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Formation of osteoclast-like cells from peripheral blood of periodontitis patients occurs without supplementation of macrophage colony-stimulating factor

Stanley T. S. Tjoa[1], Teun J. de Vries[1,2], Ton Schoenmaker[1,2], Angele Kelder[3], Bruno G. Loos[1] and Vincent Everts[2]

[1]Department of Periodontology, Academic Centre for Dentistry Amsterdam (ACTA), Universiteit van Amsterdam and Vrije Universteit, Amsterdam, The Netherlands; [2]Department of Oral Cell Biology, Academic Centre for Dentistry Amsterdam (ACTA), Universiteit van Amsterdam and Vrije Universteit, Amsterdam, Research Institute MOVE, The Netherlands; [3]Department of Hematology, VUMC, Vrije Universiteit, Amsterdam, The Netherlands

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Abstract

Aim: To determine whether peripheral blood mononuclear cells (PBMCs) from chronic periodontitis patients differ from PBMCs from matched control patients in their capacity to form osteoclast-like cells.

Material and Methods: PBMCs from 10 subjects with severe chronic periodontitis and their matched controls were cultured on plastic or on bone slices without or with macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL). The number of tartrate-resistant acid phosphatase-positive ($TRACP^+$) multinucleated cells (MNCs) and bone resorption were assessed.

Results: $TRACP^+$ MNCs were formed under all culture conditions, in patient and control cultures. In periodontitis patients, the formation of $TRACP^+$ MNC was similar for all three culture conditions; thus supplementation of the cytokines was not needed to induce MNC formation. In control cultures, however, M-CSF or M-CSF/RANKL resulted in higher numbers compared with cultures without cytokines. Upregulations of osteoclast marker mRNA cathepsin K and carbonic anhydrase II confirmed the osteoclastic character. Bone resorption was only observed when PBMCs were cultured in the presence of M-CSF and RANKL.

Conclusion: Our data indicate that PBMCs from periodontitis patients do not need priming by M-CSF to become osteoclast-like cells, suggesting that PBMCs from periodontitis patients are present in the circulation in a different state of activity.

Key words: osteoclast formation; periodontitis; peripheral blood

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Conflict of interest and source of funding statement

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Chronic periodontitis is a multi-factorial disease characterized by inflammation of the periodontium, leading to loss of soft tissue attachment and alveolar bone (Page et al. 1997). If left untreated, this condition may ultimately lead to tooth exfoliation. Although it is generally accepted that the primary aetiological factor of periodontal disease is bacterial plaque (Kinane 1999), studies have shown that not everyone is equally susceptible to periodontal disease (Loe et al. 1986). The host response has been considered as an important disease-modifying factor (Page et al. 1997).

Osteoclasts are the cells ultimately responsible for alveolar bone resorption. They are multinucleated giant cells (Roodman 1996) originating from the monocyte/macrophage lineages (Udagawa et al. 1990, Fujikawa et al. 1996). Human osteoclast precursor cells have been found in the mononuclear fraction of peripheral blood (PBMCs), and in vitro these cells differentiate into osteoclasts (Faust et al. 1999, Shalhoub et al. 1999).

Key regulators of osteoclast differentiation are macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor-κB (RANK) ligand (RANKL) and osteoprotegerin (OPG). In a mouse model, it was shown that the growth factor M-CSF is indispensable to the proliferation of osteoclast progenitors and their differentiation into mature osteoclasts (Tanaka et al. 1993). However, M-CSF alone could not induce osteoclasts to form resorption pits on dentine slices (Jimi et al. 1999). Differentiation and activation of osteoclasts is triggered by RANKL, which is a member of the tumor necrosis factor (TNF)-related cytokines, and this cytokine has a direct involvement in the differentiation, activation and survival of osteoclasts and their precursors (Lacey et al. 1998). Binding of RANKL to its receptor RANK stimulates osteoclast precursors to differentiate into osteoclasts and activates mature osteoclasts to resorb bone. OPG is a decoy receptor for RANKL, it competes with RANK for binding of RANKL, thereby acting as an inhibitor of osteoclast maturation and activity (Simonet et al. 1997, Lacey et al. 1998). Although the importance of the RANK–RANKL–OPG system in osteoclast biology was mainly explored in mice, it is believed that the regulatory mechanisms of human osteoclast differentiation are similar (Suda et al. 1999).

