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# Osteogenic effect of interleukin-11 and synergism with ascorbic acid in human periodontal ligament cells

**E. R. Leon[1,2], K. Iwasaki[1], M. Komaki[3], T. Kojima[1], I. Ishikawa[1,4]**

[1]Periodontology, Department of Hard Tissue Engineering, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan, [2]Center of Excellence Program for Frontier Research on Molecular Destruction and Reconstruction of Tooth and Bone, Tokyo Medical and Dental University, Tokyo, Japan, [3]Department of Nanomedicine (DNP), Tokyo Medical and Dental University, Tokyo, Japan and [4]Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan

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*Background and Objective:* Human periodontal ligament cells are considered to be a key cell type in the regeneration of periodontal tissues because of their unique localization and stem cell-like properties. Interleukin-11 is a multifunctional cytokine known to participate actively in bone metabolism. The purpose of this study was to examine the effect of interleukin-11 on the osteoblastic differentiation of periodontal ligament cells.

*Material and Methods:* Cultured periodontal ligament cells were stimulated with interleukin-11 and/or ascorbic acid, with or without inhibitors for type 1 collagen, janus kinase/signal transducers and activator of transcription, and mitogen-activated protein kinase (MAPK). Osteoblastic differentiation was investigated by examining the alkaline phosphatase activity and gene expression of Runx2, osteocalcin and bone sialoprotein using reverse transcription-polymerase chain reaction. Type 1 collagen and tissue inhibitor of metalloproteinase-1 production were measured using enzyme-linked immunosorbent assays.

*Results:* Interleukin-11 enhanced alkaline phosphatase activity and Runx2, osteocalcin and bone sialoprotein gene expression in the presence of ascorbic acid. Interleukin-11 induced type 1 collagen and tissue inhibitor of metalloproteinase-1 production in periodontal ligament cells. Type 1 collagen inhibitor completely inhibited the alkaline phosphatase activity enhanced by interleukin-11 and ascorbic acid. Furthermore, janus kinase/signal transducers and activator of transcription and MAPK signaling inhibitors reduced interleukin-11/ascorbic acid-induced alkaline phosphatase activity in periodontal ligament cells.

*Conclusion:* Interleukin-11/ascorbic acid induced the osteoblastic differentiation of periodontal ligament cells through type 1 collagen production and janus kinase/signal transducers and activator of transcription, and MAPK signaling pathways were involved in this process. These findings suggest that interleukin-11 may function as an osteopromotive cytokine, stimulating the osteoblastic differentiation of periodontal ligament cells mainly through the synthesis of type 1 collagen and possibly by the induction of tissue inhibitor of metalloproteinase-1.

Kengo Iwasaki, Periodontology, Department of Hard Tissue Engineering, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo, 113–8549, Japan
Tel: +81 35803 5488
Fax: +81 35803 0196
e-mail: k-iwasaki.peri@tmd.ac.jp

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The central goal of periodontal therapy is to regenerate the damaged tissues to their original structure, by re-establishing the soft tissue attachment between newly created bone and cementum on the root surface. In this context, periodontal ligament cells, owing to their unique localization and mesenchymal stem cell-like properties, are considered as a key cell type for periodontal regeneration. *In vitro* studies have shown that periodontal ligament cells share some properties with osteoblasts that gingival fibroblasts lack or only weakly express, such as high alkaline phosphatase activity, production of bone-associated proteins (including osteopontin, osteocalcin, bone sialoprotein and bone morphogenetic protein-2/4), as well as mineralized nodule formation. These attributes of periodontal ligament cells, together with their transplantability into periodontal defects with no adverse effects, have stimulated strong interest in the area of tissue engineering (1–4).

The extracellular matrix plays a key role in the connective tissue architecture and homeostasis. Type 1 collagen is the main component of extracellular matrix in bone and periodontal ligament. Ascorbic acid (vitamin C) contributes to the proper formation and maintenance of the collagen matrix by increasing the procollagen stability and secretion, which is also necessary for matrix maturation and mineralization (5–7). Type 1 collagen not only constitutes the framework for connective tissue, but it also activates cellular functions by transducing signals through its receptor integrins, including proliferation, development, apoptosis, etc. (8,9). A previous study, carried out in our laboratory, reported that ascorbic acid induced the alkaline phosphatase activity in periodontal ligament cells via the production of type 1 collagen and an increasing concentration of $\alpha 2\beta 1$ integrin, which is the main receptor for the binding of periodontal ligament cells to type 1 collagen (10). However, the mechanism of the osteoblastic differentiation of periodontal ligament cells is not still fully understood.

