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Interactions of regenerative, inflammatory and biomechanical signals on bone morphogenetic protein-2 in periodontal ligament cells

M. Nokhbehsaim[1,2], B. Deschner[1,3], J. Winter[2], C. Bourauel[1,4], B. Rath[5], A. Jäger[1,3], S. Jepsen[1,2], J. Deschner[1,2]

[1]Clinical Research Unit 208, Center of Dento-Maxillo-Facial Medicine, University of Bonn, Bonn, Germany, [2]Department of Periodontology, Operative and Preventive Dentistry, Center of Dento-Maxillo-Facial Medicine, University of Bonn, Bonn, Germany, [3]Department of Orthodontics, Center of Dento-Maxillo-Facial Medicine, University of Bonn, Bonn, Germany, [4]Department of Oral Technology, Center of Dento-Maxillo-Facial Medicine, University of Bonn, Bonn, Germany and [5]Department of Orthopedics, University of Regensburg, Regensburg, Germany

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Background and Objective: Regeneration of periodontal tissues by EMD remains a major challenge because a number of modifying factors are as yet unknown. The effects of EMD seem to be mediated, at least in part, by bone morphogenetic protein-2 (BMP-2). This *in vitro* study was performed to examine whether the effects of EMD on BMP-2 activity are modulated by inflammatory and/or biomechanical signals.

Material and Methods: Periodontal ligament cells were seeded on BioFlex® plates and exposed to EMD under normal, inflammatory or biomechanical loading conditions for 1 and 6 d. In order to mimic proinflammatory or biomechanical loading conditions *in vitro*, cells were stimulated with interleukin-1β (IL-1β), which is increased at inflamed periodontal sites, and cyclic tensile strain of various magnitudes, respectively. The synthesis of BMP-2, its receptors (BMPR-1A, BMPR-1B and BMPR-2) and its inhibitors (follistatin, matrix gla protein and noggin) were analyzed using real-time RT-PCR and ELISA.

Results: In EMD-treated cells, BMP-2 synthesis was increased significantly at 1 d. EMD also induced the expression of all BMP receptors, and of the BMP inhibitors follistatin and noggin. In general, IL-1β and biomechanical loading neither down-regulated BMP-2 nor up-regulated BMP inhibitors in EMD-stimulated cells. However, IL-1β and biomechanical loading, when applied for a longer time period, caused a down-regulation of EMD-induced BMP receptors.

Conclusion: EMD induces not only BMP-2, but also its receptors and inhibitors, in PDL cells. IL-1β and biomechanical forces may counteract the beneficial effects of EMD on BMP-2 activity via the down-regulation of BMP receptors.

James Deschner, DMD, PhD, Clinical Research Unit 208, Department of Periodontology, Operative and Preventive Dentistry, Center of Dento-Maxillo-Facial Medicine, University of Bonn, Welschnonnenstrasse 17, 53111 Bonn, Germany
Tel: +49 (0)228 287 22650
Fax: +49 (0)228 287 22081
e-mail: james.deschner@uni-bonn.de

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Regeneration of periodontal tissues remains to date a major challenge in periodontal therapy. Although a number of periodontal regenerative treatment approaches have been described and are currently performed, the outcome of regenerative therapy is still difficult to predict. One reason for this unsatisfactory situation is the fact that

a number of modifying factors, which may have an impact on cell behavior and cellular responses to bioactive molecules, are as yet unknown.

One treatment approach to attain regenerative periodontal healing comprises the application of EMD during periodontal surgery. A number of *in vitro* studies on periodontal ligament (PDL) cells have revealed that EMD promotes cell proliferation, synthesis of growth factors and matrix molecules, cell attachment and mineralization (1). The potential of EMD for periodontal regeneration has also been demonstrated clinically (2,3). Several studies have suggested that the beneficial effects of EMD on periodontal regeneration are mediated, at least in part, by growth and differentiation factors, such as bone morphogenetic protein-2 (BMP-2) and transforming growth factor-β (TGF-β). This assumption is based on the fact that fractions of EMD contain BMP-like and TGF-β-like activities and that TGF-β1 is up-regulated by EMD (4–7).

BMPs belong to the TGF-β supergene family and exert a great number of different effects, including induction of bone and cartilage formation. After secretion and cleavage, BMPs can bind to the extracellular matrix, their antagonists, co-receptors or transmembrane serine/threonine kinase receptors, which results in transcriptional and nontranscriptional responses (8). The actions of BMPs are tightly regulated by natural inhibitors, such as follistatin, matrix gla protein (MGP) and noggin. These BMP antagonists can bind to BMPs and thereby inhibit the binding of BMPs to their signaling receptors. Moreover, inhibitors of BMPs are regulated by BMPs themselves, which represents a local feedback mechanism to control the activities of BMPs (9,10). Preclinical and clinical studies have demonstrated that BMPs induce the expression of osteogenic proteins and promote the regeneration of bone and periodontal tissues, including cementum (11–14).

