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Transforming growth factor-β stimulates Interleukin-11 production by human periodontal ligament and gingival fibroblasts

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Abstract

Background: Transforming growth factor (TGF)-β is a potent multifunctional polypeptide, abundant in the bone matrix. Interleukin (IL)-11 is a pleiotropic cytokine with effects on multiple cell types. The present study was performed to evaluate the regulatory effects of TGF-β on IL-11 production by human periodontal ligament cells (PDL) and human gingival fibroblasts (HGF).

Material and Methods: The expression of TGF-β receptor in PDL and HGF were observed using flow cytometry. PDL and HGF were stimulated with TGF-β with or without protein kinase C (PKC) inhibitors and activator. IL-11, bone morphogenetic protein-2 (BMP-2) and TGF-β mRNA expression was quantified by real-time polymerase chain reaction (PCR). IL-11 production was measured using enzyme-linked immunosorbent assay.

Results: PDL and HGF expressed both TGF-β receptor I and TGF-β receptor II on the cell surfaces. IL-11 mRNA expression and IL-11 production were augmented by TGF-β in both PDL and HGF, with higher values in PDL. PKC inhibitors partially suppressed TGF-β-induced IL-11 production in PDL and HGF, whereas activator enhanced it. TGF-β mRNA and BMP-2 mRNA expression were up-regulated by TGF-β in PDL.

Conclusion: These results suggest that PDL produce IL-11 in response to TGF-β.

Key words: interleukin-11; periodontal fibroblasts; protein kinase C

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Interleukin-11 (IL-11) is a pleiotropic member of the IL-6-type cytokine family that mediates its biological activities via binding to a multimeric receptor complex that contains gp130 molecules (Kishimoto et al. 1995). Cellular and molecular components involved in the destruction of periodontal tissues are predominantly host derived. The balance of T-helper (Th)1 cytokines and Th2 cytokines is important in disease progression. IL-6 is a Th2 cytokine elevated in the periodontitis tissue, but the roles of IL-11 in periodotitis is still poorly understood (Berglundh & Donati 2005). IL-11 has many biological activities and has roles in haematopoiesis, immune responses, the nervous system and bone metabolism (Paul et al. 1990, Du & Williams 1997). IL-11 induces bone resorption by enhancing osteoclast formation in vitro (Girasole et al. 1994). In contrast, IL-11 also stimulates osteoblasts in vitro and bone formation in vivo. Overexpression of human IL-11 gene in transgenic mice results in the stimulation of bone formation (Takeuchi et al. 2002). IL-11 acts synergistically with bone morphogenetic protein-2 (BMP-2) to accelerate bone formation (Suga et al. 2001, 2003, Suga et al. 2004). IL-11 produced in human gingival fibroblasts (HGF) stimulated with butyric acid is involved in the attenuation of T-cell apoptosis by HGF (Kurita-Ochiai et al. 2002). IL-1α and tumour necrosis factor (TNF)-α stimulate HGF to produce IL-11 and the production is mainly mediated by internal prostaglandin E_2 (PGE_2) synthesis (He et al. 2004). IL-11 is a candidate molecule for therapeutic modulation of the host response in the management of periodontal diseases (Trepicchio et al. 1996, Martuscelli et al. 2000, Kinane & Attstrom 2005, Salvi & Lang 2005). IL-11 production by periodontal ligament cells (PDL) and HGF might be important for the homeostasis of alveolar bone, but so far the molecular mechanisms of IL-11 production by these cells have not been elucidated.

