BMP Inhibition with Dorsomorphin Limits Adipogenic Potential of Preadipocytes

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ABSTRACT. Previous studies revealed that bone morphogenetic protein (BMP) induces commitment to the adipocyte lineage in pluripotent stem cells. The present study explored the role of endogenous BMP activity in 3T3-L1 preadipocytes. The expression of phospho-Smad1/5/8 was monitored because BMP transmits its signal through Smad1/5/8 phosphorylation. Phosphorylated Smad1/5/8 was higher in proliferating preadipocytes, and lower in differentiating adipocytes after removing differentiation inducers. Reporter assays revealed that dorsomorphin predominantly inhibits the BMP pathway but not the structurally related TGF-β/activin pathway. The addition of dorsomorphin to the culture medium prior to treatment with differentiation inducers impaired lipid accumulation in 3T3-L1 cells. The present study indicated that activation of BMP signaling in preadipocytes is required for these cells to initiate the adipogenic program.

KEY WORDS: adipocyte differentiation, BMP, dorsomorphin, Smad.

The amount of adipose tissue in the body is an important determinant of energy metabolism in animals, and is related to various (patho-)physiological conditions [18]. Adipocytes also integrate a wide array of homeostatic processes by secreting various cytokines [22]. The size of adipocytes varies markedly and largely reflects the amount of stored triglyceride, whereas the number of adipocytes increases as a result of the proliferation of preadipocytes and their subsequent differentiation into adipocytes. Various growth factors/hormones affect adipocyte differentiation by regulating the expression and activity of adipogenic transcription factors [21].

Members of the transforming growth factor-β (TGF-β) family, which is composed of three subgroups, TGF-β, activin and bone morphogenetic protein (BMP), have diverse physiological roles, including the control of cell division, early embryonic patterning, differentiation, and cell determination [3]. They are also involved in the regulation of adipocyte differentiation; TGF-β blocked adipogenic differentiation both in vitro [4, 11] and in vivo [5]. Treatment with activin A, an activin isoform, inhibited the differentiation of 3T3-L1 preadipocytes [9]. By contrast, the roles of BMP are controversial. Treatment with BMP-2 decreased lipid accumulation in 3T3-F442A preadipocytes, suggesting the activity of BMP-2 as an inhibitor of adipocyte differentiation [19]. BMP-4 addition to the culture medium, however, conversely stimulated adipocyte differentiation of multipotential C3H10T1/2 cells [1, 20], suggesting that BMP induces to commit to the adipocyte lineage. Although endogenous BMP-4 expression is sufficient for the commitment in C3H10T1/2 cells [2], endogenous BMP signaling in preadipocytes and its role for adipocyte differentiation are unknown.

The present study examined the effects of endogenous BMP activity on adipocyte differentiation. We used 3T3-L1 preadipocytes as a cell model for adipocyte differentiation; growth-arrested confluent 3T3-L1 preadipocytes are treated with appropriate hormonal agents, which induce synchronous reentrance into the cell cycle and undergo at least 2 rounds of mitosis, referred to as mitotic clonal expansion. Subsequently, preadipocytes exit the cell cycle and begin to differentiate into adipocytes expressing adipogenic genes [14]. Here we reveal that endogenous BMP signaling is significantly activated in preadipocytes but not in differentiating cells, which is required for efficient adipocyte differentiation.

The following reagents were purchased: rabbit polyclonal antibodies against phospho-Smad1 (Ser463/Ser465)/Smad5 (Ser463/Ser465)/Smad8 (Ser426/Ser428), Smad1, phospho-AMP-activated protein kinase (AMPK) α1 (Ser485), and AMPKα2 (Cell Signaling Technology, Danvers, MA, U.S.A.); rabbit polyclonal antibody against Smad1 (Abcam, Cambridge, MA, U.S.A.); dorsomorphin (compound C: 6-[4-(2-piperidin-1-yl-ethoxy)phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a] pyrimidine; Calbiochem, San Diego, CA, U.S.A.).