Various studies were undertaken to understand the cellular mechanisms underlying bone breakdown in people with osteopenic diseases, such as autoimmune arthritis (Ritchlin et al. 2003), post-menopausal osteoporosis (D'Amelio et al. 2005) and bone tumours (Roato et al. 2005). From these studies, it appeared that differences in osteoclast recruitment exist at the level of peripheral blood. Peripheral blood mononuclear cells (PBMCs) from patients with postmenopausal osteoporosis (D'Amelio et al. 2005) and psoriatic arthritis (Ritchlin et al. 2003) exhibit a significant increase in osteoclast formation and bone resorption activity compared with non-osteoporotic postmenopausal and healthy controls, respectively. Spontaneous osteoclastogenesis, for example osteoclast formation without addition of cytokines such as M-CSF and RANKL, was higher in osteoporotic women and arthritis patients (Ritchlin et al. 2003, D'Amelio et al. 2005). Similarly, increased spontaneous osteoclastogenesis was found in cancer patients with bone metastases compared with healthy controls or cancer patients without bone metastases (Roato et al. 2005). This increase in osteoclast formation and osteoclast activity was also suggested to occur in PBMCs from periodontitis patients compared with non-periodontitis patients (Brunetti et al. 2005). However, it is not clear from the latter study whether the increased osteoclast formation was due to the higher average age of the patients (mean 42.8 ± 15.2 years) compared with the controls (mean 30.5 ± 5.2 years), because it is known that osteoclast formation from human PBMCs may increase with age (Cheleuitte et al. 1998, Jevon et al. 2002).

In the current study, we further investigated whether PBMCs from periodontitis patients have an increased capacity to form osteoclasts by analysing the dependency of M-CSF and RANKL. Osteoclastogenesis was evaluated in PBMC cultures from periodontitis patients and controls who were matched for age, sex, ethnicity and smoking.

Material and Methods

Subjects

The patient group consisted of 10 untreated patients diagnosed with generalized severe chronic periodontitis. These patients were referred to the Department of Periodontology at the Academic Centre for Dentistry Amsterdam (ACTA) for diagnosis and/or treatment of periodontitis. Generalized severe chronic periodontitis patients were defined as patients who never had periodontal therapy or at least not in the last 10 years and who presented with alveolar bone loss $\geqslant 50\%$ of the root length on at least seven teeth on a set of full mouth dental radiographs (Bizzarro et al. 2007).

Patients were carefully matched with healthy controls. Control subjects were recruited among dental patients at ACTA who received restorative dental treatments and regular dental check-ups, or were subjects from private dental clinics. A control subject was selected on basis of the following criteria: maximum of one missing tooth per quadrant (third molars excluded), a radiographic distance of <3 mm between the cementoenamel junction (CEJ) and the alveolar bone crest on all teeth on recent (not older than 1 year) bite-wings radiographs. The control subjects were matched and paired with the patient group for age, gender, ethnicity and smoking habits (Table 1), because these parameters are established risk determinants for periodontitis (Borrell & Papapanou 2005) and could possibly influence osteoclastogenesis (Jevon et al. 2002).

Table 1. Characteristics of controls and patients

Pair	C/P	Age	Gender	Ethnicity	Smoking
1	C	41	M	Caucasian	+
	P	40	M	Caucasian	+
2	C	27	M	North African	–
	P	26	M	North African	–
3	C	29	M	African black	+
	P	29	M	African black	+
4	C	45	F	African black	–
	P	44	F	African black	–
5	C	35	M	Caucasian	+
	P	35	M	Caucasian	+
6	C	32	F	North African	–
	P	31	F	North African	–
7	C	48	M	Caucasian	+
	P	49	M	Caucasian	+
8	C	40	F	Indian	–
	P	41	F	Indian	–
9	C	40	M	Indian	+
	P	43	M	Indian	+
10	C	37	F	African black	–
	P	37	F	African black	–

C, control; P, patient; M, male; F, female; +, smoker; –, no smoker.

An extensive medical history of each participant was taken by a written questionnaire and by an interview. Each subject was free from systemic diseases and had no clinical symptoms of bacterial, viral or parasitic infections at the time of the study, except for periodontitis in the patient group. None of the subjects in the study had taken any form of medication that could affect their periodontal status, such as antibiotics, anti-inflammatory agents and immune suppressants during the preceding 6 months. Women older than 46 years were excluded from the study to prevent skewing of the data due to the possible onset of menopause-related osteoporosis. All subjects received both verbal

and written information about the purpose of the study. The Medical Ethical Committee of the Academic Medical Center of the University of Amsterdam approved the study and all subjects signed an informed consent.

PBMC isolation

Venous blood was collected by venipuncture in lithium heparin-containing blood collecting tubes (Vacutainer, Becton Dickinson, Plymouth, UK) from both the patient and his corresponding control. Blood samples of the patient and his matched control were taken on the same day and time and were processed within 3 h. Two millilitres of blood was kept separate for determination of the leucocyte composition. PBMCs were isolated from whole blood with Ficoll-Paque density gradient. Heparinized whole blood was diluted in a 1:1 concentration with Hank's Balanced Salt Solution (HBSS; Gibco BRL, Paisley, Scotland, UK). Five parts of diluted blood were layered on top of three parts lymphoprep (Axisshield Po CAS, Oslo, Norway) and centrifuged without brake at 1000 $\times$ *g* for 30 min. at room temperature. The interface was carefully collected and washed two times with α-minimum essential medium (α-MEM; Gibco BRL)+5% foetal calf serum (FCS; HyClone, Logan, UT, USA)+1% antibiotics [PSF: 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B (antibiotic antimyotic solution, Sigma, St. Louis, MO, USA)].

