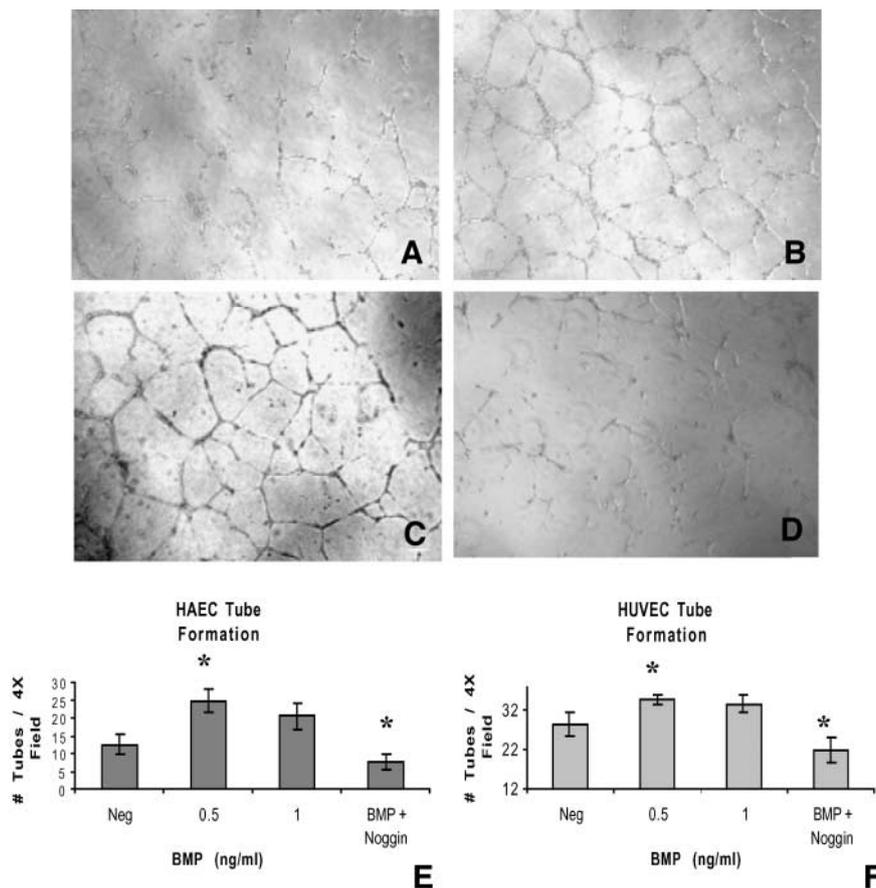
FIGURE 6. BMP-2 stimulates tube formation of endothelial cells. Representative photograph of tube-like structures formed by HAEC treated with vehicle control (**A**), 500 pg/ml BMP-2 (**B**), 1 ng/ml BMP-2 (**C**), or BMP-2 preincubated with noggin (**D**). Original magnification was 4×. Graphic representation of the number of tubes per low powered field for HAEC (**E**) and HUVEC (**F**). Each condition was repeated at least 3 times in duplicate. * $P < 0.05$ compared to control.

recombinant BMP-2 may further enhance the early development of a blood supply in these early tumors, because a sufficient concentration of BMP-2 produced from the tumors has not yet been established.

We do provide evidence that BMP-2-induced angiogenesis occurs at least, in part, by stimulating endothelial cells. The demonstration that BMP-2 promotes tube formation, induces phosphorylation of Erk-1/2, Smad 1/5, and increases Id1 expression shows that BMP-2 does activate endothelial cells. BMP-2/4 has been shown to activate Erk-1/2 in osteoblasts (32), but has not previously been shown to induce phosphorylation of Erk-1/2 in endothelial cells. VEGF, epidermal growth factor, fibroblast growth factor, and angiogenin have all been shown to mediate a cellular response in endothelial cells through the activation of Erk-1/2 (25–27). Erk-1/2 regulates several critical cellular functions in endothelial cells including proliferation and tube formation (25, 27). Studies have demonstrated that Id has an important role in mediating an angiogenic response during embryonic development and on postnatal endothelial cells (33). Mice with a double knockout of Id1-Id3 display vascular abnormalities in the forebrain (34). Id1$^{+/-}$Id3$^{-/-}$ mutant mice failed to support the growth of xenograft tumors, which is thought to occur from the inability to form a neovasculature (34). Sustained expression of Id cells delays the onset of senescence in human endothelial cells (35) and stimulates tube formation and migration of endothelial cells (36).

