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IL-1β Favors Osteoclastogenesis Via Supporting Human Periodontal Ligament Fibroblasts

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ABSTRACT

The balance between bone formation and bone resorption in inflammatory diseases is often disturbed. Periodontitis, a chronic inflammation of the tooth gums, leads to unwanted bone loss as a response to inflammatory compounds such as interleukin-1β (IL-1β). This excessive bone loss reflects an increased osteoclast formation and activity. Osteoclast formation is a multistep process driven by osteoclastogenesis supporting cells such as periodontal ligament fibroblasts. The inflammatory factors can induce osteoclastogenesis, probably also by affecting the periodontal ligament fibroblast. In this study we investigated how pre-culture of periodontal ligament fibroblasts with IL-1β affected osteoclastogenesis. Fibroblasts were pre-cultured with IL-1β and/or dexamethasone, a commonly used anti-inflammatory compound, before being co-cultured with peripheral blood mononuclear cells (PBMCs). Pre-culture with IL-1β (1–100 ng/ml) resulted in an increased number of adhered PBMCs as well as an increased mRNA expression of intercellular adhesion molecule-1 (ICAM-1), macrophage colony stimulating factor (M-CSF) and IL-1β. Pre-culture with IL-1β also caused retraction of fibroblasts and an augmented formation of $TRACP^{+}$ multinucleated cells. Our data suggest that stimulation of fibroblasts with IL-1β has a long-lasting effect, leading to a significantly increased osteoclastogenesis. These results provide new insights for understanding excessive bone loss in periodontitis. J. Cell. Biochem. 112: 1890–1897, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: IL-1β; OSTEOCLASTOGENESIS; PERIODONTAL LIGAMENT FIBROBLASTS; CELL–CELL INTERACTION

Inflammation near bony sites, such as in periodontitis, often coincides with increased osteoclast-mediated bone resorption and a favored osteoclast formation. Multinucleated osteoclasts are formed through differentiation and fusion of hematopoietic mononuclear cells. These osteoclast precursors are recruited from the monocyte population and interact with supporting osteoblast-like cells [Quinn et al., 1994; Gori et al., 2000; Kondo et al., 2001] such as periodontal ligament fibroblasts (PDLF) [Fukushima et al., 2003; de Vries et al., 2006]. In this way they receive the proper signals to further differentiate towards an osteoclast. It has been shown that intercellular adhesion molecule-1 (ICAM-1) is involved in the adhesion between those two cell types [Bloemen et al., 2009, 2010]. Interestingly, only ICAM-1 expressing osteoblast-like cells were able to support osteoclastogenesis [Tanaka et al., 2000]. ICAM-1 on the osteoblast-like cell binds to its ligand leukocyte function associated antigen-1 (LFA-1) on the osteoclast precursor and receptor activator of nuclear factor-κB (RANKL) interacts with its receptor RANK on these latter cells. In conjunction with RANKL, macrophage colony-stimulating factor (M-CSF) is also an important cytokine that, through binding to its receptor c-fms, drives the osteoclast precursor towards the osteoclast lineage [Felix et al., 1990; Hofstetter et al., 1992; Ross and Teitelbaum, 2005].

After adhesion of osteoclast precursors, the osteoblast-like cells retract to create a space for the triggered osteoclast precursor which then migrates to the bone surface and fuses with other osteoclast precursors to form a multinucleated osteoclast. Direct cell–cell interaction between osteoclast precursors and osteoblast-like cells plays herein an important role [Perez-Amodio et al., 2004]. A similar retraction was apparent in co-cultures of osteoclast precursors and periodontal ligament fibroblast [Bloemen et al., 2010; Wattanaroonwong et al., 2011].

It has been demonstrated that pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis-factor alpha (TNF-α) can modulate the expression of ICAM-1 in several cell types

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[Wheller and Perretti, 1997; Yamamoto et al., 1998; Shirasaki et al., 2004; Lin et al., 2009], including osteoblast-like cells [Tanaka et al., 2000]. The effects on mRNA expression are already visible within a short period of time (h) after administration [Zachlederova and Jarolim, 2006]. Whether an increased ICAM-1 expression results in an increased adhesion of osteoclast precursors remains to be elucidated.

