Journal of Cellular Biochemistry 98:370–382 (2006)

Gingival Fibroblasts Are Better at Inhibiting Osteoclast Formation Than Periodontal Ligament Fibroblasts

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Abstract Various studies indicate that periodontal ligament fibroblasts (PLF) have some similarities to osteoblasts, for example they have the capacity to induce the formation of osteoclast-like cells. Here, we investigated whether a second population of tooth-associated fibroblasts, gingival fibroblasts (GF), has similar osteoclastogenesis properties. PLF and GF were co-cultured with peripheral blood mononuclear cells (PBMC) in the presence and absence of dexamethasone and 1α,25dihydroxycholecalciferol (dex + vit D_3) on plastic and on cortical bone slices. Tartrate resistant acid phosphatase (TRACP) positive multinucleated cells (MNCs) were more abundant in co-cultures with PLF than in GF-PBMC co-cultures, more abundant on plastic compared to bone and more abundant in the presence of dex + vit D_3. In line with these findings was an inhibition of MNC formation and not inhibition of existing osteoclasts by medium conditioned by GF. We next investigated whether expression of molecules important for osteoclastogenesis differed between the two types of fibroblasts and whether these molecules were regulated by dex + vit D_3. OPG was detected at high levels in both fibroblast cultures, whereas RANKL could not be detected. Resorption of bone did not occur by the MNCs formed in the presence of either fibroblast subpopulation, suggesting that the fibroblasts secrete inhibitors of bone resorption or that the osteoclast-like cells were not functional. The incapacity of the MNCs to resorb was abolished by culturing the fibroblast-PBMC cultures with M-CSF and RANKL. Our results suggest that tooth-associated fibroblasts may trigger the formation of osteoclast-like cells, but more importantly, they play a role in preventing bone resorption, since additional stimuli are required for the formation of active osteoclasts. J. Cell. Biochem. 98: 370–382, 2006.

Key words: osteoclast formation; fibroblasts; dentistry; bone biology

Based on the anatomy of the soft connective tissues surrounding the teeth at least two functionally different types of fibroblasts can be distinguished: gingival fibroblasts (GF) and periodontal ligament fibroblasts (PLF). Both fibroblast populations are heterogeneous [Hassel and Stanek, 1983; Groeneveld et al., 1995] and are in close vicinity to the tooth surface as well as to the alveolar bone. GF are located supracrestally, at the most occlusal area of the tooth, just beneath the epithelium of the gums. PLF reside in the periodontal ligament space between the tooth and the alveolar bone. They are essential for the homeostasis of the periodontal ligament, a tissue responsible for anchoring the tooth in its socket [reviewed by Beertsen; McCulloch and Bordin, 1991; Beertsen et al., 1997].

Periodontal ligament fibroblasts not only play an essential role in the maintenance of the

Grant sponsor: The Royal Netherlands Academy of Arts and Sciences; Grant sponsor: PSU Collaborative Research Fund.

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Received 23 September 2005; Accepted 29 November 2005
DOI 10.1002/jcb.20795

ligament but also appear important in processes associated with bone remodeling. When periodontal ligament cells are destroyed, bone invades the periodontal space until it connects to the teeth, thus becoming ankylotic [Boyko et al., 1981]. PLF uniquely prevent invasion of bone into the periodontal space, they can also orchestrate bone degradation and formation in case of tooth movement [Oshiro et al., 2002] and physiological root resorption [Fukushima et al., 2003].

Evidence that PLF can induce the formation of multinucleated osteoclast-like cells from osteoclast precursor cells was obtained from in vitro studies. PLF express receptor activator of NF-kappa B ligand (RANKL) [Kanzaki et al., 2002; Lossdörfer et al., 2002; Hasegawa et al., 2002b], a molecule required for proper osteoclast development. Bone resorbing osteoclasts develop in a RANKL-dependent way in co-cultures of human PLF and human peripheral blood mononuclear cells (PBMC) [Kanzaki et al., 2001] or mouse bone marrow cells [Wada et al., 2001; Hasegawa et al., 2002b]. PLF not only have the capacity to induce formation of osteoclast-like cells, they can also inhibit this process by expressing high levels of RANKL decoy receptor osteoprotegerin (OPG) [Sakata et al., 1999; Kanzaki et al., 2001]. The levels of OPG are at least tenfold higher than in femoral or alveolar bone cell extracts [DeLaurier et al., 2002]. The ratio of RANKL:OPG can be indicative for a role of PLF in bone resorption or prevention of bone resorption. High ratios were observed in periodontal tissue from patients with advanced periodontitis [Liu et al., 2003] and in PLF from resorbing deciduous teeth [Fukushima et al., 2003]; situations characterized by excessive bone degradation. In experimental conditions, RANKL:OPG ratios can be elevated by the addition of dexamethasone and vitamin D_3 to the culture conditions [Quinn et al., 2000; Hasegawa et al., 2002a,b].

Although the capacity of PLF to induce formation of osteoclast-like cells has been shown in some studies, little is known about this capacity of the anatomically adjacent GF. These fibroblasts were shown to express relatively high levels of OPG [Sakata et al., 1999; Nagasawa et al., 2002] and it may thus be hypothesized that osteoclast formation is inhibited in the presence of this subset of fibroblasts. Could it be that fibroblasts that function so close to each other have such opposite effects on the formation of osteoclast-like cells? It was the aim of our study to investigate this in more detail and compare the osteoclast-inducing capacities of the two tooth-associated fibroblast populations. PLF and GF were isolated and cultured from six patients from whom wisdom teeth were extracted. PLF and GF were co-cultured with PBMC and osteoclast development was assessed.

MATERIALS AND METHODS

Fibroblasts

Gingival and PLF were obtained from six male individuals (age 22–38 years) who underwent extraction of a third molar (wisdom tooth). Informed consent was obtained from all individuals. GF and PLF were taken without overt signs of gingival inflammation and periodontitis (no plaque, periodontal probing $\leq$3 mm, no bleeding on probing, and no sign of loss of attachment).

Free gingiva and part of the intradental gingiva was cut off the tooth by means of a scalpel-knife and the tissue fragments were washed twice in Dulbecco's minimal essential medium (DMEM, Gibco BRL, Paisley, Scotland) supplemented with 10% FCS (HyClone, Logan, UT), and 1% antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B [Antibiotic antimyotic solution, Sigma, St. Louis, MO]). Fragments of periodontal ligament were obtained exclusively from the middle third of the roots by means of a scalpel knife. The biopsies were cut into small pieces and divided in a 6-well dish with 1.5 ml DMEM + 10% FCS + 1% antibiotics. The 6-well dishes were stored in a humidified atmosphere of 5% CO_2 in air at 37°C. Gingiva and PDL fibroblasts were expanded for five passages. All experiments were performed at the 6th passage.

