Interleukin-11 as a Stimulatory Factor for Bone Formation Prevents Bone Loss with Advancing Age in Mice*

Received for publication, August 1, 2002
Published, JBC Papers in Press, October 15, 2002, DOI 10.1074/jbc.M207804200

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Cytokines in interleukin (IL)-11 subfamily participate in the regulation of bone cell proliferation and differentiation. We report here positive effects of IL-11 on osteoblasts and bone formation. Overexpression of human IL-11 gene in transgenic mice resulted in the stimulation of bone formation to increase cortical thickness and strength of long bones, and in the prevention of cortical bone loss with advancing age. Bone resorption and osteoclastogenesis were not affected in IL-11 transgenic mice. In experiments in vitro, IL-11 stimulated transcription of the target gene for bone morphogenetic protein (BMP) via STAT3, leading to osteoblastic differentiation in the presence of BMP-2, but inhibited adipogenesis in bone marrow stromal cells. These results indicate that IL-11 is a stimulatory factor for osteoclastogenesis and bone formation to conserve cortical bone, possibly by enhancing BMP actions in bone. IL-11 may be a new therapeutic target for senile osteoporosis.

Aging is associated with a sustained loss of bone. Histomorphometric analyses revealed that a decline in trabecular wall thickness with a reduction in the number and functions of osteoblasts is associated with the age-related bone loss (1). Thus, a reduction in bone formation because of a decrease in the recruitment of osteoblasts is the principal feature of senile osteoporosis, whereas the age-related decrease in the activation of vitamin D may cause an increase in parathyroid hormone secretion and an elevation in bone resorption (2–4). However, it is not yet fully understood how the recruitment of osteoblasts and bone formation are impaired with aging. Accumulating evidence indicates that local factors regulate differentiation of bone marrow stromal cells into osteoblasts (5). Among them cytokines activating gp130 could be involved in the differentiation of osteoblasts (6, 7), whereas they also stimulate osteoclastogenesis in vitro (8, 9). Interleukin (IL)-11 that activates gp130 is also known to inhibit adipocytic differentiation of bone marrow stromal cells (10). An increase in the number of adipocytes in bone marrow correlates inversely with a decrease in the number of cells in an osteoblast lineage in the age-related osteopenia (11, 12). Thus, IL-11 may promote bone marrow stromal cells toward osteoblastic cells but not adipocytes.

The P6 strain of senescence-accelerated mice (SAM) established from AKR/J mice shows several characteristics of senile osteoporosis (13, 14). We have reported that there is a reduction in the expression of IL-11 in bone marrow cells of SAM-P6 and that such a change may be involved in the impaired osteoblastic differentiation and enhanced adipogenesis in the bone marrow of these mice (15). IL-11 is synthesized by bone marrow stromal cells and is shown to be an anti-inflammatory cytokine against actions of IL-1 and tumor necrosis factor-α (16, 17), both of which are bone-resorbing cytokines (18). These observations suggest that decreased IL-11 expression may be involved in the pathogenesis of bone loss and further imply a physiological role of IL-11 in bone metabolism through regulation of bone marrow cell differentiation.

We report here stimulatory effects of IL-11 on bone formation. Overexpression of human IL-11 gene in transgenic mice resulted in the stimulation of bone formation to increase cortical thickness and strength of long bones and in the prevention of cortical bone loss with advancing age. In contrast, bone resorption was not affected in the transgenic mice. IL-11 stimulated transcription of the target gene for bone morphogenetic protein (BMP) via transcription factor STAT3, leading to enhanced osteoblastic differentiation in the presence of BMP-2, but inhibited adipogenesis in bone marrow stromal cells. These results indicate that IL-11 is a stimulatory factor for osteoclastogenesis and bone formation to retain cortical bone mass, possibly by enhancing BMP actions on bone cells.

