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Hypoxia Inducible Factor-1α Directly Induces the Expression of Receptor Activator of Nuclear Factor-κB Ligand in Chondrocytes

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Receptor activator of nuclear factor-κB ligand (RANKL) is an osteoblast/stromal cell-derived essential factor for osteoclastogenesis. During endochondral bone formation, hypertrophic chondrocytes calcify cartilage matrix that is subsequently resorbed by osteoclasts in order to be replaced by new bone. Hypoxia-induced upregulation of RANKL expression has been previously demonstrated in an *in vitro* system using osteoblasts; however, the involved mechanism remains unclear in chondrocytes. In the present study, we investigated whether hypoxia regulates RANKL expression in ATDC5 cells, a murine chondrogenic cell line, and hypoxia-inducible factor-1α (HIF-1α) mediates hypoxia-induced RANKL expression by transactivating the RANKL promoter. The expression levels of RANKL mRNA and protein, as well as HIF-1α protein, were significantly increased in ATDC5 cells under hypoxic condition. Constitutively active HIF-1α alone significantly increased the levels of RANKL expression under normoxic conditions, whereas dominant negative HIF-1α reduced hypoxia-induced RANKL expression. HIF-1α increased RANKL promoter reporter activity in a HIF-1α binding element-dependent manner in ATDC5 cells. Hypoxia-induced RANKL levels were much higher in differentiated ATDC5 cells, as compared to proliferating ATDC5 cells. These results suggested that under hypoxic conditions, HIF-1α mediates induction of RANKL expression in chondrocytes; in addition, hypoxia plays a role in osteoclastogenesis during endochondral bone formation, at least in part, through the induction of RANKL expression in hypertrophic chondrocytes.

Key words: Hypoxia, HIF-1α protein, RANK ligand, Chondrocytes

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Introduction

During the development of the skeletal system, the long bones are formed by endochondral ossification, a process in which cartilage is present first then replaced subsequently by bone. In the centers of cartilage templates, the proliferating chondrocytes stop dividing and increase their volume dramatically, and differentiate into hypertrophic chondrocytes. The hypertrophic chondrocytes express cartilaginous matrix, which becomes mineralized and subsequently vascularized to form a medullary cavity. Then the calcified cartilage is partly replaced by bone through the chondroclastic/osteoclastic resorption and osteoblastic bone formation [1, 2].

Osteoblasts/stromal cells regulate osteoclastogenesis by providing a crucial signal for osteoclast recruitment and differentiation through receptor activator of nuclear factor-κB ligand (RANKL). In the presence of macrophage-colony stimulating factor, RANKL controls the differentiation, maturation and activation of osteoclasts via binding to its receptor RANK on osteoclast precursors and osteoclasts [3-5]. RANKL is mainly expressed in bone lining cells, osteoblasts, osteocytes, synovial fibroblasts, and T and B lymphocytes [1, 6-9]. The expression of RANKL by hypertrophic chondrocytes in the growth plate during embryonic development has been also reported [10]. By employing chondrocyte-specific vitamin D receptor knockout mice [11], it has been also demonstrated that vitamin D_3 stimulates RANKL expression in chondrocytes, promoting osteoclast formation. However, the regulatory mechanisms of RANKL expression and subsequent osteoclastogenesis by hypertrophic chondrocytes have not been fully studied.