PBMC Isolation

Buffy coats (Sanquin, Amsterdam, The Netherlands) were diluted 1:1 in HBSS/2% FCS. Twenty-five milliliter of diluted blood was carefully layered on 15 ml lymphoprep (Axisshield Po CAS, Oslo, Norway) and centrifuged for 30 min at 1,200*g* without brake. The interphase containing PBMC was washed two times in HBSS + 2% FCS and finally recovered in DMEM + 10% FCS + 1% antibiotics.

Co-Culture Experiments

Assessment of optimal cell density. Optimal cell densities of fibroblasts and PBMC were established in a pilot experiment ($n = 2$ GF, $n = 2$ PLF), where cultures were analyzed each week for 3 weeks. In this pilot experiment, all cells were cultured in the presence of 10^{-8}M dexamethasone and 10^{-7}M vitamin D_3. Fibroblasts were plated at cell densities 1.5, 3, and 6×10^4 in wells of a 24-well plate (Costar, Cambridge, MA) 1 day before the start of the experiment. The following day, 0.5, 1, and 2×10^6 PBMC were plated on top of the fibroblasts. Mononuclear TRACP-positive cells were present at day 7, a few small multinucleated cells (MNCs) were present after 14 days of co-culturing and large MNCs were present after 3 weeks of co-culturing. Optimal cell condition was 1.5×10^4 fibroblasts and 1×10^6 PBMC. This culture condition resulted in the highest number of MNC at 21 days of co-culturing. These cell numbers were used in all experiments.

Osteoclastogenesis. Co-culture experiments were performed on plastic and on 650 μm thick bovine cortical bone slices, either without or with the addition of 10^{-8}M dexamethasone and 10^{-7}M vitamin D_3 (dex + vit D_3). Each condition was plated in triplicate. Culture medium (1 ml per well) was replaced twice a week. In addition, PBMC were co-cultured with fibroblasts in the presence of dex + vit D_3 and 25 ng/ml recombinant human M-CSF (R&D Systems, Minneapolis, MN) with or without 40 ng/ml recombinant human RANKL (PreProtech, Rocky Hill, NJ) for 3 weeks. Medium was changed twice a week.

Formation of osteoclast-like cells in the presence of conditioned medium obtained from GF and PLF conditioned medium was performed in a mouse osteoclastogenesis assay, analogous to a study by Wada et al. [2001], who studied this for PLF conditioned medium only. GF and PLF were cultured in the presence or absence of dex + vit D_3 in 6-well plates at a density of 2×10^5 cells per well for 3 days. Medium was collected and centrifuged for 10 min at 300*g* to remove cells. Supernatant was used in osteoclastogenesis assays. Based on DNA measurements, no differences in cell number were observed between GF and PLF. Mouse bone marrow cells were isolated and cultured as previously described [De Vries et al., 2005]. Bone marrow was either cultured in the presence of 30 ng/ml murine recombinant M-CSF (R&D Systems) and 20 ng/ml RANKL-TEC (R&D Systems) or in the presence of these cytokines supplemented with conditioned medium (1/2 or 1/20 volume). Medium was replaced after 3 days and the experiment was finished after 5 days by fixing the cells.

At the end of each osteoclastogenesis assay, cells were fixed in PBS buffered 4% formaldehyde and stained for TRACP activity using the leukocyte acid phosphatase kit (Sigma, St. Louis, MO). Nuclei were stained with diamidino-2phenylindole dihydrochloride (DAPI). Five micrographs from fixed positions per well or per bone slice were taken with a digital camera (Leica, Wetzlar, Germany) and analyzed for the number of MNCs containing three or more nuclei. Cell size and number of nuclei per MNC were measured using Image-Pro plus (Media Cybernetics, Silver Spring, ML) software.

Bone resorption. Co-cultures on bone were analyzed for bone resorption after a culture period of 4 weeks. After this period, the cells present on the bovine cortical bone slices were removed with 0.25M NH_4OH. The slices were washed in distilled water, incubated in a saturated alum solution, washed in distilled water, and stained with Coommassie Brilliant blue [Everts et al., 1999].

In an additional bone resorption experiment, PBMC were cultured on bone slices for 5 weeks in the presence of conditioned medium from PLF and GF cultures cultured either without or with dex + vit D_3. For this experiment, medium was collected from confluent cell cultures, which had conditioned the medium for 7 days. This conditioned medium was diluted 1:1 and frozen in aliquots and used in the experiment. Freshly defrosted conditioned medium was applied twice a week. Finally, PBMC were cultured on bone in the presence of M-CSF or M-CSF and RANKL. Bone resorption was assessed after 21 and 28 days of culturing.

Actin staining. F-actin was stained according to the method previously described [Perez-Amodio et al., 2004] using ALEXA-conjugated phalloidin (Molecular Probes, Eugene, OR).

Flow Cytometric Analysis of RANK Expression

PBMC isolated from buffy coats from six donors were cultured in the presence or absence of dex + vit D_3 for 7 days on a confluent layer of PLF. Upon two washes with PBS, both

fibroblasts and PBMC were detached after a brief incubation with cell dissociation solution (Sigma, Steinheim, Germany). All incubations and washes were in PBS containing 1% BSA at 4°C. Upon centrifugation (5 min. 200*g*, at 4°C), cell pellets were recovered and incubated for 1 h in a concentration of 20 μg/ml goat anti-human RANK antibody (Santa Cruz, Santa Cruz, CA) or non-immune IgG (Sigma, Steinheim, Germany). After one wash with FACS-buffer, cells were labeled with FITC-conjugated mouse anti-goat IgG (Jackson Immunoresearch Laboratories, West Grove, PA). Fluorescence was measured on a FACSVantage (Becton and Dickinson, Franklin Lakes, NJ). Only cells with PBMC forward and sideward scatter characteristics were analyzed.

RNA Analysis and Real-Time Quantitative PCR

RNA from cultured cells was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA concentration was measured with the RiboGreen kit (Molecular Probes). One-hundred nanogram 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.

Real-time PCR primers were designed using the Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA) (Table I). To avoid amplification of genomic DNA, each amplicon spanned at least one intron.

The external standard curve used in the PCR reactions is cDNA from the quantitative PCR human reference total RNA (Stratagene, La Jolla, CA), which is composed of total RNA obtained from 10 human cell lines.

Real-time PCR was performed on the ABI PRISM 7000 (Applied Biosystems). The reactions were performed with 5 ng cDNA in a total volume of 25 μl containing SYBR Green PCR Master Mix, consisting of SYBR Green I Dye, AmpliTaq Gold DNA polymerase, dNTPs with dUTP instead of dTTP, passive reference and buffer (Applied Biosystems) and 300 nM of each primer.