Flow cytometry

Two millilitres of blood was analysed by flow cytometry to differentiate leucocytes. Four-colour flow cytometry was performed at the Hematology Laboratory of the Vrije Universiteit Medical Center (VUMC). The immunophenotypic labelling was performed as follows: the erythrocytes were lysed for 10 min. using Pharmlyse (Becton Dickinson, Franklin Lakes, NJ, USA) and washed once with phosphate-buffered saline (PBS) containing 0.1% human serum albumin. The cells were incubated for 15 min. at room temperature with either fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinyl chlorophyllin (PerCP) or allophycocyanin (APC) conjugated monoclonal antibodies (Becton Dickinson) and washed once with PBS containing 0.1% human serum albumin. The pan-leucocyte marker CD45 (PerCP-labelled) was used to discriminate between white blood cells and unlysed red cells or debris. Lymphoid markers (CD19 PE, CD3 FITC or CD3 APC) were used to discriminate between B-cells and T-cells. CD8 (FITC-labelled) and CD4 (PE-labelled) were included to identify the cytotoxic/suppressor T-cells and T-helper/inducer cells, respectively. CD14 (APC-labelled) was included to identify monocytes. Data acquisition was performed using a FACScalibur equipped with an argon and red diode laser and analysis was performed using Cellquest software (Becton Dickinson). Cells negative for CD19, CD3 and CD14 were classified as 'other, e.g. granulocytes and NK cells'. Based on the forward–sideward scatter of cells, an estimate was made of the total number of granulocytes present. These cells are lost after Ficoll density gradients and consequently not included in the cell cultures.

Osteoclastogenesis assays

PBMCs were cultured in 96-well plates at a density of 10^6 cells per well on plastic and on 650-μm-thick bovine cortical long bone slices. The formation of multinucleated cells (MNCs) was assessed after 3 weeks of culturing on plastic, and bone resorption was evaluated after 4 weeks. The PBMCs were cultured under three conditions: (1) α-MEM (Gibco BRL) supplemented with 5% FCS, (2) α-MEM+FCS+1% PSF and M-CSF (R&D Systems, Minneapolis, MN, USA), (3) α-MEM+5% FCS+1% PSF and M-CSF and RANKL (PreProtech, Rocky Hill, NJ, USA). M-CSF was used at a concentration of 25 ng/ml and RANKL was used at a concentration of 40 ng/ml, concentrations previously found to be optimal in these assays (Olivier et al. 2008). Although a broad range of M-CSF can be used in osteoclastogenesis assays, a concentration of 25 ng/ml M-CSF proved to be optimal (Hodge et al. 2007). Cells were cultured in duplicate and the culture media was changed twice a week. The cultures were incubated in a humidified 5% CO_2 atmosphere at 37°C.

Tartrate-resistant acid phosphatase staining and cell count

TRACP is widely used as a marker enzyme of osteoclast-like cells. Therefore, the number of $TRACP^+$ MNCs was determined after 21 days of culturing. The cultured cells were washed with PBS and fixed in 4% PBS-buffered formaldehyde for 5 min. and stained for TRACP activity using the leucocyte acid phosphatase kit (Sigma). The nuclei were visualized by incubating the cell cultures with diamidino-2-phenylindole-dihydrochloride (DAPI) in PBS. Overall, adhered cells after 21 days seemed viable, because apoptotic cells (shrunken nuclei) were scarcely seen after DAPI staining.

To assess the number of $TRACP^+$ MNCs, five micrographs were taken from pre-determined positions per well with a digital camera (Leica, Wetzlar, Germany) mounted on an inverted light microscope (Leica) equipped with a fluorescence lamp and appropriate filters to visualize blue stained nuclei, using 20 $\times$ magnification and quantified manually using Image-Pro plus software (Media Cybernetics, Silver Spring, ML, USA). The centre of the well was first found with a low magnification, then with an objective of 20 $\times$, this middle micrograph was taken, next the adjacent fields to the left, right and to the top and bottom were photographed. Only cells with three or more nuclei were considered as MNC. The number of nuclei may reflect the maturity of osteoclasts (Piper et al. 1992). Based on the number of nuclei per cell, each MNC was assigned to one of the three groups: 3–5 nuclei or 6–10 nuclei or $>$10 nuclei. The data were expressed as mean numbers of $TRACP^+$ MNCs per mm^2.

RNA analysis and real-time quantitative PCR

RNA from cells before culture and from cells cultured for 3 weeks 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, Eugene, OR, USA). One hundred nanograms of RNA was used in the reverse transcriptase reaction which was performed according to the MBI Fermentas cDNA Synthesis Kit (Vilnius, Lithuania), using both Oligo(dT)18 and D(N)6 primers.

Real-time PCR primers were designed using the Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA, USA) and have been used in a previous study (de Vries et al.