Interleukin-11 belongs to the interleukin-6-type cytokine family (interleukin-6, leukemia inhibitor factor, oncostatin M, ciliary neurotrophic factor and cardiotropin-1), sharing the transmembrane glycoprotein-130 as a signal transducer receptor subunit. In interleukin-11-responding cells, signaling transduction also requires binding of the cytokine to its membrane-bound receptor – interleukin-11 receptor-$\alpha$ – and subsequent homodimerization of the signal transducer glycoprotein-130, leading to activation of the Janus kinase/signal transducer and activator of transcription and mitogen-activated protein kinase (MAPK) cascade (11–13). Interleukin-11 is produced by a number of cell types, including fibroblasts, osteoblasts and leukocytes, in response to inflammatory cytokines, growth factors and hormones (14,15). Interleukin-11 is a functionally pleiotropic cytokine which is currently used for the treatment of patients undergoing chemotherapy-induced thrombocytopenia because of its thrombopoietic effect (NEUMEGA®) (16). However, interleukin-11 also exerts different functions in other cell types and systems, such as inhibitor of adipogenesis (17,18), pro-inflammatory cytokines (19,20) and glucocorticoids (21), and as an inducer of tissue inhibitor of metalloproteinase (TIMP) production (22,23). Nevertheless, special interest has arisen from the involvement of interleukin-11 in bone metabolism (24–31).

Glycoprotein-130 signaling activation by interleukin-11 was originally regarded as a key pathway for osteoclastogenesis (27,31). However, a growing body of evidence supports the ability of interleukin-11 to stimulate osteoblastogenesis and bone formation. Takeuchi *et al.* reported that bone volume and osteoblastic activity were enhanced in interleukin-11 transgenic mice, whereas the osteoclastic function was not affected (28). Moreover, a series of studies from Suga *et al.* demonstrated a significant role for interleukin-11 in osteogenesis by promoting osteoblastic differentiation of mouse mesenchymal progenitor cells *in vitro*, and accelerating bone formation in both ectopic models and segmental bone defect models in rats (26,29,30). From those previous studies, the physiological function of interleukin-11 in bone metabolism is still controversial. In periodontal disease, Martuscelli *et al.* have reported that the subcutaneous injection of interleukin-11 prevented ligature-induced periodontal attachment loss and bone resorption in a beagle dog model (25). More recently, Yashiro *et al.* showed that periodontal ligament cells produced a significantly higher amount of interleukin-11 than gingival fibroblasts in response to transforming growth factor-$\beta$ (15). Based on this background, it could be considered that interleukin-11 plays a significant role in the homeostasis of the periodontal tissues, and we hypothesized that interleukin-11 may function as an inducer of osteoblastic differentiation of periodontal ligament cells.

## Material and methods

### Collection of periodontal ligament cells

Periodontal ligament cells were obtained from healthy periodontal ligament tissues of premolar teeth extracted for orthodontic reasons, after obtaining informed consent. The experimental protocol was previously approved by the Ethics Committee from the Tokyo Medical and Dental University. Briefly, periodontal ligament tissue was removed from the center of the root surface and cultured in Dulbecco's modified Eagle's medium (Sigma, St Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (MultiSer, Melbourne, Victoria, Australia) and 1% (v/v) antibiotic-antimycotic (Gibco, Gaithersburg, MD, USA) until the cells grew out from the explants and became nearly confluent. The cells were subcultured and used from passages 3 to 5 in order to minimize the possible changes which can occur during prolonged culture *in vitro*.

### Culture and stimulation of periodontal ligament cells

Twenty-five thousand periodontal ligament cells were plated onto a 48-well tissue culture plate (Falcon,

Lincoln Park, NY, USA) in Dulbecco's modified Eagle's medium/10% fetal bovine serum/1% antibiotic-antimycotic. For all experiments, the periodontal ligament cells were treated with 10 ng/mL of interleukin-11 (R & D, Minneapolis, MN, USA), alone or in combination with 50 μg/mL of L-ascorbic acid 2-phosphate (Sigma). The exception to this was the test for alkaline phosphatase activity, in which different concentrations of interleukin-11 were applied alone or in combination with ascorbic acid at 50 μg/mL. Experiments were run for 1 wk and test solutions were changed twice, on days 3 and 6, except in those prepared for reverse transcription-polymerase chain reaction (RT-PCR). For some experiments, periodontal ligament cells were previously incubated for 30 min with 500 μM 3,4-dehydro-l-proline (Sigma), 10 μM U0126 (LC Laboratory, Woburn, MA, USA), 15 nM janus kinase-1 inhibitor (Calbiochem, La Jolla, CA, USA), or 20 μM AG-490 (Calbiochem), prior to stimulation. In these experiments, all inhibitors were diluted using 0.1% dimethylsulfoxide (Sigma), and their control and test solutions were also diluted in order to maintain the same conditions for all samples. In the case of interleukin-11 receptor-α identification by RT-PCR, $1.25 \times 10^5$ periodontal ligament cells were seeded in six-well tissue culture plates (Falcon), and cultured for several days, until confluent, without any stimulation prior to total RNA extraction. For identification of the osteoblastic differentiation markers (Runx2, osteocalcin and bone sialoprotein) and interleukin-11 receptor-α, $1.25 \times 10^5$ periodontal ligament cells were seeded in six-well tissue culture plates and stimulated with interleukin-11 (10 ng/mL), ascorbic acid (50 μg/mL), or interleukin-11 (10 ng/mL)/ascorbic acid (50 μg/mL), with the test solutions every 3 d for 3 wk prior to total RNA extraction.