IL-1β induces also the expression of ICAM-1 by periodontal ligament fibroblasts [Joe et al., 2001]. Whether a short stimulation of these fibroblasts with IL-1β has an effect at a later time point and can also affect the later stages of osteoclast formation is not known. There is ample evidence that the anti-inflammatory agent dexamethasone can inhibit IL-1β-induced expression of adhesion molecules such as ICAM-1 and directly temper the expression of IL-1β itself [Tessier et al., 1996; Wheller and Perretti, 1997; Liu et al., 2000; Han et al., 2001].

In this study we investigated whether a relatively short pre-incubation with IL-1β affects osteoclast formation via its action on the periodontal ligament fibroblasts. We therefore stimulated periodontal ligament fibroblasts with IL-1β for 6 h prior to the addition of osteoclast precursors and analyzed the effects of this stimulation on (i) the adhesion of the latter cells, (ii) the mRNA expression of osteoclastogenesis-related genes at different time points, and (iii) the ultimate formation of TRACP+ multinucleated cells. In addition, we examined whether the effects of IL-1β were influenced by the presence of dexamethasone.

MATERIALS AND METHODS

CELL CULTURES

Human periodontal ligament fibroblasts were retrieved from five patients undergoing third molar extractions at the Department of Maxillofacial Surgery (Academic Centre for Dentistry (ACTA)/VU University Medical Center (VUMC)). The molars were obtained with informed consent and the use of periodontal ligament fibroblasts for this study was approved by the Medical Ethical Committee of the VU University. Fibroblasts were expanded as described previously [de Vries et al., 2006].

Peripheral blood mononuclear cells (PBMCs) were isolated from a buffy coat (Sanquin, Amsterdam, The Netherlands) according to previously described protocols [Bloemen et al., 2009].

Periodontal ligament fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Paisley, UK) containing 10% fetal calf serum (FCS, Hyclone, Logan, UT) and were seeded at passage 6 (1.5×10^4 cells per well) in a 48-well plates (Costar, Cambridge, MA) for 2 days. Thereafter, the cells were stimulated for 6 h with IL-1β (1, 10, and 100 ng/ml) in the absence or presence of dexamethasone (10^{-8} M) prior to co-culture with PBMCs. After stimulation, the different media were removed and all cell cultures were washed extensively with PBS. PBMCs (5×10^5) per well were seeded on top of the fibroblasts. The co-cultures were further cultured for an additional 3 days (RNA analysis and adhesion analysis) or 21 days (osteoclast formation) in DMEM containing 10% FCS and 1% antibiotics (100 U/ml penicillin, 100 g/ml streptomycin, and 250 ng/ml amphotericin B [Antibiotic antimyotic solution, Sigma, St. Louis, MO]). Since we previously showed that osteoclastogenesis genes are measurable in co-cultures of fibroblasts and PBMCs [Bloemen et al., 2010], only the co-culture conditions were analyzed in this study.

Cultures used for RNA analysis were washed twice with phosphate-buffered saline PBS at the end of the culture period and stored in lysis buffer (Qiagen, Hilden, Germany) containing 1% β-mercaptoethanol at −80°C until RNA extraction.

Cultures used for the quantification of adhesion were washed three times with PBS and cells were fixed in 4% formaldehyde in PBS for 10 min. Images were taken with a Leica phase contrast microscope (Leica, Wetzlar, Germany) and the PBMCs adhered to fibroblasts were counted.

RNA ANALYSIS AND REAL-TIME QUANTITATIVE PCR

RNA from cultured cells was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. One hundred nanograms RNA was used in the reverse transcriptase reaction which was performed according to the MBI Fermentas cDNA synthesis kit (Vilnius, Lithuania), using both the Oligo(dT)18 and the D(N)6 primers. A previously described protocol was used [Bloemen et al., 2009]. Porphobilinogen deaminase (PBGD) was used as a housekeeping gene. Expression of this gene was not affected by the experimental conditions. For the PCR analysis, samples were normalized for the expression of PBGD by calculating the ΔCt ($Ct_{\text{gene of interest}} - Ct_{PBGD}$) and expression of the different genes is expressed as $2^{-(\Delta Ct)}$.

Real-time PCR was performed on an ABI PRISM 7000 (Applied Biosystems, Foster City, CA). Primer sequences are listed in Table I, all PCR efficiencies were comparable.

