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Fundamental study of osteoclast chemotaxis toward chemoattractants expressed in periodontitis

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Background and Objective: Periodontitis is a chronic inflammatory disease that leads to bone resorption by osteoclasts (OCs). Several factors contribute to the differentiation of OCs from hematopoietic precursors. Cellular chemotactic factors are expressed in periodontitis tissue, but the effects of these chemoattractants on OCs are not well understood. Here we examined the effects of chemoattractants produced in inflamed periodontal tissue on OC chemotaxis.

Material and Methods: Rat bone-marrow OCs were cultured in OC culture medium for 3 or 6 d. Using EZ-TAXIScan™, the chemotactic response of these OCs to several chemoattractants [monocyte chemotactic protein-1; macrophage inflammatory protein 1α; regulated on activation, normal T-cell expressed and secreted; stromal cell-derived factor-1α; and complement activation product 5a (C5a)] was measured. In addition, we measured the effect of C5a-specific inhibitors on chemotactic responses toward C5a. The recorded chemotactic responses were quantitatively analysed using ImageJ software.

Results: Chemoattractants associated with periodontal disease significantly increased the chemotactic activity of differentiated rat OCs in a concentration-dependent manner, with C5a inducing the highest chemotactic activity of OCs cultured for 3 or 6 d. The C5a-specific inhibitor significantly inhibited chemotaxis toward C5a in a concentration-dependent manner.

Conclusion: We suggest that C5a plays an important role in pathologic bone resorption in periodontal disease by stimulating the chemotaxis of OCs. Therefore, C5a is a potential target for the treatment of periodontal disease.

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Periodontitis, a chronic inflammatory disease associated with localized bone resorption, is one of the most frequent causes of tooth loss. Chemoattractants, such as monocyte chemotactic protein 1 (MCP-1) (1,2); macrophage inflammatory protein-1 alpha (MIP-1α) (3,4); regulated on activation, normal T-cell expressed and secreted (RANTES) (3,5,6); stromal cell-derived factor-1 alpha (SDF-1α) (7); and complement intermediate product 5a (C5a) (8–10), are detected in periodontal tissue or in gingival crevicular fluid following prolonged inflammation. Chemoattractants increase the infiltration of inflammatory cells into periodontitis tissues (1). The inflammatory cytokines secreted by such inflammatory cells promote osteoclast (OC) differentiation in periodontitis tissues, leading to bone resorption (11,12). The effect of chemoattractants on OCs to enhance OC differentiation and increase OC-mediated bone resorption is currently a hot topic in dentistry (13,14).

Studies of the effects of chemoattractants on cellular chemotaxis have mainly used cell-membrane permeation tests, such as the typical Boyden chamber test (15), in which a multiperforated membrane separates an upper chamber from a lower chamber. Cells are added to the upper chamber, chemoattractants are added to the lower chamber, and the number of cells that migrate into the lower chamber or attach to the membrane is measured. However, several problems associated with this membrane-permeability test exist, such as the need for a relatively large number of cells, the inability to observe the chemotactic reaction directly, the inability to distinguish chemotaxis from random migration, the effects of gravity on the assay and difficulty in maintaining a concentration gradient and in determining concentration-dependent responses. The principles underlying the measurement of chemotactic responses in the recently developed EZ-TAXIScan™assay (16,17) differ from those of traditional membrane-permeability tests. The EZ-TAXIScan™results in the formation of a stable and highly reproducible chemoattractant concentration gradient between a glass plate and a thin microfabricated silicon chip (channel). Cells are chemoattracted toward and into the channel. Images of the horizontal chemotaxis of the cells can be captured using a charge-coupled device camera, which can measure the chemotactic speed and angle. The use of the EZ-TAXIScan™therefore allows for a more quantitative analysis of cellular chemotactic responses than other methods. The purpose of the present study was to examine and measure the effects of chemoattractants, expressed in periodontitis tissues, on OC chemotaxis using the EZ-TAXIScan™ method.

