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Research paper

Cilomilast enhances osteoblast differentiation of mesenchymal stem cells and bone formation induced by bone morphogenetic protein 2

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ABSTRACT

A rapid and efficient method to stimulate bone regeneration would be useful in orthopaedic stem cell therapies. Rolipram is an inhibitor of phosphodiesterase 4 (PDE4), which mediates cyclic adenosine monophosphate (cAMP) degradation. Systemic injection of rolipram enhances osteogenesis induced by bone morphogenetic protein 2 (BMP-2) in mice. However, there is little data on the precise mechanism, by which the PDE4 inhibitor regulates osteoblast gene expression. In this study, we investigated the combined ability of BMP-2 and cilomilast, a second-generation PDE4 inhibitor, to enhance the osteoblastic differentiation of mesenchymal stem cells (MSCs). The alkaline phosphatase (ALP) activity of MSCs treated with PDE4 inhibitor (cilomilast or rolipram), BMP-2, and/or H89 was compared with the ALP activity of MSCs differentiated only by osteogenic medium (OM). Moreover, expression of *Runx2*, *osterix*, and *osteocalcin* was quantified using real-time polymerase chain reaction (RT-PCR). It was found that cilomilast enhances the osteoblastic differentiation of MSCs equally well as rolipram in primary cultured MSCs. Moreover, according to the H89 inhibition experiments, Smad pathway was found to be an important signal transduction pathway in mediating the osteogenic effect of BMP-2, and this effect is intensified by an increase in cAMP levels induced by PDE4 inhibitor.

1. Introduction

Bone regeneration is one of the most important issues in regenerative medicine. Since the effect of drugs that inhibit bone resorption is not satisfactory, the development of bone anabolic molecules is necessary in patients who have suffered substantial bone loss. Therefore, the enhancement of bone formation is a technology of utmost importance in scaffold-based tissue engineering.

Bone morphogenetic proteins (BMPs) play critical roles in osteoblast differentiation of mesenchymal stem cells (MSCs). In numerous clinical studies, BMP-2 has been used to stimulate the differentiation of osteoprogenitor cells and has been employed as an effective alternative to autogenous bone graft [1–4]. In fact, BMP-2 controls the expression and function of *Runx2* through Smad signalling [5], which directs multipotent MSCs to an osteoblastic lineage and inhibits them from differentiating into adipocytic and chondrocytic lineages. Although several studies have reported that the possibility to use agents capable of increasing the effect of BMPs, the mechanisms on these effects are uncertain [6–9]. In different experimental osteopaenia models, therapeutic effects were achieved by using specific or nonspecific phosphodiesterase (PDE) inhibitors [10,11]. Sugama et al. [12] studied the combinative effect of PeTx, a nonspecific PDE inhibitor, and BMP-4 on ST2 cells, and suggested that cyclic adenosine monophosphate (cAMP) may be involved in the intensification of the BMPs signalling. However, Rawadi et al. [13] showed that the osteoblast differentiation promoted by PeTx is protein kinase A (PKA) independent. These contrasting observations may be resulted from the different model cell features used in these works [12,13]. Hence, we investigated the significant role in the rapid mineralization of the combination of BMP-2 and a specific PDE4 inhibitor using primary cultured MSCs. We hypothesised that a specific PDE4 inhibitor might increase the osteogenetic effect of BMP-2, in a dose-dependent manner, through the enhancement of the cAMP levels. Thus, we tested the combined effect of BMP-2 and two different PDE4 inhibitors (the first-generation PDE4 inhibitor rolipram or the second-generation PDE4 inhibitor cilomilast) on primary MSC differentiation, with or without H89, a PKA inhibitor. Moreover, by evaluating alkaline phosphatise (ALP) activity levels and *Runx2*, *osterix* (*Osx*), and

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osteocalcin (*OCN)* gene expression, we investigated the differentiation mechanism underlying that the key factor in the enanhced bone formation of primary MSCs, is PKA. In particular, our study showed that only the combination of BMP-2 and a specific PDE4 inhibitor activates both the Smad and cAMP pathways, and thereby enhances the mineralization.

2. Materials and methods

2.1. Reagents

Standard medium (SM) consisted of alpha minimal essential medium (α-MEM) supplemented with antibiotics/antimycotics (50 U/mL and 50 mg/mL, respectively; Gibco-Invitrogen, Carlsbad, CA, USA) and 15% foetal bovine serum (FBS). Osteogenic medium (OM) consisted of standard medium supplemented with 50 μM ascorbate, 1 μM dexamethasone, 2 mM glutamine, and 10 mM β-glycerophosphate. Recombinant human BMP-2 (1 mg/300 μL in acetic acid; Creative BioMart, Shirley, NY, USA) was stored at −20 °C. Standard medium containing 30 or 300 ng/mL BMP-2 was freshly prepared. Cilomilast (Ariflo®/SB-207499, S1455; Selleckchem, Houston, TX, USA) and rolipram (R6520; Sigma-Aldrich, Japan) were dissolved in 1 mL of dimethylsulphoxide (DMSO) to obtain 4 mM solutions. Subsequently, the aliquots of the solutions were added to standard medium, with or without BMP-2, for a final concentration of 10 μM or 40 μM.