ELISA ASSAY

Measurements of M-CSF and IL-1β protein levels were performed using highly sensitive enzyme-linked immunoassay from R&D Systems (Abingdon, United Kingdom) and Sanquin respectively according to the manufacturer's instruction. All ELISA measurements were performed on cell culture supernatants of PDLF-PBMC co-cultures.

TRACP STAINING

After 3 weeks of culture, cells were fixed in PBS buffered 4% formaldehyde and stained for TRACP activity using the leukocyte acid phosphatase kit (Sigma). Nuclei were stained with diamidino-2-phenylindole dihydrochloride (DAPI).

OSTEOCLAST QUANTIFICATION AND FIBROBLAST-FREE AREA ASSESSMENT

Micrographs were taken from five fixed areas per well. For each micrograph (i) the number of TRACP-positive multinucleated cells (cells with 3 or more nuclei) was counted and (ii) the surface area of the well devoid of fibroblasts was assessed.

TABLE I. Primers Used for Quantitative PCR

Primer	Sequence 5′ → 3′	Amplicon	Ensembl Gene ID
ICAM-1	TgAgCAATgTgCAAgAAgATAgC CCCgTTCTggAgTCCAgTACA	104	ENSG00000090339
LFA-1	gAgCTggTgggAgAgATCgA gAggCgTTgCTgCCATAgA	106	ENSG00000005844
M-CSF	CCgAggAggTgTCggAgTAC AATTTggCACgAggTCTCCAT	100	ENSG00000184371
RANK-L	CATCCCATCTggTTCCCATAA gCCCAACCCCgATCATg	60	ENSG00000120659
OPG	CTgCgCgCTCgTgTTTC ACAgCTgATgAgAggTTTCTTCgT	100	ENSG00000164761
IL-1β	CTTTgAAgCTgATggCCCTAAA AgTggTggTCggAgATTCgT	100	ENSG00000125538
PBGD	TgCAgTTTgAAATCATTgCTATgTC AACAggCTTTTCTCTCCAATCTTAgA	84	ENSG00000113721

STATISTICAL ANALYSIS

One way-ANOVA of repeated measures was performed to assess whether there was a significant difference in mRNA expression, adhesion, and osteoclast formation between groups. As a post-hoc test, a Bonferroni comparison between pairs of groups was used. A Kruskal–Wallis test followed by a Dunn's multiple comparison test was used to test significance in the protein gene expression data. In Figures 1–4 results are expressed as means ± SEM. Differences were regarded significant when $P < 0.05$.

RESULTS

IL-1β STIMULATES THE NUMBER OF ADHERED PBMCs TO PERIODONTAL LIGAMENT FIBROBLASTS

Stimulation of periodontal ligament fibroblasts with IL-1β prior to the addition of PBMCs resulted in an increase in the number of adhered PBMCs (Fig. 1A,B). A dose-dependency study revealed that all concentrations of IL-1β used (1, 10, and 100 ng/ml) significantly and dose-dependently augmented this number (Fig. 1B) (correlation analysis: $r = 0.46$, $P = 0.03$).