Material and methods

OC culture

OC cultures were performed according to the instructions of the OC culture kit (Primary Cell Co., Ltd., Sapporo, Japan), as follows. OCs were derived from bone marrow cells of Sprague–Dawley rats. These cells were purchased as part of the OC culture kit. The bone marrow cells were cultured in α-modified Eagle's minimum essential medium containing 10% fetal bovine serum, 50 ng/mL of RANKL, and 50 ng/mL of macrophage colony-stimulating factor to which penicillin (100 U/mL) and streptomycin (100 μg/mL; Wako Pure Chemical Industries Ltd., Osaka, Japan) were added. The bone marrow-derived cells were seeded onto Upcell® temperature-responsive culture dishes (CellSeed Inc., Tokyo, Japan) (18) and incubated at 37°C in the presence of 5% CO_2 in 100% humidity. After incubation for 24 h, nonadherent cells were washed away with phosphate-buffered saline (Sigma-Aldrich Japan Corp., Tokyo, Japan). The medium was replaced every 2 d, and the cells were allowed to differentiate for 3 d (3-d group) or 6 d (6-d group). After culture, the medium was replaced with RPMI-1640 (Nacalai Tesque Inc., Kyoto, Japan) supplemented with 0.1% bovine serum albumin (Sigma-Aldrich Japan Corp.). OCs were harvested by incubating the Upcell® dish at 4°C for 5 min. The cell density of the harvested OCs was adjusted to 2×10^6 cells/mL in RPMI-1640.

Horizontal chemotactic assay

Cellular chemotactic responses were measured using the EZ-TAXIScan™(ECI, Inc., Kawasaki, Japan). A type I collagen-coated coverslip (BD Bioscience, Bedford, MA, USA) was placed between the glass plate and the silicon chip. The thickness of the silicon chip used to analyse the 3-d group was 6 μm, and the thickness was 8 μm for the 6-d group. The assay medium used was RPMI-1640 containing 0.1% bovine serum albumin. An OC cell suspension (1 μL) was applied to each well on one side of the apparatus and preincubated for 1 h at 37°C. A chemoattractant (1 μL) was then injected into the well on the other side, and chemotaxis was immediately recorded for 2 h at 37°C. We used the following recombinant rat chemoattractants: MCP-1, MIP-1α, RANTES, SDF-1α (all from Peprotech Inc., Rocky Hill, NJ, USA) and C5a (Hycult Biotech, Uden, the Netherlands). Each protein was dissolved (in RPMI-1640 containing 0.1% bovine serum albumin) at concentrations ranging from 1 nM to 1 μM, except for C5a, which was dissolved at concentrations ranging from 1 nM to 10 μM. One channel also recorded random migration without chemoattractant, as a control.

The highest chemotactic response observed was toward C5a; therefore, we examined the inhibitory effect of H-8135 (Bachem Distribution Services GmbH, Weil am Rhein, Germany), a specific C5a inhibitor, on chemotaxis toward C5a. H-8135 was dissolved in trifluoroacetic acid (TFA) (Nacalai Tesque Inc.). After an OC cell suspension was applied in the same manner as described above, 1 nM to 1 μM H-8135 was added and preincubated for 1 h at 37°C. In one channel, 0.0001% TFA was also added, and, in another channel, no H-8135 or TFA was added. C5a (1 μL) was then injected into the well on the other side, and chemotaxis was immediately recorded for 2 h at 37°C. For the 3-d culture group, 1 μM C5a was used; and for the 6-d culture group, 100 nM C5a was used.

All procedures were repeated three independent times.

Analysis of chemotactic responses

Images recorded by the EZ-TAXIScan™ were imported to ImageJ (National Institutes of Health, Bethesda, MD, USA), and the chemotactic angle of each cell was calculated by a centroid trajectory once every minute. Cells with a mean chemotactic angle of $\leq 45°$ were considered chemotactic, and the percentage of chemotactic cells in the population was calculated. Thirty chemotactic cells were selected from each channel for calculation of their chemotactic speed.

Statistical analysis

Data are shown as mean ± SE. Significant differences between two groups were analysed using the Student's *t*-test, and differences among at least three groups were tested with one-way ANOVA. The Tukey–Kramer test was

used for post-hoc analysis. A value of $p < 0.05$ was considered significant.

