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Biochemical and Biophysical Research Communications 338 (2005) 872–879
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Mechanism of osteogenic induction by FK506 via BMP/Smad pathways

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Received 1 October 2005
Available online 14 October 2005

Abstract

FK506 is an immunosuppressant that exerts effects by binding to FK506-binding protein 12 (FKBP12). Recently, FK506 has also been reported to promote osteogenic differentiation when administered locally or in vitro in combination with bone morphogenetic proteins (BMPs), although the underlying mechanism remains unclarified. The present study initially showed that FK506 alone at a higher concentration (1 μM) induced osteogenic differentiation of mesenchymal cell lines, which was suppressed by adenoviral introduction of Smad6. FK506 rapidly activates the BMP-dependent Smads in the absence of BMPs, and the activation was blocked by Smad6. Overexpression of FKBP12, which was reported to block the ligand-independent activation of BMP type I receptor A (BMPRIA), suppressed Smad signaling induced by FK506, but not that induced by BMP2. BMPRIA and FKBP12 bound to each other, and this binding was suppressed by FK506. These data suggest that FK506 promotes osteogenic differentiation by activating BMP receptors through interacting with FKBP12.

Keywords: FK506; BMP; Smad; FKBP12; Osteogenic differentiation; Osteoblast

FK506 is an immunosuppressive agent with an increasing number of clinical applications [1–4]. FK506 exerts its immunosuppressive effects by binding to the FK506-binding protein 12 (FKBP12) [5]. The complex of FK506 and FKBP12 inactivates calcineurin, resulting in the inhibition of the cytokine expression including interleukin-2, interleukin-3, and γ-interferon in T cells and the consequent immunosuppression [6]. In addition to its immunosuppressive activity, FK506 has been shown to exert a variety of actions on bone metabolism. When administered systemically, FK506 causes osteopenia in mice, rats, and humans [7–11]. When administered locally or in vitro in combination with bone morphogenetic proteins (BMPs), FK506 promotes osteogenic differentiation [12–14].

BMPs are members of secreted signaling proteins that belong to the transforming growth factor-β (TGF-β) superfamily. BMPs were originally identified as molecules that induced ectopic bone formation when implanted into the rodent muscle [15,16]. In accordance with such in vivo effects, the BMPs have been shown to regulate osteogenic differentiation in vitro [17]. They bind to a characteristic pair of transmembrane serine/threonine kinase receptors, BMP type I and type II receptors (BMPRI and BMPRII). They first bind to the BMPRII, which phosphorylates the GS region of the BMPRI [18]. The activated BMPRI subsequently recruits and phosphorylates Smad1, Smad5, and Smad8 (the BMP-dependent Smads) through the GS region. The BMP-dependent Smads then physically associate with Smad4, translocate into the nucleus, and activate the target genes. Smad6 blocks BMP signaling by inhibiting the phosphorylation of the BMP-dependent Smads by the BMPRI. FKBP12 has been reported to block the ligand-independent activation of the BMPRI [19], but whether FKBP12 mediates the interactions of BMP signaling and FK506 remains unknown. The current study investigated

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doi:10.1016/j.bbrc.2005.10.024

the regulation and the mechanism of action of FK506 on osteogenic differentiation of mesenchymal cell lines using the in vitro culture systems.

Materials and methods

In vitro osteogenic differentiation assay. MLB13MYC clone 17 (C17), a limb bud-derived cell line, which differentiates into osteoblasts upon treatment with BMP signaling [20], was a generous gift from V. Rosen (Harvard University, MA). C2C12, a mouse myoblastic cell line, which differentiates into osteoblasts upon treatment with BMP signaling [21], was obtained from the Riken Cell Bank (Tsukuba, Japan). These cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS) (Sigma–Aldrich) and 1% penicillin/streptomycin (Sigma–Aldrich). For the alkaline phosphatase (ALP) staining, the cells were fixed in 70% ETOH and stained for 10 min with a solution containing 0.01% naphthol AS-MX phosphate disodium salt (Sigma–Aldrich), 1% *N*,*N*-dimethylformamide (Wako Pure Chemicals Industry Tokyo), and 0.06% fast blue BB (Sigma–Aldrich). FK506, cyclosporin A (CsA), recombinant human BMP2 (rhBMP2), and Noggin were purchased from Sigma–Aldrich.

Real-time RT-PCR. The total RNA was extracted using an ISOGEN Kit (Wako) and an RNeasy Mini Kit (QIAGEN, Hilden, Germany), and treated with DNaseI (QIAGEN), according to the manufacturer's instructions. One microgram of RNA was reverse-transcribed using a Takara RNA PCR Kit (AMV) ver.2.1 (Takara, Shiga, Japan) to generate the single-stranded cDNA. PCR was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster-city, CA). Each PCR consisted of 1XQuantiTect SYBR Green PCR Master Mix (QIAGEN), 0.3 μM specific primers, and 500 ng DNA. The mRNA copy number of a specific gene in the total RNA was calculated using a standard curve generated with serially diluted plasmids containing PCR amplicon sequences and normalized to the human or rodent total RNA (Applied Biosystems) with mouse actin as the internal control. The standard plasmids were synthesized using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instruction. All reactions were run in triplicate. The primer sequences are available upon request.