The beneficial actions of EMD on periodontal cells may be jeopardized by the local cellular environment, such as inflammation caused by an inadequate control of the microflora. In addition, the periodontium represents load-bearing tissues, and teeth affected with periodontitis are often subject to comparatively high biomechanical forces during mastication or functional dental habits. However, the role of biomechanical signals in the response of periodontal cells to bioactive molecules has been very much neglected. A broader and deeper understanding of the interactions among regenerative molecules, inflammatory mediators and biomechanical forces is essential to improve the outcome of currently applied regenerative treatment approaches in periodontally diseased patients. This *in vitro* study was performed to examine whether the beneficial effect of EMD on BMP-2 is modulated by inflammatory and/or biomechanical signals.

Material and methods

Cell culture

PDL cells from six periodontally healthy donors, who had to undergo extraction of teeth for orthodontic reasons, were used. Approval of the Ethics Committee of the University of Bonn and informed parental consent were obtained. Cells dissected from the mid-third portion of the roots were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM; Invitrogen®, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (Invitrogen®), 100 units of penicillin and 100 μg/mL of streptomycin (Biochrom®, Berlin, Germany) at 37°C in a humidified atmosphere of 5% CO_2. Cells between passages 3 and 5 were seeded (50,000 cells/well) on BioFlex® collagen-coated culture plates (Flexcell International, Hillsborough, NC, USA) and grown to 80% confluence. One day before the experiments were started, the fetal bovine serum concentration was reduced to 1%. The medium was changed every other day during the course of the experiment.

In order to mimic regenerative, inflammatory, or biomechanical loading conditions *in vitro*, cells were stimulated with EMD (0.1 mg/mL; Straumann AG, Basel, Switzerland), interleukin-1β (IL-1β, 1 ng/mL; Calbiochem, San Diego, CA, USA), or equibiaxial cyclic tensile strain (CTS) of low (3%, CTSL) and high (20%, CTSH) magnitudes at a rate of 0.05 Hz, respectively. For the application of biomechanical forces to cells, a strain device developed at the University of Bonn was used. This system has already been used for the application of static strain (15,16). Briefly, the BioFlex® culture plates were positioned in such a way that posts were centered directly beneath the flexible-bottom wells of the plates. By cyclic upward and downward movements of a moving table, which was located directly above the culture plate, the flexible membrane of each well was pulled over the posts, which caused the cells grown on the flexible membrane to be dynamically stretched. In the present study, PDL cells were exposed to EMD, IL-1β and CTS, and to their combinations (Table 1). In order to unravel the intracellular mechanisms of BMP-2 regulation, cells were pre-incubated with specific inhibitors of MEK1/2, p38 and SMA- and MAD-related protein (SMAD) 1/5/8 signaling (U0126, SB203580 and dorsomorphin, respectively, all purchased from Calbiochem) 1 h before the start of the experiments.

Real-time RT-PCR

RNA was extracted using a Qiagen® RNA extraction kit (Qiagen®, Hilden, Germany) and reverse transcribed using an iScriptTM Select cDNA Synthesis kit (Bio-Rad, Munich, Germany) at 42°C for 90 min and then at 85°C for 5 min. The expression of *BMP2*, its receptors (*BMPR-1A*, *BMPR-1B* and *BMPR-2*), its natural regulators (follistatin, *MGP* and noggin) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were analyzed by real-time RT-PCR using the iCycler iQ detection system (Bio-Rad), SYBR Green (Qiagen®) and specific primers (QuantiTect Primer Assay; Qiagen®). One microliter of cDNA was amplified as a template in a 25-μL reaction mixture containing 12.5 μL of 2× QuantiFast SYBR Green PCR Master Mix (Qiagen®), 2.5 μL of primers and deionized water. The mixture was heated initially at 95°C for 5 min, which was followed

Table 1. Treatment regimens employed to study the regulation of bone morphogenetic protein-2 (BMP-2), and its receptors and inhibitors, by EMD, interleukin-1β (IL-1β), cyclic tensile strain of low (CTSL) and high (CTSH) magnitudes, and their combinations

Groups	Target molecules		
	BMP-2	BMP receptors	BMP inhibitors
Control	X	X	X
EMD	X	X	X
IL-1β	X		X
CTSL	X		X
CTSH	X		X
EMD + IL-1β	X	X	X
EMD + CTSL	X	X	X
EMD + CTSH	X	X	X
IL-1β + CTSL	X		
IL-1β + CTSH	X		
EMD + IL-1β + CTSL	X		
EMD + IL-1β + CTSH	X		

by 40 cycles of denaturation at 95°C for 10 s and combined annealing/extension at 60°C for 30 s. Data were analyzed using the comparative threshold cycle method (17).

ELISA

The concentration of BMP-2 in cell supernatants was measured using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions, and a microtiter plate reader (PowerWave x; BioTek Instruments, Winooski, VT, USA) at 450 nm. Data were normalized by cell number.