Results

Chemotaxis of OCs in response to various chemoattractants

Chemotactic responses of OCs to different concentrations of the chemoattractants analysed in this study were compared by measuring the cellular chemotactic speed and angle using the EZ-TAXIScan™, and the percentage of chemotactic cells was calculated. We examined the chemotactic responses of the 3- and 6-d groups of OCs toward MCP-1, MIP-1α, RANTES, SDF-1α (all 1 μM to 1 nM) and C5a (10 μM to 1 nM), chemokines that have been confirmed to be expressed in periodontitis tissue. The 3-d group exhibited a significantly higher chemotactic response to MCP-1 (1 μM and 100 nM), MIP-1α (100 nM), SDF-1α (100 nM and 10 nM) and C5a (1 μM and 100 nM) compared with other concentrations or with the control (no chemoattractant); however in Table 1 there is no indication of significance for chemotactic cells with MCP-1 (1 μM and 100 nM), MIP-1α (100 nM) or SDF-1α (10 nM). RANTES (100 nM) also tended to enhance the chemotactic response, but the increase was not statistically significant. The 6-d group also exhibited a significantly higher chemotactic response to MIP-1α (1 μM and 100 nM), SDF-1α (100 nM) and C5a (1 μM and 100 nM) compared with the control. MCP-1 and RANTES (each at 100 nM) also tended to enhance the chemotactic response, but these increases were not statistically significant. The migration speed of the control group (no chemoattractant) did not differ significantly from that of other groups toward chemoattractants other than C5a in either the 3- or 6-d groups. The percentage of cells showing random migration was low; most control cells showed only deformation of the cell shape in both the 3- and 6-d groups (data not shown), but migration was directed toward the chemoattractants (Tables 1 and 2, Fig. 1).

Comparison of rat OCs at different culture time-points

In consideration of the results from the measurement of either chemotactic speed or chemotactic angle or percentage of chemotactic cells, all chemoattractants that induced a chemotactic response did so at a concentration of 100 nM. We therefore used chemoattractant to compare the chemotactic responses of OCs cultured for 3 or 6 d. The 6-d culture group of cells moved toward SDF-1α and C5a with a significantly higher chemotactic speed than did the 3-d culture group. The 3-d culture group of cells moved toward RANTES with a significantly higher chemotactic speed than did the 6-d culture group. We observed no significant difference in the chemotactic angle or in the percentage of chemotactic cells between the two groups. A significantly higher chemotactic speed was also observed in the 6-d control group compared with the 3-d control group (Fig. 2).

Comparison of the effect of different chemoattractants

The chemotactic response of the 3- and 6-d groups to each chemoattractant at 100 nM was then compared. Both the 3- and 6-d groups had significantly higher chemotactic speeds and lower chemotactic angles toward C5a, and C5a induced a higher percentage of chemotactic cells compared with the other chemoattractants (Tables 1 and 2).

Table 1. Chemotactic responses of osteoclasts cultured for 3 d

Variable	Concentration of chemotactic factor 10 μM	1 μM	100 nM	10 nM	1 nM	Control
Speed (μm/h)						
MCP-1		31.3 ± 1.6[a]	31.7 ± 2.3[a]	25.4 ± 2.1	18.5 ± 1.5[b]	24.4 ± 2.9
MIP-1α		26.1 ± 1.6	34.2 ± 2.6[c]	30.4 ± 2.3	23.5 ± 1.7[d]	23.0 ± 2.5[d]
RANTES		24.4 ± 1.7	31.8 ± 2.0	25.1 ± 1.7	26.3 ± 2.1	23.8 ± 2.4
SDF-1α		31.3 ± 2.1	28.2 ± 1.9	35.2 ± 2.3[e]	30.2 ± 1.5	24.7 ± 2.2[f]
C5a	36.7 ± 2.0[g]	54.1 ± 2.3[h]	41.0 ± 2.6[g, i]	29.5 ± 2.3[g, j]	31.1 ± 2.3[g]	32.4 ± 3.2[g]
Angle (°)						
MCP-1		25.6 ± 2.2	17.3 ± 2.0	23.5 ± 2.5	24.4 ± 2.4	
MIP-1α		29.1 ± 3.0	19.2 ± 2.8[k]	24.1 ± 3.1[l]	34.5 ± 3.0	
RANTES		27.9 ± 3.6	26.7 ± 4.0	28.7 ± 2.8	28.4 ± 3.4	
SDF-1α		25.4 ± 2.4	23.3 ± 1.6	20.3 ± 1.9	20.5 ± 1.7	
C5a	27.3 ± 3.0[m]	15.2 ± 1.6[n]	8.8 ± 1.3[n]	22.3 ± 2.6[m]	26.6 ± 2.6[m]	
Chemotactic cells (%)						
MCP-1		23.0 ± 3.4	23.0 ± 3.4	21.5 ± 3.5	15.5 ± 3.4	
MIP-1α		22.5 ± 2.4	24.4 ± 8.4	23.3 ± 6.4	15.0 ± 3.2	
RANTES		14.4 ± 2.2	21.4 ± 4.0	18.1 ± 2.8	18.6 ± 1.4	
SDF-1α		16.1 ± 0.3[o]	32.7 ± 4.3[p]	23.1 ± 4.8	30.8 ± 5.5	
C5a	24.5 ± 3.1[q]	77.2 ± 1.3[r]	69.3 ± 4.9[r]	26.4 ± 0.9[q]	28.1 ± 2.9[q]	