Consistent with our findings, BMP-6 was recently shown to directly activate endothelial cells, which occurred through the stimulation of Id1/5 (24). BMP-6 also induced phosphorylation of Smad 1/5 and/or 8 in BAEC. Furthermore, activation of either type IA or type IB BMP receptor was sufficient to induce BAEC migration and tube formation (24). We were unable to show that BMP-2 enhanced the migration of human endothelial cells. These observed differences seen in endothelial migration might vary depending on the particular BMP or the cell type. Our data would suggest that different endothelial cells respond differently to BMP-2. We were only able to see an increase in proliferation in HAEC, but not HUVEC. The activation of Erk-1/2 was more robust in HAEC compared to HUVEC, which may explain the differences in proliferation. Because it did not stimulate endothelial cell migration, it may be that BMP-2 is required more for the maturation of the blood vessel rather than as a sole initiator of angiogenesis.

We suspect that BMP-2 may promote angiogenesis by mechanisms other than directly stimulating endothelial cells. BMP-2, BMP-4, BMP-6, and BMP-7 have all been shown to induce VEGF expression in osteoblasts (37, 38). Therefore, it is possible that BMP-2 may be stimulating VEGF expression from tumor cells and/or surrounding normal parenchymal cells, which also contributes to the angiogenic response. BMP-2 may also enhance angiogenesis by serving as a chemotactic factor for monocytes (3). Monocytes are known to be present in lung tumors, which can secrete cytokines that promote an angiogenic

response (39). Postnatal stem cells in mice have been shown to migrate to developing tumors and differentiate into endothelial and myeloid cells (40). Up to 90% of endothelial cells in early tumors have been shown to originate from bone marrow-derived stem cells (40). Because BMPs promote vasculo-genesis, it will be interesting to see if BMP-2/4 are required for postnatal stem cell differentiation in tumors.

The autocrine effects of BMP-2 on tumor growth have not been fully elucidated. Studies have shown that recombinant BMP-2/4 inhibits the growth of tumors cells *in vitro* (41, 42). Recombinant BMP-2 has been shown to inhibit the growth of A549 cells cultured in serum-free medium (42). However, BMP-2 enhanced tumor growth of A549 cells injected s.c. into nude mice (12). This would suggest that BMP-2 acts predominately in a paracrine manner.

However, transfection of a dominant-negative BMP-2 type receptor inhibited the growth of breast cancer cell lines, suggesting that BMP-2 may actually stimulate proliferation of tumor cells. BMP-2 inhibited the growth of the LNCap prostate carcinoma cell in the presence of androgen, while in the absence of androgen, BMP-2 stimulated cell growth (43). BMP-2 has also been shown to enhance proliferation during early wound healing (44) and chondrocytes in costrochondral growth plates (45). These studies suggest that the autocrine effects of BMP-2 on the proliferation of tumor cells may be dependent on the presence of specific cytokines. During embryogenesis, BMP-mediated responses are dependent on concentration gradients and the presence of other specific cytokines (46). This may also explain why BMP-2 does not induce bone formation in lung tumors.

This study demonstrates that BMP-2 enhances angiogenesis in an *in vivo* tumor model. BMP-2 mediates in endothelial cells an activation of Id1 and Erk-1/2 with a corresponding stimulation of proliferation and tube formation. These data suggest that BMP-2 mediates angiogenesis in part by activating postnatal endothelial cells. This study provides further evidence that BMP-2 expression in lung tumors has an important role in the tumorigenic process of lung and possibly other tumors.

Materials and Methods

Cell Culture and Reagents

HAEC and HUVEC were obtained from Bio-Whittaker (Walkersville, MD). Endothelial cells were maintained in EBM-2 supplemented with 2% FCS and growth factors supplied in the bullet kit (Bio-Whittaker). The A549, A549-AS, and A549-vector lung cancer cell lines were prepared and maintained as described (12). Recombinant BMP-2, noggin, and VEGF were purchased from R&D Systems (Minneapolis, MN). Primary antibodies against BMPR IA, BMPR IB, BMPR II (R&D Systems), phosphorylated Smad 1 (Upstate Biotechnology, Lake Placid, NY), Id1 (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated Erk-1/2 (Promega, Madison, WI), and Actin (Sigma, St. Louis, MO) were used for Western blot analysis. Antibody recognizing Smad 1, 5, and/or 8 was used in immunofluorescence and immunoprecipitation studies (Santa Cruz Biotechnology) and platelet-endothelial cell adhesion molecule-1 (PECAM-1) for immunohistochemistry (BD PharMingen, San Diego, CA).

Migration Assay

3.0×10^4 HAEC cells in EBM-2 supplemented with 0.5% FCS and growth factors excluding VEGF (Bio-Whittaker) were placed in the upper chamber of a transwell migration chamber (Becton Dickinson, Bedford, MA). Medium containing 1, 10, or 100 ng/ml of BMP-2, 50 ng/ml VEGF, or 4 mM HCl/0.1% BSA (vehicle control) was added to the lower chamber. After 24 h, the cells that had migrated were stained with Giemsa (Sigma) and ten 40× fields counted.