After an initial activation step of the AmpliTaq Gold DNA polymerase for 10 min at 94°C, 40 cycles were run of a two step PCR consisting of a denaturation step at 95°C for 30 s and annealing and extension step at 60°C for 1 min. Subsequently the PCR products were subjected to melting curve analysis to test if any unspecific PCR products were generated. The PCR reactions of the different amplicons had equal efficiencies. β2-microglobulin was used as the housekeeping gene. Expression of this gene was not affected by the experimental conditions. Samples were normalized for the expression of β2-microglobulin by calculating the ΔC_t ($C_{t,\beta 2\text{-microglobulin}} - C_{t,\text{gene of interest}}$) and expression of the different genes is expressed as $2^{-(\Delta C_t)}$.

TABLE I. Primers Used for Quantitative RT-PCR

	Sequence 5′ → 3′	Ensembl gene ID[a]
Osteoclastogenesis genes		
c-Fms	CCCTCATgTCCgAgCTgAA AggTCgCCATAgCAACAgTACTC	ENSG00000113721
M-CSF	CCGAGGAGGTGTCGGAGTAC AATTTGGCACGAGGTCTCCAT	M11038 (exon 1)[b] M11295 (exon 2)[b]
RANK	CCTggACCAACTgTACCTTCCT ACCgCATCggATTTCTCTgT	ENSG00000141655
RANKL	CATCCCATCTggTTCCCATAA gCCCAACCCCgATCATg	ENSG00000120659
OPG	CTgCgCgCTCgTgTTTC ACAgCTgATgAgAggTTTCTTCgT	ENSG00000164761
Osteoclast-related genes		
TRACP	CACAATCTgCAgTACCTgCAAgAT CCCATAgTggAAgCgCAgATA	ENSG00000102575
Cathepsin K	CCATATgTgggACAggAAgAgAgTT TgCATCAATggCCACAgAgA	ENSG00000143387
Carbonic anhydrase II	TggACTggCCgTTCTAggTATT TCTTgCCCTTTgTTTTAATggAA	ENSG00000104267
Calcitonin receptor	gCATACCAAggAgAAggTCCATAT ATACTCCAgCCggTgTgTCAT	ENSG00000004948

[a]Sequences were retrieved from the ENSEMBL genome browser (http://www.ensembl.org).
[b]For M-CSF the NCBI accession numbers were used for primer design.

ELISA

ELISAs for the detection of human OPG (R&D Systems) and human RANKL (PreProtech, Rocky Hill, NJ) were performed following the manufacturer's instructions.

Statistical Analysis

Paired t tests (two-tailed) were used to compare means of two groups. Differences were considered significant when $P < 0.05$. To compare mean mRNA expression of two groups, all (time) data points of two groups were compared in a paired t-test (two-tailed).

RESULTS

Formation of Osteoclast-Like Cells

PBMC were co-cultured with PLF and GF for 3 weeks in the presence or absence of dex + vit D_3 on plastic or on bone slices and the formation of TRACP-positive MNCs were analyzed. Both PLF (Fig. 1A) and GF (Fig. 1B) induced the formation of TRACP-positive MNCs, but the number of MNCs differed considerably among the conditions tested. First, more TRACP-positive MNCs were generated by PLF compared to GF. Second, more were found on plastic than on bone and finally, more were observed in the presence of dex + vit D_3 than in the absence (Fig. 1C). Not only a higher number of MNCs was apparent in the presence of dex + vit D_3, also the cells were larger (Fig. 1D) and the number of nuclei per MNC (Fig. 1E) was higher under these conditions. The presence of fibroblasts was required for the formation of MNCs; in PBMC cultures without fibroblasts only TRACP-positive mononuclear cells were present.

It could be that GF secrete (more) inhibitors of MNC formation than PLF, thus explaining the lower numbers of MNCs found in GF-PBMC compared to PLF-PBMC cultures. Therefore we analyzed the effect of conditioned medium of PLF and GF on the formation of osteoclast-like cells in an assay previously used by Wada et al. [2001]. Mouse bone marrow cells were cultured in the presence of murine M-CSF and RANKL and conditioned medium of PLF and GF. Formation of TRACP-positive MNCs was severely inhibited when high concentrations of GF-conditioned medium were added. PLF-conditioned medium, however, did not affect formation of TRACP-positive MNCs (Fig. 2).

mRNA and Protein Analysis

Since expression levels of RANKL and M-CSF by cells that orchestrate osteoclastogenesis are considered critical for proper osteoclast development [Boyle et al., 2003], we next investigated the quantities of the mRNAs of the M-CSF/c-Fms and RANKL/RANK/OPG systems in the co-culture experiments. Possible differences in expression of these components could explain some of our findings, such as enhanced osteoclastogenesis in PLF versus GF or in cultures with versus without dex + vit D_3.

First, we determined the expression levels in monocultures 1 day after seeding (Table II). As expected, M-CSF was mainly expressed by fibroblasts, whereas its receptor c-Fms was expressed by PBMC. RANKL and RANK were expressed by both fibroblasts and PBMC, whereas OPG was exclusively expressed by fibroblasts.

The osteoclast-related genes TRACP and carbonic anhydrase II were mainly expressed in PBMC, whereas cathepsin K was highly expressed in fibroblasts (C_t value of 21.9–24.1 cycles). Calcitonin receptor was hardly detectable (C_t value of 35–37 cycles) in both fibroblast and PBMC monocultures.

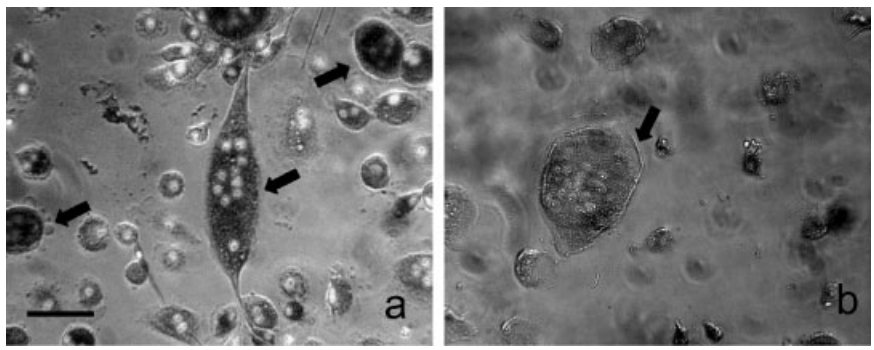
Co-culture experiments were performed to determine the transcription levels of mRNAs of M-CSF and its receptor c-Fms and of RANKL, its receptor and its decoy receptor OPG (Fig. 3). The cultures were performed in the presence or absence of dex + vit D_3. In addition, expression of osteoclast-related genes calcitonin receptor, carbonic anhydrase II, cathepsin K, and TRACP was studied (Fig. 4).