Reduced availability of oxygen, i.e. hypoxia, activates a transcriptional response that has an important role in various physiological and pathological conditions. A reduction in pericellular oxygen tension within bone occurs during bone development [12], disuse [13] and fracture [14]. Cartilage tissue is avascular in nature. The microenvironment of chondrocytes is hypoxic with oxygen tension as low as 1%. Highly adaptive mechanisms exist for deeply embedded chondrocytes to live in hypoxic environment, i.e. to control genes regulating energy metabolism, glucose metabolism and pH, and allow anaerobic energy generation [15, 16]. The most important component of this hypoxic response is mediated by the transcription factor hypoxia-inducible factor-1 (HIF-1) [17]. Hypoxic signaling in skeleton initiates a series of cellular events that induce bone loss. Hypoxia-enhanced osteoclastogenesis via increased RANKL expression has been well demonstrated in *in vitro* system using osteoblasts and periodontal ligament fibroblasts [18, 19]. We previously demonstrated that hypoxia induces RANKL expression in human periodontal ligament fibroblasts and that HIF-1α mediates hypoxia-induced RANKL expression by directly binding to the RANKL promoter [19]. However, the molecular mechanisms how chondrocytes in hypoxic environment regulate osteoclastogenesis have remained unclear. In the present study, we investigated whether and how hypoxia regulates RANKL expression in chondrocytes. Here we present that hypoxia induces RANKL expression and that HIF-1α mediates hypoxia-induced RANKL expression in ATDC5 murine chondrogenic cell line.

Materials and Methods

Reagents and antibodies

A GasPak EZ CO_2 Pouch System was purchased from BD (San Jose, CA, USA). The PCR primers were synthesized by CosmoGenetech (Seoul, Korea). HIF-1α antibody was purchased from NOVUS (Littleton, CO, USA), RANKL antibody was from R&D Systems (Minneapolis, MN, USA), actin antibody was from Santa Cruz Biotechnology (Dallas, TX, USA) and HRP-conjugated secondary antibodies were from Thermo Fisher Scientific (Waltham, MA, USA). LipofectAMINE 2000 reagent was purchased from Invitrogen (Waltham, MA, USA).

Cell culture and hypoxic treatment

ATDC5 cells, a murine chondrogenic cell line, were maintained in growth medium (Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture (Invitrogen) supplemented with 5% fetal bovine serum (Hyclone, Logan, UT, USA)). Chondrocytic differentiation of ATDC5 cells were induced by incubating in differentiation medium (growth medium supplemented with 10 μg/ml insulin (Sigma, St. Louis, MO, USA), 5.5 ng/ml transferrin (Sigma), 5 ng/ml sodium selenite (Sigma) and 10 ng/ml bone morphogenetic protein 2 (R&D Systems)).

To induce hypoxia, the ATDC5 cells were transferred to a GasPak pouch, where the total oxygen concentration was reduced to less than 1% [20] or incubated for 24 h in the presence of desferoxamine (DFO, 200 μM) (Sigma), a high affinity iron chelator [21].

Reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis

Semi-quantitative RT-PCR or quantitative real-time PCR was performed to evaluate mRNA expression levels as previously described [19]. The primer sequences used for semi-quantitatitve RT-PCR were as follows: aggrecan-f 5′ -CGA GAA TGA CAC CTG CTA GG-3′, aggrecan-r 5′-AAG AAG ACA GGA CCA GGA AGG-3′; type II collagen (Col II)-f 5′-AAG ATG GTC CCA AAG GTG CTC G-3′, Col II-r 5′-AGC TTC TCC TCT GTC TCC TTG C-3′; type X collagen (Col X)-f 5′-CCA CCT GGG TTA GAT GGA AAA-3′, Col X-r 5′-AAT CTC ATC AAA TGG GAT GGG-3′; RANKL-f 5′-CAG GTT TGC AGG ACT CGA-3′,

RANKL-r 5′-AGC AGG GAA GGG TTG GAC A-3′; alkaline phosphatase (ALP)-f 5′-AGG CAG GAT TGA CCA CGG-3′, ALP-r 5′-TGT AGT TCT GCT CAT GGA-3′; Runx2-f 5′-CCG CAC GAC AAC CGC ACC AT-3′, Runx2-r 5′-CGC TCC GGC CCA CAA ATC TC-3′; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-f 5′-TCA CCA TCT TCC AGG AGC G-3′, GAPDH-r 5′-CTG CTT ACC ACC TTC TTG A-3′.