2006). To avoid amplification of genomic DNA, each amplicon spanned at least one intron. The primers used were as follows: cathepsin K: forward primer CCA TATgTgggACAggAAgAgAgTT, reverse primer TgCATCAATggCCACAgAgA; Carbonic anhydrase II: forward primer TggACTggCCgTTCTAggTATT, reverse primer TCTTgCCCTTTgTTTTAATg gAA; β2-microglobulin: forward primer AAgATTCAggTTTACTCACgTC, reverse primer TgATgCTgCTTACATg TCTCg.

The external standard curve used in the PCR reactions is cDNA from the quantitative PCR human reference total RNA (Stratagene, La Jolla, CA, USA), 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 of a two-step PCR were run 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 ΔCt ($Ct_{\beta 2\text{-microglobulin}} - Ct_{\text{gene of interest}}$), and expression of the different genes is expressed as $2^{-(\Delta Ct)}$. Expression data at 21 days of culture were expressed as fold increase from $t = 0$.

Osteoclast resorption assay

PBMCs were cultured in 96-well plates at a density of 10^6 cells per well on bovine cortical long bone slices and analysed for resorption pits. PBMCs were cultured as indicated in the previous paragraph. Cells were cultured for 28 days, after which they were lysed with demineralized water. Cell remnants were removed mechanically after sonicating the bone slices in 10% ammonium hydroxide for 30 min. The bone slices were then thoroughly washed with demineralized water and subsequently incubated in a 10% saturated alum ($KAl(SO_4)_2 \cdot 12H_2O$) solution for 10 min. Finally, the bone slices were washed again with demineralized water and the resorption pits were stained with Coomassie brilliant blue. Five micrographs from pre-determined positions in the centre of the bone slices were taken with a digital camera mounted on a light microscope using 20 × magnification and the percentage of resorbed bone area was quantified using Image-Pro plus software (Media Cybernetics).

Statistical analysis

Statistics were performed using GraphPad InStat version 3.00 (GraphPad Software, San Diego, CA, USA). A paired Student's *t*-test (two tailed) was performed for the comparisons between patients and controls regarding number of teeth present, blood cell counts, number of $TRACP^+$ MNCs on tissue culture plates or bovine cortical bone slices, as well as comparisons between culturing on tissue culture plates or bovine cortical bone slices.

Comparisons between the formation of $TRACP^+$ MNCs of control and patient groups under three different culture conditions on the tissue culture plates were performed by means of repeated-measures ANOVA and, if appropriate, followed by Tukey's multiple comparison post-test. We constructed a general linear model (ANCOVA) to analyse the influence of smoking, gender, sex and ethnicity on the formation of multinucleated cells. Within the patient/control comparison, we have used the values of multinuclearity as a fixed factor, and age, gender, ethnicity and smoking as co-variables.

The level of bone resorption was analysed by the Mann–Whitney (*U*) test.

Results

Population characteristics

The ethnicity, gender, age and smoking habits data of the control and the patient group are presented in Table 1. There was no statistical difference between the patient and the matched control group regarding the number of teeth present. Further, the patient group presented on average 26.0 (SD ± 3.7) teeth with ⩾30% bone loss and 13.9 (SD ± 5.5) teeth with ⩾50% bone loss, attesting to the fact that substantial periodontal destruction was present in these patients. Per definition, no bone loss was found in controls (Table 2).

Table 2. Teeth present and description of severity of periodontal destruction in patients and controls

	Patients	Controls
Total # teeth present	29.0 ± 2.1	28.8 ± 2.4
# teeth with ⩾30% bone loss	26.0 ± 3.7	0 ± 0
# teeth with ⩾50% bone loss	13.9 ± 5.5	0 ± 0

Values are reported as mean ± standard deviation. Percentage bone loss was based on measurements on the periapical radiographs.

Table 3. Blood cell composition of controls and patients

	Controls ($n = 10$)	Patients ($n = 10$)
Whole blood		
T-cells	21.6 ± 7.3	19.8 ± 6.6
B-cells	5.2 ± 4.9	3.8 ± 1.7
Monocytes	7.4 ± 4.0	7.1 ± 2.6
Other (e.g. granulocytes and NK cells)	62.4 ± 10.5	65.4 ± 15.1
In culture		
T-cells	51.1 ± 11.2	49.3 ± 9.6
B-cells	11.7 ± 9.4	9.8 ± 4.8
Monocytes	19.3 ± 12.8	19.1 ± 9.8
Other (e.g. NK cells)	9.9 ± 4.9	11.5 ± 11.0

Values reported are mean percentage ± SD. Percentage of each peripheral blood cell type was determined by flow cytometry. For cells in culture, cell percentages were corrected for the percentage of granulocytes, because this cell type is lost during the density gradient separation. No significant differences were found in blood cell composition between the control and the patient group (paired Student's *t*-test, two-tailed) in both whole blood as well as in culture.