Bone homeostasis is regulated by osteoclastic bone resorption and osteoblastic bone formation. Osteoclasts and

osteoblasts are tightly regulated by each other (Rodan & Martin 1981, Suda et al. 1992). Osteoblasts are fibroblastic cells of mesenchymal origin, and osteoclasts differentiate from the monocyte/macrophage lineage cells (Katagiri & Takahashi 2002). Recently, receptor activator of nuclear factor κ B ligand (RANKL) was identified as an important factor involved in osteoclast development by osteoblasts (Lacey et al. 1998, Yasuda et al. 1998b). Expression of RANKL enhances osteoclast differentiation from osteoclast precursors, and its decoy receptor osteoprotegerin (OPG) suppresses osteoclast differentiation through the inhibition of cognate interaction between osteoblasts and osteoclast precursors (Simonet et al. 1997, Yasuda et al. 1998a). Periodontal ligament is the unique tissue connecting alveolar bone and tooth cementum. PDL and gingival fibroblasts (HGF) are two major fibroblasts of mesenchymal origin in periodontal tissue. PDL have similar functions as osteoblasts, as they regulate osteoclast differentiation through the expression of RANKL and OPG (Kanzaki et al. 2001). On the contrary, HGF produce large amounts of OPG, but rarely express RANKL (Nagasawa et al. 2002). Although the regulation of osteoclast differentiation by osteoblastic cells has been extensively studied, little is known about the regulation of osteoblast function by osteoclasts or macrophage lineage cells.

Transforming growth factor β (TGF-β) is produced by various cells including macrophages, as well as non-immune cells like gingival fibroblasts, in the periodontal tissue (Wahl et al. 1993, Cotrim et al. 2002). TGF-β is abundant in the bone matrix, and is released from the matrix during bone resorption (Seyedin et al. 1986). Macrophage cell lines secrete TGF-β and enhance alkaline phosphatase activity in osteoblastic cells (Champagne et al. 2002). TGF-β might be a candidate molecule for monocyte/macrophage lineage cells to regulate osteoblast functions (Champagne et al. 2002).

The aim of the present study was to investigate the regulatory effects of TGF-β on IL-11 production by both PDL and HGF.

Material and Methods

Reagents

Recombinant human TGF-β1 was purchased from Techne (Minneapolis, MN, USA). Stock solution of TGF-β1 (2 μg/ml) was reconstituted in 4 mM HCl containing 1 mg/ml bovine serum albumin according to the manufacturer's instructions and stored at -20°C until use. Flourosene isothiocyanate (FITC)-conjugated rabbit polyclonal antibody against human TGF-β receptor I, receptor II and FITC-conjugated normal rabbit Immunoglobulin G (IgG) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Staurosporine streptomyces (ST) was purchased from Sigma Chemicals (St Louis, MO, USA). Myristoylated protein kinase C (PKC) (Myr) peptide inhibitor and phorbol 12-myristate 13-acetate (PMA) were purchased from Promega (Madison, WI, USA). Stock solutions of ST (400 mM), Myr (4 mM) and PMA (30 μM) were prepared in dimethyl sulphoxide and stored at -20°C until use.

Cell culture

HGF were isolated from six systemically healthy patients (three females and three males, aged 21–29 years old, mean 30.5 ± 8.5) who had no sites with clinical attachment loss greater than 2 mm and no radiographic bone loss. Six healthy gingival samples were collected during routine crown lengthening surgery. The washed explants were placed in a sterile dish and minced into smaller pieces with a sterile scalpel blade. Attempts were made to remove the epithelium and leave only connective tissue. PDL were isolated from the teeth of six systemically healthy patients (four females and two males, 17–29 years old, mean 21.8 ± 4.7). These clinically healthy pre-molar teeth were extracted for orthodontic reasons. The mid-root surfaces of the teeth were scraped lightly with a sterile scalpel blade. Before starting, the study protocol was approved by the Ethics Committee of Tokyo Medical and Dental University. Informed consent was obtained from all 12 subjects, after verbal and written explanation regarding the nature of the study. All cell lines were prepared as described previously (Hayashi et al. 1994). Fibroblastic cells were allowed to grow out from the explant at 37°C in a humidified atmosphere with 5% CO_2 in the air until they formed a confluent layer, at which point they were subcultured. Cells subcultured to the fifth passage were used for these experiments.

Cell stimulation

PDL and HGF were seeded in 24-well culture plates at 1×10^5 cells per well, and were grown to confluence. Once confluent, the fibroblasts were cultured with or without 2 ng/ml TGF-β1 or an equal volume of vehicle. RNA was extracted from the cultured cells.