The primer sequences used for the real time-PCR were as follows: RANKL-f 5′-CAG AAG ATG GCA CTC ACT GCA-3′, RANKL-r 5′-CAC CAT CGC TTT CTC TGC TCT-3′; vascular endothelial growth factor (VEGF)-f 5′-TTA CTG CTG TAC CTC CAC C-3′, VEGF-r 5′-ACA GGA CGG CTT GAA GAT G-3′; HIF-1α-f 5′-GGT TCC AGC AGA CCC AGT TA-3′, HIF-1α-r 5′-AGG CTC CTT GGA TGA GCT TT-3′; and GAPDH-f 5′-CCA TCT TCC AGG AGC GAG ATC-3′, GAPDH-r 5′-GCC TTC TCC ATG GTG GTG AA-3′.

To examine the expression levels of protein, ATDC5 cells were scraped into lysis buffer (10 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM sodium fluoride, 0.2 mM sodium orthovanadate, 1 mM PMSF, 1 μg/ml aprotinin, 1 μM leupeptin and 1 μM pepstatin) and sonicated briefly. Equal amounts of protein were subjected to 10% SDS-PAGE and subsequently electro-transferred onto a PVDF membrane. For HIF-1α detection, confluent cells were lysed in 2× Laemmli sample buffer, and the proteins were separated using a 6% SDS-PAGE gel. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 and incubated with the relevant primary antibody, followed by incubation with the corresponding HRP-conjugated secondary antibody. Immune complexes were visualized using WEST-ZOL (plus) reagent and luminescence was detected with a LAS1000 (Fuji PhotoFilm; Japan) [19].

Plasmid constructs

The constitutively active and dominant negative HIF-1α expression plasmids were generous gifts from Prof. J.W. Park at Seoul National University [22, 23]. The reporter constructs containing the mouse RANKL promoter (RANKL-WT-luc, RANKL-MT-luc) were prepared as previously described [19].

Transient transfection and reporter assay

ATDC5 cells were transfected with the indicated vectors using LipofectAMINE 2000 reagent in accordance with the manufacturer's instructions. In each transfection, 0.2 μg of expression vectors (pcDNA, constitutively active or dominant negative HIF-1α), 0.2 μg of reporters (pGL3, RANKL-WT-luc or RANKL-MT-luc) and 0.1 μg of *Renilla* luciferase plasmid were used as indicated. After 24 h, the cells were harvested, and luciferase activity was measured using the Dual-Glo luciferase assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The relative luciferase activity was calculated after normalizing the transfection efficiency by *Renilla* luciferase activity.

Statistical analysis

The data from the real-time PCR and reporter assays were expressed as the mean ± S.D. The statistical significance was analyzed by Student's *t*-test. A p value less than 0.05 was considered statistically significant.

Results and Discussion

Hypoxia increases the levels of RANKL mRNA and protein in ATDC5 cells

We first examined whether hypoxia regulates the expression levels of RANKL in proliferating and differentiated chondrocytes. To induce chondrogenic differentiation, ATDC5 cells were cultured in differentiation medium for 11 days. Both proliferating (0 D) and differentiated (11 D) cells were incubated for an additional 24 h in normoxic or hypoxic condition. Then, mRNA levels of chondrocytes marker genes and RANKL were examined by RT-PCR. As expected, expression levels of type II and type X collagen, Runx2 and ALP were increased by chondrogenic differentiation stimuli (Fig. 1A). Hypoxia for 24 h further enhanced expression levels of Runx2, ALP and aggrecan in differentiated chondrocytes. Considering that chondrogenic differentiation of mesenchymal stem cells is further enhanced under the hypoxic conditions compared to that under normoxic conditions and that expression of Runx2 and ALP are induced in hypertrophic chondrocytes [24], our data suggest that hypoxia further enhance chondrogenic differentiation of ATDC5 cells toward hypertrophic chondrocytes. Conventional RT-PCR data showed that RANKL mRNA was barely detectable in both proliferating and differentiated ATDC5 cells under the normoxic conditions, but hypoxia for 24 h clearly induced RANKL mRNA expression