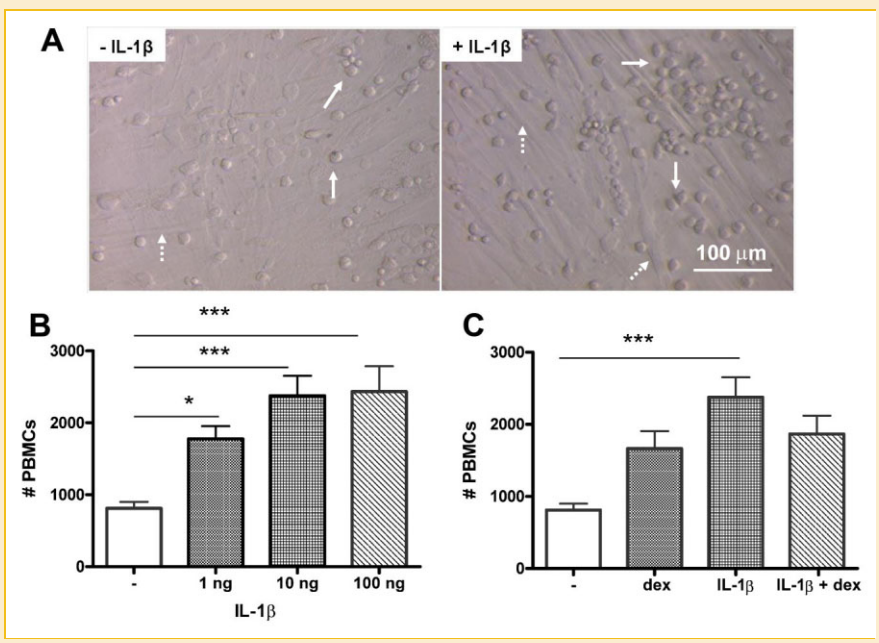

Fig. 1. IL-1β stimulates adhesion of PBMCs to periodontal ligament fibroblasts (A). After 6 h incubation with IL-1β, human periodontal ligament fibroblasts (dashed arrows) were co-cultured with peripheral blood mononuclear cells (full arrows) for 3 days. Examples of PBMCs adhered to a confluent layer of control and IL-1β primed fibroblasts are shown. More adhered PBMCs were found in the IL-1β stimulated culture compared to the control. B: Adhesion of PBMCs to periodontal ligament fibroblasts was analyzed in the presence of various IL-1β concentrations. The total number of adhered PBMCs/well is depicted. C: The effect of dexamethasone (10^{-8} M; dex), with or without IL-1β (10 ng/ml), on the number of adhered PBMCs. The total number of adhered PBMCs/well is depicted for each culture condition. Data are expressed as mean number ± SEM per 11 micrographs in co-cultures of cells from five different donors. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Comparing the number of adhered PBMCs in cultures with dexamethasone and with and without IL-1β showed that there was no significant effect of dexamethasone (Fig. 1C). Since a near maximal response was found with 10 ng/ml IL-1β, all following experiments were performed using this concentration.

EXPRESSION OF OSTEOCLASTOGENESIS-RELATED GENES BY PERIODONTAL LIGAMENT FIBROBLASTS INCREASES AFTER IL-1β STIMULATION

Effect after 6 h on periodontal ligament fibroblasts. The expression of both ICAM-1 and M-CSF was augmented up to 30-fold after IL-1β stimulation. The addition of dexamethasone had no significant effect on the expression of these genes and also no effect was found when dexamethasone was added alone.

The expression of IL-1β (Fig. 2A) or RANKL (Fig. 2B) was not significantly different in the presence of IL-1β, with or without dexamethasone.

OPG mRNA expression was induced by IL-1β and this induction was inhibited by dexamethasone (Fig. 2B).

Effect after 3 days on PDLF-PBMC co-cultures. In order to obtain insight into the priming effects of IL-1β, we also analyzed the mRNA expression of the genes described above in the co-culture after 3 days. This revealed that the IL-1β-induced increased expression of ICAM-1 seen at the 6 h time point was no longer present at the 3 days time point (Fig. 2A). ICAM-1's counterpart LFA-1 was not detectable in PDL cells and not significantly altered by the various conditions in co-cultures (data not shown). A pattern similar to the ICAM-1 induction was found for the mRNA expression of M-CSF. In contrast, at the 3 days time point expression of IL-1β was highly increased in the co-culture with IL-1β stimulated fibroblasts; the level being approximately 150-fold higher compared to the controls (Fig. 2A). Interestingly, this increased expression was completely inhibited by dexamethasone (Fig. 2A; note the different scale of the *Y*-axis of the two IL-1β graphs).

mRNA expression of RANKL was increased in the co-culture, but no additional effect of IL-1β and/or dexamethasone was found (Fig. 2B). OPG mRNA expression was strongly up-regulated In the presence of IL-1β and this was not changed by the addition of dexamethasone (Fig. 2B). Calculating the RANKL/OPG ratios for the different culture conditions revealed that this ratio was extremely low (less than 0.01 in all comparisons) and not affected by IL-1β after 6 h (mono-culture PDLF) nor after 3 days (co-culture PDLF-PBMCs). Though, this ratio was markedly higher in the co-culture of 3 days compared to the 6 h culture of periodontal ligament fibroblasts.