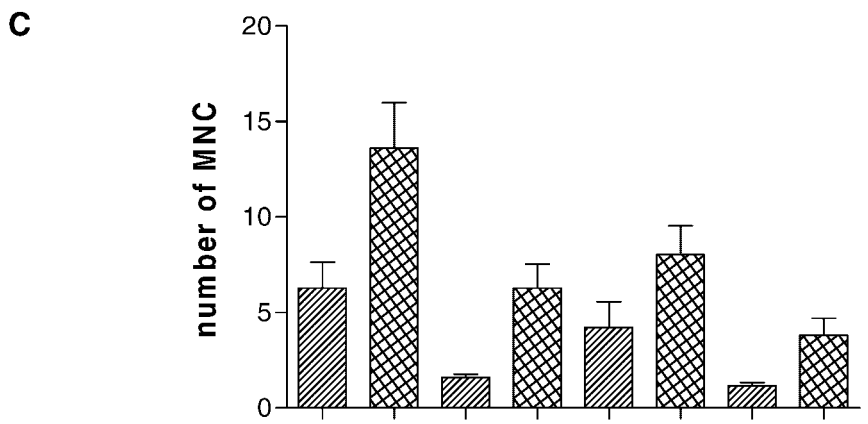
Expression of both c-Fms (more than 10-fold, $P < 0.001$) and M-CSF (2.5-fold, $P < 0.01$) was significantly increased in the presence of dex + vit D_3 in both PLF-PBMC and GF-PBMC co-cultures (Fig. 4).

A constant expression of RANK was observed over time in co-cultures containing dex + vit D_3, whereas significantly lower expression was seen ($P < 0.01$ for both PLF-PBMC and GF-PBMC) in the absence of these additives. No differences were observed in RANK expression between PLF and GF co-cultures (Fig. 3). The presence of dex + vit D_3 increased the number of RANK-expressing osteoclast precursor cells in PLF-PBMC co-cultures (Fig. 5) and in GF-PBMC co-cultures (not shown). RANKL transcripts were barely detectable in any of the co-cultures (C_t value >35).

OPG was expressed at higher levels in GF-PBMC co-cultures compared to the PLF-PBMC co-cultures, both in the absence (4×, $P < 0.01$) and in the presence (3×, $P < 0.05$) of dex + vit D_3, resulting in significantly lower RANKL/OPG mRNA ratios (7.8×, $P < 0.01$ and 3.6×, $P < 0.05$, respectively). Overall RANKL:OPG mRNA ratio was very low (0.1–0.01) throughout the co-culture period (Fig. 3).

At the protein level, no significant differences in OPG expression in the medium could be detected between PLF and GF cultured in the





Cell	PLF				GF			
Substrate	plastic		bone		plastic		bone	
-/+ vit. D + dex	-	+	-	+	-	+	-	+
PLF vs. GF	*		*		*			
Plastic vs. bone	*		**			*		
- vs. + vit. D+dex	*		*				*	

Fig. 1. Formation of TRACP-positive MNCs is lower in GF-PBMC than in PLF-PBMC co-cultures. TRACP-positive MNCs were analyzed at 21 days and were found under all co-culture conditions. Examples of TRACP-positive MNCs in a PLF-PBMC co-culture (**A**) and in a GF-PBMC co-culture (**B**) are shown. Mean number (±SEM) of TRACP-positive MNCs (>2 nuclei per cell) per five microscope fields were counted for each condition (n = 6, plated in triplicate) (**C**). Braces show the significant differences (*$P < 0.05$; **$P < 0.01$) between the various co-culture conditions. Cell size (mean ± SEM) of TRACP-positive MNCs (**D**) and number of nuclei (mean ± SEM) per MNC (**E**) were determined from PLF-PBMC and GF-PBMC co-cultures. Dex + vit D_3 significantly induce larger MNCs with more nuclei, *$P < 0.05$; **$P < 0.01$. PLF and GF from six patients were co-cultured with PBMC, each condition was plated in triplicate, 57, 67, 69, and 43 TRACP-positive MNCs were analyzed for PLF without and with dex + vit D_3, GF without and with dex + vit D_3, respectively.

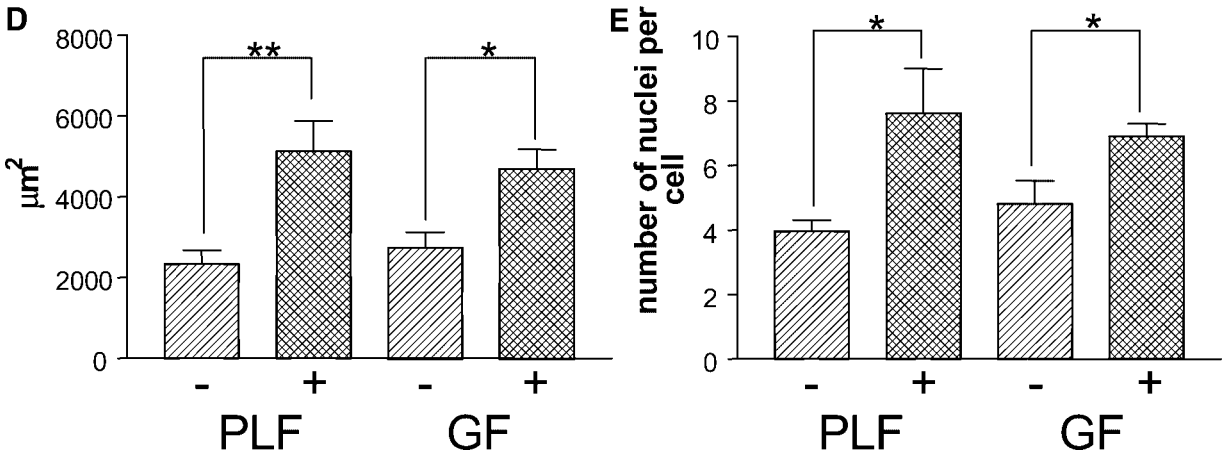


Fig. 1. (*Continued*)

absence or presence of dex + vit D_3: (PLF without: 333 ± 84; PLF with: 500 ± 236; GF without: 571 ± 233; GF with: 471 ± 123, data are expressed as mean ± SEM pg/ml; n = 6 patients). OPG concentrations were approximately 10 times lower in cell PLF and GF lysates. No OPG was detectable in PBMC. RANKL could not be detected with ELISA (detection limit: 15 pg/ml) in any of the samples (supernatants or cell lysates).