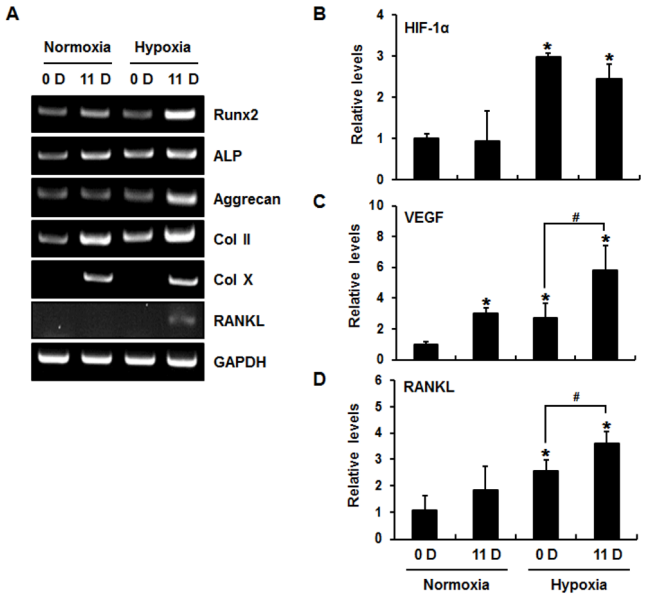

Fig. 1. Hypoxia enhanced the expression levels of RANKL in proliferating and differentiated ATDC5 cells. To induce chondrogenic differentiation, ATDC5 cells were cultured in differentiation medium for 11 days. Both proliferating (0 D) and differentiated (11 D) cells were incubated for an additional 24 h under the normoxic or hypoxic conditions. Then, the mRNA levels of chondrocyte differentiation marker genes and RANKL were evaluated by conventional RT-PCR (A) or quantitative real time-PCR analyses (B-D). Quantitative data were presented as the mean ± S.D. of three independent experiments (*p<0.05, compared to 0 D cells under the normoxic conditions; #p<0.05, for the indicated comparison).

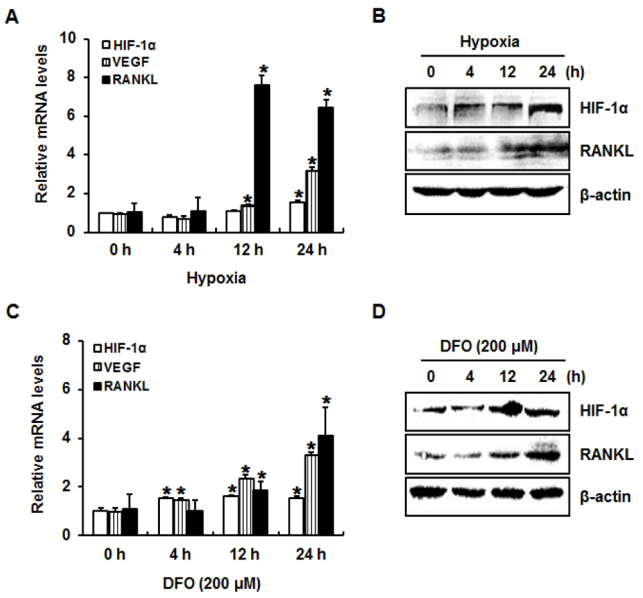

Fig. 2. Stabilization of HIF-1α protein by hypoxic culture conditions or by desferoxamine treatment increased expression levels of RANKL mRNA and protein in a time-dependent manner. ATDC5 cells were incubated in the GasPak pouches (A, B) or in the presence of desferoxamine (DFO, 200 μM) for the indicated periods (C, D). Real time-PCR (A, C) and western blot analysis (B, D) were then performed. Quantitative data were presented as the mean ± S.D. of three independent experiments (*p<0.05, compared to 0 h).

in differentiated chondrocytes (Fig. 1A). Quantitative real time-PCR data demonstrated that hypoxia increased mRNA expression levels of HIF-1α both in proliferating and differentiated chondrocytes (Fig. 1B). In addition, hypoxia increased RANKL mRNA expression in both proliferating and differentiated chondrocytes but RANKL mRNA levels were significantly higher in differentiated cells than in proliferating cells (Fig. 1D). These results suggest that hypoxia enhanced RANKL expression in chondrocytes and that hypoxia-induced RANKL response was much higher in differentiated cells than in proliferating cells.