Preparation of adenoviruses and plasmids. Adenoviruses expressing LacZ and Smad6 were generous gifts from K. Miyazono (The University of Tokyo, Tokyo, Japan). Adenoviruses were amplified in HEK293 cells and purified using an AdenoX Virus Purification Kit (Clontech, Palo Alto, CA). Viral titers were determined by the end-point dilution assay. Plasmids expressing the HA-tagged BMP type I receptor A (HA-BMPRIA) and Flag-tagged FKBP12 (Flag-FKBP12), and the luciferase reporter construct responding to BMP-dependent Smad signaling (12xGCCG-luc) were generous gifts from K. Miyazono.

Luciferase assay. The human hepatoma cell line HuH-7 was obtained from the Riken Cell Bank. HuH7 cells were plated onto 24-well plates and then transfected with 0.1 μg of the reporter plasmid construct alone or in combination with the plasmid expressing HA-BMPRIA and Flag-FKBP12 for 1 day, then treated with FK506 (0.1 or 1 μM) or rhBMP2 (200 ng/ml) and cultured for 2 days. The luciferase assay was performed 48 h after transfection using a PicaGene Dual SeaPansy Luminescence Kit (Toyo Ink, Tokyo, Japan) and the Lumat LB 9507 (Berthold Technologies GmbH, KG, Wildbad, Germany). The level of luciferase activity was normalized to the level of the *Renilla* luciferase activity. All data were expressed as means ± SE ($n = 6$).

Immunoblot and immunoprecipitation assay. The cells were washed twice with ice-cold PBS, and the proteins were extracted using an M-PER Kit (Pierce Chemical, Rockford, IL) according to the manufacturer's instructions. The protein concentrations of the cell lysates were measured using a Protein Assay Kit II (Bio-Rad, Hercules, CA). For the immunoblot analysis, the lysates were fractionated by SDS–PAGE with 4–20% Tris–glycine gradient gel or 18% Tris–glycine gel (Invitrogen) and transferred onto nitrocellulose membranes (Bio-Rad). After being blocked with 6% milk/TBS-T, the membranes were incubated with the anti-Smad1 mouse monoclonal antibody (1:1000; Cell Signaling Technology, Beverly MA), anti-phospho-Smad 1/5/8 (1:1000; Cell Signaling Technology), anti-HA mouse monoclonal antibody (1:1000; Santa Cruz Biolaboratories, Santa Cruz, CA), or anti-Flag rabbit antibody (1:1000; Sigma–Aldrich). The secondary antibodies, i.e., HRP-conjugated goat anti-mouse IgG (Promega, Madison, WI) and goat anti-rabbit IgG (Promega), were used at dilutions of 1:10,000. The immunoreactive bands were visualized using an ECL Plus Kit (Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

For immunoprecipitation, 293 cell extracts were incubated with 5 μg of anti-HA and anti-Flag antibodies at 4 °C overnight. The immune complexes were recovered with protein G–Sepharose (Sephadex G-50 Fine; Amersham Life Sciences), subjected to SDS–PAGE, and then transferred onto nitrocellulose membranes. Immunoblotting was performed as already described.

Statistical analysis. Means of groups were compared by ANOVA, and the significance of differences was determined by post hoc testing using Bonferroni's method.

Results

Induction of osteogenic differentiation by FK506

To clarify the effect of FK506 on osteogenic differentiation, we treated the limb bud-derived cell line C17 with FK506 at concentrations ranging from 0.01 to 10 μM. This cell line has been shown to rapidly undergo osteogenic differentiation upon treatment with BMP signaling [20]. Consistent with the report by Tang et al. [12], the treatment with FK506 at 0.1 μM for 3 days induced the ALP activity determined by the ALP staining, but not the osteocalcin mRNA expression determined by the real-time RT-PCR analysis (Figs. 1A and B). When we treated the cells with FK506 at 1 μM, however, both the ALP activity and osteocalcin expression were induced. When the cells were treated with FK506 at 10 μM, neither the ALP activity nor osteocalcin expression was induced (data not shown), probably due to the toxic effect of the drug. In contrast, CsA, an immunosuppressive drug, which also inhibits calcineurin, did not increase the ALP activity or osteocalcin expression at the concentrations tested (Figs. 1A and B). The induction of the ALP activity and osteocalcin expression by FK506 (1 μM) was also seen in C2C12 cells (Figs. 1C and D). These data suggest that FK506 alone is able to induce osteogenic differentiation and that the effect of FK506 on osteogenic differentiation is not dependent on calcineurin signaling.

Interactions of FK506 and BMP signaling during osteogenic differentiation

Since FK506 has been reported to enhance BMP signaling [12], we investigated the interactions of FK506 and BMP signaling. We treated C17 cells with rhBMP2 at various concentrations in the presence or absence of FK506 (1 μM). The ALP activity induced by rhBMP2 was dose-dependent and reached the maximal intensity at 500 ng/ml (Fig. 2A). The ALP activity induced by FK506 alone was more intense than that induced by rhBMP2 at