**EXPERIMENTAL PROCEDURES**

Generation of Human IL-11 Transgenic Mice—The present studies were approved by the institutional animal care and oversight committee according to the guideline principles in the “Care and Use of Animals.” The Mx-human IL-11 transgene was constructed. A 762-bp BamHI-BglII fragment containing the coding region of human IL-11 (597 bp) was isolated from the pCD-20-2 plasmid (19). The fragment was inserted into the pCMV-v5 medium; ALP, alkaline phosphatase; BMPRE, BMP-response element; PPARγ, peroxisome proliferator-activated receptor-γ; rh, recombinant human; TRAP, tartrate-resistant acid phosphatase.
Mechanical Strength of Femurs—Mechanical strength of femurs was evaluated with 3-point bending test using a physical property analyzing system (SHIMAZU AG-2000E; Shimazu Co., Tokyo, Japan) as described (28).

Preparation of Bone Marrow Cells and Cultures—Bone marrow cells were obtained from femurs and tibiae of 6-week-old male ddY mice (Japan SCL, Shizuoka, Japan) or 10-week-old IL-11 transgenic mice by flushing with standard minimum essential medium (αMEM), and red blood cells in marrow cells were hemolyzed in 17 mM Tris-HCl, pH 7.5, containing 0.8% ammonium chloride. Hemolyzed bone marrow cell suspensions were rinsed twice with phosphate-buffered saline (PBS). Resuspended bone marrow cells were cultured on plastic dishes in αMEM containing 10% fetal bovine serum and 50 mg/liter ascorbic acid at 37°C in a 5% CO2-in-air atmosphere. Cells were freshly replaced twice a week.

Mouse bone marrow stromal cell line ST2 (RIKEN Cell Bank, Tsukuba, Japan) and osteoblastic cell line MC3T3-E1 provided by Dr. Kodama (Oho Dental College, Japan) were cultured in αMEM supplemented with 10% fetal bovine serum and 50 mg/liter ascorbic acid at 37°C in 5% CO2-in-air atmosphere.

Formation of TRAP-positive Multinucleated Osteoclast-like Cells—The whole bone marrow cell suspensions after hemolysis (1.5 × 10^6 cells/well) were cultured in 24-well plates. On the 2nd day, they were treated with 1 μM 1,25-dihydroxyvitamin D3 in the presence of indicated concentrations of recombinant human (r) IL-11 (Genzyme Co., Cambridge, MA). On the 8th day of culture, cells were fixed in 10% formalin neutral buffer solution for 10 min, and TRAP-positive cells were stained with 5% nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyphosphate in 0.1 M sodium carbonate buffer, pH 10, supplemented with 1 mM MgCl2 and was incubated at 37°C for 30 min. After adding 0.1 mM NaOH, the amount of p-nitrophenyl phosphate released was measured by spectrophotometry.

Oil Red O Staining—Bone marrow cells (1 × 10^6 cells/well) and ST2 cells were seeded on 8-well slide chambers (Lab-Tek chamber, Nunc). Cells were grown with αMEM supplemented with 10% FBS and 1 μg/ml Mx (The Source, CA). For induction of adipocytic differentiation for 7 days in the presence of indicated concentrations of rH-L11 (Genzyme Co., Cambridge, MA). On the 8th day of culture, cells were fixed in 10% formalin neutral buffer solution for 10 min, and TRAP-positive cells were stained with 5% nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyphosphate in 0.1 M sodium carbonate buffer, pH 10, supplemented with 1 mM MgCl2 and was incubated at 37°C for 30 min. After adding 0.1 mM NaOH, the amount of p-nitrophenyl phosphate released was measured by spectrophotometry.

Plasmid Construction and cDNA Transfection—Two kinds of plasmids with luciferase gene provided by Dr. Miyazono (University of Tokyo, Tokyo, Japan) were transfected as reporter for BMP signaling. One reporter construct containing an 8.5-kb fragment of the promoter region of mouse Smad6 (−9.5 kb/−1 kb) was generated by inserting the upstream of the core promoter of type X collagen and luciferase gene (33). A full-length rat Smad1 was cloned into an expression vector pCDNA3 (Invitrogen) (34). Expression plasmids for wild-type and dominant negative PPARg (Life Sciences). Murine glyceraldehyde-3-phosphate dehydrogenase mRNA expression was also examined by RT-PCR as an internal control.