VEGF, a representative target gene of HIF-1α is the primary mediator of angiogenesis [25] and is known to stimulate osteoclastogenesis [26]. Thus, we also examined VEGF mRNA levels in hypoxic conditions. Hypoxia or differentiation stimuli increased VEGF mRNA levels, and combination of hypoxia and differentiation stimuli further enhanced VEGF expression (Fig. 1C). During endochondral bone formation, Runx2 functions together with HIF-1α to stimulate expression of angiogenic genes including VEGF in bone cells [27]. In addition, Runx2 enhances VEGF expression and its angiogenic activity in human umbilical vein endothelial cells [28]. Considering these reports, increased levels of Runx2 may in part explain higher levels of VEGF mRNA in differentiated ATDC5 cells under hypoxic conditions in our study. Osteoblast-derived VEGF is known to stimulate osteoclasts formation and activation [29]. Given that hypoxia induces both VEGF and RANKL expressions in ATDC5 cells, it could be proposed that additive mechanism of VEGF and RANKL to stimulate osteoclastogenesis under hypoxic conditions exists.

We next examined time-dependent profile of hypoxia-induced VEGF and RANKL expression. Hypoxia-induced stabilization of HIF-1α protein was observed after a 4 h-hypoxia and further increased until 24 h (Fig. 2B). Increase in VEGF and RANKL levels was significant after 12 h- and 24 h-hypoxia (Fig. 2A, 2B). When the stabilization of HIF-1α protein was induced by treating cells with DFO, a prolyl hydroxylase inhibitor, similar induction of VEGF and RANKL expression was observed (Fig. 2C, 2D), further suggesting that hypoxia and subsequent accumulation of HIF-1α contribute to RANKL

induction in chondrocytes.

HIF−1α directly stimulates RANKL transcription

To clarify whether HIF-1α mediates hypoxia-induced RANKL expression in chondrocytes, constitutively active or dominantly negative HIF-1α were overexpressed in ATDC5 cells and RANKL expression levels were examined (Fig. 3). Overexpression of constitutively active HIF-1α increased expression levels of RANKL mRNA and protein in normoxic conditions, whereas overexpression of dominantly negative HIF-1α attenuated hypoxia-induced RANKL expression. These results imply that stabilized HIF-1α upregulates RANKL expression under hypoxic conditions in chondrocytes.

We next examined whether HIF-1α transactivates RANKL transcription in ATDC5 cells using the reporter constructs containing mouse RANKL promoter sequences; RANKL-WT-luc contains wild-type HIF-1α binding element but RANKL-MT-luc contains mutations in HIF-1α binding element sequence (CGTG→AAAA) [30]. Hypoxia induced by GasPak pouch system significantly increased RANKL-WT-luc reporter activity but not RANKL-MT-luc activity (Fig. 4). These data further support that hypoxia-induced HIF-1α directly transactivates the RANKL promoter in ATDC5 chondrocytes.

Previous reports have shown that hypoxia induces osteoclastogenesis via direct action on osteoclast precursor cells or via indirect action on osteoblasts and bone marrow