In addition, PDL and HGF were seeded in 96-well flat-bottomed culture plates at 1×10^5 cells per well, and were grown to confluence. Once confluent, the fibroblasts were stimulated with TGF-β1 with or without PKC inhibitors, 400 nM ST and 4 μM Myr or an equal volume of vehicle. In some experiments, PDL and HGF were stimulated with PKC activator, 30 nM PMA. After 24 h, the supernatants were collected.

Enzyme-linked immunosorbent assay (ELISA)

IL-11 in the cultured supernatants was measured using commercially available ELISA kits (IL-11, R&D Systems Inc., Minneapolis, MN, USA), according to the manufacturer's instructions.

RNA extraction and real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Cultured HGF and PDL were washed three times with PBS, and RNA-Bee solution (Tel-Test, Inc. Friendswood, TX, USA) was added to the culture dish. RNA was extracted with phenol and chloroform, precipitated with isopropanol, washed with 75% ethanol and suspended in diethylpyrocarbonate-treated distilled water. Samples containing 5 μg of RNA were used for RT-PCR. First-strand cDNA was synthesized using a kit (Super-Script II First-Strand cDNA Synthesis Kit, Invitrogen, Grand Island, NY, USA). Real-time PCR analyses were performed in a fluorescent temperature cycler (LightCycler, Roche Molecular Biochemicals, Mannheim, Germany). For IL-11 and BMP-2, LightCycler-Primer Sets (Roche Molecular Biochemicals) were used according to the manufacturer's instructions. For TGF-β the primers were designed on the basis of previously described sequences (Champagne et al. 2002). The following primers were used:

TGF-β1 primer R, 5′-GCCCTGGACACCAACTATTGCT;

TGF-β1 primer F, 5-AGGCTCCAAATGTAGGGGCAGG.

One-tenth (2 μl) of each RT reaction served as template in a 20 μl PCR reaction mixture containing 1.5 mM $MgCl_2$, 0.5 μM of each primer and 1 U LightCycler DNA Master SYBR Green I mix (Roche Molecular Biochemicals). After initial denaturation at 94°C for 30 s, reactions were cycled 35 times using the following parameters for detection: 95°C for 1 s, primer annealing at 61°C for 7 s and primer extension at 72°C for 11 s. SYBR Green I fluorescence was detected at the end of each cycle to monitor the amount of PCR product formed during that cycle. At the end of each run, melting curve profiles were analysed (cooling the sample to 68°C and heating slowly to 95°C with continuous measurement of fluorescence) to confirm amplification of specific cDNA.

Detection of TGF-β receptor in PDL and HGF

In our preliminary study, we could not detect cell surface receptors on cells detached by using trypsin-EDTA. Confluent cultures of PDL and HGF were mechanically detached by using cell scrapers so as to avoid possible proteolytic destruction of cell surface antigens. The cells were then centrifuged and fixed with 1% para-formaldehyde in phophate-buffered solution (PBS) for 30 min. They were washed with PBS supplemented with 2% fetal bovine serum (FBS) (PBS/FBS) and centrifuged at 400 × *g* for 10 min. after each step. The cells were incubated for 30 min. with an FITC-conjugated rabbit polyclonal antibody against human TGF-β receptor I and receptor II (Santa Cruz Biotechnology Inc.). FITC-conjugated normal rabbit IgG (Santa Cruz Biotechnology Inc.) was used as a negative control. The expression of TGF-β receptors was observed using flow cytometry (Nagasawa et al. 2002).

Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) using StatView. Fisher's protected least significance test was used for the post hoc comparison of specific groups.

Results

Expression of TGF-β receptors on PDL and HGF

4.1 ± 1.1% of PDL expressed TGF-β receptor I and 5.3 ± 0.2% of PDL expressed TGF-β receptor II on the cell surfaces. 5.6 ± 0.2% of HGF expressed TGF-β receptor I and 4.8 ± 2.3% of HGF expressed TGF-β receptor II on the cell surfaces.