3T3-L1 preadipocytes are cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C under a humidified 5% CO2 atmosphere. Two days after reaching confluence (day 0), cells were cultured in DMEM containing 10% FBS and antibiotics in the presence of differentiation inducers (3-isobutyl-1-methylxanthine (0.5 mM; Sigma), dexamethasone (0.25 μM; Sigma, St. Louis, MO, U.S.A.), and insulin (10 μg/ml; Wako, Tokyo, Japan)) for 2 days, known as mitotic clonal expansion [14], followed by culture with insulin (5 μg/ml) in DMEM containing 10% FBS and antibiotics. To examine the effects of dorsomorphin on adipocyte differentiation, dorsomorphin (5 μM) dissolved in DMSO was added to the culture medium for the indicated period. Lipid accumulation was examined by Oil Red O
staining on day 8. Cells were washed twice with PBS, fixed with 10% formalin, and stained with 0.5% filtered Oil Red O in 2-propanol:water (3:2). The images were obtained by scanning stained wells (GT-9400UF; EPSON, Tokyo, Japan). Subsequently, dye was extracted with 2-propanol. Absorbance of the solution was measured at 510 nm for quantification [17].

B16 cells were cultured and transfected as described previously [16]. The following plasmids were kindly provided: constitutively active ALK4 by Dr. L.S. Mathews; constitutively active ALK6 by Dr. K. Miyazono; BRE-luc and CAGA-luc by Dr. P. ten Dijke; constitutively active ALK5 by Dr. X.-F. Wang. After 24 hr of transfection, cells were treated with or without dorsomorphin for 16 hr. Luciferase reporter assays were performed as described previously [16].

To examine the expressions of Smad and AMPK during adipocyte differentiation, recovered cells were resuspended in hypertonic buffer (200 mM phosphate buffer (pH 7.4), 2 M NaCl, 2 mM Na2VO4, 1 mM PMSF, 1% aprotinin), followed by lysis by ultrasonication. DNA content was measured by the method of Labarca and Paigen [13]. Protein samples containing 1 µg DNA were loaded and electrophoresed on 10% SDS-polyacrylamide gels, followed by immunoblotting as described previously [7]. The reacted proteins were visualized using the ECL Plus Western blotting detection system (GE Healthcare, Piscataway, NJ, U.S.A.) according to the manufacturer’s protocol, and band intensity was quantified by an image analyzer (LAS-4000; Fujifilm, Tokyo, Japan).

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer’s protocol. Recovered RNA was used as a template for reverse transcriptase with random primers (ABI high capacity cDNA reverse transcription kit, Applied Biosystems, Foster City, CA, U.S.A.) according to the manufacturer’s protocol. qRT-PCR was carried out using a SYBR premix Ex Taq II kit (TaKaRa, Otsu, Japan) in a Rotor-Gene 6000 (Corbett Research, Mortlake, Australia). PCR was performed as follows: an initial denaturation step consisting of 10 sec at 95°C, followed by 40 cycles of 5 sec at 95°C and 20 sec at 60°C. Subsequently, the dissociation (melting) curve of qRT-PCR products was examined by changes in ramp temperature from 60°C to 94°C. As a result, each sample showed a single peak, suggesting the expected PCR products. The following oligonucleotides were used as PCR primers for Bmp-4, Bmp-5 and Hprt1: S'-gaggagtctcatacgaga-3' and S'-gtctgcggaggagta-3' for Bmp-4, S'-atcagacctccctagag-3' and S'-tgcagctgttggtagt-3' for Bmp-5, and S'-tcctcctcagccctt-3' and S'-cctggttcatactc-3' for Hprt1. The PCR primers used to detect Id1 were described previously [16]. The Ct value was determined, and the abundance of gene transcripts was calculated from the Ct value.