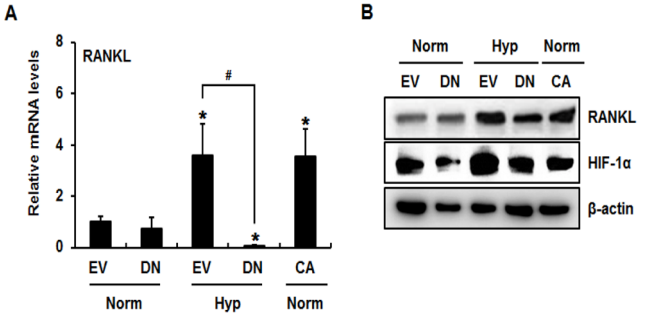

Fig. 3. Constitutively active HIF-1α increased RANKL expression under the normoxic conditions, whereas dominant negative HIF-1α suppressed hypoxia-induced RANKL expression. ATDC5 cells were transiently transfected with pcDNA (EV), constitutively active HIF-1α (CA) or dominant negative HIF-1α (DN) expression plasmids and then incubated for 24 h under the normoxic (Norm) or hypoxic (Hyp) conditions. Real time-PCR (A) and western blot analysis (B) were then performed. Quantitative data were presented as the mean ± S.D. of three independent experiments (*p<0.05, compared to EV-transfected cells under normoxic conditions; #p<0.05, for the indicated comparison).

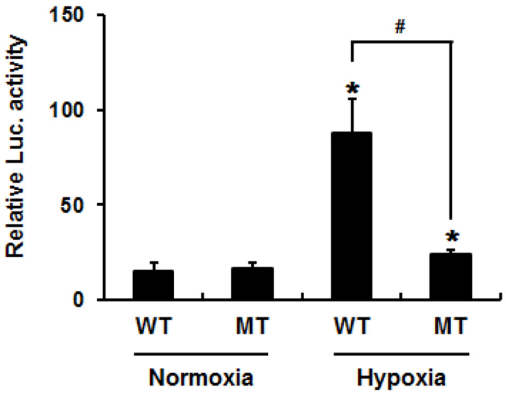

Fig. 4. Hypoxia enhanced RANKL promoter activity in a HIF-1α binding element-dependent manner. Reporter plasmids containing the 1 kb mouse RANKL promoter sequence with wild-type (WT) or mutated HIF-1α binding site (MT) were prepared. ATDC5 cells were transiently transfected with the indicated reporter plasmids and incubated for 24 h under the normoxic or hypoxic conditions. Data represent mean ± S.D. of the firefly luciferase activity normalized to *Renilla* luciferase activity (N=6; *p<0.05, compared to WT under the normoxic conditions; $^{\#}p < 0.05$, for the indicated comparison).

cells [26, 31]. The results from our study further support the indirect regulatory role of hypoxia in osteoclastogenesis via increased RANKL expression in hypertrophic chondrocytes. The cross-talk between HIF-1α and Runx2 in the hypertrophic chondrocytes during endochondral bone formation has been reported that Runx2 caused the accumulation and nuclear translocation of HIF-1α protein in ATDC5 chondrocytes [28]. Furthermore, physical and functional interactions between Runx2 and HIF-1α during endochondral bone formation and angiogenesis has been also reported [27]. Increased levels of Runx2 in differentiated ATDC5 cells were observed in this study, which may be a clue to understand why hypoxia- and subsequently HIF-1α-induced RANKL expression is much higher in differentiated chondrocytes.

Although Martínez-Calatrava *et al.* recently reported that increased RANKL synthesis by articular chondrocytes contributes to arthritic juxta-articular bone loss [32], there have been little reports about the transcription factors that transactivate RANKL expression in chondrocytes during fetal bone development. In the present study, we demonstrated that hypoxia induces RANKL expression and RANKL is also a target gene of HIF-1α in chondrocytes. This result is consistent with our previous report showing that hypoxia induces RANKL expression in mouse MLO-Y4 and human periodontal ligament fibroblasts and that HIF-1α mediates hypoxia-induced RANKL expression by directly binding the

RANKL promoter [19, 30]. Given that the microenvironment of calcified cartilage is hypoxic, it is suggested that hypoxic hypertrophic chondrocytes themselves contribute to resorption of calcified cartilage matrix via induction of RANKL and VEGF expression in a HIF-1α-dependent manner and subsequent osteoclast recruitment and differentiation.

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Conflict of interest

The authors have no conflicts of interest.

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