Data are expressed as mean ± SE. [a]$p < 0.05$ vs. [b], [c]$p < 0.05$ vs. [d], [e]$p < 0.05$ vs. [f], [g]$p < 0.05$ vs. [h], [i]$p < 0.05$ vs. [j], [k]$p < 0.05$ vs. [l], [m]$p < 0.05$ vs. [n], [o]$p < 0.05$ vs. [p], [q]$p < 0.05$ vs. [r].

C5a, complement activation product 5a; MCP-1, monocyte chemotactic protein-1; MIP-1α, macrophage inflammatory protein-1 alpha; RANTES, regulated on activation, normal T-cell expressed and secreted; SDF-1α, stromal cell derived factor-1 alpha.

Table 2. Chemotactic responses of osteoclasts cultured for 6 d

Variable	Concentration of chemotactic factor 10 μM	1 μM	100 nM	10 nM	1 nM	Control
Speed (μm/h)						
MCP-1		30.5 ± 1.4	35.7 ± 2.0	29.4 ± 1.6	33.2 ± 2.2	33.9 ± 3.0
MIP-1α		36.6 ± 1.8	39.6 ± 2.3	36.4 ± 2.6	23.0 ± 2.2	28.7 ± 3.3
RANTES		24.3 ± 1.5	24.9 ± 1.6	22.6 ± 1.5	24.9 ± 2.0	25.9 ± 1.9
SDF-1α		36.7 ± 2.4	39.0 ± 1.5[a]	29.5 ± 1.4[b]	31.3 ± 2.2	33.1 ± 2.5
C5a	27.8 ± 2.4[c]	54.1 ± 2.8[d]	63.6 ± 2.1[d]	34.9 ± 1.6[c]	35.4 ± 2.2[c]	33.7 ± 1.8[c]
Angle (°)						
MCP-1		25.9 ± 3.6	19.3 ± 2.1	21.7 ± 2.5	29.3 ± 3.3	
MIP-1α		26.1 ± 1.7	16.5 ± 3.8	21.2 ± 1.8	28.5 ± 6.2	
RANTES		22.6 ± 2.7	20.8 ± 3.1	23.8 ± 3.5	21.4 ± 2.9	
SDF-1α		34.2 ± 3.5[e]	15.4 ± 2.3[f]	18.5 ± 2.4[f]	22.7 ± 3.0	
C5a	19.5 ± 2.0[g]	11.0 ± 1.3[h]	9.8 ± 1.3[h]	19.3 ± 2.2[g]	26.8 ± 2.0[g]	
Chemotactic cells (%)						
MCP-1		17.8 ± 2.8	21.9 ± 2.6	18.8 ± 5.9	12.5 ± 3.5	
MIP-1α		22.8 ± 2.6[i]	25.9 ± 1.3[i]	19.3 ± 1.1[j]	13.8 ± 1.5[k]	
RANTES		17.9 ± 3.2	18.5 ± 2.2	18.2 ± 4.6	18.0 ± 1.5	
SDF-1α		19.5 ± 6.0	25.1 ± 2.7	21.6 ± 2.3	14.8 ± 0.6	
C5a	23.9 ± 0.3[l,n]	71.3 ± 0.7[m]	76.4 ± 0.5[m]	34.6 ± 2.3[l, o]	19.5 ± 3.3[l]	

