BMP-2 inhibits proliferation of human aortic smooth muscle cells via p21\textsuperscript{Cip1/Waf1}

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Bone-morphogenetic proteins (BMP)-2 and -7, multifunctional members of the transforming growth factor (TGF)-β superfamily with powerful osteoinductive effects, cause cell cycle arrest in a variety of transformed cell lines by activating signaling cascades that involve several cyclin-dependent kinase inhibitors (CDKIs). CDKIs in the cip/kip family, p21\textsuperscript{Cip1/Waf1} and p27\textsuperscript{Kip1}, have been shown to negatively regulate the G1 cyclins and their partner cyclin-dependent kinase proteins, resulting in BMP-mediated growth arrest. Bone morphogens have also been associated with antiproliferative effects in vascular tissue by unknown mechanisms. We now show that BMP-2-mediated inhibition of platelet-derived growth factor (PDGF)-stimulated human aortic smooth muscle cell (HASMC) proliferation is accompanied by increased levels of p21 protein. Antisense oligodeoxynucleotides specific for p21 attenuate BMP-2-induced inhibition of proliferation when transfected into HASMCs, demonstrating that BMP-2 inhibits PDGF-stimulated proliferation of HASMCs through induction of p21. Whether p21-mediated induction of cell cycle arrest by BMP-2 sets the stage for osteogenic differentiation of vascular smooth muscle cells, ultimately leading to vascular mineralization, remains to be investigated.

Bone morphogenetic protein-2; vascular smooth muscle cell; antisense oligodeoxynucleotides; human aortic smooth muscle cell; osteoblast-like cell

ONE OF THE EARLY EVENTS in the development of atherosclerotic lesions is the proliferation of vascular smooth muscle cells (VSMCs) in response to a variety of luminal injuries (24). A number of growth-promoting proteins, including platelet-derived growth factor (PDGF), fibroblast growth factor, and insulin-like growth factor, have been identified in VSMCs as being important in the pathogenesis of these vascular lesions (2, 25). Elucidating the mechanism by which VSMCs commit to either cellular proliferation or cell cycle arrest is therefore critical to understanding the genesis of the cellular response to vascular injury and its subsequent contribution to atherosclerotic plaque formation, angioplasty restenosis, and vascular calcification.

The cyclin-dependent kinases (CDKs) are a family of serine-threonine kinases that act in conjunction with their partner cyclins in cascade fashion in response to mitogenic stimulation, ultimately leading to cell cycle progression through the G1/S phase transition (3). In many cell systems, cell cycle arrest is associated with p21\textsuperscript{Cip1/Waf1}-mediated inhibition of CDK and cyclin activities and subsequent downstream dephosphorylation of retinoblastoma protein (8). More recently, we have shown that p21 is important in promoting assembly of cyclin D/CDK4 complexes in VSMCs (31).

In this context, p21 may be permissive for cell cycle progression through the G1/S phase and therefore significant in maintaining cellular integrity and survival (16, 31). Thus p21 may act differently in various cell types in response to specific cytokines to promote growth arrest or cell cycle progression. Because multiple cyclin-CDK complexes are present simultaneously in the local environment, it is possible that different signaling pathways can be concurrently invoked and result in divergent effects in the same target cells.

Bone-morphogenetic proteins (BMPs) are powerful osteoinductive proteins thought to be integral to the process of differentiation, proliferation, and osteoblastic activation by which new bone is made (22, 23). BMP-2 is the most potent of the multiple cytokines and growth factors that interact sequentially to induce the differentiation of osteoprogenitor cells into osteoblasts (28, 30). Additionally, it is known that BMP-2 can elicit diverse responses in a number of cell systems, ranging from differentiation to apoptosis (12, 14, 20). Recent studies have implicated the CDK inhibitors (CDKIs) of the cip/kip family, p21 and p27 (6, 7, 12), in BMP-mediated actions. Subpopulations of aortic smooth muscle cells have been shown to have osteoblastic potential under specific conditions. These cells resemble pericytes morphologically and can be induced to differentiate into osteoblast-like cells, able to produce mineralized bone nodules in culture (29). In this study,

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we examined the potential of BMP-2 to inhibit PDGF-stimulated proliferation in a human aortic smooth muscle cell (HASMC) line and asked whether p21 plays a causative role in this process. In light of its powerful osteoinductive properties, the actions of BMP-2 on HASMCs in response to mitogenic triggers may thus have important implications with respect to the potential of a selected population of VSMCs to differentiate into osteogenic cells. p21 May therefore prove to be a useful target for future therapies aimed at preventing vascular calcification.