In general, dex + vit D_3 caused a highly significant increase ($P < 0.001$) of expression of the osteoclast-related genes TRACP, cathepsin K and carbonic anhydrase II both in PLF-PBMC and in GF-PBMC co-cultures. This higher expression possibly reflects the larger number of osteoclast-like cells found in co-cultures containing dex + vit D_3. Relative expression of calcitonin receptor was very low (below 10^{-5}, at least 35 cycles were needed for detection) compared to the other osteoclast-related genes (between 10^{-3} and 10^{-1}, Fig. 4).

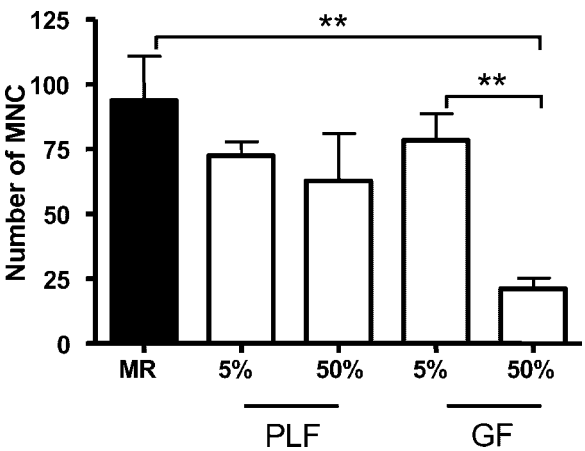


Fig. 2. GF-conditioned medium inhibits the formation of osteoclast-like cells. Mouse bone marrow cells were cultured in the presence of M-CSF and RANKL (MR) and in the presence of M-CSF and RANKL supplemented with 5% or 50% volume PLF- or GF-conditioned medium from cell cultures of five patients. Number of TRACP-positive MNCs was severely inhibited by GF-conditioned medium, $^{**}P < 0.01$. Data are expressed as mean number of MNC ± SEM.

Bone Resorption

Although co-cultures on bone revealed the presence of TRACP-positive MNCs, no signs of bone resorption were observed in any of the PLF or GF co-cultures, not even after 4 weeks of co-culturing. We tested whether this was due to the presence of fibroblasts that somehow prevented the MNCs to resorb bone.

Absence of bone resorption in fibroblast-PBMC co-cultures can be attributed to high levels of OPG (see above). Therefore, we tested whether addition of M-CSF or RANKL alone or a combination of M-CSF and RANKL could overcome the lack of bone resorption. Fibroblasts and PBMC were co-cultured for 3 weeks in the presence of dex + vit D_3 on cortical bone slices, without and with M-CSF, RANKL or M-CSF + RANKL. Simultaneous visualization of TRACP activity and actin staining demonstrated that TRACP-positive (mainly mononuclear) cells remained on top of the fibroblast layer in cultures without cytokines or with alone M-CSF or RANKL. Under these conditions, no cell free areas were seen and no penetration through the fibroblast layer and subsequent migration to the bone surface was

TABLE II. Relative Contribution of Periodontal Ligament Fibroblasts (PLF), Gingival Fibroblasts (GF), and Peripheral Blood Mononuclear Cells (PBMC) to the mRNA Levels of Osteoclastogenesis and Osteoclasts-Related Genes at t = 1 Day

			Percentage (PLF or GF + PBMC = 100%)			
	Relative expression range	C_t values	PLF	PBMC	GF	PBMC
Osteoclastogenesis genes						
c-Fms	$(1 \times 10^{-2}–2 \times 10^{-2})$[a]	24.7–26.1	3	97	1	99
M-CSF	$(5 \times 10^{-2}–9 \times 10^{-2})$[b]	24.8–26.1	99	1	99	1
RANK	$(1 \times 10^{-4}–3 \times 10^{-4})$	30.1–36.9	59	41	73	27
RANKL	$(4 \times 10^{-5}–2 \times 10^{-4})$	30.9–38.8	38	62	30	70
OPG	$(5 \times 10^{-2}–2 \times 10^{-1})$[b]	23.5–26.3	100	0	100	0
Osteoclast-related genes						
TRACP	$(5 \times 10^{-4}–1 \times 10^{-3})$[a]	28.1–30.0	9	91	12	88
Cathepsin K	$(2 \times 10^{-1}–6 \times 10^{-1})$[b]	21.9–24.1	100	0	100	0
Carbonic anhydrase II	$(4 \times 10^{-4}–5 \times 10^{-3})$	26.4–35.2	23	77	23	77
Calcitonin receptor	$(1 \times 10^{-5}–5 \times 10^{-4})$[b]	35.0–37.5	94	6	95	5

Similar data were found at t = 1 day when cultured in the presence of dexamethasone and vitamin D_3.
[a]Expression only in PBMC is shown, expression in fibroblasts is negligible.
[b]Expression only in the fibroblast cultures is shown, expression in PBMC is negligible.

observed (Fig. 6A–D). Also, no bone resorption took place (Fig. 6G,H). Larger TRACP-positive MNCs formed in fibroblast-PBMC co-cultures where M-CSF and RANKL was added. These cells were able to reach the bone surface (Fig. 6E,F). The presence of actin rings (Fig. 6F) and resorption pits (Fig. 6I) indicated that these cells were actively resorbing osteoclasts.

DISCUSSION

In the present article, we demonstrate that two different populations of tooth-associated fibroblasts, periodontal ligament, and gingival fibroblasts (GF), tend to inhbit the formation of osteoclast-like cells. Nevertheless these fibroblasts also play a role in the onset of osteoclast formation, since no TRACP-positive MNC formed in the absence of fibroblasts. Although it has been recognized that fibroblasts from various non-skeletal sites of the body can induce osteoclast formation [Quinn et al., 2000; Perez-Amodio et al., 2004], our study is the first to describe that differences between fibroblast populations exist with regard to their osteoclastogenic potential. A central dogma is that osteoblasts at the alveolar bone surface surrounding our teeth are essential for the formation of osteoclasts. Interestingly, our study shows that tooth-associated fibroblasts can also induce the formation of osteoclasts. PLF induced higher numbers of TRACP-positive MNCs than GF. This difference is in accordance with the respective functions of PLF and GF: osteoclast formation mediated by PLF is pivotal for remodeling of alveolar bone which occurs throughout life but is particularly active during tooth movement. GF, on the other hand, are essential for the maintenance of the gingival connective tissue and they play a role in protection against oral pathogens. In line with this more protective role of GF are the findings by Nagasawa et al. [2002] who showed that LPS increases OPG expression by GF, resulting in a decreased induction of osteoclastogenesis by these cells. These and our data suggest that GF help to prevent the formation of active osteoclasts.