### Measurement of alkaline phosphatase activity

The cells were lysed in Tris-buffered saline containing 0.9% NaCl (Wako, Osaka, Japan) and 0.2% Triton X-100 (Sigma) on ice. The alkaline phosphatase activity in the cell lysates was assayed at 37°C for 30 min using the alkaline phosphatase B-test (Wako). The protein concentrations were measured using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Both procedures were carried out according to the manufacturer's instructions. The alkaline phosphatase activities were expressed as the rate of hydrolysis of 1 pmol of p-nitrophenol/mg of protein/minute.

### Alkaline phosphatase staining

To examine alkaline phosphatase activity histochemically, after 1 wk of stimulation with interleukin-11, ascorbic acid or interleukin-11/ascorbic acid, periodontal ligament cells were fixed for 10 min with 4% paraformaldehyde (Wako). After washing with distilled water, the cells were incubated for 20 min with 0.1 mg/mL of naphtol AS-MX phosphate (Sigma), 0.5% *N*,*N*-dimethylformamide (Wako), 2 mM $MgCl_2$ (Wako) and 0.6 mg/mL of Fast Blue BB Salt (Sigma) in 0.1 M Tris-HCl (Wako), pH 8.5. All procedures were performed at 37°C.

### Procollagen type I c-peptide and TIMP-1 assays

Twenty-five thousand periodontal ligament cells were plated onto a 48-well tissue culture plate with interleukin-11 and/or ascorbic acid. Solutions were changed on day 3, and supernatants from days 3 and 7 were collected together for each condition in order to measure the total amount of procollagen type-I c-peptide and TIMP-1 produced in 1 wk, using the procollagen type-I c-peptide enzyme-linked immunosorbent assay (ELISA) Kit (Takara-Bio, Shiga, Japan) and the TIMP-1 ELISA Kit (Daichi Fine Chemical, Toyama, Japan), respectively, according to the manufacturer's instructions.

### RT-PCR for interleukin-11 receptor-α and the osteoblastic markers Runx2, osteocalcin and bone sialoprotein

Total RNA of periodontal ligament cells was isolated using the RNeasy® Mini Kit (Qiagen, German Town, MD, USA). Single-strand cDNA synthesis and PCR were performed with the Superscript™ First-Stand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA), using 1 μg of total RNA, and the BD Advantage™ 2 PCR Enzyme System (BD Biosciences-Clontech, Mountain View, CA, USA), respectively, following the manufacturer's instructions. Amplification reactions were performed using the following primers and protocols: human interleukin-11 receptor-α: forward, 5′-atgagggcacctacatctgc-3′; reverse, 5′-gtcgggggtaacctggtact-3′ (annealing at 60°C, 30 cycles, 441-bp product, and GenBank accession no.: NM_004512); Runx2: forward, 5′-aacccacgaatgcactatcca-3′; reverse, 5′-cggacataccgagggacctg-3′ (annealing at 63°C, 30 cycles, 75-bp product, and GenBank accession no.: NM_001024630); osteocalcin: forward, 5′-ttgtgtccaagcaggagggca-3′; reverse, 5′-catccatagggctgggaggt-3′ (annealing at 66°C, 30 cycles, 304-bp product, and GenBank accession no.: NM_199173), bone sialoprotein: forward, 5′-gcagaagtggatgaaaacga-3′; reverse, 5′-tggtggagtaattctgacca-3′ (annealing at 61°C, 30 cycles, 448-bp product, and GenBank accession no.: NM_004967) and glyceraldehyde-3-phosphate dehydrogenase: forward, 5′-gtcagtggtggacctgacct-3′; reverse, 5′-aggggtctacatggcaactg-3′ (annealing 60°C, 30 cycles, 420-bp product, and GenBank accession no.: NM_002046) as the housekeeping control. Reaction products were analyzed and visualized by electrophoresis of 10-μL samples in a 1% agarose gel containing 0.5 μg/mL of ethidium bromide (Wako) using the GENE GENIUS BIO IMAGING SYSTEM with GENE SNAP version 6.03 (Syngene, Cambridge, EN).

### Statistical analysis

Analyses were performed using STATVIEW (version 5.0) software for one-way analysis of variance, with Fisher's protected least significance test as a posthoc test for comparison of specific groups. Data shown are expressed as the mean ± standard deviation, with significance at a $p$-value of $< 0.05$.

## Results

### Interleukin-11 receptor-α expression in periodontal ligament cells

Before investigating the effect of interleukin-11 in periodontal ligament cells, it is obligatory to demonstrate the expression of interleukin-11 receptor-α in their membranes. Hence, we first examined the mRNA expression of interleukin-11 receptor-α using RT-PCR. As shown in Fig. 1, periodontal ligament cells from four different donors clearly expressed the mRNA of interleukin-11 receptor-α.