MATERIAL AND METHODS

Materials

HASMCs and SmGM growth medium were purchased from Clonetics (San Diego, CA). These cells are isolated from the aortic arch, stain positively for α-smooth muscle actin and negatively for von Willebrand’s factor. Dulbecco’s modified Eagle’s medium (DMEM), Opti-MEM medium, Fungizone, and lipofectin were purchased from Invitrogen (Carlsbad, CA). Recombinant human (rh) PDGF-BB and mouse monoclonal p21Waf1/Cip1 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-caspase-3 rabbit polyclonal and horseradish peroxidase (HRP)-conjugated anti-mouse antibodies were purchased from Santa Cruz Biotechnology (Lake Placid, NY). The p21 antisense sequence 5'-ATCCCCAGC-3' and the random control sequence 5'-TGGATCCGACATGTCAGA-3' were synthesized by Oligos, Etc. (Wilsonville, OR). The p21 antisense sequence 5'-ATCCCCAGC-CGTTCTGACAT-3' was designed around the start codon of human p21Waf1/Cip1. The randomly generated control sequence was 5'-TGATCCGACATGTCAGA-3'. HASMCs were grown in SmGM medium on 60-mm dishes until 70–80% confluent. Cells were synchronized in G0 in serum-free medium supplemented with 10% FBS on 6-well plates until 70–80% confluent. The cells were synchronized in G0; in serum-free medium for 24 h and then incubated in 40 ng/ml PDGF-BB and/or 150 ng/ml BMP-2 for 24 h. The cells were fixed with methanol for 10 min. After the methanol was aspirated, 1 μg/ml Hoechst 33258 in H2O with 10 μg of nonfat dry milk was added to the wells. The cells’ nuclear morphology was viewed under a Zeiss Axioskop fluorescence microscope.

Measurement of Apoptosis

Hoechst staining. HASMCs were grown in growth medium supplemented with 10% FBS on 6-well plates until 70–80% confluent. The cells were synchronized in G0 in serum-free medium for 24 h and then incubated in 40 ng/ml PDGF-BB and/or 150 ng/ml BMP-2 for 24 h. The cells were rinsed with PBS and incubated with 10 μg/ml Hoechst 33258 in H2O with 10 μg of nonfat dry milk. The cells were rinsed with PBS and then incubated with 10 μg/ml Hoechst 33258 in H2O with 10 μg of nonfat dry milk. The cells were rinsed with PBS and then incubated with 10 μg/ml Hoechst 33258 in H2O with 10 μg of nonfat dry milk.

Caspase-3 activation. HASMCs were grown in growth medium until 70–80% confluent. Cell lysates were prepared as discussed above after an overnight incubation in serum-free medium, followed by a 24-h incubation in 40 ng/ml PDGF-BB and/or BMP-2. Western analysis of procaspase-3 cleavage to caspase-3 was performed as described above, using 1:1,000 dilutions of anti-caspase-3 rabbit polyclonal antibody as the primary antibody (1:1,000 dilution) and goat anti-rabbit IgG as the secondary antibody (1:40,000). A Jurkat HLM cell lysate was used as a positive control for caspase cleavage.

Cell Transfections with p21Waf1/Cip1 Antisense Oligonucleotide

Phosphorothioate antisense and random sequence oligodeoxynucleotides (ODN) were synthesized by Oligos, Etc. (Wilsonville, OR). The p21 antisense sequence 5’-ATCCCCAGCCGTTCTGACAT-3’ was designed around the start codon of human p21Waf1/Cip1. The randomly generated control sequence was 5’-TGATCCGACATGTCAGA-3’. HASMCs were grown in supplemented SmGM growth medium on 60-mm dishes until 70–80% confluent and rinsed with sterile PBS. Random sequence or p21 antisense ODNs were mixed in 5 μl lipofectin/ml Opti-MEM medium to a final concentration of 200 nM. The cells were transfected with lipofectin/Opti-MEM alone, random sequence ODN, or p21 antisense ODN for 4 h at 37°C. Control cells were not transfected. After a rinse and an overnight incubation in serum-free medium, cells were incubated with or without PDGF-BB (40 ng/ml) for the indicated times in the presence and absence of BMP-2 (150 ng/ml) and used to prepare either cell lysates for immunoblotting of p21 protein or in cell proliferation experiments as described.
RESULTS