In addition, we investigated the effects of IL-1β and dexamethasone on the release of osteoclastogenesis-related proteins after 3 days of co-culture (Fig. 3). Dexamethasone increased M-CSF protein release whereas no significant effect of IL-1β was found. The concentration of IL-1β in conditioned medium was highly up-regulated when the periodontal ligament fibroblasts were pre-incubated with IL-1β (Fig. 3). No significant effect of dexamethasone was found on the IL-1β-induced IL-1β protein release (Fig. 3).

STIMULATION OF IL-1β CAUSES AN INCREASED RETRACTION OF PERIODONTAL LIGAMENT FIBROBLASTS AND SUBSEQUENT FORMATION OF OSTEOCLAST-LIKE CELLS

Based on the significant changes in mRNA expression found after a relatively short (6 h) incubation with IL-1β, we wondered whether this could also affect the fibroblasts at a later stage during osteoclast formation. Since it has been shown that the retraction of osteoblast-like cells is essential for the migration of osteoclast precursors to the bone surface [Perez-Amodio et al., 2004], we analyzed the surface area where the fibroblasts had retracted (fibroblast-free area) in conjunction with the number of osteoclasts formed under the different culture conditions (Fig. 4). Examples of the different cultures are shown in Figure 4A.

The 6 h pre-culture with IL-1β led to a significant increase in fibroblast-free areas after a subsequent culture period of 21 days (Fig. 4B). Concomitantly, the number of $TRACP^+$ multinucleated cells was markedly increased. When dexamethasone was added, both parameters were significantly inhibited (Fig. 4C). Further analysis by pair wise comparing fibroblast-free area (Fig. 4B) and osteoclast-like cell outcomes (Fig. 4C) revealed a positive correlation between these two parameters ($r^2 = 0.67$; $P < 0.001$).

DISCUSSION

In this study we show that a pre-culture of periodontal ligament fibroblasts with IL-1β for 6 h leads to a significant increase in the number of PBMCs adhered to the fibroblasts, suggesting that IL-1β stimulates the adhesion of the PBMCs to periodontal ligament fibroblasts. One likely candidate responsible for this adhesion is ICAM-1, a cell adhesion molecule essential for the attachment of osteoclast precursors to osteoblast-like cells [Tanaka et al., 2000]. Here we show that IL-1β stimulated the expression of ICAM-1. The observed increased expression of ICAM-1 was established within hours and is in line with data presented by others using osteoblasts [Tanaka et al., 2000] or periodontal ligament fibroblasts [Joe et al., 2001]. Once PBMCs were adhered to the fibroblasts, the enhanced expression of M-CSF due to IL-1β could then further induce osteoclast differentiation. After 3 days of co-culture no differences were observed anymore in ICAM-1 or M-CSF mRNA expression between the cultures. This indicates that the effects of IL-1β on mRNA expression of those molecules disappear over the time frame of 3 days after withdrawal of the cytokine. Together, these findings suggest that the fibroblasts can differentiate in a population that is more prone to support osteoclastogenesis under the influence of IL-1. IL-1β can thus shift the phenotype of osteoblast-like cells from one that participates in bone formation [Agarwal et al., 1998] to one that favors—albeit indirectly—bone destruction (this study).

In the present study, we confirm the high expression of OPG by periodontal ligament cells and the relatively low expression of RANKL, which was previously seen in mono-cultures of periodontal ligament fibroblasts [Hasegawa et al., 2002; Fukushima et al., 2003] and in co-cultures with PBMC [de Vries et al., 2006]. OPG expression was further significantly increased in co-cultures that included IL-1β. This, combined with the extremely low RANKL/OPG ratio in the presence of IL-1β suggests that osteoclastogenesis may occur, at