### Effect of interleukin-11, with or without ascorbic acid, on the alkaline phosphatase activity in periodontal ligament cells

To evaluate the effect of interleukin-11, ascorbic acid and interleukin-11/ascorbic acid on the alkaline phosphatase activity of periodontal ligament cells, we performed a quantitative alkaline phosphatase activity test and alkaline phosphatase staining. Figure 2A shows the effect of interleukin-11 on alkaline phosphatase activity alone or combined with ascorbic acid. As previously reported, periodontal ligament cells express a low level of alkaline phosphatase activity in the control group and ascorbic acid significantly increased this activity (10). In the present study, interleukin-11 increased alkaline phosphatase activity in a dose-dependent manner and this effect was synergistically enhanced when interleukin-11 was applied with ascorbic acid. The effect of interleukin-11 on alkaline phosphatase activity was enhanced fourfold when combined with 50 μg/mL of ascorbic acid. Figure 2B shows that interleukin-11 induced a higher number of alkaline phosphatase-positive cells compared with the control, and that the combination of interleukin-11/ascorbic acid dramatically increased the number of alkaline phosphatase-positive cells compared with any of the other groups.

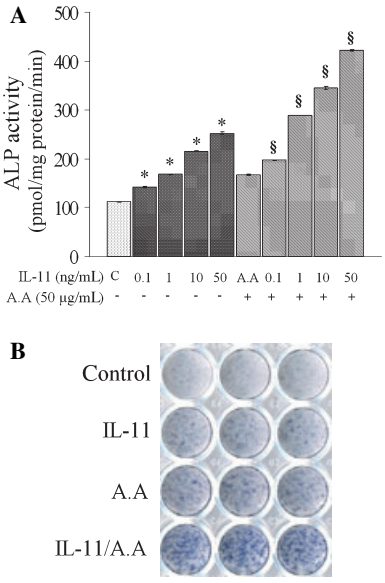


*Fig. 2.* Alkaline phosphatase activity of periodontal ligament cells stimulated with interleukin-11 and/or ascorbic acid (A) Periodontal ligament cells were incubated for 1 week in presence of various concentrations of interleukin-11 with or without ascorbic acid. Interleukin-11 induced a dose-dependent upregulation of the alkaline phosphatase activity in periodontal ligament cells. Values are means ± SD (N = 3). The data are representative of three separate experiments. *Significantly different from control ($p < 0.05$) and §significantly different from ascorbic acid ($p < 0.05$). (B) Alkaline phosphatase staining for periodontal ligament cells stimulated with interleukin-11 (10 ng/ml) and/or ascorbic acid (interleukin-11 50 μg/ml) for 1 week. The data are representative of three separate experiments. A.A, ascorbic acid; ALP, alkaline phosphatase; IL-11, interleukin-11.

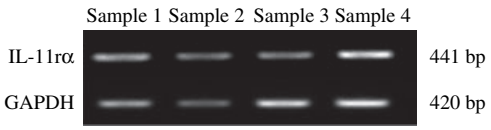


*Fig. 1.* Identification of mRNA expression for interleukin-11 receptor α in periodontal ligament cells by RT-PCR. Total RNA was extracted from periodontal ligament cells from 4 different donors. cDNA was synthesized and PCR was performed as described in Materials and Methods. All samples demonstrated the mRNA expression of interleukin-11 receptor-α. Glyceraldehyde-3-phosphate dehydrogenase was used as the housekeeping control. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-11rα, interleukin-11 receptor-α.

### mRNA expression of interleukin-11 receptor-α and the osteoblastic differentiation markers in periodontal ligament cells after application of interleukin-11, with or without ascorbic acid

In order to examine whether there is any autocrine modulation of interleukin-11 receptor-α by interleukin-11, ascorbic acid or interleukin-11/ascorbic acid, and to confirm whether the periodontal ligament cells develop osteoblastic differentiation after stimulation, we performed RT-PCR to identify the mRNA expression of interleukin-11 receptor-α and of osteoblastic markers such as Runx2, osteocalcin and bone sialoprotein. As shown in Fig. 3, interleukin-11 receptor-α expression in periodontal ligament cells was induced after treatment with interleukin-11 or ascorbic acid. Moreover, this expression was more significant when interleukin-11 and ascorbic acid were applied together. The same tendency was observed with mRNA expression of the osteoblastic differentiation markers Runx2, osteocalcin and bone sialoprotein. In agreement with the observation in the alkaline phosphatase activity test (Fig. 2), a combination of interleukin-11 and ascorbic acid showed the most potent expression of the osteoblast-related genes Runx2, osteocalcin and bone sialoprotein.