BMP-2 is Associated with Decreased PDGF-Stimulated and Unstimulated HASMC Proliferation

To determine whether BMP-2 decreases cell cycle transit in HASMCs, we examined \(^{3}H\)thymidine incorporation in an established line of HASMCs under PDGF-stimulated and unstimulated conditions in the presence or absence of simultaneously added BMP-2 at physiological concentrations. HASMCs were serum starved overnight and then stimulated to proliferate with PDGF (40 ng/ml) in the presence of increasing concentrations of rhBMP-2. DNA synthesis, as a measure of cell cycle transit, was assessed by \(^{3}H\)thymidine incorporation into DNA. rhBMP-2 at 50, 150, and 300 ng/ml inhibited PDGF-induced HASMC proliferation by 35, 46, and 64%, respectively, compared with PDGF-stimulated control cells (Fig. 1A). A similar inhibitory effect was seen in serum-starved cells in the absence of PDGF stimulation (Fig. 1B). Thus BMP-2 decreases cell cycle progression in serum-starved HASMCs under both unstimulated and PDGF-stimulated conditions.

BMP-2-mediated cell cycle arrest is not associated with increased apoptosis. A possible explanation of the observed decrease in \(^{3}H\)thymidine incorporation in response to BMP-2 is that BMP-2 is causing apoptosis of HASMCs. To address this possibility, we utilized two distinct methods to assess apoptosis. Hoechst staining and caspase-3 activation in HASMCs were performed in PDGF-treated and untreated cells after 24 h of incubation in the presence and absence of 150 ng/ml BMP-2, a concentration of BMP-2 that resulted in significant growth arrest (see Fig. 1, A and B). There were no changes in Hoechst staining under all conditions examined (Fig. 2). In addition, no activation of procaspase-3 to caspase-3 was apparent under all tested conditions (Fig. 3). Thus, in HASMCs, BMP-2 treatment results in decreased PDGF-mediated cell proliferation by directly inhibiting new DNA synthesis and subsequent G1/S phase progression, rather than by causing apoptosis.

BMP-2 Increases p21 Protein Levels

The CDK inhibitor p21 is associated both with cell cycle arrest through its inhibitory effects on CDK2 (8) and with cell cycle progression by both promoting assembly of cyclin D1-CDK4 complexes and by its anti-apoptotic actions (16, 31). To determine whether p21 is increased by BMP-2, HASMCs were treated with 150 ng/ml rhBMP-2 for 4, 8, and 24 h under PDGF-stimulated and unstimulated conditions. Exposure to PDGF caused an increase in p21 protein levels in the absence of BMP-2 after 4, 8, and 24 h of treatment, consistent with our previous observations (10). BMP-2 treatment of HASMCs resulted in increased p21 protein levels above those seen with PDGF at both 4 and 8 h (Fig. 4), with the effect disappearing in PDGF-stimulated cells after 24 h of incubation. p21 Levels increased in response to BMP-2 at 4, 8, and 24 h in unstimulated cells. Identification of the p21 protein band was confirmed by immunoblotting using HeLa cell lysate as a positive control.
control for p21 (Fig. 4), and smooth muscle α-actin was used as a loading control.