Human IL-11 Assay—Human IL-11 in sera and culture supernatants was measured with an enzyme immunoassay method using Quantikine human IL-11 immunoassay (R & D Systems, Minneapolis, MN). X-ray Analysis, Histological Analysis, and Histomorphometry—Radiographic studies were performed using a soft x-ray apparatus (type SRO-M50; Sofron, Tokyo, Japan). Computed tomography scans at the diaphysis of excised femurs were taken using a composite x-ray apparatus (type SRO-M50; Sofron, Tokyo, Japan). Computed tomography scans at the diaphysis of excised femurs were performed using a soft x-ray apparatus (type SRO-M50; Sofron, Tokyo, Japan) and αMEM containing 10% fetal bovine serum and 50 mg/liter ascorbic acid at 37°C in a 5% CO2-in-air atmosphere. Media and reagents were freshly replaced twice a week.

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Contact x-ray analysis showed proportional changes of femurs in either dimension until 52 weeks of age and loss of trabeculae in the metaphysis in both IL-11 transgenic and wild-type mice with advancing age as expected (Fig. 2b). In contrast to trabecular bone, cortical bone thickness of femurs increased in IL-11 transgenic mice but decreased in wild-type with aging (Fig. 2, b and c). In accordance with this observation, bone mineral density of femurs increased in IL-11 transgenic mice, whereas it decreased in wild-type with advancing age (Fig. 2d). Histological analysis of proximal tibiae demonstrates similar changes with aging (Fig. 2e). There was no osteoclastic change in long bones of IL-11 transgenic mice until 52 weeks after birth. There were no differences in serum level of calcium, phosphorus, and ALP between transgenic mice and wild-type littermates (data not shown).

To determine whether the overexpression of human IL-11 affected the strength (maximal load) or flexibility (stiffness) of the cortical bone, biomechanical properties of the femurs from IL-11 transgenic and wild-type mice were measured in a 3-point bending test. There were no significant differences in the biomechanical properties of femurs from 10-week-old mice. Femurs from 52-week-old wild-type mice lost their strength as a result of normal aging, whereas the strength of bones from IL-11 transgenic mice increased to the level significantly higher than that in wild-type littermates (Table I). These results suggest that IL-11 overexpression caused an increase in cortical bone mass with intact overall architecture and a comparable increase in mechanical strength.

Histomorphometry of Tibiae of IL-11 Transgenic Mice—To study the cellular mechanisms that account for the progressive increase of bone mass, we performed static and dynamic histomorphometric analyses on the proximal tibiae of 10-week-old transgenic and wild-type mice. Trabecular bone volume (Fig. 3a) and osteoblast-covered bone surfaces (Fig. 3b) were higher in transgenic mice than in wild-type littermates. Histomorphometric parameters measuring the amount of newly formed bone matrix, such as an osteoid-covered surface, was also increased in transgenic mice (data not shown). Dynamic histomorphometry after the double labeling with calcein revealed a significant increase in mineral apposition rates (Fig. 3c) in transgenic mice, resulting in increased bone formation rates. On the other hand, the number of osteoclasts as determined by TRAP staining (Fig. 3d) and eroded surface per bone surface (Fig. 3e) was comparable with wild-type littermates. The structure of the epiphyseal growth plates seemed to be normal in transgenic mice (Fig. 2e). These results indicate that the increase in bone mass observed in transgenic mice is due to increased bone formation with no change in bone resorption.

Functional Characterization of Bone Marrow Cells of IL-11 Transgenic Mice in Cultures—To investigate the consequences of IL-11 overexpression on the cellular level, we prepared and examined primary bone marrow cells from long bone in vitro. The number of ALP-positive colonies was higher in bone marrow cells of IL-11 transgenic mice than that of wild-type littermates (Fig. 4a). Addition of 10–100 pM rhIL-11 increased the number of ALP-positive colonies in wild-type bone marrow cell cultures but had no effect in transgenic cells (Fig. 4a). Adherent bone marrow cells were propagated for examination of osteoblastic differentiation. ALP activities associated with cell layers were higher in adherent bone marrow cells of IL-11 transgenic mice than those of wild-type littermates (15.1 ± 2.5 versus 7.4 ± 0.2 nmoL/min/μg of protein, p < 0.01). In transgenic cell cultures, areas of mineralized extracellular matrix monitored by von Kossa staining were apparent at day 18 of culture, whereas few mineralized nodules were visible in wild-type cell cultures (Fig. 4b). Accordingly, the expression of
mRNA for osteocalcin was higher in transgenic cells than wild-type cells, whereas mRNAs for BMP-2 and BMP-4 were equally expressed in transgenic and wild-type cells (Fig. 4c).

