Dextromethorphan inhibits osteoclast differentiation by modulating RANKL signalling
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INTRODUCTION:
Osteoclasts are bone-resorptive multinucleated cells derived from hematopoietic stem cells. Differentiation of osteoclasts is regulated by multiple molecules within the bone microenvironment. Among these factors, receptor activator of nuclear factor-κB ligand (RANKL) plays an essential role in osteoclastogenesis. Dextromethorphan (DMX) is the d-isomer of the codeine analog levorphanol, a dextrorotatory morphinan. DXM is widely used as a cough-suppressant in cold and cough. In recent studies, dextromethorphan showed to have anti-inflammatory effect both in vivo and in vitro. In this study, we studied the effect and role of DXM in osteoclastogenesis using two in vitro models, the murine myelomonocytic RAW264.7 cell line and mouse bone marrow cells, both of which differentiate into osteoclasts in the presence of macrophage colon-stimulating factor (M-CSF) and RANK ligand (RANKL).

METHODS:
Cell cultures
Cells were cultured in α-MEM supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 g/ml streptomycin (pH 7.6). Primary osteoclasts were derived from bone marrow cells in femurs of 8-10 week-old (300-350 g) male Wistar rats. Bone marrow cells were harvested by flushing the bone marrow cavity with MEM and DMEM. Cells were plated at a density of 10^6 per well (0.5 ml) with human recombinant soluble RANKL (50 ng/ml) and murine M-CSF (20 ng/ml) at 37°C in 5% CO2 in humidified air. After 6 days culture, the cells were stained with TRAP staining kit at 37°C for 1 hour in the dark. Cells were then washed with distilled water and air dried for photography and counting. TRAP positive cells with more than 3 nuclei were defined as osteoclasts.
RAW264.7, was obtained from American Type Culture Collection (ATCC, Manassas, VA). RAW264.7 cells were plated at a concentration of 2000/well of a 48-well culture dish in the presence of 10 ng/ml RANKL. After 24 hours, different concentrations of DXM were added to the cultures to assess its effect on osteoclastogenesis. After 3 days culture, the cells were fixed and stained for TRAP using TRAP staining kit according to the manufacturer’s instructions.
Pit formation assay.
Pit formation was examined by using RANKL-stimulated RAW264.7 cells cultured on BioCoat OsteologicTM slide (BD,Biosciences) in the presence or absence of DXM. After 3 days culture, the slides were washed with 6% sodium hypochlorite solution to remove the cells. The resorbed areas on the slides were photographed with an Olympus camera, and were quantified by Metaview Image Analysis System. Assay of IκB-α phosphorylation, p65 translocation, and NFATc1 expression
Nuclear extracts (NE) and cytoplasmic extracts (CE) of the RAW264.7 cells were prepared with a NE-PER nuclear and cytoplasmic reagents (Pierce, Rockford). The levels of phospho-IκB-α and p65 in NE and CE were analyzed by Western blotting with specific antibodies (Santa Cruz Biotechnology). RAW264.7 cells were treated with RANKL (100 ng/ml) for 30 minutes, and then were added different concentrations of DXM for 24 hours. Cell lysates were subjected to SDS-PAGE, and were analyzed by Western blotting with anti-NFATc1 antibody (Santa Cruz Biotechnology).

RESULTS:
In our in vivo study using both primary cell culture and RAW264.7, we have demonstrated dose-dependently inhibition of RANKL-induced osteoclast differentiation by DXM. DXM showed inhibition not only on the decrease of number of TRAP positive cell formation, but also of the total TRAP activity was reduced significantly. Furthermore, in our RAW cell model, the bone resorption activity was strongly inhibited by admistration of DXM. Using RT-PCR analysis with specific probes, we showed DXM decreases the RANKL signalling pathway through decreasing phosphorylation of IκB-α and nuclear translocation of p65. DXM inhibited RANKL-induced tumor necrosis factor α production and osteoclast differentiation. DXM also inhibits RANKL-induced osteoclastogenesis through down-regulation of NFATc1, an essential transcription factor of osteoclastogenesis. Collectively, our study demonstrates for the first time

Fig 1. (A) Inhibition of osteoclast differentiation in primary bone cell. Osteoclast precursors isolated from long bone of adult male rats were plated at a density of 10^6 per well (0.5 ml) with human recombinant soluble RANKL (50 ng/ml), murine M-CSF (20 ng/ml) and different concentration of DXM. TRAP-positive cells in each well were scored by counting the number of TRAP-positive multinucleated cells containing three or more nuclei. (B) DXM inhibits osteoclastogenesis in RAW264.7 cells. RAW264.7 cells were cultured treated with different concentration of DXM and RANKL (10 ng/ml) for 3 days. At the end, cells were fixed and stained for TRAP. TRAP activity was assayed and the optical density at 405 nm was determined by spectrophotometry. (C) Osteoclast-like TRAP-positive cells in each well were scored by counting the number of TRAP-positive multinucleated cells (MNC) containing three or more nuclei.

Fig 2. DXM inhibits RANKL-induced NF-κB activation in RAW 264.7 cells. RAW 264.7 cells were incubated with DXM (200 μM) for 1 hour, treated with RANKL (100 ng/ml) for 30 minutes, and then lysed. Nuclear extracts (NE) and cytoplasmic extracts (CE) were prepared using a commercial kit. (A) p-IκB-α in CE was detected by Western blot assay. (B) P65 in NE was detected by Western blot assay. (C) The cells lysates were fractionated by 10% polyacrylamide gel electrophoresis and immunoblotted with anti-NFATc1 and anti-b-actin

DISCUSSION:
DXM showed inhibition on osteoclastogenesis in both primary cell culture model and cell line model. Furthermore, in our RAW cell model, the bone resorption activity was strongly inhibited by admistration of DXM. These data showed DXM not only inhibits osteoclast differentiation but also inhibits osteoclast’s activation as well. NF-κB signalling pathway an essential and important pathway for RANKL induces osteoclast differentiation. In our study, we have demonstrated DXM inhibits osteoclast differentiation by affecting the NF-κB signalling pathway.