p21 Antisense ODN Attenuates BMP-2-Mediated Inhibition of Cell Proliferation

To determine whether inhibition of HASMC proliferation by BMP-2 involves p21, we generated a phosphorothioate-modified antisense ODN sequence to human p21. PDGF-stimulated and unstimulated HASMCs were untransfected (control) or transfected with lipofectin alone (no ODN), random sequence (scrambled) ODN control, or antisense ODN specific for human p21 (anti-p21 ODN). Cell lysates were prepared, and p21 protein levels were then analyzed by...
Western immunoblotting. Partial but significant inhibition of p21 protein was seen in cells transfected with p21 antisense ODN after 4 h of PDGF exposure, a time before the G1/S restriction point (Fig. 5). We then asked whether BMP-2-mediated inhibition of HASMC proliferation was attenuated in cells transfected with p21 antisense ODN. Cell proliferation by [3H]thymidine incorporation was measured in PDGF-stimulated (Fig. 6A) and unstimulated (Fig. 6B) HASMCs in the presence and absence of BMP-2 (150 ng/ml). Cells were untransfected (control) or transfected with lipofectin alone (no ODN), random sequence (scrambled control) ODN, or p21-antisense ODN. BMP-2 inhibited [3H]thymidine incorporation in untransfected cells (control) and after transfection with lipofectin and random sequence ODN by 48, 35, and 46%, respectively, in PDGF-stimulated cells. However, after transfection with antisense to p21, the degree of BMP-2-mediated inhibition decreased to 7% in PDGF-stimulated HASMCs. In unstimulated HASMCs, BMP-2 similarly caused a decrease in cell proliferation in untransfected cells (control) and after transfection with lipofectin alone and scrambled control ODN. However, no significant attenuation of BMP-2-mediated growth inhibition was seen in unstimulated cells after transfection with p21 antisense ODN (Fig. 6B). Therefore, p21 is a significant mediator of antiproliferative effects of BMP-2 in PDGF-stimulated HASMCs but not in unstimulated, serum-starved cells. In addition, PDGF stimulation appears to be a requirement for p21-mediated BMP-2 antiproliferative actions in this system. In unstimulated cells, BMP-2 growth-inhibitory actions appear to be p21 independent.

**DISCUSSION**

BMP-2 has strong osteoinductive properties and, in addition, appears to mediate growth inhibition via the CDK inhibitors in a number of cell types. Whether BMP-2 is important in inducing osteogenesis in vascular cells in vivo, subsequently contributing to vascular calcification, however, is unknown. Intuitive to the progression of cells from one phenotype to another is the induction of growth inhibition of the precursor
population. Vascular cells proliferate in response to injury and inflammation, but that proliferation must be arrested if they are to become osteoblast-like in nature (21).

In light of the antiproliferative effects of BMP-2 and other members of the TGF-β family of cytokines in a number of cell systems (18, 19, 27), we asked whether BMP-2 also inhibits the proliferation of HASMCs. We observed a strong antiproliferative effect of BMP-2 on VSMC proliferation under both PDGF-stimulated and serum-free conditions. BMP-2 treatment of HASMCs resulted in decreased PDGF-stimulated DNA synthesis and cell proliferation in a dose-dependent manner over a wide physiological range of concentrations. BMP-2-mediated antiproliferative effects were also appreciated in unstimulated cells but were independent of p21. The decrease in proliferation mediated by BMP-2 was not due to increased apoptosis but appeared to result directly from the inhibition of new DNA synthesis. This is the first report of BMP-2-mediated inhibition of HASMC proliferation in vitro and thus may have broad implications for addressing the mechanism by which osteogenesis occurs in vascular tissue in response to pathogenic insult.

BMP-2, a member of the TGF-β superfamily, is a powerful osteoinductive protein with diverse effects in a variety of cell lines. For example, BMP-2 and a member of the interleukin-6 (IL-6) family, leukemic inhibitory factor, act in synergy to induce neuroprogenitor cells to differentiate into astrocytes (20). In contrast, BMP-2 and IL-6 interact to cause apoptosis in mouse hybridoma cells (14). BMP-7, which has close sequence and structural homology to BMP-2, induces cell cycle arrest of anaplastic thyroid cancer cells (6). Studies in vascular tissue suggest a requirement for cycle arrest vs. cell death in order to allow proper assembly, stabilization, and transport of cyclin D/CDK4 complexes, thus promoting cellular viability and integrity (15, 31). Furthermore, p21 can function as an antiapoptotic survival protein in tumor cells (16) as well as in VSMCs (Davis BB and Weiss RH, unpublished observations). Thus it appears that p21 can function differentially, depending on the cell’s requirement for cycle arrest vs. cycle progression, in response to diverse conditions as well as time of activation relative to cell cycle events. In addition, the behavior of p21 may be different in cells transformed by mutagenic DNA damage than in its response to vascular insult.

We employed an established cell line of HASMCs in our experiments as one model for VSMC phenotype. These cells are derived from the aortic arch and stain positively for α-actin and negatively for von Willebrand factor. HASMCs have been used by other investigators in a number of published reports examining regulatory events in cell cycling (5, 9, 26). Using these cells, we found p21 protein increased in PDGF-stimulated cells after 4, 8, and 24 h of treatment, consistent with our previous work in rat VSMCs and demonstrating a growth-permissive role of p21 in this context (31). BMP-2 treatment resulted in an additional increase in p21 above that seen with PDGF after 4 and 8 h. This increase was not observed after 24 h of treatment of BMP-2, suggesting that p21-mediated BMP-2 effects occur earlier in the cell cycle, likely at the G7/S boundary.