Time course of IL-11 mRNA and BMP-2 mRNA expression in PDL stimulated with TGF-β

In the preliminary experiments, different concentrations of TGF-β were used to stimulate PDL and HGF (0.02, 0.2, 2 and 10 ng/ml TGF-β), and optimal response was observed at ≥ 2 ng/ml of TGF-β (data not shown). An increase of IL-11 mRNA in PDL was apparent in 6 h, with the addition of 2 ng/ml TGF-β. The level of IL-11 mRNA peaked between 6 and 12 h and then returned to a baseline level by 24 h (Fig. 1). Similarly, an increase of BMP-2 mRNA was apparent in 6 h with the addition of TGF-β and the level of BMP-2 mRNA peaked between 6 and 12 h and then returned to a baseline level by 24 h (Fig. 1).

Effect of TGF-β on IL-11 mRNA expression in PDL and HGF

Real-time PCR analysis showed that IL-11 mRNA expression was significantly up-regulated by TGF-β in both PDL and HGF (6 h after stimulation). The IL-11 mRNA was significantly higher in PDL than in HGF in both stimulated and non-stimulated conditions (Fig. 2a).

Effect of TGF-β on IL-11 production in PDL and HGF

PDL produced significantly higher amounts of IL-11 than HGF in either stimulated or non-stimulated conditions (Fig. 2b).

Effect of TGF-β1 on TGF-β1 mRNA expression in PDL and HGF

TGF-β mRNA expression was significantly up-regulated by TGF-β in PDL (Fig. 3).

The up-regulation of TGF-β mRNA was not statistically significant in HGF (Fig. 3).

Effect of PKC inhibitors on IL-11 production in PDL and HGF stimulated with TGF-β1

Staurosporine streptomyces (ST) and myristoylated (Myr) PKC significantly suppressed IL-11 production in PDL stimulated with TGF-β1 (Fig. 4a). The effect of the inhibitors was 31.9% and 37.4%, respectively. Staurosporine streptomyces (ST) and (Myr) PKC significantly suppressed IL-11 production by HGF stimulated with TGF-β (Fig. 4a). The effect of the inhibitors was 65.4% and 53.4%, respectively.

Effect of PKC activator on IL-11 production in PDL and HGF

PMA significantly enhanced IL-11 production in PDL, but the amount of IL-11

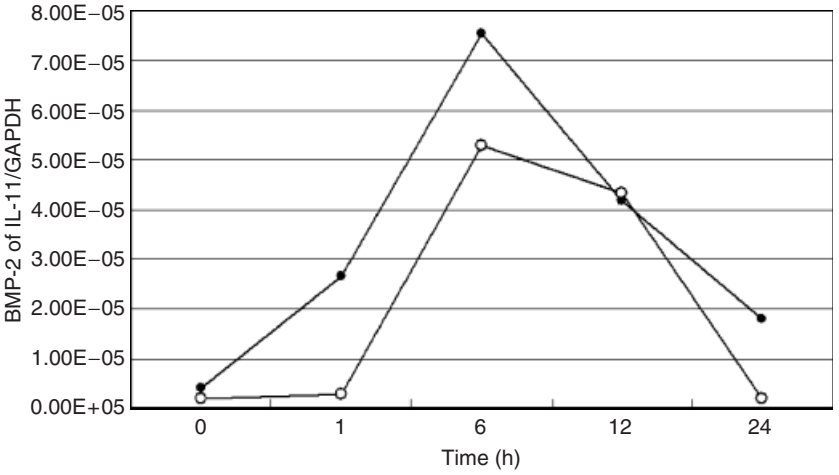

Fig. 1. Time course of Interleukin (IL)-11 mRNA and bone morphogenetic protein-2 (BMP-2) mRNA expression in periodontal ligament cell (PDL) stimulated with transforming growth factor (TGF)-β PDL was cultured in the presence of 2 ng/ml TGF-β. RNA harvested at 0, 1, 6, 12, 24 h, and reverse transcriptase polymerase chain reaction (RT-PCR) was performed by real-time PCR to evaluate IL-11 and BMP-2 mRNA expression. An increase of IL-11 mRNA and BMP-2 mRNA was apparent in 6 h after the addition of TGF-β. Open and closed circles indicate the increase of IL-11 mRNA (●) and BMP-2 mRNA (○), respectively. Data are representative of three separate experiments.