### Stimulation of procollagen type-I c-peptide production in periodontal ligament cells by interleukin-11 and ascorbic acid, alone and in combination

As previously reported, ascorbic acid is able to increase the alkaline phosphatase activity of periodontal ligament cells through the stimulation of type 1 collagen production (10). To examine the mechanism of synergistic alkaline phosphatase activity enhancement produced by interleukin-11 in ascorbic acid-stimulated periodontal ligament

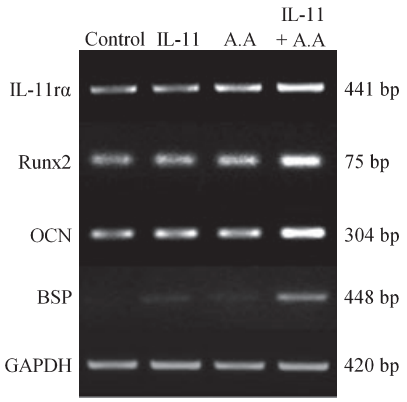


*Fig. 3.* mRNA expression of interleukin 11 receptor α and the osteoblastic markers Runx2, osteocalcin and bone sialoprotein in periodontal ligament cells stimulated with interleukin-11 and/or ascorbic acid by RT-PCR. Periodontal ligament cells were treated with interleukin-11 (10 ng/ml) and/or ascorbic acid (50 μg/ml) for 3 weeks. Interleukin 11 receptor α and osteoblastic marker RNA expression were induced by interleukin-11 and further enhanced in the interleukin-11 + ascorbic acid samples. Glyceraldehyde-3-phosphate dehydrogenase was used as the housekeeping control. The data are representative of three separate experiments. A.A, ascorbic acid; BSP, bone sialoprotein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-11, interleukin-11; IL-11rα, interleukin-11 receptor-α; OCN, osteocalcin.

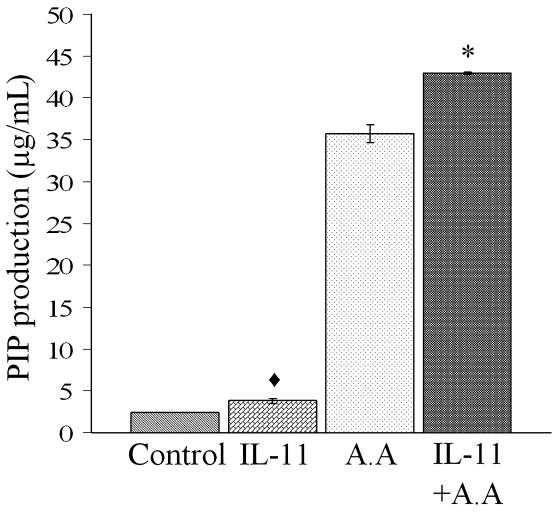


*Fig. 4.* Stimulation of procollagen type 1 c-peptide production in periodontal ligament cells by interleukin-11 and/or ascorbic acid. Periodontal ligament cells treated with interleukin-11 (10 ng/ml) and/or ascorbic acid (50 μg/ml) for 1 week. Addition of interleukin-11 to ascorbic acid-stimulated periodontal ligament cells significantly increased the production of procollagen type 1 c-peptide to 5-fold higher than that of interleukin-11 alone. Values are means ± SD (N = 3). The data are representative of three separate experiments. ♦Significantly different from control ($p < 0.05$) and *significantly different from ascorbic acid ($p < 0.05$). A.A, ascorbic acid; IL-11, interleukin-11; PIP, procollagen type I c-peptide.

cells, we investigated the effect of interleukin-11 on type 1 collagen production in periodontal ligament cells, with or without ascorbic acid. Given that the amount of the free procollagen type-I c-peptide reflects the total type 1 collagen synthesized from procollagen, we quantified the procollagen type-I c-peptide content in the supernatant of periodontal ligament cell cultures after stimulation with interleukin-11, ascorbic acid or interleukin-11/ascorbic acid. As shown in Fig. 4, the addition of interleukin-11 to ascorbic acid-stimulated periodontal ligament cells significantly increased the production of procollagen type-I c-peptide. This increase was fivefold higher than that induced by interleukin-11 alone, indicating a positive effect of interleukin-11 on type 1 collagen production and a clear synergism with ascorbic acid when applied together.

### 3,4-Dehydro-l-proline attenuated alkaline phosphatase activity in periodontal ligament cells stimulated with interleukin-11/ascorbic acid

3,4-Dehydro-l-proline is known to interfere in the triple helical conformation of procollagen, through inhibition of the intracellular synthesis of proline and hydroxyproline amino acids (32,33). In order to examine whether the increase of alkaline phosphatase activity after stimulation with interleukin-11/ascorbic acid was caused by type 1 collagen synthesis, we investigated the effect of 3,4-dehydro-l-proline in periodontal ligament cells. As shown in Fig. 5A, the application of 3,4-dehydro-l-proline inhibited the alkaline phosphatase activity of periodontal ligament cells, after stimulation with interleukin-11/ascorbic acid, to a level similar to that of the control group. Figure 5B clearly revealed how the number of alkaline phosphatase positive-stained cells was completely reduced when 3, 4-dehydro-l-proline was added to interleukin-11/ascorbic acid, suggesting that enhancement of the alkaline phosphatase activity after the application of interleukin-11/ascorbic acid was mainly a result of type 1 collagen production in our experiment.