Proliferation Assay

HAEC or HUVEC cells were plated at 2.0×10^5 cells/well in six-well tissue culture plates (Falcon, Franklin Lakes, NJ) in EBM-2 with 2% FCS. Cells were treated with 1, 10, or 100 ng/ml BMP-2 or vehicle control for 24 h then pulsed with 4 μCi/ml [^{3}H]thymidine for 1 h. Cells were washed twice with cold PBS and fixed with 5% trichloroacetic acid for 10 min. Cells were treated with 70% ethanol, lysed with 1% SDS/10 mM NaOH, and incorporated [^{3}H]thymidine measured in a scintillation counter.

Smad Localization

HAEC or HUVEC cultured on glass cover slips were serum starved in EBM-2 deficient of FCS and VEGF for 6 h, then treated with 10 ng/ml BMP-2 for 0, 15, 30, 45, and 60 min. Cells were fixed with 3.7% formaldehyde, permeabilized with 0.5% Triton X, and blocked with 1% BSA/PBS (29). Cells were incubated overnight with a 1:50 dilution of anti-pSmad 1/5/8 antibody at 4°C and AlexaFluor 488 anti-goat was used as the secondary antibody (Molecular Probes, Eugene, OR). To inhibit BMP-2, 40 ng of recombinant noggin were added to the BMP-2-treated cells. Negative controls included primary antibody incubated with blocking peptide (Santa Cruz Biotechnology) and AlexaFluor 488 without primary antibody. Smad 1/5/8 localization was assessed by immunofluorescent microscopy.

Western Blot Analysis and Immunoprecipitation

HAEC or HUVEC in EMB-2 with 2% FCS were treated with 10 ng/ml of recombinant BMP-2 for 0, 10, 20, 40, 60, and 120 min. Western blot analysis was performed on the cell lysate as previously described (12). Each blot was probed with one antibody specific for that protein. For immunoprecipitation studies, HAEC were treated for 20 min with 20 ng/ml of BMP-2, cell lysate incubated with anti-pSmad 1/5/8 antibody, and separated by SDS-PAGE. Immunoblots were probed with anti-phosphoserine antibody (Zymed Laboratories, San Francisco, CA).

Tube Assay

Twenty-four-well tissue culture plates were coated with 250 μl of Matrigel (BD Biosciences). HAEC or HUVEC were plated at 5.0×10^4 in 1 ml of EBM-2 supplemented with 1% FCS and all growth factors excluding VEGF. Cells were then treated with vehicle control, 500 pg/ml BMP-2, or 1 ng/ml

BMP-2 for 18 h at 37°C. To inhibit BMP-2, 40 ng/ml of recombinant noggin were added to samples treated with BMP-2. Cells were photographed at 4× magnification and the formation of complete tubes counted in three fields.

BMP-2 Tumor Angiogenesis Assay

1×10^7 A549 cells were s.c. injected into the flanks of NCR nude mice with Affi-Blue agarose beads (Bio-Rad, Hercules, CA) coated with BSA or recombinant human BMP2 as previously described (12). In brief, 25 μg of Affi-Blue agarose beads were incubated with 2 μg of BSA or recombinant BMP-2 for 2 h and washed with PBS (12, 47, 48). Tumors were collected 4–6 or 12–14 days later. Tumors were placed in optimal cutting temperature (OCT) and frozen in liquid nitrogen. Blood vessels were detected by immunohistochemistry using anti-PECAM-1 antibody. Vessels within the tumors were photographed and five random 40× fields counted as previously described (49). The mice studies were approved by the Robert Wood Johnson Medical School Institutional Animal Care and Use Committee.

Matrigel Angiogenesis Assay

2×10^6 A549, A549-AS, or A549-vector cells in 100 μl DMEM with 5% FBS were mixed with 400 μl Matrigel and injected s.c. into nude mice. The Matrigel was supplemented with 835 ng of BSA or 670 ng recombinant BMP-2. To inhibit BMP-2, 670 ng of recombinant BMP-2 were incubated with 835 ng noggin for 2 h at 37°C, before mixing with the Matrigel. The Matrigel plugs were harvested after 6 days, placed in OCT, and frozen in liquid nitrogen. Blood vessels were detected by immunochemistry. All of the vessels within the Matrigel plugs were photographed and counted.

Immunohistochemistry

Immunohistochemistry was performed as previously described (12). In brief, frozen samples were cut with a dermatome to obtain 5-μm sections. The samples were incubated with a rat anti-PECAM-1 antibody at a 1:20 dilution overnight at 4°C. The anti-rat IgG was incubated at a 1:300 dilution at room temperature for 20 min. Positive control included a mouse liver. Negative controls consisted of samples treated with secondary antibody alone.

Statistical Analysis

To evaluate multiple groups, a one-way ANOVA, followed by Fisher LSD post hoc test was used to compare individual means. To compare two groups, a Student t test was used. Differences with P values ≤ 0.05 were considered statistically significant.

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