Data are expressed as the mean ± SEM. Differences between treatments were examined by Duncan’s multiple range test for qRT-PCR analyses or by Student’s t-test for dye content. Differences of P<0.05 were considered significant.

BMP transmits signals through a complex formation consisting of a ligand, type I (ALK2/3/6) and type II receptors (ActRIIA/IB and BMPRII). Activation of receptor complexes resulting from ligand binding induces the phosphorylation of C-terminal serines of Smad1/5/8. Subsequently, phosphorylated Smad1/5/8 form complexes with Smad4 and accumulate in the nucleus where they participate in transcriptional regulation of target genes. Thus, serine phosphorylation of Smad1/5/8 at the C-terminus is a key event in BMP signaling [15].

To examine changes in BMP signaling during adipocyte differentiation, we first examined the phosphorylation of Smad1/5/8 by Western blot analyses using an antibody that recognizes the phosphorylation of C-terminal serines (Fig. 1A). Band intensity was higher in proliferating 3T3-L1 preadipocytes (lane 1). Although band intensity transiently increased on day 1 (lane 5), it gradually decreased with time. In particular, it was lower after removing differentiation inducers (lanes 7–9); however, it should be mentioned that this antibody recognizes the unphosphorylated form of Smad1/5/8 as well as the phosphorylated form, but is not structurally related to the other Smads [16]. Thus, it is possible that increased band intensity in preadipocytes resulted from an increase in the unphosphorylated form of Smad1/5/8. However, it is unlikely; total Smad1 expression was relatively constant throughout the examined period (Fig. 1A).

Id1 is transcriptionally activated in response to BMP treatment, which is mediated by phosphorylated Smad1 [12, 15, 16]. Changes in the gene transcript level of Id1 during adipocyte differentiation were then examined (Fig. 1B). Consistent with the results of Western blot analyses using the anti-phospho-Smad1/5/8 antibody, the gene transcript level of Id1 was significantly higher on day -4, and gradually decreased with the culture time. Taken these results together, we concluded that endogenous BMP activity is higher in preadipocytes and is decreased during adipocyte differentiation.

To explore ligand(s) responsible for higher BMP activity in preadipocytes, expression of Bmp-4 and Bmp-5 was examined by qRT-PCR (Fig. 1C and D). Gene transcript levels of Bmp-4 were significantly higher in preadipocytes, and were decreased after day 1 (Fig. 1C). By contrast, expression of Bmp-5 was transiently higher on day 0 (Fig. 1D). Neither Bmp-2 nor Bmp-7 was significantly expressed (data not shown). In view of parallel changes in gene transcript of Bmp-4 to phosphorylated Smad1/5/8 and Id1 expression, Bmp-4 expression may be a determinant for endogenous BMP activity during adipocyte differentiation.

Dorsomorphin is an inhibitor of the BMP pathway, which was identified in screening compounds disrupting dorsoventral axis formation governed by BMP in zebrafish [23]. Although dorsomorphin blocked BMP-induced Smad1/5/8 phosphorylation [20], it is not known whether dorsomorphin affects the transcriptional activation mediated by structurally related TGF-β and activin. Thus, the effects of dorsomorphin on pathway-specific gene transcription were evaluated by reporter assays in B16 cells, which are highly
sensitive to the TGF-β family [16]. We used BRE-luc as a BMP-responsive reporter gene [12], and CAGA-luc [6] as a TGF-β/activin-responsive reporter genes. Dorsomorphin treatment unexpectedly inhibited transcriptional activation induced by the expression of constitutively active ALK6 in a dose-dependent manner when BRE-luc was used (Fig. 2A). By contrast, constitutively active ALK4 or ALK5, type I receptor for activin or TGF-β, respectively, potentiated the transcriptional activation of CAGA-luc, but dorsomorphin hardly affected the transcription (Fig. 2B). The expression of constitutively active ALK6 also increased the expressions of both reporter genes, which may be related to the fact that B16 cells transmitted BMP and TGF-β/activin signals in response to TGF-β and BMP, respectively [16]. Constitutively active ALK6-mediated transcriptional activation was sensitive to dorsomorphin in a dose-dependent manner. These results suggest that dorsomorphin predominantly inhibits signaling mediated by BMP receptors but not TGF-β/activin receptors.