Statistical analysis

Statistical analysis of the data was performed using spss 17.0 software (SPSS Inc., Chicago, IL, USA). For quantitative analysis, mean values and standard errors of the mean were calculated. In order to test for significant ($p < 0.05$) differences between groups, the Student's *t*-test, analysis of variance, and the *post-hoc* Dunnett's and Tukey's tests were applied.

Results

Regulation of BMP-2 by EMD, IL-1β and biomechanical loading

As EMD has been shown to support regeneration of lost alveolar bone, we studied the effect of EMD on the expression of *BMP2*, a morphogen endowed with the striking prerogative to initiate the induction of bone formation. One day after application, EMD had significantly up-regulated the expression of *BMP2* mRNA (2.4-fold; $p < 0.05$), whereas the expression of *BMP2* mRNA was only slightly increased by IL-1β (1.5-fold; $p > 0.05$) (Fig. 1A). As the PDL is subjected to biomechanical loading during chewing and functional habits, we also studied the influence of biomechanical loading on the expression of *BMP2* mRNA. Although CTSL exerted no significant effect, CTSH caused a significant up-regulation of *BMP2* mRNA (2.6-fold; $p < 0.05$) (Fig. 1A).

When cells were exposed to EMD or to CTSL for 6 d, the constitutive expression of *BMP2* was decreased nearly two-fold to 59% of the control, but this was not statistically significant. By contrast, IL-1β, and again CTSH, induced a significant up-regulation of *BMP2* (2.4- and 1.9-fold, respectively; $p < 0.05$) at 6 d (Fig. 1B). The stimulation of BMP2 by EMD at 1 d and the EMD-induced inhibition of BMP2 at 6 d were also observed at the protein level, as analyzed using an ELISA (Fig. 1C).

Next, we examined how the EMD-induced expression of *BMP2* is modulated by IL-1β and/or biomechanical loading. At 1 d, IL-1β significantly enhanced the EMD-induced expression of *BMP2* (3.4-fold; $p < 0.05$). Similarly, a further increase in the EMD-upregulated expression of *BMP2* was caused by CTSL (1.4-fold; $p > 0.05$) and was even more pronounced by CTSH (4.0-fold; $p < 0.05$). Interestingly, the strongest expression of *BMP2* was found in cells that were exposed to a combination of EMD, IL-1β and CTSH (15.2-fold of control; $p < 0.05$) (Fig. 1D).

At 6 d, the expression of *BMP2* in EMD-treated cells was increased by IL-1β (7.6-fold; $p < 0.05$), CTSL (2.1-fold; $p > 0.05$) and CTSH (6.1-fold; $p < 0.05$). After 1 d, the strongest expression of *BMP2* was observed for EMD-treated cells in the presence of IL-1β and CTSH (14.6-fold of control; $p < 0.05$) (Fig. 1E). The stimulatory and magnitude-dependent effects of biomechanical forces on BMP-2 in EMD-treated cells were also found at the protein level (Fig. 1C,F).

Pre-incubation of cells with a specific MEK1/2 inhibitor (U0126) significantly reduced the increase in *BMP-2* expression caused by EMD, IL-1β and the combination of EMD and IL-1β, by 29, 48 and 67%, respectively, at 1 d (Fig. 1G). Furthermore, the pretreatment of cells with a specific p38 inhibitor (SB203580) abrogated the stimulation of *BMP2* by IL-1β, either alone or in combination with EMD, by 78 and 88%, respectively (Fig. 1G). However, a specific inhibitor of SMAD 1/5/8 signaling did not interfere with the actions of EMD and/or IL-1β on *BMP2* (data not shown).

As the EMD-induced expression of *BMP2* was further increased by IL-1β and/or biomechanical loading, we next studied the interactions of these two stimulants. After 1 d, IL-1β had slightly stimulated (by 1.2- to 1.5-fold; $p > 0.05$) the expression of *BMP2* in stretched and unstretched cells. The strongest expression of *BMP2* was observed in cells exposed simultaneously to IL-1β and CTSH (2.8-fold; $p < 0.05$) (Fig. 2A). At 6 d, the highest levels of *BMP2* mRNA were measured in IL-1β-stimulated cells (2.4-fold; $p < 0.05$) in comparison with CTSL- (0.6-fold; $p > 0.05$) and CTSH- (1.9-fold; $p < 0.05$) treated cells. CTSL and CTSH slightly enhanced (by 1.2- and