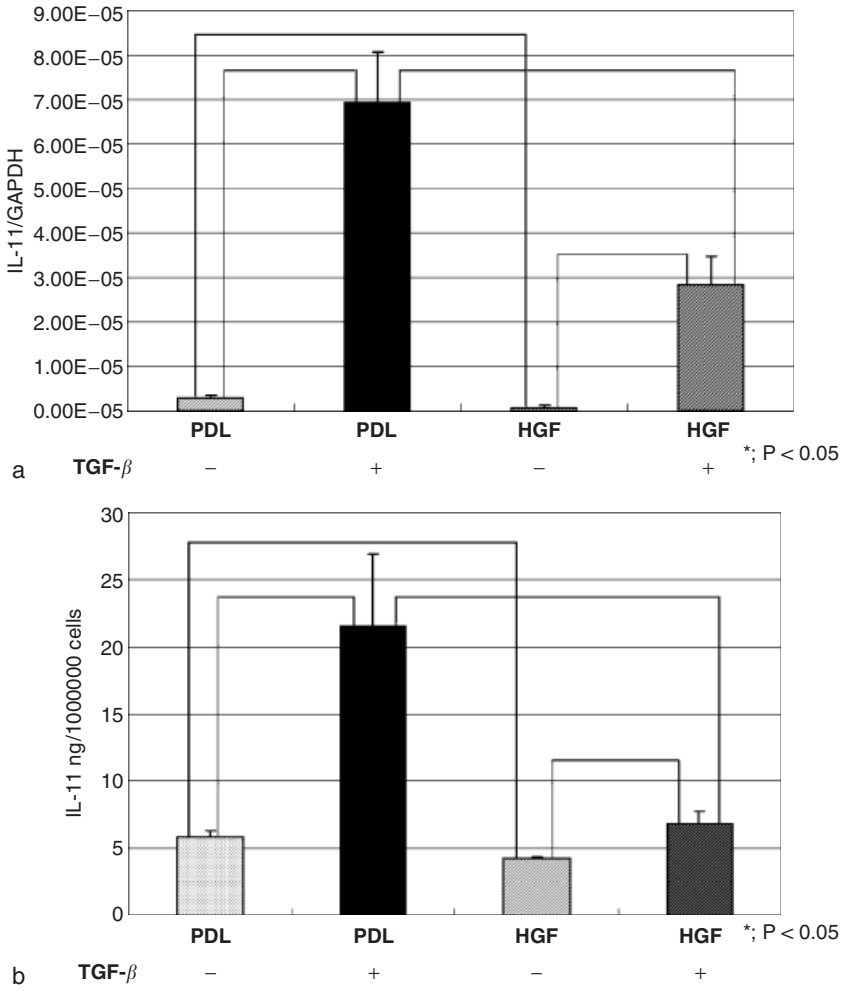

Fig. 2. (A) Effect of transforming growth factor (TGF)-β on Interleukin (IL)-11 mRNA expression in PDL and human gingival fibroblasts (HGF). The cells were stimulated with 2 ng/ml TGF-β for 6 h. After incubation, RNA was extracted and reverse transcriptase polymerase chain reaction (RT-PCR) was performed. IL-11 mRNA expression was evaluated by real-time PCR, as described in Material and Methods. Values shown are mean ± SD. Data are representative of three separate experiments. *Significantly different from control ($p < 0.05$). (B) Effect of TGF-β on IL-11 production in PDL and HGF. The cells were stimulated with 2 ng/ml TGF-β for 24 h. After incubation, IL-11 levels in the culture media were determined by enzyme-linked immunosorbent assay, as described in Materials and Methods. Values shown are mean ± SD. Data are representative of three separate experiments. *Significantly different from control ($p < 0.05$).

was less than that after TGF-β stimulation (Fig. 4b). The percent of the TGF-β response obtained with PMA alone was 45.3%. PMA-enhanced IL-11 production in HGF. The amount of IL-11 was also less than that after TGF-β stimulation (Fig. 4b). The percent of the TGF-β response obtained with PMA alone was 29.1%.