The numbers of TRAP-positive multinucleated osteoclast-like cells generated in bone marrow cell cultures were comparable in wild-type and transgenic cells in the presence of 1 nM 1,25-dihydroxyvitamin D3 (Fig. 4d). Treatment with rhIL-11 did not further increase the number of TRAP-positive osteoclast-like cells in wild-type cell cultures in the presence of 1,25-dihydroxyvitamin D3 (Fig. 4d). Addition of rhIL-11 alone did not stimulate the formation of osteoclast-like cells (data not shown). Consistent with this, the expression of mRNA for RANK ligand was similar in bone marrow cells from either mouse (Fig. 4c).

**Effect of IL-11 on Differentiation of Bone Marrow Stromal Cells**—Bone marrow stromal cells contain precursor cells for both osteoblasts and adipocytes. We further investigated effects of IL-11 on bone marrow cells obtained from mice with a different genetic background from founders of IL-11 transgenic mice and on clonal bone marrow stromal cells. IL-11 at 10–1000 pM increased the number of ALP-positive colonies that include osteoblast precursors in bone marrow cell primary cultures derived from long bones of 6-week-old male ddy mice (Fig. 5a). In contrast, IL-11 decreased the number of Oil Red-O positive adipocytes (data not shown) as reported (37). A mouse bone marrow stromal cell line ST2 has the potential to differentiate into either osteoblastic or adipocytic cells in vitro with specific stimulants (32, 38). We examined the effects of IL-11 on osteoblastic differentiation induced by BMP-2 and on adipocytic differentiation induced by troglitazone in ST2 cells. Treatment with 1000 pM IL-11 enhanced the increase in ALP activity in ST2 cells in the presence of 100 ng/ml BMP-2, although IL-11 had no effect on ALP activity in the absence of BMP-2 (Fig. 5b). In contrast, treatment with 10–1000 pM IL-11 decreased the number of Oil Red-O positive cells in the presence of 1 μM troglitazone (Fig. 5c). There were no Oil Red-O positive cells formed in the absence of troglitazone.

**Effect of IL-11 on Transcription of Target Genes of BMP-2 and Troglitazone**—BMP-2 signals that promote osteoblastic differentiation are in large part mediated by ligand-restricted Smads, such as Smad1, as transcription factors. Treatment of ST2 cells with 100 ng/ml BMP-2 increased the promoter activity of the mouse Smad6 gene that contains BMPRE where Smad1 directly binds (22). Treatment with 1000 pM IL-11 along with BMP-2 significantly enhanced the transcriptional activity driven by BMP-2, although it had no effect in the absence of BMP-2 (Fig. 6a). When the cells were co-transfected with dominant negative STAT3, the stimulatory effect of IL-11 was abrogated, although the effect of BMP-2 was still observed (Fig. 6a). Similar results were obtained in mouse osteoblastic cells and on clonal bone marrow stromal cells. IL-11 at 10–1000 pM increased the number of ALP-positive colonies that include osteoblast precursors in bone marrow cell primary cultures derived from long bones of 6-week-old male ddy mice (Fig. 5a). In contrast, IL-11 decreased the number of Oil Red-O positive adipocytes (data not shown) as reported (37). A mouse bone marrow stromal cell line ST2 has the potential to differentiate into either osteoblastic or adipocytic cells in vitro with specific stimulants (32, 38). We examined the effects of IL-11 on osteoblastic differentiation induced by BMP-2 and on adipocytic differentiation induced by troglitazone in ST2 cells. Treatment with 1000 pM IL-11 enhanced the increase in ALP activity in ST2 cells in the presence of 100 ng/ml BMP-2, although IL-11 had no effect on ALP activity in the absence of BMP-2 (Fig. 5b). In contrast, treatment with 10–1000 pM IL-11 decreased the number of Oil Red-O positive cells in the presence of 1 μM troglitazone (Fig. 5c). There were no Oil Red-O positive cells formed in the absence of troglitazone.