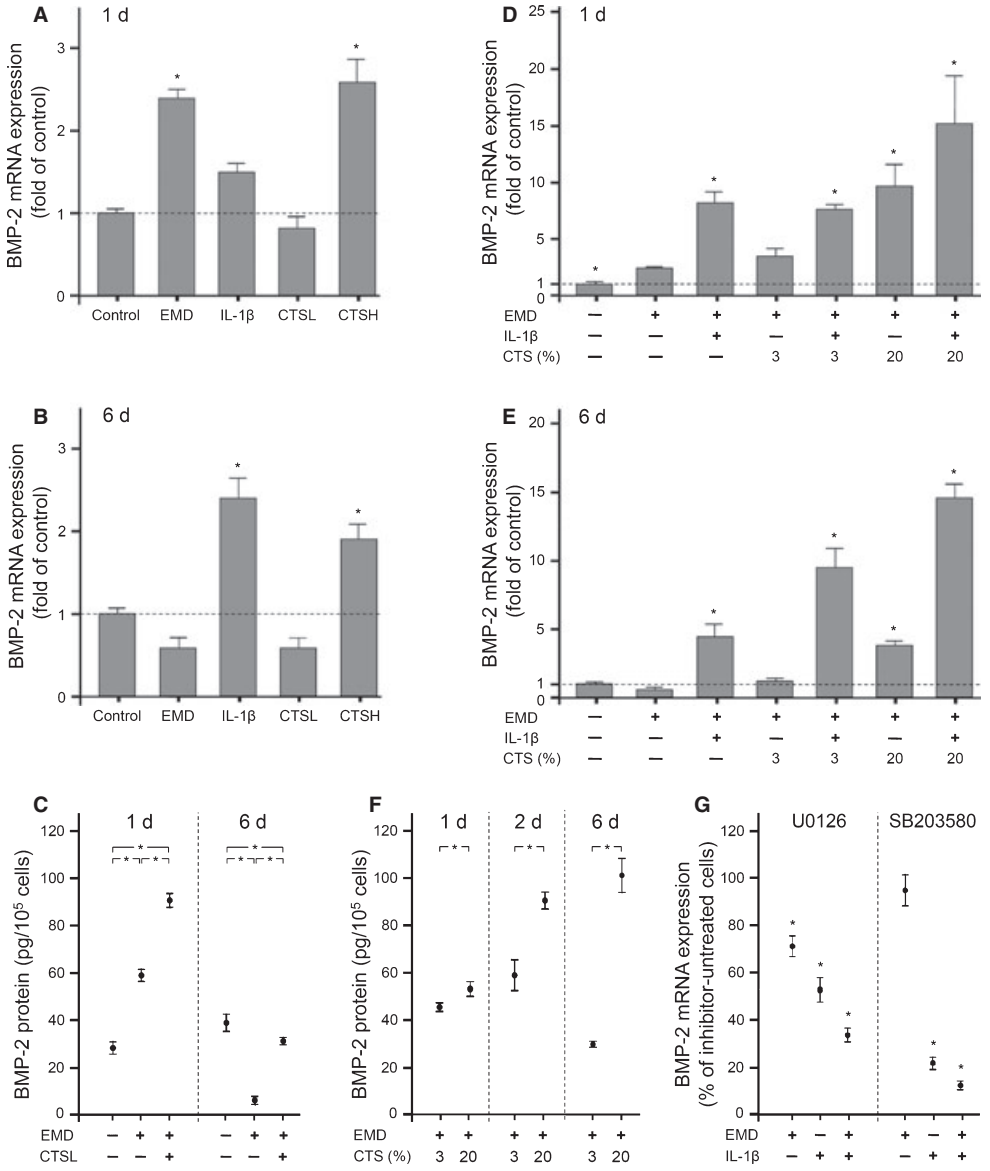

Fig. 1. Effect of EMD, interleukin-1β (IL-1β) and biomechanical forces [cyclic tensile strain of low magnitude (CTSL), 3%; and cyclic tensile strain of high magnitude (CTSH), 20%] on bone morphogenetic protein-2 (*BMP2*) mRNA expression in periodontal ligament (PDL) cells at 1 d (A) and 6 d (B). Regulation of BMP-2 protein synthesis by EMD in the presence and absence of CTSL at 1 and 6 d (C). Influence of IL-1β and/or biomechanical forces (CTS) of 3 and 20% on the EMD-induced expression of *BMP2* mRNA at 1 d (D) and 6 d (E). BMP-2 protein synthesis in EMD-treated cells subjected to CTS of 3 and 20% at 1, 2 and 6 d (F). Effect of U0126, a specific MEK1/2 inhibitor, and of SB203580, a specific p38-MAPK inhibitor, on the expression of *BMP2* mRNA in cells exposed to EMD and/or IL-1β at 1 d (G). *Significantly different from control (A,B), significantly different from EMD-treated cells in the absence of IL-1β and CTS (D,E), significant difference between groups (C,F), or significantly different from cells treated in the absence of the inhibitor (G).

1.4-fold, respectively; $p > 0.05$) the IL-1β-induced expression of *BMP2* (Fig. 2B).

Taken together, these data show that BMP-2 is not only up-regulated by regenerative molecules such as EMD, but also by proinflammatory and biomechanical signals. Both IL-1β and biomechanical strain enhanced, rather than inhibited, the expression of *BMP2* in EMD-treated cells.