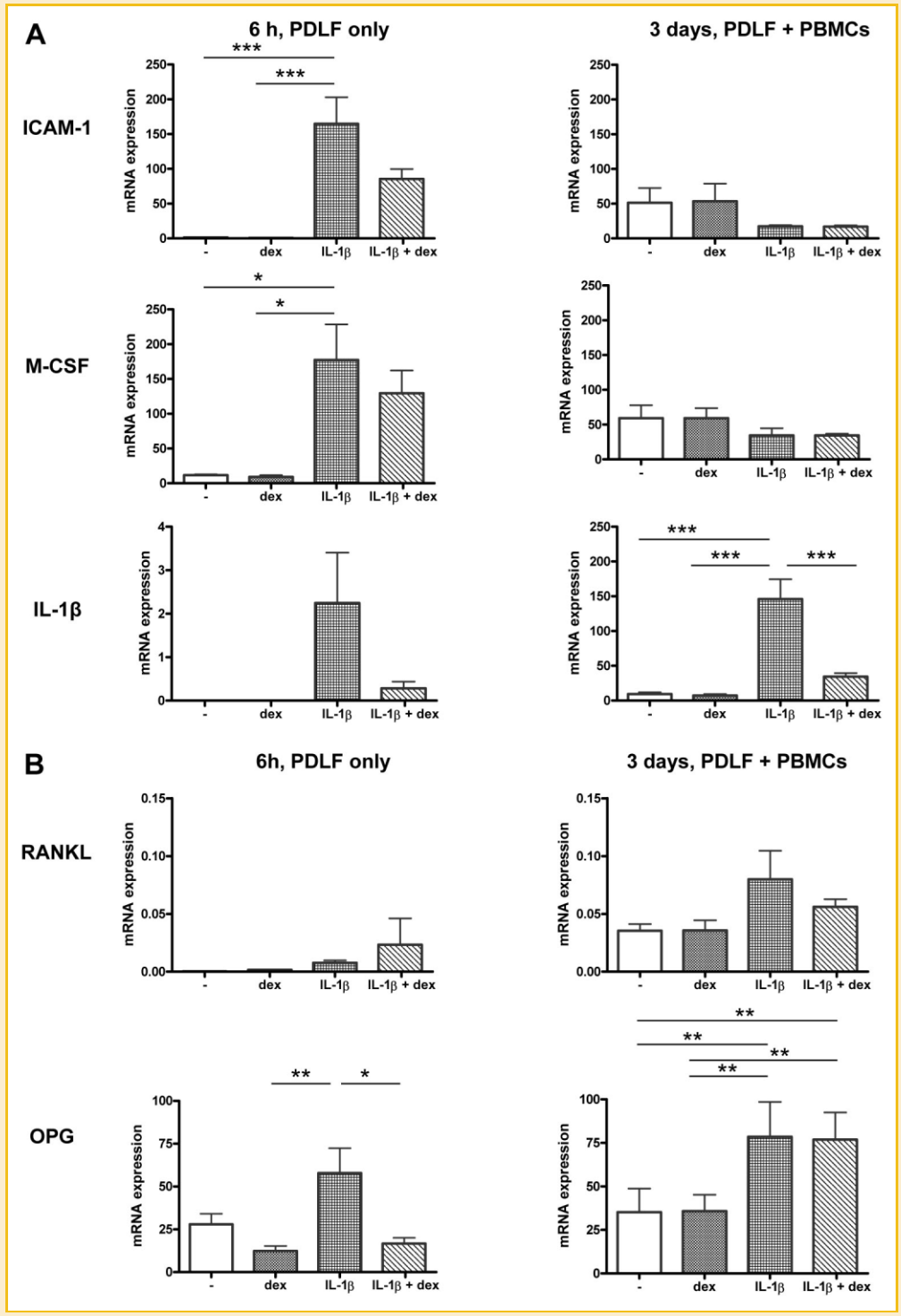

Fig. 2. mRNA expression of osteoclastogenesis related genes by periodontal ligament fibroblasts increases after IL-1β stimulation Human periodontal ligament fibroblasts were incubated for 6 h (left column) with IL-1β (10 ng/ml) and/or dexamethasone (10^{-8} M; dex) and gene expression of osteoclastogenesis related genes (A) ICAM-1, M-CSF and IL-1β and (B) RANKL and OPG was analyzed and compared to the gene expression in their subsequent co-cultures with PBMCs after 3 days (right column) of culture. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data are expressed as mean expression ± SEM in mono-cultures and co-cultures of cells from five different donors.

least in part, in a RANKL-RANK-OPG independent manner. Kim et al. [2005] demonstrated that osteoclasts could form in mice lacking RANKL or RANK where IL-1β substituted for RANKL. Further studies are needed to explore this in more detail.