To examine the role of endogenous BMP activity in preadipocytes, dorsomorphin was administered for the indicated period (Fig. 3A), and lipid accumulation on day 8 was evaluated by Oil Red O staining. Since the degree of staining was proportional to the extent of adipocyte differentiation [17], the dye content was quantified. Treatment with dorsomorphin before treatment with differentiation inducers severely inhibited lipid accumulation, but treatment during mitotic clonal expansion minimally affected staining (Fig. 3B). These results suggest that increased BMP activity before stimulation of differentiation, i.e., both the proliferating period and postconfluent period, is required for adipogenic differentiation.

Dorsomorphin was originally isolated from the compound library as an inhibitor of AMPK [24]. Thus, dorsomorphin-induced decrease in lipid accumulation possibly resulted from the inhibition of AMPK activity. Changes in
Fig. 2. Dorsomorphin inhibits BMP receptor-mediated transcriptional activation but not TGF-β and activin receptors-mediated transcriptional activation. Effects of dorsomorphin on transcriptional activation mediated by activated receptors for the TGF-β family. B16 cells were transiently transfected with a reporter (BRE-luc (A) or CAGA-luc (B)), β-galactosidase, and constitutively active ALK4, ALK5 or ALK6. Luciferase activity was normalized to β-galactosidase activity and luciferase activity in cell lysates in the absence of dorsomorphin and exogenous for each reporter was set to 1. Data were expressed as the mean ± SE of triplicates from a representative experiment.

Fig. 3. Inhibition of BMP signaling prior to clonal expansion inhibits adipocyte differentiation. Dorsomorphin (5 μM) was added to the culture medium for the indicated period (A), and lipid accumulation on day 8 was examined by Oil Red O staining (B, upper panel). After staining, the dye was extracted in 2-propanol, and the amount was quantified by measuring absorbance at 510 nm. The absorbance of the extracted dye from cells treated with vehicle was set at 100 (B, lower panel). Data are expressed as the mean ± SE of quadruplicates. * and **: P<0.05 and P<0.001 versus control cells treated with vehicle, respectively.
the phosphorylation of Ser485 of AMPKα1, a site of auto-phosphorylation of AMPK α1, was constant after reaching confluence, implying that higher AMPK activity is not restricted to preadipocytes. The pattern of changes in AMPK activity is definitely different from that of BMP activity. Considering the effective inhibition of adipocyte differentiation by dorsomorphin treatment in proliferating 3T3-L1 cells, the dorsomorphin effect on adipocyte differentiation is suggested to result from the inhibition of BMP activity but not AMPK activity. This is also supported by a previous result that the activation of AMPK by treatment with AICAR, a synthetic activator of AMPK, for the entire differentiation period (day 0 to 8) inhibited adipocyte differentiation in 3T3-L1 preadipocytes [8]. Nevertheless, effects of dorsomorphin on AMPK signaling in preadipocytes and adipocytes should be clarified in future studies.

The present study indicates that endogenous BMP activity is required for adipocyte differentiation. Bower et al. showed the importance of endogenous BMP expression for the commitment to the preadipocytes lineage; A33 cell line, a subline of multipotential C3H10T1/2 cells, expressing BMP-4 differentiated into adipocytes without exogenous BMP, and the addition of noggin, an antagonist of BMP, to the culture medium throughout proliferation and postconflu-ence, blocked adipocyte differentiation [2]. The present results provide additional role of BMP for adipocyte differentiation; a factor necessary to maintain the preadipocytic property in preadipocytes.

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