Discussion

In this study, TGF-β augmented IL-11 mRNA expression, resulting in the enhancement of IL-11 production. IL-11 mRNA was constitutively expressed in PDL and HGF, and it was significantly higher in PDL.

TGF-β is an important local regulator of metabolism (Centrella et al. 1994), and is produced by various kinds of cells including macrophages, as well as mesenchymal fibroblastic cells. Two classes of receptors (type I and type II) have been identified as signal-transducing receptors for TGF-β (Yamashita et al. 1996). We confirmed that TGF-β RI and RII were expressed on both PDL and HGF. Expression of TGF-β receptors on HGF and PDL suggests that PDL and HGF might be regulated by TGF-β released from the matrix during bone resorption or produced by various cells including macrophages in the periodontal tissue. It has been demonstrated using flow cytometry that both PDL and HGF express TGF-β RI and TGF-β RII, although in only a portion of the total cells (Parkar et al. 2001). In our results, percentage of the positive cells was lower than their report. It might be because of the different antibodies used. Cells expressing a low amount of TGF-β receptors might be counted as negative cells in our study.

Although a large amount of TGF-β is released from the bone matrix or produced by various cells including macrophages in the periodontal tissue, it is difficult to determine the physiological TGF-β concentration in periodontal tissue. It has been reported in vivo that 0.05 μg of TGF-β plus bone marrow cells induced bone formation in rabbit skull defect (Tabata et al. 2000). Our dose of TGF-β (2 ng/ml) is lower than their study, and the concentration might be closer to the physiological concentration.

IL-11 mRNA expression was significantly higher in PDL than in HGF in both TGF-β-stimulated and non-stimulated conditions (Fig. 2a). The production of IL-11 was also significantly higher in PDL than in HGF in both TGF-β-stimulated and non-stimulated conditions (Fig. 2b), suggesting that the production of IL-11 by PDL was transcriptionally regulated.

Somerman et al. (1988) compared PDL and HGF derived from the same patient, same passage, in vitro. They found greater protein and collagen production and higher alkaline phosphatase levels in PDL than in HGF. Although all the six lines showed similar responses in the present study, we should take into consideration that the PDL and HGF were derived from different individual. There is considerable heterogeneity within each group of gingival or periodontal ligament fibroblasts cell lines (Hassell & Stanek 1983, Dongari-Bagtzoglou et al. 1997).

Earlier reports indicate that IL-11 augments osteoclastogenesis in vitro (Hill et al. 1998, Yasuda et al. 1998b). It has been repoted that IL1-α and TNF-α stimulate HGF to produceIL-11 (He et al. 2004). IL-11 produced by IL-1-stimulated HGF might augment osteoclastogenesis. However, gingival concentrations of IL-11 were highest within gingiva adjacent to