In the case of BMP-2, reduction of p21 levels by antisense techniques attenuated growth-inhibitory actions when observed 24 h after PDGF stimulation. In addition, treatment of PDGF-stimulated HASMCs with p21 antisense in the absence of BMP-2 resulted in a nonsignificant decrease in p21 levels and proliferative response, consistent with previous observations that p21 is important in PDGF-mediated cell proliferation. No attenuation of BMP-2-mediated growth arrest by p21 antisense occurred in unstimulated cells, however, suggesting that BMP-2 may act to modulate the PDGF-mediated upregulation of p21 that occurs early in the cell cycle. Our findings suggest that BMP-2...
acts to promote cell cycle arrest in HASMCs stimulated by PDGF by further induction of the CDK inhibitor p21Clp1/Waf1 to promote growth arrest of these cells. It is conceivable that p21 may act differentially depending on its location. PDGF may induce cytoplasmic p21, promoting its function as a survival factor (1) and mediating PDGF-associated cell proliferation. However, further induction of p21 by BMP-2 and subsequent nuclear translocation of p21 from the cytoplasm may result in growth arrest in this system. This is consistent with the findings of two independent studies in a balloon injury model in the rat showing that adenovirally mediated transfer of either BMP-2 (19) or p21 (4) results in decreased intimal hyperplasia of carotid artery in response to luminal insult. Thus p21 is a critical downstream mediator of BMP-2-induced antiproliferative actions on PDGF-stimulated HASMCs. In contrast, BMP-2-mediated growth-inhibitory actions on cells in the basal state occur via a p21-independent mechanism and may be autocrine in nature.

Both endogenous and exogenous factors have been recently identified as causing growth arrest in VASMCS. p21 Has been invoked as an important mediator in suppression of VSMC proliferation by salicylates, nitric oxide, and cAMP (9, 11, 17). The mechanism is thought to be similar to that in nonvascular cells, i.e., a direct p21-mediated inhibition of cyclin E/CDK2 responses during G1/S progression leading to cell cycle arrest. Our findings and these results, taken together, suggest that p21 may act under specific conditions as a common mediator upon which disparate pathways converge to activate downstream signaling events that lead ultimately to growth arrest of VSMCs. This pathway may be induced by factors that differ from each other as widely as do salicylates, BMPs, and nitric oxide. Clearly, induction of such a pathway has important clinical implications in the regulation of vasculoproliferative response to arterial injury in vivo.

The mechanism by which TGF-β, and BMPs in particular, cause cell cycle arrest has not been clearly elucidated. Induction of osteoblast differentiation and regulation of embryogenesis by BMP-2 occurs by signaling through the family of transcription factors known as the Smads (21). Recently, however, MAPK pathways have been demonstrated to be important in BMP-mediated actions. Activation of the TAK1-p38 pathway is required for BMP-2-mediated apoptosis of mouse hybridoma cells (14). Moreover, recent evidence suggests that cross talk between the Smad and MAPK pathways plays a significant role in downstream signaling events. In hybridoma cells, Smad6 has been shown to physically interact with TGF-β-activated kinase-1 (TAK1), preventing activation of the TAK1-p38 pathway and abolishing apoptosis induced by BMP-2 (14). Notably, a recent report showing that p38 and Jnk1 stabilize p21 by phosphorylation (13) lends support to the potential role of upstream MAPK-signaling proteins in modulating BMP-2-mediated antiproliferative effects in VSMCs through p21. In this study, we show that BMP-2 causes cell cycle arrest in human VSMCs and that its growth-inhibitory action is mediated by p21, a CDK inhibitor involved in the mechanism utilized by other growth-inhibitory factors in VSMCs.

BMP-2 has been shown to induce osteoblastic phenotype in a number of pluripotent cell lines and indeed is one of the most osteoinductive cytokines known. We now show that BMP-2 acts to promote growth inhibition in PDGF-stimulated HASMCs through a p21-mediated mechanism. Whether these observations can
be extended to explain osteogenic differentiation of VSMCs in general, or whether induction by BMP-2 of p21-mediated growth arrest is a requirement for subsequent differentiation of mitogen-stimulated human VSMCs into those with osteoblastic potential, is currently under investigation in our laboratory.

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