Regulation of receptors for BMP-2 by EMD, IL-1β and biomechanical loading

The biological effects of BMP-2 depend on the availability of its receptors, which might also be subject to regulation by regenerative, inflammatory and biomechanical signals. PDL cells showed constitutive expression of *BMPR-1A*, *BMPR-1B* and *BMPR-2*. At the 1-d time-point, *BMPR-1A* and *BMPR-1B* were not significantly regulated by EMD, alone or in combination with IL-1β, CTSL

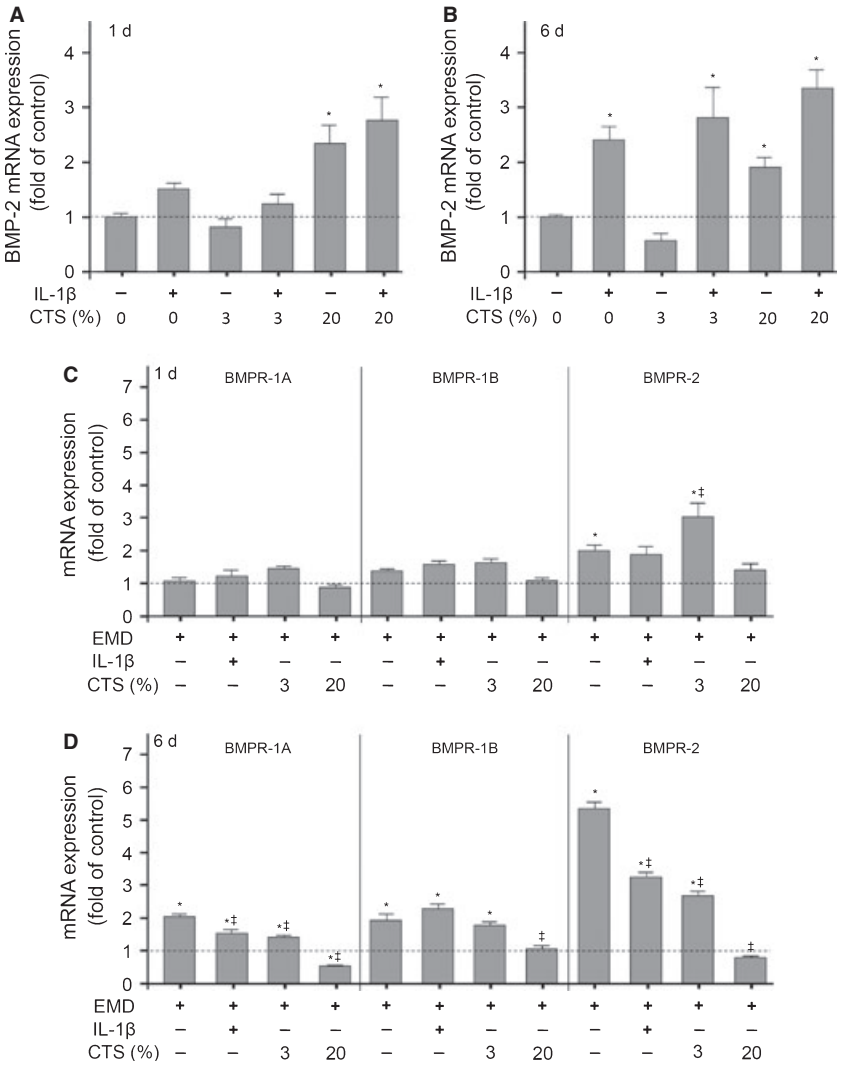

Fig. 2. Interactions of interleukin-1β (IL-1β) and/or of biomechanical forces [cyclic tensile strain (CTS)], of low (3%) and high (20%) magnitudes, on bone morphogenetic protein-2 (*BMP2*) mRNA expression in periodontal ligament (PDL) cells at 1 d (A) and 6 d (B). Effect of EMD in the presence and absence of IL-1β or CTS of 3 and 20% on the mRNA expression of BMP receptors (*BMPR-1A*, *BMPR-1B* and *BMPR-2*) at 1 d (C) and 6 d (D). *Significantly different from control (A–D); ‡significantly different from EMD-treated cells in the absence of IL-1β and CTS (C,D).

or CTSH (Fig. 2C). By contrast, *BMPR-2* was significantly increased (2.0-fold; $p < 0.05$) by EMD. Although no additional effect was observed when cells were simultaneously exposed to IL-1β, CTSL significantly enhanced (by 1.5-fold; $p < 0.05$) and CTSH slightly decreased (to 70%; $p > 0.05$) the EMD-induced expression of *BMPR-2* in PDL cells at 1 d (Fig. 2C). In general, the lowest expression of *BMPR-1A*, *BMPR-1B* and *BMPR-2* was found when EMD-treated cells were exposed to CTSH.

At the 6-d time-point, EMD had stimulated a significant increase in the levels of expression of *BMPR-1A* (2.0-fold; $p < 0.05$), *BMPR-1B* (1.9-fold; $p < 0.05$) and *BMPR-2* (5.4-fold; $p < 0.05$) (Fig. 2D). Interestingly, the EMD-stimulated expression of *BMPR-1A* and *BMPR-2* was significantly ($p < 0.05$) decreased by IL-1β to 75 and 61%, respectively, by CTSL to 70 and 50%, respectively, and by CTSH to 27 and 15%, respectively. The levels of *BMPR-1B* in EMD-treated cells were significantly reduced only by CTSH (to 56%; $p < 0.05$) (Fig. 2D).