### TIMP-1 production after interleukin-11, ascorbic acid or interleukin-11/ascorbic acid stimulation of periodontal ligament cells

There are four members of the TIMP family. Of these, TIMP-1 is the most predominant and able to inhibit most matrix metalloproteinases (MMPs), including MMP-2, MMP-3 and MMP-13 (34). To elucidate if TIMP-1 production is enhanced in interleukin-11- and/or ascorbic acid-stimulated periodontal ligament cells, we quantified the amount of TIMP-1 in periodontal ligament cell cultures using ELISA. As shown in Fig. 6, interleukin-11 and ascorbic acid induced the production of TIMP-1 in periodontal ligament cells. This production was threefold higher in the interleukin-11/ascorbic acid group than in the control, suggesting that interleukin-11 and ascorbic acid stabilized the extracellular matrix, not only by inducing type 1 collagen synthesis, but possibly also by inhibiting the extracellular matrix degradation by MMPs.

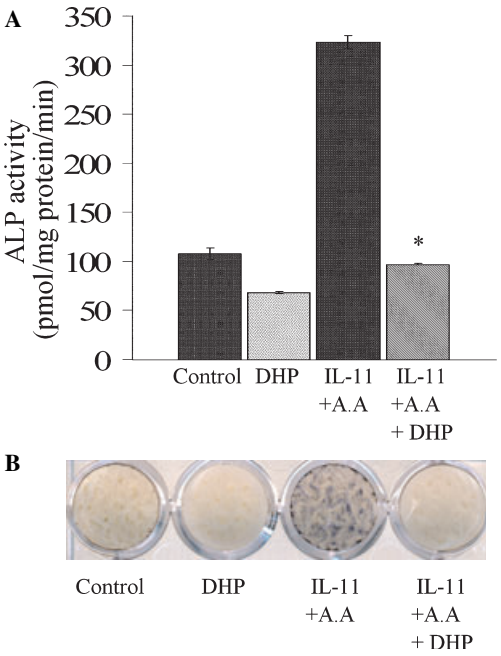


*Fig. 5.* Inhibition of alkaline phosphatase activity in interleukin-11/ascorbic acid-stimulated periodontal ligament cells by 3-4-dehydro-1-proline. (A) Periodontal ligament cells were pretreated with 3-4-dehydro-1-proline (500 $\mu$M) for 30 minutes, prior to the stimulation of the cells with interleukin-11 (10 ng/ml) and ascorbic acid (50 $\mu$g/ml). Addition of 3-4-dehydro-1-proline inhibited the alkaline phosphatase activity of interleukin-11/ascorbic acid-stimulated periodontal ligament cells to a similar level as the control. Values are means ± SD (N = 3). The data is representative of three separate experiments. *Significantly different from interleukin-11 + ascorbic acid ($p < 0.05$). (B) Alkaline phosphatase staining after inhibition of collagen production by 3-4-dehydro-1-proline in periodontal ligament cells stimulated for 1 week with interleukin-11 + ascorbic acid. The data are representative of three separate experiments. A.A, ascorbic acid; ALP, alkaline phosphatase; DHP, 3,4-dehydro-l-proline; IL-11, interleukin-11.

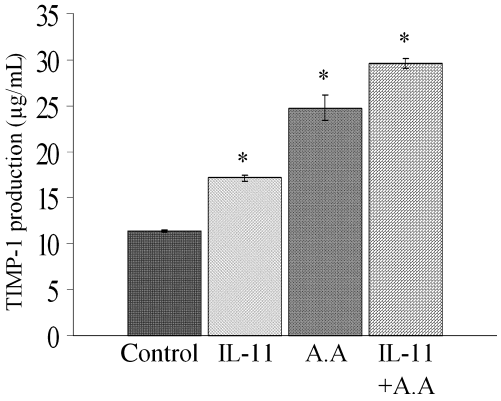


*Fig. 6.* Stimulation of the tissue inhibitor of metalloproteinase-1 (TIMP-1) production in periodontal ligament cells by interleukin-11 and/or ascorbic acid. Periodontal ligament cells were treated with interleukin-11 (10 ng/ml) and/or ascorbic acid (50 $\mu$g/ml) for 1 week. Interleukin-11 and ascorbic acid induced TIMP-1 production in periodontal ligament cells. This production was 3-fold higher in the interleukin-11 + ascorbic acid group compared to the control. Values are means ± SD (N = 3). The data are representative of three separate experiments. *Significantly different from control ($p < 0.05$). A.A, ascorbic acid; IL-11, interleukin-11; TIMP-1, tissue inhibitor of metalloproteinase-1.

### Involvement of MAPK and janus kinase/signal transducers and activator of transcription inhibitors in interleukin-11-induced alkaline phosphatase activity in periodontal ligament cells

To investigate, in greater detail, the signaling pathways through which interleukin-11/ascorbic acid induces the alkaline phosphatase activity within the periodontal ligament cells, we proceeded to inhibit their two potential intercellular signaling cascades: janus kinase/signal transducers and activator of transcription; and MAPK (11,35). For this purpose we used three different inhibitors: U-0126, a selective inhibitor of MAPK kinases (18,36); janus kinase-1 inhibitor, which inhibits the janus kinase signaling pathway (37); and AG-490, which is known to be a potent inhibitor of both the janus kinase/signal transducers and activator of transcription and MAPK signaling pathways (38). As demonstrated in Fig. 7, all of these inhibitors restrained the alkaline phosphatase activity of interleukin-11/ascorbic acid-stimulated periodontal ligament cells, suggesting that the janus kinase/signal transducers and activator of transcription and MAPK pathways are necessary to transmit the signals required to increase the alkaline phosphatase activity after stimulation with interleukin-11/ascorbic acid.