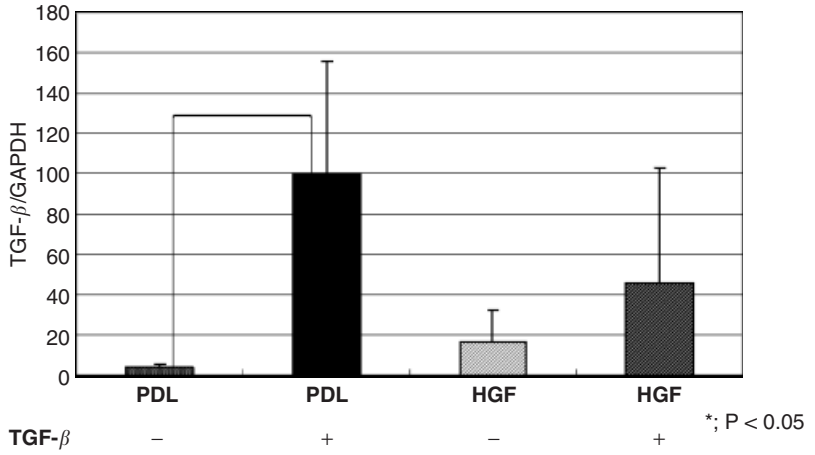

Fig. 3. Effect of transforming growth factor (TGF)-β on the expression of TGF-β mRNA in PDL and human gingival fibroblasts (HGF). The cells were stimulated with 2 ng/ml TGF-β for 6 h. After incubation, RNA was extracted and reverse transcriptase polymerase chain reaction (RT-PCR) was performed. TGF-β mRNA expression was evaluated by real-time PCR, as described in Material and Methods. Values shown are mean ± SD. Data are representative of three separate experiments. *Significantly different from control ($p < 0.05$).

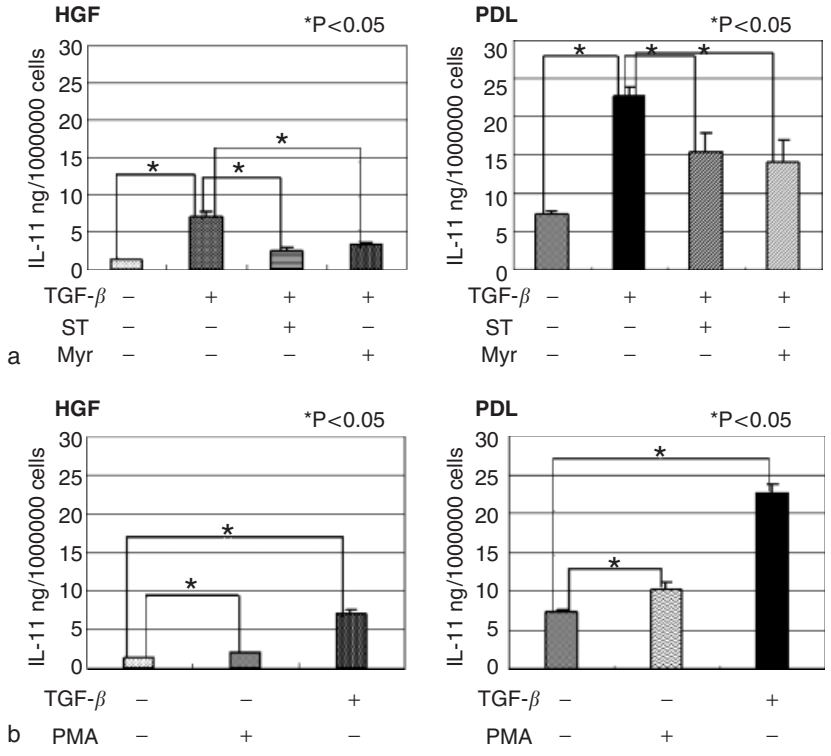

Fig. 4. (a) Effect of protein kinase C (PKC) inhibitors staurosporine streptomyces (ST) and myristoylated PKC (Myr) on transforming growth factor (TGF)-β-stimulated Interleukin (IL)-11 production in periodontal ligament cells (PDL) and human gingival fibroblasts (HGF). PDL and HGF were cultured with or without 2 ng/ml TGF-β1 and PKC inhibitors (400 nM ST and 4 μM Myr) were simultaneously added. After 24 h, supernatants were collected and enzyme-linked immunosorbent assay was performed. Values shown are mean ± SD. Data are representative of three separate experiments. *Significantly different from control ($p < 0.05$). (b) Effect of PKC activator phorbol 12-myristate 13-acetate (PMA) on TGF-β-stimulated IL-11 production in PDL and HGF. PDL and HGF were cultured with or without 2 ng/ml TGF-β or PKC activator (30 nM PMA). After 24 h, supernatants were collected and enzyme-linked immunosorbent assay was performed. Values shown are mean ± SD. Data are representative of three separate experiments. *Significantly different from control ($p < 0.05$).