Significantly more osteoclast-like cells were formed in the presence of dex + vit D_3. In previous studies, it was shown that a combination of dexamethasone and vitamin D_3 is pivotal for the proper development of TRACP-positive, bone resorbing MNCs in co-culture systems [Itonaga et al., 1999; Atkins et al., 2000; Quinn et al., 2000; Hasegawa et al., 2002b]. In our study, using fibroblasts instead of osteoblasts, higher numbers of TRACP-positive MNCs were formed in co-cultures with dex + vit D_3. We found that the addition of dex + vit D_3 caused increased expression of M-CSF, c-Fms, and RANK, genes important for proper osteoclast formation, confirming previous reports in which osteoblasts were studied. Both dexamethasone [Rubin et al., 1998] and vitamin D_3 [Kaneki et al., 1994] were shown to upregulate M-CSF in osteoblasts and monocytes, respectively. Dex + vit D_3 treatment also increased expression of

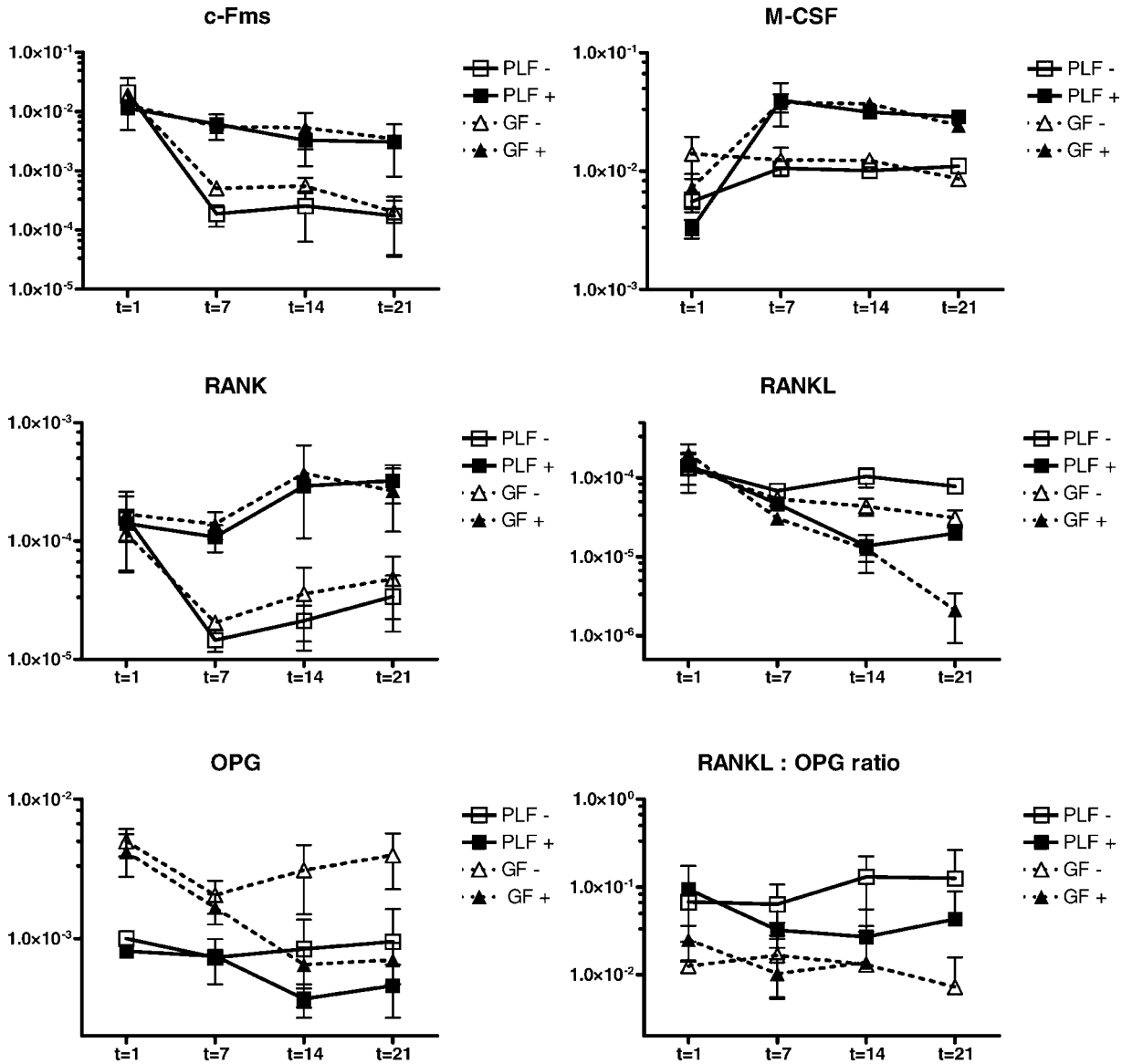


Fig. 3. mRNA expression c-Fms, M-CSF, RANK, RANKL, OPG, and the ratio of RANKL/OPG in PLF-PBMC and GF-PBMC co-cultures. PLF-PBMC (squares) and GF-PBMC (triangles) co-cultures were cultured either in the presence (+, filled line) or in the absence (−, dotted line) of dex + vit D_3. Mean ± SEM expression from three fibroblast-PBMC co-cultures is represented.

osteoclast-related genes cathepsin K, TRACP, and especially carbonic anhydrase II in the PLF-PBMC and GF-PBMC co-cultures, possibly reflecting the higher numbers of osteoclast-like cells formed in these cultures. Cell size and number of nuclei per TRACP-positive MNC were also significantly increased by dex + vit D_3, implying that these substances also play a role in fusion of pre-osteoclasts. Our results are in line with in vivo observations by Holtrop et al. [1981], who showed that rats receiving a vitamin D supplemented diet contained larger osteoclasts than control animals. Although alterations in gene expression by dex + vit D_3 could account for increased formation of osteoclast-like cells in these culture conditions, mRNA expression data do not reflect differences in the formation of osteoclast-like cells between PLF and GF.