## Discussion

Interleukin-11 is a pleiotropic cytokine, which has various functions, such as inhibitor of adipogenesis (17,18), pro-inflammatory cytokines (19,20) and glucocorticoids (21). In the present study, we demonstrated that interleukin-11 increased alkaline phosphatase activity through the enhancement of type 1 collagen production in ascorbic acid-stimulated periodontal ligament cells and suggested that interleukin-11 had a positive effect on the osteoblastic differentiation of periodontal ligament cells.

The process of osteoblastic differentiation consists of three phases: proliferation with matrix formation (1–7 d); maturation (7–12 d); and mineralization (12–21 d) (39). Type 1 collagen

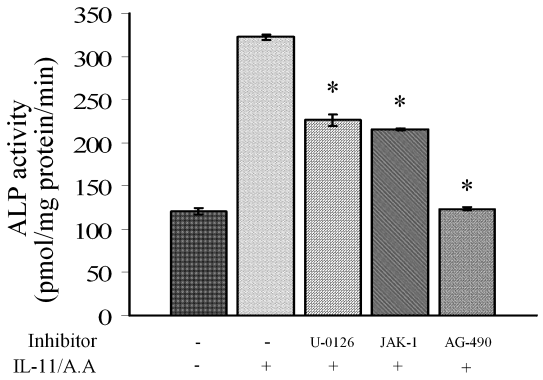


*Fig. 7.* Inhibition of the alkaline phosphatase activity in interleukin-11/ascorbic acid-stimulated periodontal ligament cells by inhibitors of the JAK/STAT and MAPK signaling pathways. Periodontal ligament cells were incubated with U0126 (10 μM), JAK-1 Inhibitor (15 nM) or AG-490 (20 μM) for 30 minutes prior to the stimulation with interleukin-11 (10 ng/ml) and ascorbic acid (50 μg/ml). The inhibitors restrained the effect of interleukin-11/ascorbic acid on alkaline phosphatase activity, with AG-490 exhibiting the most restrictive effect. Values are means ± SD (N = 3). The data are representative of three separate experiments. *Significantly different from interleukin-11/ascorbic acid ($p < 0.05$). A.A, ascorbic acid; ALP, alkaline phosphatase; IL-11, interleukin-11; JAK-1, janus kinase-1.

synthesis and alkaline phosphatase production are two closely related mechanisms and characterize the first and second phases of this process, respectively (40). We previously reported that ascorbic acid induced alkaline phosphatase activity in periodontal ligament cells via the production of type 1 collagen and increase of α2β1 integrin, which is the main receptor for the binding of periodontal ligament cells to type 1 collagen, and suggested that establishment of the type 1 collagen-based extracellular matrix is important in the osteoblastic differentiation of periodontal ligament cells (10). Moreover, in the present study we demonstrated that interleukin-11 synergistically enhanced the ascorbic acid-induced alkaline phosphatase activity in periodontal ligament cells (Fig. 2) and showed how this enhancement could be completely inhibited by the application of 3,4-dehydro-l-proline, the most potent type 1 collagen synthesis inhibitor (Fig. 5). These data strongly suggested that interleukin-11-enhanced alkaline phosphatase activity mainly depends on the stimulation of type 1 collagen production by interleukin-11. Previously, Tang *et al.* reported that an increased accumulation of type 1 collagen was observed in interleukin-11 transgenic mice, suggesting that interleukin-11 may be involved in type 1 collagen production (41).

Type 1 collagen is the main component of periodontal ligament and bone. The interaction between type 1 collagen and cells through integrins regulates many cellular functions, such as enhancement of proliferation and induction of differentiation and apoptosis (8,9). In osteoblastic cells, the binding of cells to type 1 collagen induced the expression of specific genes associated with the osteoblastic phenotype, alkaline phosphatase, osteocalcin and bone sialoprotein, which are selectively activated by the transcription factor, Runx2, to be expressed in the differentiated state (40,42,43). As shown in Fig. 3, the addition of interleukin-11 to ascorbic acid-stimulated periodontal ligament cells increased the mRNA expression of Runx2 and osteocalcin, and the most noteworthy point was that only their combination induced the mRNA expression of bone sialoprotein. As previously demonstrated, expression of the bone sialoprotein gene induced in newly formed osteoblasts is known to be up-regulated by hormones and cytokines, and also to coincide with *de novo* mineralization in bone and cementum (44). Suga *et al.*, who demonstrated that interleukin-11 increased alkaline phosphatase activity and osteocalcin, bone sialoprotein and parathyroid hormone receptor gene expression in mouse mesenchymal progenitor cells C3H10T1/2 (26), supported our findings, which suggest the involvement of interleukin-11 in osteoblastic differentiation in ascorbic acid-stimulated periodontal ligament cells.