3 mm diseased pockets and gingival concentrations of IL-11 were significantly lower in gingiva adjacent to 6 mm pockets (Johnson et al. 2004). They suggest that the elevated concentration of IL-11 is a consequence of changes in the gingival microenvironment during early stages of gingival inflammation. The lower IL-11 concentration within gingiva adjacent to 6 mm pockets could minimize the potential protective effect of IL-11. IL-11 has been reported to down-regulate synthesis of TNF-α and IL-1 β in vitro, which are potent proinflammatory cytokines (Trepicchio et al. 1996). In a ligature-induced beagle dog model, subcutaneous injection of IL-11 slowed down the progression of attachment and radiographic alveolar bone loss (Martuscelli et al. 2000), suggesting that IL-11 might ameliorate inflammatory response in the gingival tissue.

Several investigators reported the involvement of IL-11 in osteoblast differentiation and bone formation in vivo and in vitro. Transgenic mice expressing the IL-11 gene exhibited enhanced ratios of bone volume/tissue volume and increased bone formation rates, indicating that IL-11 positively affects bone formation (Takeuchi et al. 2002). IL-11 has been observed to induce osteoblastic differentiation of C3H10T1/2 mouse mesenchymal progenitor cells, to act synergistically with BMP-2, and to be an early acting mediator of bone formation (Suga et al. 2001). These cytokines acted synergistically on bone formation in vivo, in a rat ectopic model (Suga et al. 2003) and rabbit model (Suga et al. 2004).

In this study, increase of BMP-2 mRNA was apparent at 6 h with the addition of TGF-β in PDL, and this was similar to the increase of IL-11 mRNA stimulated with TGF-β1 (Fig. 1). PDL might augment bone formation through the combined production of IL-11 and BMP-2. IL-11 and BMP-2 might act synergistically on osteoblasts. Furthermore, TGF-β mRNA expression was up-regulated in PDL stimulated with TGF-β (Fig. 3). This suggests that autocrine TGF-β production may further augment IL-11 induction.

PKC-dependent activities have been found to participate in TGF-β1-induced gene expression (Halstead et al. 1995, Mucsi et al. 1996). For example, TGF-β activates PKC to induce collagen I expression in mesangial cells (Runyan et al. 2003). In PDL stimulated with TGF-β, both PKC inhibitors ST and Myr suppressed IL-11 production

(Fig. 4a), suggesting that the production of IL-11 was ,at least in part, mediated by PKC. The PKC activator PMA enhanced IL-11 production, suggesting that PKC activation directly augmented IL-11 production (Fig. 4b) in PDL. However, other pathways might regulate IL-11 production, as the inhibitors did not completely inhibit IL-11 production, nor did the PDL stimulated with PKC activators secrete comparable amounts of IL-11 to TGF-β stimulation.

In conclusion, PDL produced higher levels of IL-11 than HGF in response to TGF-β. Simultaneous production of IL-11 and BMP-2 by PDL might be a pathway for bone formation by periodontal ligament fibroblasts stimulated with TGF-β.

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Clinical Relevance

Scientific rationale: Periodontal ligament is involved in bone and cementum formation in periodontal tissue. IL-11 has anti-inflammatory properties and augments bone formation collaborating with BMP-2. TGF-β suppresses the osteoblast-supported osteoclast differentiation. However, little is known about the effects of TGF-β on IL-11 production in periodontal ligament cells, and this needs to be investigated.

Principal findings: IL-11 mRNA expression and IL-11 production were augmented by TGF-β in both PDL and HGF, with higher values in PDL.

Practical implications: TGF-β might stimulate periodontal ligament fibroblasts to ameliorate inflammation and regenerate alveolar bone through the production of IL-11.