When comparing previous osteoclastogenesis studies using PLF-PBMC co-cultures and our study, the view emerges that the source of PLF determines whether bone degrading osteoclasts are formed in co-cultures with osteoclast

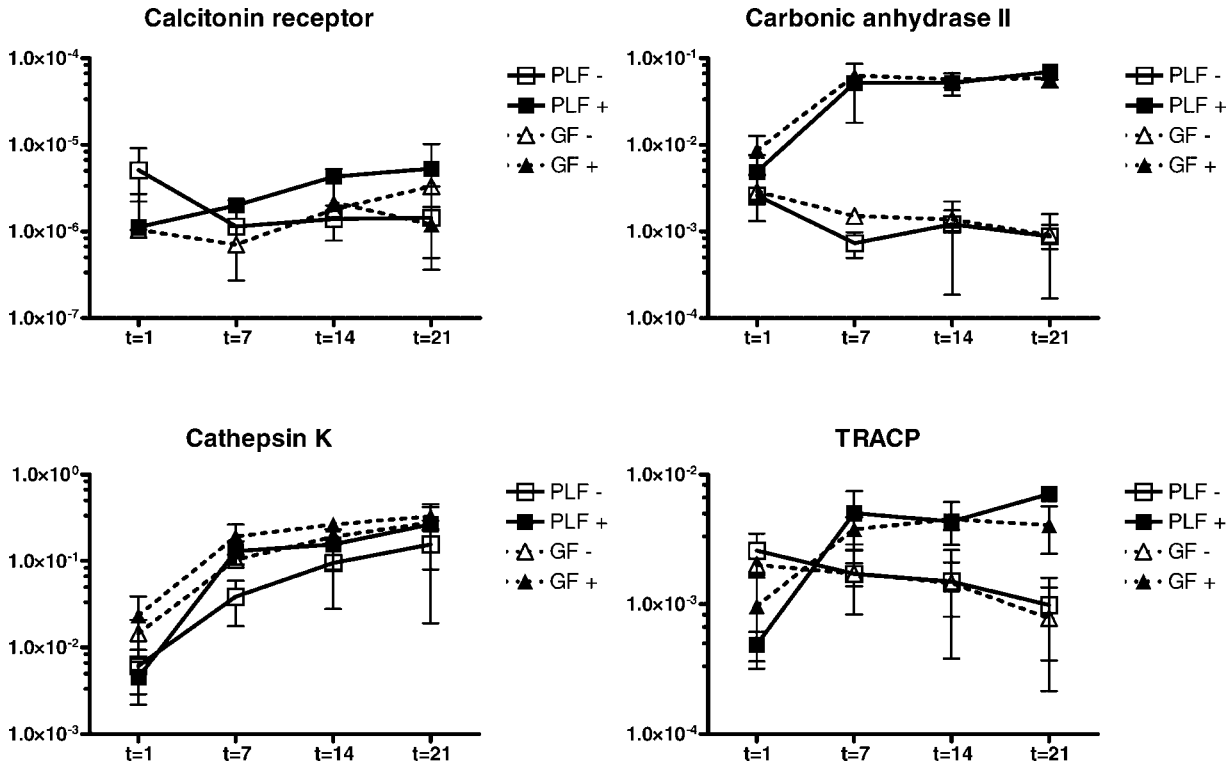


Fig. 4. mRNA expression of osteoclast-related genes TRAP, cathepsin K, calcitonin receptor, and carbonic anhydrase II in PLF-PBMC and GF-PBMC co-cultures. PLF-PBMC (squares) and GF-PBMC (triangles) co-cultures were cultured either in the presence (+, filled line) or in the absence (−, dotted line) of dex + vit D_3. Mean ± SEM expression from three fibroblast PBMC co-cultures is represented.

progenitor cells. Fukushima's study contributed greatly to this understanding by showing that periodontal ligament cell isolates from permanent and deciduous teeth without signs of inflammation or resorption hardly expressed RANKL, whereas periodontal ligament cell isolates from resorbing deciduous teeth expressed high levels of RANKL which correlated with the occurrence of odontoclasts on the tooth surface [Fukushima et al., 2003]. PLF from deciduous teeth also express high levels of OPG. When OPG was captured with a neutralizing antibody, a more than tenfold increase in osteoclast formation was observed in human PLF-mouse bone marrow co-cultures [Hasegawa et al., 2002b]. Kanzaki et al. used PLF from teeth, which were extracted for orthodontic reasons and showed that bone resorbing osteoclasts were formed only in co-cultures. PBMC cultured in PLF conditioned medium, however, hardly developed into osteoclast-like cells, possibly due to high OPG levels present in conditioned medium [Kanzaki et al., 2001]. These findings imply that cell–cell contact between PLF and osteoclast precursor cells is necessary for proper osteoclast development. Importantly, mechanical loading applied previously to extraction of the teeth possibly gave the required signal for the generation of bone resorbing osteoclasts, since it was shown by others that mechanical loading increases RANKL expression in PLF,

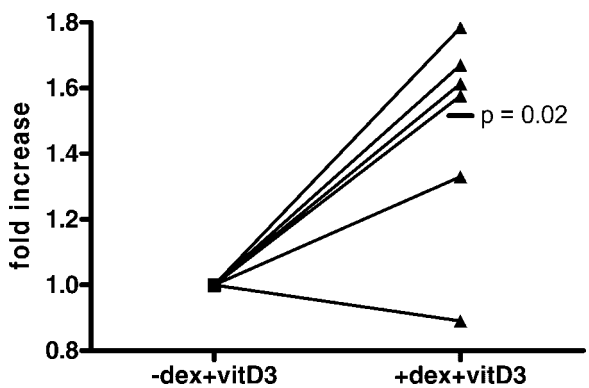


Fig. 5. Relative increase of RANK expressing osteoclast precursor cells after addition of dex + vit D_3. Dexamethasone and vitamin D_3 present in PLF-PBMC and GF-PBMC (not shown) co-cultures increase the number of RANK expressing PBMC in co-cultures 1.5-fold as determined by flowcytometry. Results of six different buffy coats are shown, $P < 0.05$.