Leucocyte composition

Statistical analyses revealed that there were no differences between the control and the patient group in numbers of T-lymphocytes, B-lymphocytes, NK cells, monocytes or granulocytes (Table 3).

Formation of osteoclast-like cells on tissue culture plates

$TRACP^+$ MNCs formed in both the patient and the control group under the

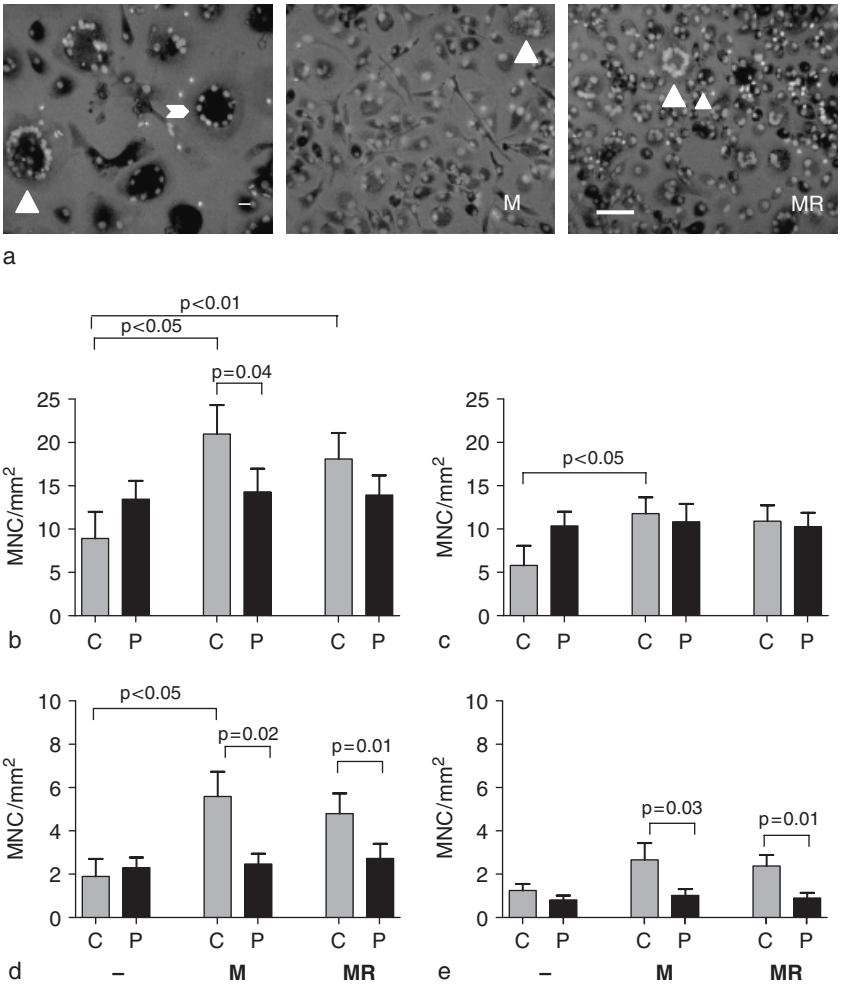

Fig. 1. Formation of tartrate-resistant acid phosphatase-positive ($TRACP^+$) multinucleated cells (MNCs) on tissue culture plates. (a) Micrographs taken after 21 days of culturing and consecutive TRACP staining under the conditions: without cytokines (–), macrophage colony-stimulating factor (M-CSF) (M) and M-CSF and receptor activator of nuclear factor-κB ligand (RANKL) (MR). Small triangle: multinucleated cell containing 3–5 nuclei; arrowhead: cell containing 6–10 nuclei; large triangle: multinucleated cell containing >10 nuclei. (b) Number of multinucleated cells from control (C) and patient (P) peripheral blood mononuclear cells under the following culture conditions: without cytokines (–), M-CSF (M) and M-CSF+RANKL (MR). (c–e) Results as in (b), subdivided as number of nuclei per cell category, number of $TRACP^+$ MNC containing 3–5 nuclei (c), 6–10 nuclei (d) and >10 nuclei (e). Data are represented as mean number of $TRACP^+$ MNCs per $mm^2 \pm$ SEM (for culturing without cytokines and with M-CSF: $n = 9$ per group; for culturing with M-CSF+RANKL: $n = 10$ per group).

three culture conditions (Fig. 1a). The arrangement of the nuclei in large multinucleated cells was often in a circular configuration (Fig. 1a). In the presence of M-CSF in the control group, more $TRACP^+$ MNCs with $\geqslant 3$ nuclei were found compared with the patient group. Culturing without the addition of cytokines or with M-CSF+RANKL did not differ between the groups (Fig. 1b). No differences in smaller (3–5 nuclei per cell; Fig. 1c) $TRACP^+$ MNCs existed. However, significantly larger $TRACP^+$ MNCs, with 6–10 nuclei (Fig. 1d) per cell and >10 nuclei per cell (Fig. 1e), were formed in the control group under culture conditions with M-CSF (M-CSF; M-CSF+RANKL). General linear model considering the formation of multinucleated cells as dependent factor and age, gender, race and smoking as co-variables indicated that none of these variables contributed to the observed differences between patients and controls.