2.2. Preparation of bone marrow cells and cell cultures

Rat stromal cells were isolated according to the protocol of Kopen et al. [14]. Bone marrow was obtained from the tibias and femurs of male Wistar rats (4 weeks old) by flushing femurs and tibias with α-MEM medium and penicillin/streptomycin (50 U/mL and 50 mg/mL, respectively; Invitrogen) supplemented with heparin. Cells were then washed in medium without heparin, centrifuged, and plated in a dish coated with fibronectin. One day later, non-adherent cells were removed by 3 washes with PBS. Adherent cells were further cultured in standard medium for 3 days. MSCs were grown in SM at 37 °C in a 5% CO_2 atmosphere. The medium was change twice a week, and a subculture was performed every week. At passage 3, the cells were trypsinised and plated at a density of 38,000/cm^2 for use in experiments. After 3 days, medium, with and without reagents, was added. The treatment was performed for 11 days (or 21 days for Alizarin Red staining), changing the medium every 3 days.

2.3. RNA preparation and RT-PCR

Total RNA was isolated from cultured cells in the presence of PDE4 inhibitors, BMP-2, standard and osteogenic medium by using QuickGene-Mini80 (Fujifilm Co., Tokyo, Japan), according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed using the SuperScript™ III CellsDirect cDNA Synthesis system (Invitrogen). The reaction program was 10 min at 25 °C, 2 h at 37 °C, and 5 s at 85 °C. Aliquots of the cDNA were subjected to PCR and amplified in a 20-μL reaction mixture using SYBR® Green Real-time PCR Master Mix (Toyobo Co., Tokyo, Japan). Amplifications were performed in StepOne Plus (Applied Biosystems, Foster, CA, USA) with an initial denaturation step at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing for 60 s at the specified temperature, and extension at 72 °C for 60 s. The PCR primer sequences and annealing temperatures are presented in Table 1.

Table 1
Primers used in this study.

	Primer sequence (5′–3′)	Annealing temperature (°C)
Runx2	F: CAGTTCCCAGGCATTTCATC	60
	R: CAGCGTCAACACCATCATTC	
Osterix (*Osx*)	F: CACTGGCTCCTGGTTCTCTC	60
	R: CCACTCCTCCTCTTCGTGAG	
Osteocalcin (*OCN*)	F: TCTCTGCTCACTCTGCTGGC	57
	R: TCCAGGGCAACACATGCCCTA	

2.4. Assay for alkaline phosphatase activity

Cell cultures were processed for ALP activity after 11 days of treatment with the reagents. Intracellular ALP activity was measured using the TRACP & ALP Assay Kit (MK301 colorimetric kit; Takara Bio Inc., Tokyo, Japan). Cells were washed 3 times with PBS, and then, lysed with 500 μL of lysis buffer containing physiological saline and 1% NP-40. Lysates were also prepared from cells grown in DMSO, standard and osteogenic medium as controls. The cells lysates were then mixed with an assay mixture containing *p*-nitrophenyl phosphate and incubated at 37 °C for 30 min, at which time the reaction was stopped by the addition of 0.4 M NaOH. After incubation, the amount of *p*-nitrophenol released by the reaction was measured with a spectrophotometer at 405 nm. All values were normalised against cell number. Protein in the cell lysates was determined using the micro Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA). Data were expressed as a ratio of ALP activity per milligram of protein.

2.5. Alizarin Red staining

Cells were washed twice with PBS and fixed in 10% formalin for 10 min. The cells were then stained with 1% Alizarin Red solution for 10 min at 37 °C and washed 3 times with PBS.

2.6. Statistical analysis

Data are expressed as the mean (SD) for each group. Statistical differences among treatment groups were evaluated by Student's *t*-test. A p value of <0.05 was considered significant.

3. Results and discussion

Since osteoblasts can be differentiated from pluripotent MSCs, a bone marrow stromal progenitor cells, in our study, we modelled this process of bone formation using primary MSCs. In fact, the osteogenic induction of MSCs by BMPs has been extensively studied. BMP-2 binds to a heterotetrameric complex of type I and type II transmembrane serine/threonine kinase receptor proteins. Upon activation by BMP-2, these receptors phosphorylate the intracellular signalling molecule receptor-regulated Smad (R-Smad). After activation, R-Smad associates with Smad4 (C-Smad), and the R-Smad/C-Smad complex accumulates in the nucleus where it interacts with the transcription factor *Runx2* and upregulates osteoblastic gene expression (Fig. 1). This differentiation process can be divided into 3 stages (proliferation, matrix development and, maturation and mineralisation), which are characterized by several markers. The markers most frequently used are ALP, *Runx2*, *Osx*, and *OCN*. In general, ALP, an early marker of the osteoblastic phenotype, is upregulated at the early stages and decreases as the cell differentiation progresses, while *OCN* is considered an advanced marker for maturation and a link to bone mineralisation. Therefore, since pre-osteoblasts are histologically similar to osteoblasts, but do not acquire all the characteristics of