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MC3T3-E1 cells (data not shown). The overexpression of STAT3 in ST2 cells enhanced the transcriptional activity of 3GC2-lux that contains 3 tandem repeats of BMPRE when the Smad1 construct was transfected in the presence and absence of BMP-2 (Fig. 6b). These observations suggest that IL-11 stimulates the transcriptional activity of BMP target gene through the activation of STAT3.

Troglitazone induces adipogenesis through its binding to and activation of PPARγ that is a key transcription factor for adipocytic differentiation. Treatment of ST2 cells with 1 μM troglitazone increased the transcriptional activity of PPARγ, and simultaneous treatment with IL-11 completely abolished the induction of PPARγ transactivation (Fig. 6c) as reported (32).

**DISCUSSION**

Our observations shown here indicate that IL-11 is a stimulatory factor for bone formation to conserve cortical bone in vivo and for osteoblastogenesis in vitro possibly through enhancement of BMP actions. Transgenic mice expressing the human IL-11 gene that we have developed are suitable for investigation of topical effects of IL-11 on bone, because they show higher expression of IL-11 in bone than wild-type littersmates, and human IL-11 is immunologically detected in bone marrow but not in circulating blood. It is also important to note that they are fertile and show no apparent abnormalities. Cortical thickness and mineral density of long bones were higher in IL-11 transgenic mice than those of their wild-type littersmates at 10 weeks after birth. Bone histomorphometric analyses indicate no change in bone resorption but an increase in bone formation in IL-11 transgenic mice compared with their wild-type littersmates. *Ex vivo* cultures of bone marrow cells obtained from IL-11 transgenic mice showed no difference in the degree of formation of osteoclast-like cells but enhanced expression of osteoblastic markers and *in vitro* mineralization compared with those of wild-type littersmates, whereas BMP expressions were similar in cells from both mice. These observations suggest that IL-11 functions as an anabolic factor for bone in vivo through its stimulatory effects on cells in an osteoblast lineage. Observations that increased actions of LIF and oncostatin M, both of which activate gp130, result in overgrowth of bone in vivo (39, 40) suggest that signaling molecules activated by gp130 are common mediators for changes in bone among these experimental animals including IL-11 transgenic mice.

To clarify how IL-11 stimulates osteoblastogenesis, we examined effects of IL-11 signals on BMP actions that are essential for osteoblastic differentiation (38). STAT3 is a critical signaling molecule downstream of gp130 activated by IL-11 (41). We demonstrated that IL-11 stimulated the transcription of the BMP-responsive gene via STAT3 in bone marrow stromal ST2 and osteoblastic MC3T3-E1 cells, because transient transfection of dominant negative STAT3 construct abrogated the stimulatory effect of IL-11, although it had no effect on the transcriptional activity by BMP-2. The fact that STAT3 overexpression in ST2 cells enhanced the transcriptional activity of BMP-specific transcription factor Smad1 further elaborates
this notion. This stimulatory effect of IL-11 on the transcription of target genes for BMP might lead to osteoblastic differentiation in the presence of BMP-2. Because cells in an osteoblast lineage can produce BMPs, IL-11 could cooperate with endogenous BMPs in osteoblast precursors even in the absence of exogenous BMPs. Hence, cytokines in an IL-11 subfamily may have the potential to stimulate bone formation via the gp130-STAT3 pathway. Recently, Nakashima et al. (42) have reported synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300. Taken together, it is suggested that crosstalks between BMP-Smad and gp130-STAT3 signals are common and critical in several aspects of cellular differentiation including osteoblasts.

The gp130-STAT3 pathway is also reported to be involved in the stimulation of RANK ligand expression in osteoblastic cells in vitro (43). This observation may be inconsistent with our results. Although IL-11 by itself did not stimulate osteoclast-like cell formation in vitro and histomorphometric parameters for bone resorption were similar in IL-11 transgenic mice and their wild-type littermates in the present study, further investigations are needed to clarify the role of IL-11 for bone resorption in vivo.