When comparing the formation of $TRACP^+$ MNCs under the three culture conditions, two patterns became apparent. First, higher numbers of $TRACP^+$ MNCs were present when culturing control PBMCs in the presence of M-CSF (M-CSF; M-CSF+RANKL; Fig. 1b–e). In contrast, in the patient group, addition of M-CSF or M-CSF+RANKL did not increase the formation of $TRACP^+$ MNCs (Fig. 1b–e). Overall Fig. 1 mainly appears to show that the cells from patients are refractory to M-CSF stimulation.

We further investigated the osteoclastic character of the cells cultured for 3 weeks under the three conditions by analysing expression of the osteoclast-related genes cathepsin K and carbonic anhydrase II. For all culture conditions, these markers were 120–493 × and 10–46 × higher, respectively (Fig. 2), than in PBMCs just after isolation. M-CSF alone caused a significant increase compared with cultures without cytokines. Expression levels of cultures where M-CSF was added were comparable with expression levels of cultures that had received both M-CSF and RANKL. No differences in expression were observed between controls and patients.

Formation of osteoclast-like cells on bovine cortical bone slices

In contrast to the abundant $TRACP^+$ MNCs generated when cultured on tissue culture plates, very few $TRACP^+$ MNCs were detected when cells were cultured on bone slices (Fig. 3). On bone, no differences were observed in the formation of $TRACP^+$ MNCs between patient and control for any of the culture conditions.

We next compared the formation of $TRACP^+$ MNCs on tissue culture plastic and on bone slices (Table 4; derived from Figs 1 and 3). Numbers of $TRACP^+$ MNCs were significantly higher on tissue culture plates than those on cortical bone slices.

Bone resorption

PBMCs were cultured for 28 days on bovine cortical bone slices under the three culture conditions and resorption pits were visualized (Fig. 4). Bone resorption was found exclusively when cells were cultured in the presence of M-CSF and RANKL, albeit in a low number of control (3 out of 10) and patient (3 out of 10) PBMC cultures, not allowing any further statistical analysis.

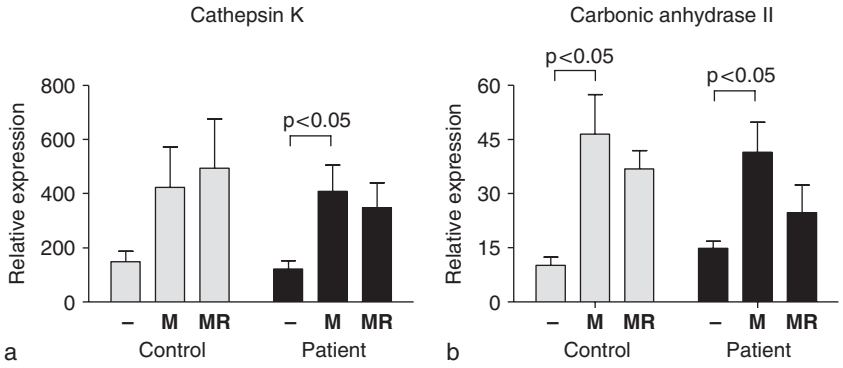

Fig. 2. Expression of osteoclast-related genes. Expression of cathepsin K (a) and carbonic anhydrase II (b) was assessed in peripheral blood mononuclear cell (PBMC) cultures from periodontitis patients and matched controls ($n = 5$) who either received no cytokines (–), macrophage colony-stimulating factor (M-CSF) alone (M) or M-CSF and receptor activator of nuclear factor-κB ligand (MR). Data are represented as fold increase compared with expression by PBMCs at isolation (mean increase ± SEM).