Taken together, these findings suggest that the stimulatory effects of IL-1β and biomechanical loading on *BMP2* expression are antagonized by the inhibitory influences of these signals on the BMP receptors in EMD-treated cells at 6 d.

Regulation of inhibitors of BMP-2 actions by EMD, IL-1β and biomechanical loading

Next, we examined whether expression of the natural inhibitors of BMP actions are influenced by EMD, IL-1β and/or biomechanical loading. Interestingly, EMD caused a significant up-regulation of follistatin (1.5-fold; $p < 0.05$) and noggin (1.7-fold; $p < 0.05$), and a significant down-regulation of *MGP*, to 65% of the control, at 1 d (Fig. 3A–C). Similar results were observed at 6 d (Fig. 3D–F). Neither IL-1β nor biomechanical forces increased significantly the expression of follistatin, *MGP* or noggin, at any time-point. In general, their effect on these molecules was inhibitory (Fig. 3A–F). The strongest inhibition (to 3% of the control; $p < 0.05$) was found for the effect of IL-1β on the expression of *MGP* at 6 d (Fig. 3E).

We then studied whether the EMD-stimulated expression of follistatin and noggin was modulated by IL-1β and/or biomechanical loading. CTSL significantly ($p < 0.05$) decreased the EMD-induced expression of follistatin and noggin to 56 and 57%, respectively, at 1 d and to 68 and 66%, respectively, at 6 d (Fig. 4A–D). Moreover, CTSH caused a reduction in the EMD-stimulated expression of follistatin and noggin to 57 and 60%, respectively, at 6 d ($p < 0.05$) (Fig. 4B,D). While IL-1β up-regulated (1.5-fold; $p < 0.05$) the expression of noggin in EMD-treated cells at 1 d, no such stimulatory effect was observed at 6 d (Fig. 4C,D). Furthermore, IL-1β did not exert any significant effect on the expression of follistatin in EMD-stimulated cells at either time-point (Fig. 4A,B).

Taken together, these data demonstrate that EMD up-regulates the natural inhibitors of BMPs and,