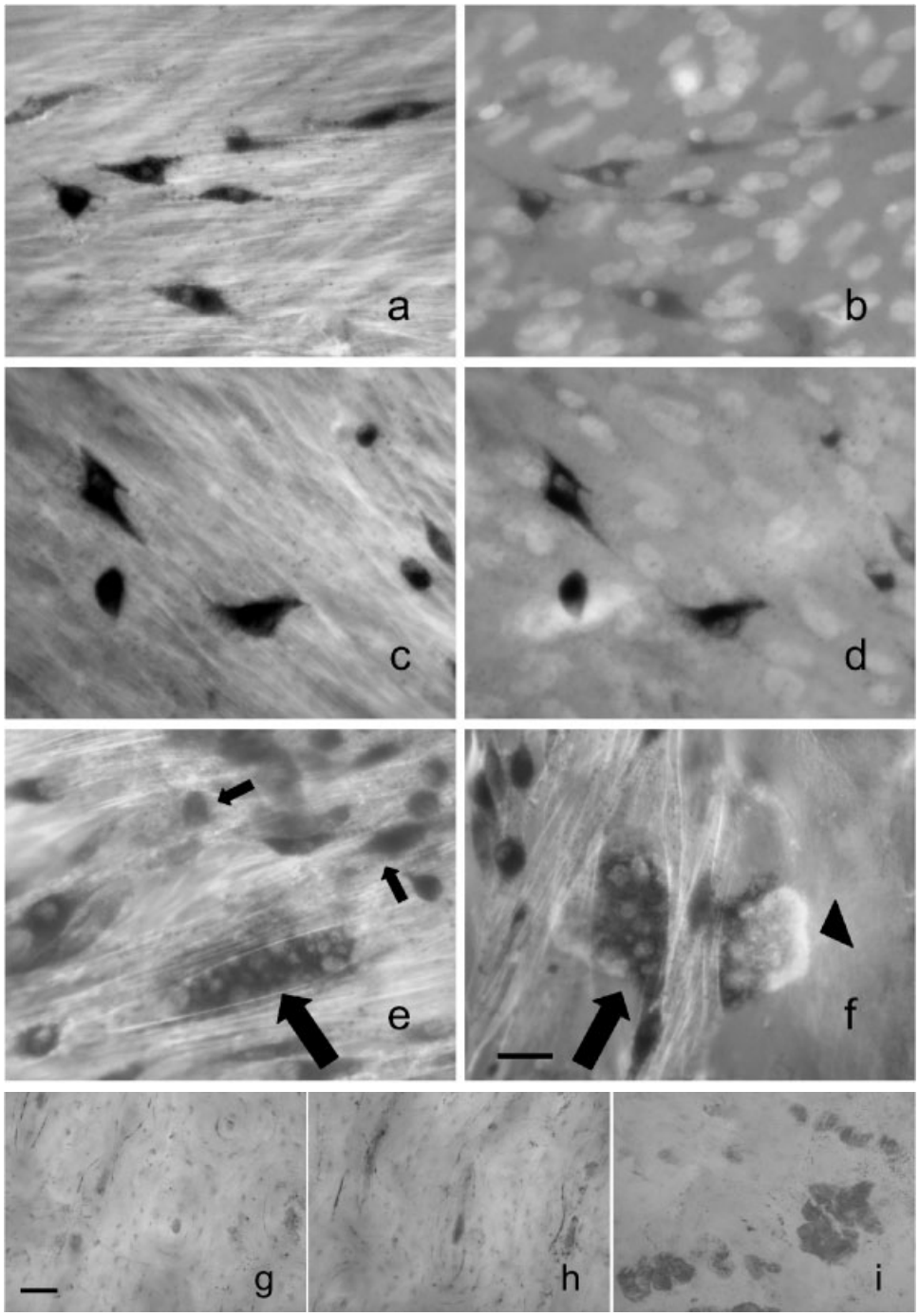


Fig. 6. M-CSF + RANKL are required for the formation of active osteoclasts in fibroblast PBMC co-cultures. PLF-PBMC (**C**, **D**, **F–I**) and GF-PBMC (**A**, **B**, **E**) co-cultures (n = 4) were cultured without cytokines (A, B, G) with M-CSF (C, D, H) or with M-CSF + RANKL (E, F, I) for 3 weeks. TRACP-activity, actin (A, C, E, F) and nuclei (B, D) were visualized. Primarily mononuclear TRACP-positive cells were exclusively located on top of a confluent layer of fibroblasts (A, C) when cultured without cytokines (A, B) or with M-CSF (C, D). Large multinucleated TRACP-positive cells (large arrows) were seen cultured with M-CSF and RANKL (E, F). Mononuclear TRACP-positive cells (small arrow) were still located on top of fibroblasts. Occasionally actin ring formation was observed in multinucleated cells (F, arrowhead). Bone resorption was absent in co-cultures without cytokines (G), and in co-cultures where M-CSF was added (H) and present in both fibroblast-PBMC co-cultures when M-CSF + RANKL was added (I). Bar = 25 μm.

through a PGE_2-dependent mechanism [Kanzaki et al., 2002]. Fibroblasts in our study were from wisdom teeth, quiescent teeth, which have experienced a totally different regime of mechanical loading than the previous studies. These fibroblasts expressed very low levels of RANKL mRNA and a 10- (PLF) to 100- (GF) fold excess of OPG at the mRNA level. At the protein level, RANKL could not be detected. Since RANKL directly activates osteoclasts [Burgess et al., 1999], and no active osteoclasts were found, RANKL present in our co-cultures is possibly immediately neutralized by OPG. Although a minimal cellular RANKL:RANK signaling between periodontal ligament or GF and osteoclast precursors cannot be excluded in the presence of an excess of OPG, it is nevertheless remarkable that formation of osteoclast-like cells took place in our co-cultures. It seems likely that formation of TRACP-positive MNC can occur despite the absence or very low levels of RANKL. RANKL and M-CSF were required, however, for the migration of TRACP-positive MNCs towards the bone surface and for subsequent bone resorption. It is likely that lack of sufficient RANKL caused the absence of bone resorption. In line with this, actively resorbing odontoclasts were only formed on teeth where PLF expressed detectable levels of RANKL mRNA [Fukushima et al., 2003].

Our study indicates that GF secrete agents, which inhibit the formation of osteoclast-like cells. The nature of the secreted inhibitors remains to be elucidated. OPG is not a likely candidate, since protein levels of OPG are comparable for both cell types.

One of the hallmarks of osteoclasts, which discriminates them from other MNCs, is the expression of calcitonin receptor [reviewed by Anderson, 2000]. In contrast to the other osteoclast-related genes studied (TRACP, carbonic anhydrase II and cathepsin K), expression of calcitonin receptor was hardly detectable and did not increase over time, implying that the TRACP-positive cells formed in our co-culture system were not fully matured osteoclasts.

As a general concept of a role for GF and PLF in osteoclastogenesis, we would like to propose that both types of fibroblasts are primarily driven towards preventing the formation of *active* osteoclasts as previously formulated for PLF [Wise et al., 2002]. High expression of OPG by these two types of fibroblasts [Sakata et al., 1999; Ogasawara et al., 2004] prevents the formation of active osteoclasts. More in particular: high OPG levels in the gingival tissue could play a protective role in inflammation-regulated osteoclastogenesis, whereas high levels of OPG in the periodontal ligament could play a role in modulating the speed of tooth movement [DeLaurier et al., 2002; Kanzaki et al., 2004]. Disturbance of this homeostasis by bacterial challenge [Belibasakis et al., 2005], periodontal disease [Teng et al., 2000; Liu et al., 2003; Mogi et al., 2004] or by (experimental) tooth movement [Kanzaki et al., 2002; Oshiro et al., 2002; Ogasawara et al., 2004] could cause an increase of RANKL expression followed by osteoclast formation and activity.

ACKNOWLEDGMENTS

This study is supported by The Royal Netherlands Academy of Arts and Sciences (T.J.d.V., K.N.A.W.) and PSU Collaborative Research Fund (N.W.)

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