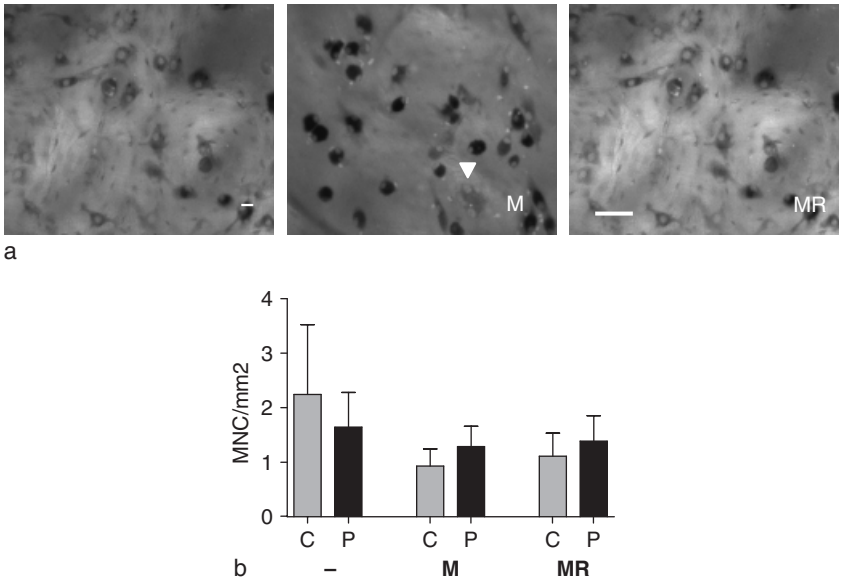

Fig. 3. Formation of tartrate-resistant acid phosphatase-positive ($TRACP^+$) multinucleated cells (MNCs) on bovine cortical bone slices. (a) Micrographs taken after 21 days of culturing under the conditions: without cytokines (–), macrophage colony-stimulating factor (M-CSF) (M) and M-CSF and receptor activator of nuclear factor-κB ligand (RANKL) (MR). Nuclei were visualized with diamidino-2-phenylindole-dihydrochloride. Bar = 50 μm. (b) Number of $TRACP^+$ MNCs of controls (C) and patients (P) under the following culture conditions: without cytokines (–), M-CSF (M) and M-CSF+RANKL (MR). Data are mean number of $TRACP^+$ MNCs per mm^2 ± SEM (for culturing without cytokines and with M-CSF: $n = 9$ per group; for culturing with M-CSF+RANKL: $n = 10$ per group). No significant differences were observed for any of the culture conditions.

Table 4. Excess of osteoclasts formed on plastic *versus* bone

	–	M	MR
Controls			
Plastic/bone	4.0*	22.5***	17.0***
Patients			
Plastic/bone	8.2***	11.1***	10.6***

*Not significant.
***$p < 0.001$.
Plastic/bone: ratio of number of osteoclast-like cells formed on plastic compared with that on bone ($n = 9$).
–, M, MR: culture conditions without cytokines, with M-CSF and with M-CSF+RANKL.

Discussion

In the present study, we found that the formation of $TRACP^+$ MNCs from PBMCs of chronic periodontitis patients may differ from the formation of these cells by blood cells of control subjects: PBMCs from periodontitis patients did not require supplementation of M-CSF or M-CSF/RANKL for the formation of osteoclast-like cells, whereas in case of controls, addition of these cytokines was needed to induce a higher number of MNCs. A possible explanation might be that in chronic periodontitis patients, priming of the precursors of osteoclasts already takes place in the peripheral blood, possibly by $CD4^+$ cells present in the circulation (Brunetti et al. 2005); and therefore additional stimulation with cytokines does not influence osteoclast formation. Although cultures of controls and patients contained comparable numbers of $CD14^+$ cells, properties (e.g. RANKL expression) of B- and T-lymphocytes could differ between controls and patients. Precursors of healthy controls, however, can still be influenced by these cytokines. A similar insensitivity for priming was observed by Roato et al. (2005) who compared osteoclast formation from PBMCs from healthy controls and cancer patients with secondary bone loss due to metastatic disease. When PBMCs from cancer patients without bone-associated metastases or from healthy controls were stimulated with increasing concentrations of RANKL, a dose-dependent increase in osteoclast-like cells was found, while this dose dependency was not seen when PBMCs of cancer patients with bone breakdown were stimulated with increasing concentrations of RANKL (Roato et al. 2005).

Previously, it was found in various studies comparing osteoclast formation from PBMCs from various bone destructive (osteopenic) diseases that PBMCs from patients gave rise to more $TRACP^+$ MNCs than controls. This was found, for instance, in postmenopausal osteoporosis (D'Amelio et al. 2005), bone tumors (Roato et al. 2005) and also in periodontitis (Brunetti et al. 2005). Also, in another study in which we assessed osteoclast formation from peripheral blood of chronic liver disease patients, we found that osteoclast formation from patients with chronic liver disease with osteoporosis is higher than that in similar patients without osteoporosis (Olivier et al. 2008). To our surprise, and not in line with the mentioned bone diseases with concomitant excessive bone degradation, more

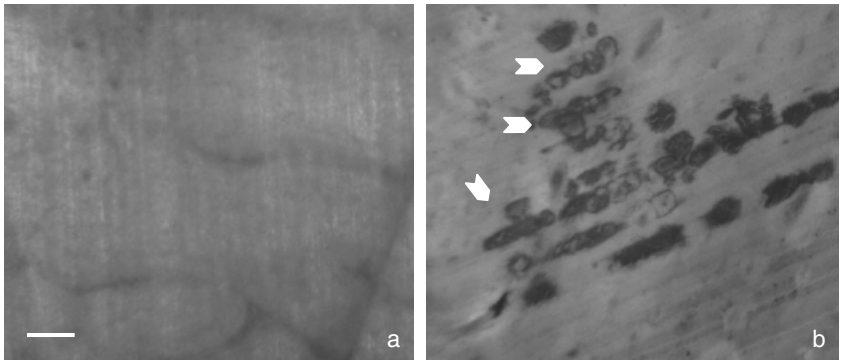

Fig. 4. Bone resorption micrographs taken after 28 days of culturing with macrophage colony-stimulating factor and receptor activator of nuclear factor-κB ligand: (a) no bone resorption, (b) resorption pits are present (arrows) on the bone surface. Scale bar = 30 μm.