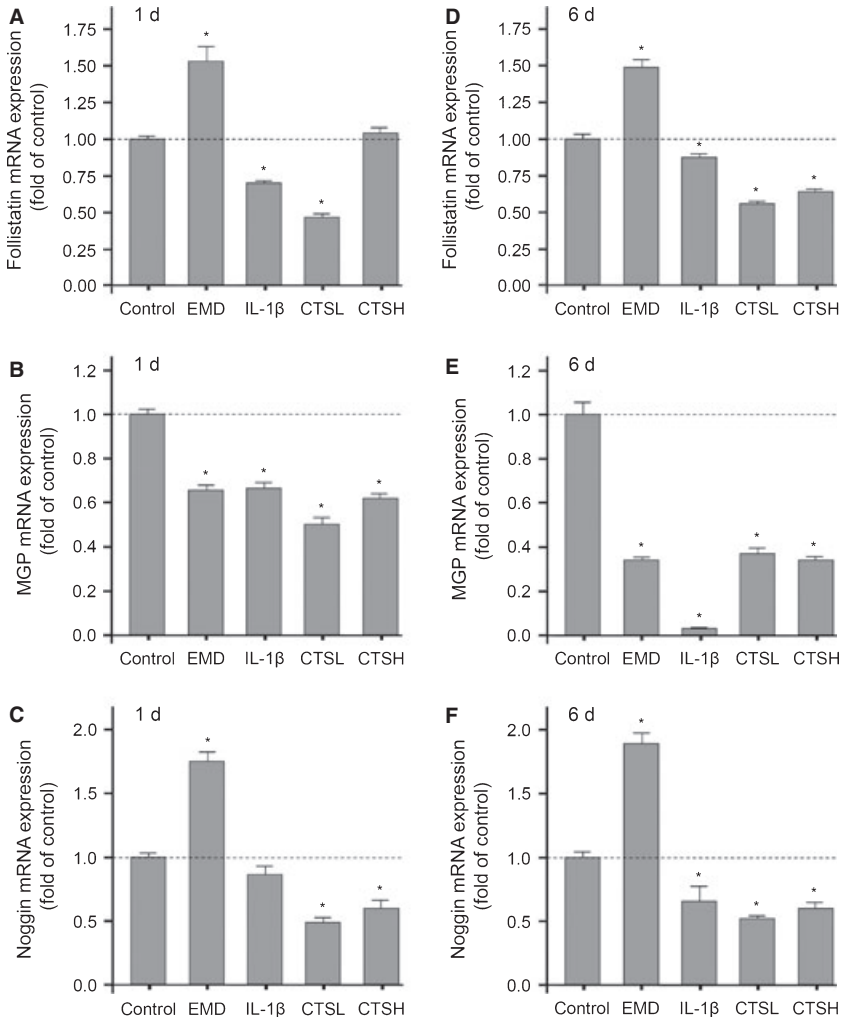

Fig. 3. Effect of EMD, interleukin-1β (IL-1β) and biomechanical forces [cyclic tensile strain of low magnitude (CTSL), 3%; and cyclic tensile strain of high magnitude (CTSH), 20%] on the expression of follistatin mRNA in periodontal ligament (PDL) cells at 1 d (A) and 6 d (D), on the expression of matrix gla protein (*MGP*) mRNA at 1 d (B) and 6 d (E), and on the expression of noggin mRNA at 1 d (C) and 6 d (F). *Significantly different from control (A–F).

additionally, that these osteoinhibitory effects of EMD are in general not enhanced by IL-1β or by biomechanical loading.

Discussion

To our knowledge, this study shows, for the first time, that EMD not only induces BMP-2, but also its receptors and inhibitors, in PDL cells. Furthermore, our experiments revealed that the effects of EMD on BMP-2, and on its receptors and inhibitors, are modulated by IL-1β and by biomechanical loading.

EMD has been shown to promote periodontal regeneration by stimulating PDL cell proliferation and osteogenic differentiation, as well as the synthesis of growth factors and matrix molecules (1). As the EMD-stimulated mineralization was reduced by IL-1β and biomechanical strain in our previous experiments (Nokhbehsaim M, Deschner B, Bourauel C, Reimann S, Winter J, Rath B, Jäger A, Jepsen S, Deschner J., unpublished data), and BMP-2 is closely involved in bone formation, we speculated whether IL-1β and biomechanical strain would interfere with the possible effects of EMD on BMP-2. In the present study, EMD stimulated the expression and synthesis of BMP-2. Interestingly, the expression of *BMP2* in EMD-treated cells was enhanced under inflammatory and biomechanical loading conditions, suggesting that the inhibitory effects of inflammatory and biomechanical signals on the EMD-stimulated mineralization are not mediated via the down-regulation of *BMP2*.

Few studies have focused on the effect of proinflammatory mediators on the expression of *BMP2* (18–20). Although the synthesis of BMP-2 was up-regulated in response to IL-1β in synovial fibroblasts, which is in accordance with our results, BMP-2 was down-regulated by IL-1β in human alveolar bone cells and osteosarcoma cell lines. These findings indicate that the regulatory effect of IL-1β on the production of BMP-2 may depend on the cell type (18–20).

Very little is also known about the biomechanical regulation of BMP-2 in PDL cells (21,22). Wescott *et al.* (21) examined the effect of cyclic tensile strain at a magnitude of 12% on PDL cells and found an up-regulation of BMP-2, which was also demonstrated in another study and supports our findings, despite different strain regimens (22).

Although the effects of EMD have been widely studied, the signal transduction pathways involved in the actions of EMD are only partially disclosed. EMD has been shown to activate the ERK1/2, p38 and SMAD pathways in a variety of cell types (23–26). In our experiments, EMD up-regulated *BMP-2* via MEK1/2 signaling, but not via p38 or SMAD signaling, which emphasizes the role of ERK1/2 in the effects induced by EMD on PDL cells.

BMP-2 binds to BMPR-1, upon which BMPR-2 is recruited into the complex, or binds to a preformed complex of BMPR-1 and BMPR-2, which leads to activation of SMAD and non-SMAD pathways (8). In the present study, PDL cells expressed receptors for BMP-2, which is in accordance with findings by other investigators (27,28). However, to our knowledge, this is the first study to report the induction of BMP receptors by EMD. Biomechanical forces and IL-1β, when applied for a longer time-period, caused a down-regulation of EMD-induced BMP receptors, suggesting that IL-1β and biomechanical strain may counter-