IL-1β administration also significantly increased mRNA expression of the cytokine itself. This transcriptional auto-regulation of IL-1β may facilitate long term effects after a relatively short IL-1β stimulation. Here we show that this short activation of periodontal

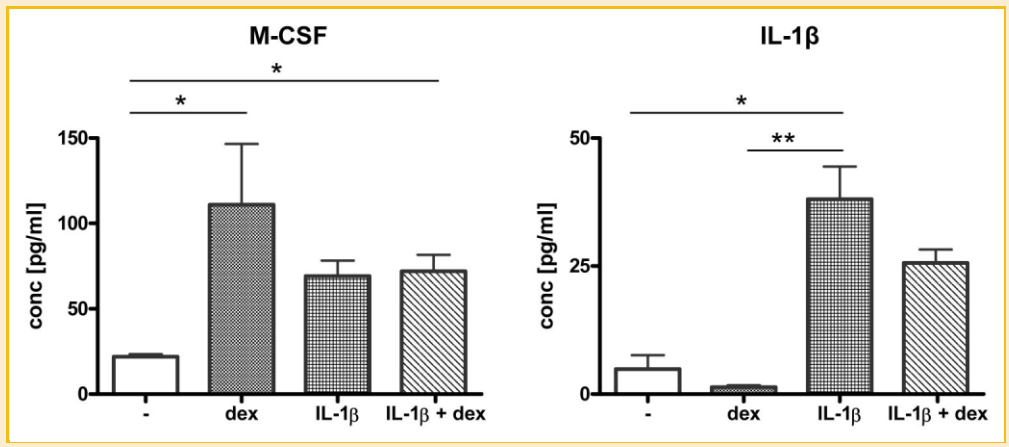

Fig. 3. M-CSF and IL-1β proteins are secreted in a co-culture of periodontal ligament fibroblasts and PBMCs Human periodontal ligament fibroblasts were pre-cultured for 6 h with IL-1β (10 ng/ml) and/or dexamethasone (10^{-8} M; dex) before co-culture with peripheral blood mononuclear cells (PBMCs). Protein expression of M-CSF and IL-1β in the supernatant was analyzed after 3 days of co-culture by ELISA and data are expressed as mean expression ± SEM in medium of cultures of cells from five different donors. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

ligament fibroblasts by IL-1β resulted in intermediate (3 days) and long term (21 days) effects facilitating osteoclast differentiation. This facilitation was reflected in an increased adhesion of precursors at 3 days, an endured effect on IL-1β and OPG gene expression at 3 days and even an increased formation of osteoclast-like cells at 21 days. The present study shows that dexamethasone can inhibit especially the long term effects (formation of osteoclast-like cells at 21 days) of the IL-1β auto-regulating signaling in periodontal ligament fibroblasts and PBMCs cultures.

At a later stage during osteoclastogenesis osteoblast-like cells retract and create a space for primed osteoclast precursors to migrate to the surface [Perez-Amodio et al., 2004]. We demonstrate that an initial stimulation with IL-1β leads to an increased fibroblast-free area at a later time point (21 days). Fibroblast migration can be