TIMPs constitute the major biological regulator of extracellular matrix through inhibition of the collagen matrix degradation produced by metalloproteinases (45). In Fig. 6, we showed enhanced production of TIMP-1 by interleukin-11 and ascorbic acid in periodontal ligament cells. Our results are consistent with previous studies by Herman *et al.* and Maier *et al.*, who showed that interleukin-11 induced TIMP-1 production in synoviocytes and chondrocytes (22,23). From these data, together with our results, interleukin-11 is thought to be a regulator of extracellular matrix by controlling TIMP-1 and following inhibition of MMP activity. Geoffroy *et al.* showed that the endogenous inhibition of MMPs by TIMP-1 overexpression resulted in higher alkaline phosphatase activity and increased trabecular bone mass in transgenic mice (34). More recently, Hayami *et al.* demonstrated that exogenous TIMP-1 application inhibited MMP activity and enhanced alkaline phosphatase activity in periodontal ligament cells, and suggested that TIMP-1 could enhance the osteoblastic differentiation of periodontal ligament cells by inhibition of collagenases (7). However, we were unable to clarify whether enhanced TIMP-1 production controlled type 1 collagen-based extracellular matrix formation and the subsequent alkaline phosphatase activity. Further experiments using TIMP-1 siRNA or TIMP-1 antibody are necessary to determine, in detail, whether interleukin-11/ascorbic acid-induced TIMP-1 production functionally avoids the degradation of type 1 collagen by MMPs and consequently induces the osteoblastic differentiation of periodontal ligament cells.

As with other members of the interleukin-6 cytokine family, interleukin-11 uses the transmembrane glycoprotein-130 as a signal-transducing

receptor subunit. However, interleukin-11 responsiveness is restricted to cells that also express the interleukin-11 receptor-α subunit (12,31,35,46). In our study, we identified the gene expression of interleukin-11 receptor-α. This result, together with the ability of interleukin-11 to increase the alkaline phosphatase activity in periodontal ligament cells (Fig. 2), suggests that periodontal ligament cells may act as target cells for interleukin-11. Moreover, we also found that the addition of interleukin-11 or ascorbic acid, alone or in combination, up-regulated the gene expression of interleukin-11 receptor-α (Fig. 3), which also suggests an autocrine loop, allowing an enhanced response of periodontal ligament cells to interleukin-11.

It is known that the interleukin-11/interleukin-11 receptor-α complex triggers the activation of glycoprotein-130 by enforcing glycoprotein-130 homodimerization. Consecutively, janus kinase 1, janus kinase 2 and TYK2 are activated, and the cytoplasmic tail of glycoprotein-130 is phosphorylated. The phosphorylation of glycoprotein-130 activates signal transducers and activator of transcription 1/3, which are also phosphorylated, form dimers and translocate to the nucleus, where they regulate the transcription of target genes. Phosphorylated residues of glycoprotein-130 also bind to SHP2, thereby forming the link SHP2/ras/MAPK and activate the MAPK cascade (11,12,46). To establish the involvement of janus kinase/signal transducers and activator of transcription and MAPK in the alkaline phosphatase activity of interleukin-11/ascorbic acid-stimulated periodontal ligament cells, we examined the effect of inhibitors of those signaling pathways. As demonstrated in Fig. 7, U0126, janus kinase-1 inhibitor and AG490 diminished interleukin-11/ascorbic acid-elicited alkaline phosphatase activity, suggesting the involvement of janus kinase/signal transducers and activator of transcription and MAPK in alkaline phosphatase activity. Previously, Shin *et al*. had shown a decreased number of osteoblasts and alkaline phosphatase activity in glycoprotein-130 knockout mice, indicating a crucial role of the signaling pathways associated with glycoprotein-130 in osteoblastic differentiation (47). Furthermore, Xiao *et al*. demonstrated that ascorbic acid induced osteoblastic differentiation through type 1 collagen production and subsequent MAPK activation via type 1 collagen–integrin interaction in murine MC3T3-E1 cells (48). From these studies, and together with our findings, it is probable that the janus kinase/signal transducers and activator of transcription and MAPK may be the main signaling pathways related to the osteoblastic differentiation of periodontal ligament cells induced by interleukin-11 and ascorbic acid.

In summary, we have demonstrated that interleukin-11 synergistically increased the alkaline phosphatase activity and gene expression of the osteoblastic differentiation markers Runx2, osteocalcin and bone sialoprotein in ascorbic acid-stimulated periodontal ligament cells. We proved that these effects of interleukin-11 and ascorbic acid were mainly associated with the stimulation of type 1 collagen production. Furthermore, this alkaline phosphatase activity was enhanced via the janus kinase/signal transducers and activator of transcription and MAPK signaling pathways. Our conclusion is that interleukin-11 may function as an osteopromotive cytokine, stimulating the osteoblastic differentiation of periodontal ligament cells through stimulating the synthesis of type 1 collagen and possibly, to some extent, by the induction of TIMP-1 production.

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