$TRACP^+$ MNCs were formed by control PBMCs than by PBMCs from severe periodontitis patients. Noteworthy, these higher numbers of $TRACP^+$ MNCs were only observed when cultured with M-CSF or M-CSF/RANKL, suggesting that osteoclast precursors of healthy controls are more susceptible to M-CSF or M-CSF/RANKL.

Our results are in conflict with the results of Brunetti et al. (2005), who observed a higher number of $TRACP^+$ MNCs and bone resorption by PBMCs of periodontitis patients compared with controls. In the latter study, increased osteoclast formation in periodontitis patients was due to increased RANKL and TNF-α production by peripheral T-lymphocytes. Although these characteristics were not investigated in our patient and control groups, a possible explanation for the discrepancies between the latter study and the current investigation could be due to different matching procedures. Both age and sex influence the osteoclastogenic potential of peripheral blood cells (Jevon et al. 2002). In our study, the patient group consisted of untreated subjects with generalized severe chronic periodontitis, and in order to control known confounding factors, we meticulously matched the healthy controls for age, gender, ethnicity and smoking. Moreover, control and patient blood samples were processed simultaneously; thus, patient and control were matched within each experiment.

Morphologically, the circular orientation of nuclei within a multinucleated cell (Fig. 1a) is normally not seen in osteoclasts and is suggestive that these cells are in fact related to Langhans' giant cells (Anderson 2000). Like osteoclasts, Langhans' giant cells are derived from monocytes or monocyte progenitor cells, but both cell types are differently induced by cytokines. Our RT-PCR further reveals that osteoclast markers cathepsin K and carbonic anhydrase II were increased (120–493 × and 10–46 ×, respectively) at the time point where multinucleated cells were analysed, indicating that these cell cultures contained some characteristics typical of osteoclasts. Remarkably, this increase was seen in cell cultures where no cytokines were added and in cell cultures containing M-CSF (M-CSF or M-CSF+RANKL). M-CSF alone caused an upregulation, while extra supplementation with RANKL did not further upregulate osteoclast markers. Supplementation with RANKL also did not increase the number of multinucleated cells, but RANKL was obligatory for resorption.

In accordance with other studies, where the number of $TRACP^+$ MNCs on cortical bone slices was counted and compared with cells cultured on plastic, we found a lower number of $TRACP^+$ MNCs on bone (Faust et al. 1999, de Vries et al. 2006). Moreover, these osteoclast-like cells contained fewer nuclei compared with cells cultured on tissue culture plates. Consistent with this is the low number of patients and controls where bone resorption was observed on bovine cortical bone slices. It might well be that inhibitory factors present in the cortical bone could cause this effect. One of these factors could be transforming growth factor-β (TGF-β), which is found in high levels in bone. Osteoclasts can activate the latent form of TGF-β (Oursler 1994), and the activated form of TGF-β could act as a natural inhibitor of osteoclast activity by inhibiting osteoclast formation (Kukita et al. 1990). Especially long exposure time of human CD14-positive osteoclast precursors to TGF-β – possibly occurring on bone slices – results in downregulation of RANK, and subsequently osteoclast formation and resorption was inhibited (Karsdal et al. 2003).

In conclusion, the current data strongly suggest that peripheral blood of chronic periodontitis patients contains relatively mature precursors of osteoclasts that have the capacity to become osteoclast-like cells, also without stimulation with added M-CSF and/or RANKL. Possibly, because these precursors are relatively mature, they need less triggering at the inflammatory site to further differentiate into osteoclasts.

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Address:
Teun de Vries
Experimental Periodontology
c.o. Department of Oral Cell Biology
ACTA, Universiteit van Amsterdam and Vrije Universiteit
Van der Boechorststraat 7
1081 BT Amsterdam
The Netherlands
E-mail: teun.devries@vumc.nl

Clinical Relevance

Scientific rationale for the study: Peripheral blood contains precursor cells of osteoclasts, the bone-degrading cell responsible for alveolar bone degradation in periodontitis. Osteoclast formation from precursors in peripheral blood is altered in a variety of bone destructive diseases. Here, we investigated osteoclast formation from peripheral blood of periodontitis patients. *Principle findings:* Peripheral blood-derived osteoclast formation in periodontitis patients occurs without induction by the cytokines M-CSF and RANKL. *Practical implications:* Osteoclast formation may occur more rapidly from blood of periodontitis patients. The precursors of these cells seem already primed in the circulation before entry of the site of periodontal bone breakdown.