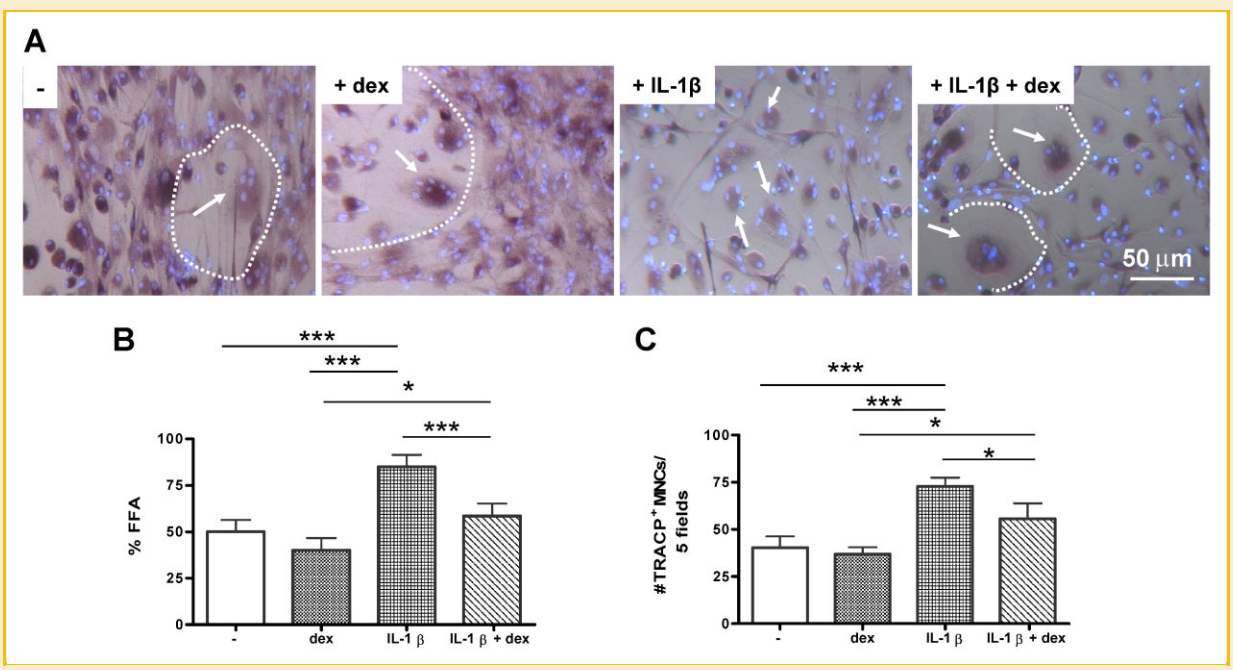

Fig. 4. IL-1β stimulated fibroblasts favor retraction and the subsequent formation of osteoclasts (A). The retraction of fibroblasts (fibroblast free areas are enclosed with a dashed line) and the subsequent formation of TRACP-positive, multinucleated cells (full arrows) was analyzed in co-cultures with fibroblasts pre-treated with or without IL-1β (10 ng/ml) and/or dexamethasone (10^{-8} M; dex). B: The surface area devoid of fibroblasts (fibroblast free areas, FFA) was analyzed under the different culture conditions (IL-1β, 10 ng/ml; dex 10^{-8} M). The surface area without fibroblasts (% FFA) was increased in the presence of IL-1β compared to controls (C). The number of TRACP-positive multinucleated cells was analyzed and expressed as average number of TRACP+ multinucleated cells per 5 random sites in the well. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data are expressed as mean ± SEM in mono-cultures and co-cultures of cells from four different donors.

induced either directly, via an effect of IL-1β on the fibroblasts themselves, or indirectly, via the PBMCs. The latter cells may exert a different response on stimulated fibroblasts and may promote migration. IL-1β-associated migration has also been proposed for other cell types [Kudo et al., 2002; Striedinger and Scemes, 2008; Lin et al., 2009]. In A549 epithelial cells, IL-1β induced the expression of matrix metalloproteinases which supported cell migration [Lin et al., 2009]. It is possible that in periodontal ligament fibroblasts IL-1β also stimulates cell migration through an increase in MMP expression since it was shown previously that (i) IL-1 stimulates the expression of MMPs by periodontal ligament fibroblasts [Nakaya et al., 1997; Rossa et al., 2005], and (ii) the retraction of osteoblast-like cells is mediated by these proteinases [Perez-Amodio et al., 2004]. In this regard MMP-3 (stromelysin-1) appears to be an attractive candidate. This enzyme can cleave homotypic interactions mediated by adhesion molecules such as E-cadherin [Noe et al., 2001] and it has been described that IL-1β increases the expression of MMP-3 by periodontal ligament fibroblasts at both mRNA and protein level [Nakaya et al., 1997].

We next examined the number of osteoclasts formed and this correlated positively with the surface area of fibroblast-free areas. Thus it appears that even such a short interaction (6 h) between the cytokine and the fibroblasts results in changes at later stages of osteoclast differentiation.

In conclusion, our data indicate that IL-1β can favor osteoclastogenesis by altering the phenotypic characteristics of the supporting periodontal ligament fibroblasts; an effect apparent already after a short and single exposure of these cells to the cytokine. This may have implications for the understanding of affected bone remodeling in periodontitis.

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