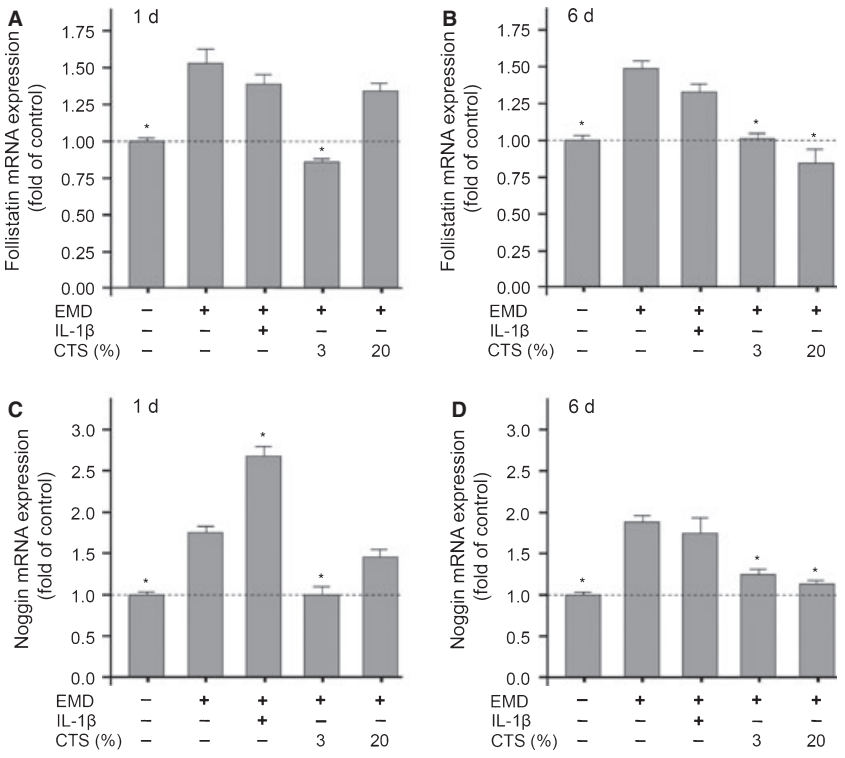

Fig. 4. Influence of EMD in the presence and absence of interleukin-1β (IL-1β) or biomechanical forces [cyclic tensile strain (CTS)], of low (3%) and high (20%) magnitudes on the expression of follistatin mRNA at 1 d (A) and 6 d (B) and on the expression of noggin mRNA at 1 d (C) and 6 d (D). *Significantly different from EMD-treated cells in the absence of IL-1β and CTS (A–D).

regulate their stimulatory effect on BMP-2 by the down-regulation of BMP receptors.

We also examined the effects of EMD, IL-1β and biomechanical loading on the expression of BMP inhibitors in PDL cells. While EMD up-regulated noggin and follistatin, it caused a significant down-regulation of *MGP*. These findings are novel and may indicate a possible mechanism whereby EMD controls hard-tissue formation. Except for a significant up-regulation of the EMD-stimulated expression of noggin by IL-1β at 1 d, neither IL-1β nor biomechanical forces increased the constitutive or EMD-induced expression of BMP inhibitors. In general, their effects on noggin, follistatin and *MGP* were inhibitory. Our findings demonstrate that EMD not only induces BMP-2, but also BMP inhibitors, and that their expression in EMD-treated cells is regulated by inflammatory and biomechanical signals.

In the present study, EMD was used to simulate regenerative conditions *in vitro*, and the concentration applied was based on the results from other studies (29,30). As in previous experiments, IL-1β was used to mimic an inflammatory environment *in vitro* because the levels of this proinflammatory cytokine are increased at inflamed periodontal sites (15,31,32). In order to simulate biomechanical loading conditions *in vitro,* cells were exposed to cyclic tensile strain. The strain regimens, which were chosen in our experiments, have also been used in PDL cells by other investigators (33).

In our experiments, BMP-2 was induced by EMD at 1 d. BMP-2 can stimulate its own synthesis and regulate the expression of its receptors and inhibitors (34,35). It is therefore possible that the regulatory effects of EMD on the BMP receptors and inhibitors observed in our experiments were mediated, at least in part, by BMP-2. Interestingly, EMD does not seem to increase the levels of BMP-2 in osteoblasts, which is in contrast to our findings in PDL cells (36). It is therefore conceivable that EMD exerts a different osteogenic effect on PDL cells vs. osteoblasts.

In the present study, *BMP2* expression was up-regulated by two- to three-fold by EMD, IL-1β or biomechanical loading. Similarly, the effect of these regenerative, inflammatory and biomechanical signals on the expression of BMP-2 inhibitors and receptors was also moderate. However, when cells were exposed simultaneously to EMD, IL-1β or biomechanical loading, the expression of *BMP2* was increased by up to 15-fold relative to the control. Furthermore, the effects of biomechanical loading were partially magnitude-dependent. Compared with low biomechanical forces, high forces caused a greater up-regulation of BMP-2 and a stronger down-regulation of its receptors in EMD-stimulated cells. In addition, low biomechanical forces inhibited the EMD-induced expression of follistatin and noggin, whereas high forces did not exert a significant effect on these molecules in EMD-treated cells at 1 d. It remains to be elucidated whether the magnitude of effects observed in our study is sufficient to be clinically significant.

In summary, our study showed that EMD not only induces BMP-2, but also its receptors and inhibitors, in PDL cells. Furthermore, IL-1β and biomechanical strain up-regulated BMP-2 in EMD-treated cells and, in most cases, down-regulated BMP inhibitors. In addition, IL-1β and biomechanical loading, when applied for a longer time-period, caused a down-regulation of EMD-induced BMP receptors. Within the limits of this study, we conclude that EMD stimulates the expression of *BMP-2*, BMP receptors and BMP inhibitors in PDL cells. Moreover, IL-1β and biomechanical forces may counteract the beneficial effects of EMD on BMP-2 activity via the down-regulation of BMP receptors.

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