Recently several transgenic mice have been reported to show higher bone mass than their controls due to the increased bone formation. Mice overexpressing a certain component of AP-1 family, Fra-1 or ΔFosB, show the increased bone formation and higher bone mass than their controls (44, 45); however, they demonstrate osteosclerosis, which is narrowing bone marrow spaces, with aging. IL-11 transgenic mice demonstrate enhanced bone formation and thickening of cortical bone with no signs of osteosclerosis, indicating that bone metabolism in this animal model is physiologically relevant, whereas that in transgenic mice overexpressing the fra-1 or ΔfosB gene may not. We observed no cortical bone loss with advancing age in IL-11 transgenic mice, whereas osteopenia progressed with time in their wild-type littermates. In addition, the physical strength against bending force of long bone was correlated with its bone mineral density and was higher in IL-11 transgenic mice than that in wild-type mice at 52 weeks after birth. Similar observations are reported in tob-deficient mice that show a greater bone mass without osteosclerosis with aging (46). Tob inhibits BMP-Smad signals in the nucleus, suggesting that Tob deficiency enhances BMP actions to stimulate osteoblastic functions (46). Taken together with our observations, stimulation of BMP-Smad signals in bone may generally underlie the increased bone formation in both IL-11 transgenic and tob-deficient mice.

Because molecular mechanisms for bone loss with aging are not yet fully understood, we have no therapeutic strategy specific to bone loss with advancing age. Results in the present

**FIG. 4.** Functional characterization of bone marrow cells of IL-11 transgenic mice in cultures. *a,* primary cultures of mouse bone marrow cells from femurs and tibiae of 10-week-old wild-type and IL-11 transgenic (TG) mice were treated with indicated concentrations of IL-11 for 10 days and were subjected to ALP staining. The number of ALP-positive colonies was counted. Data are means ± S.E., n = 4. *, significantly higher than values of wild-type bone marrow cell cultures in the absence of IL-11. *b,* primary bone marrow cells as described above were cultured for 18 days without IL-11. *c,* primary bone marrow cells from wild-type (WT) and IL-11 transgenic (TG) mice. Cells were cultured for 10 days without IL-11. Each set of primers was described under “Experimental Procedures.” *d,* primary bone marrow cells as described above were cultured for 7 days in the presence of 1 nM 1,25-dihydroxyvitamin D3 (1,25-D3). The number of osteoclast-like TRAP-positive multinucleated cells (MNC) was counted. Data are means ± S.E., n = 4. *, significantly higher than values of wild-type cultures without 1,25-dihydroxyvitamin D3.
study may provide an example of preventing bone loss with aging with a persistent expression of a certain cytokine in bone marrow. Because bone loss is associated with an increase in bone marrow adipocytes along with a decrease in osteoblasts in several conditions such as aging and glucocorticoid-induced osteoporosis (47), anti-adipogenic effects of IL-11 may also advance the recruitment of osteoblasts to stimulate bone formation. Given that the continuous expression of IL-11 in bone marrow stimulates bone formation to preclude bone loss, an impaired expression of IL-11 may be involved in bone loss in elderly people. The P6 strain of SAM is an example that suggests the linkage between the IL-11 cytokine family and bone metabolism. SAM-P6 has been shown to be a model of senile osteoporosis with reduced bone turnover (13, 48). We have reported that the impaired expression of IL-11 in bone marrow cells in SAM-P6 is involved in the decrease in osteoblastogenesis and in the increase in adipogenesis (15), both of which may contribute to the impaired bone formation. This observation may not be specific to SAM-P6 but physiologically ubiquitous, because the expression of IL-11 in bone marrow cells decreases with aging in normal mice, and the same may be true for humans when examined in autopsy femoral heads (49).

In conclusion, the present study demonstrates that the persistent expression of human IL-11 gene in bone marrow cells results in the stimulation of bone formation to increase cortical thickness and the strength of long bone and to prevent bone loss with advancing age in mice. It is also shown that IL-11 augments BMP-2 actions via STAT3 in mouse ST2 bone marrow stromal cells to stimulate osteoblastic differentiation but inhibits adipogenesis in vitro. Thus, stimulation of IL-11 actions in bone marrow would provide a new strategy for thera-

2 E. Tohjima, D. Inoue, N. Yamamoto, S. Kido, Y. Ito, S. Kato, Y. Takeuchi, S. Fukumoto, and T. Matsumoto, submitted for publication.