Data are expressed as mean ± SE, [a]$p < 0.05$ vs. [b], [c]$p < 0.05$ vs. [d], [e]$p < 0.05$ vs. [f], [g]$p < 0.05$ vs. [h], [i]$p < 0.05$ vs. [j], [j]$p < 0.05$ vs. [k], [l]$p < 0.05$ vs. [m], [n]$p < 0.05$ vs. [o].

C5a, complement activation product 5a; MCP-1, monocyte chemotactic protein-1; MIP-1α, macrophage inflammatory protein-1 alpha; RANTES, regulated on activation, normal T-cell expressed and secreted; SDF-1α, stromal cell derived factor-1 alpha.

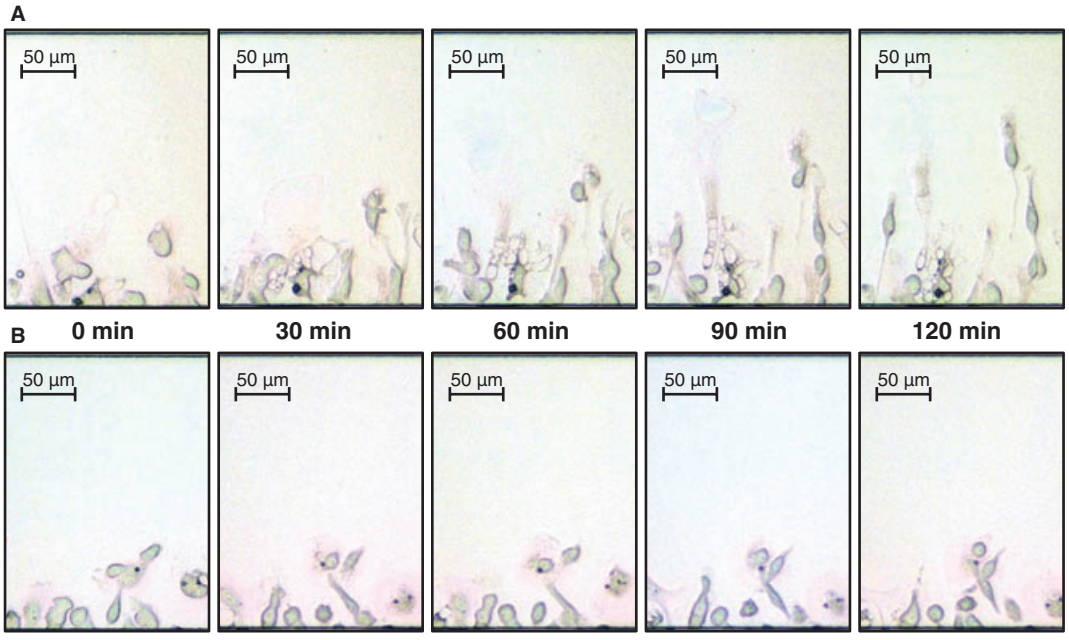

Fig. 1. Chemotactic effect of complement activation product 5a (C5a) on rat osteoclasts, visualized using the EZ-TAXIScan™. Rat osteoclasts cultured for 6 d were placed in one compartment of an EZ-TAXIScan™ holder and 100 nM C5a was then placed in the opposite compartment. Directed migration toward C5a (A), and random osteoclast migration toward the control (B), was visualized over time.

Inhibitory effect of the C5a-specific inhibitor

Because C5a induced the highest chemotactic response, we examined the inhibitory effect of H-8235 on chemotaxis toward C5a. We used the C5a concentration that induced the highest chemotactic reaction in both the 3-d (1 μM) and 6-d (100 nM) groups. In both groups, the chemotactic speed was inhibited by the addition of 1 μM H-8135. Regardless of the concentration of H-8135, we observed no significant differences in the percentage of chemotactic cells. TFA is cytotoxic, but we confirmed that 0.0001% TFA, which was the final

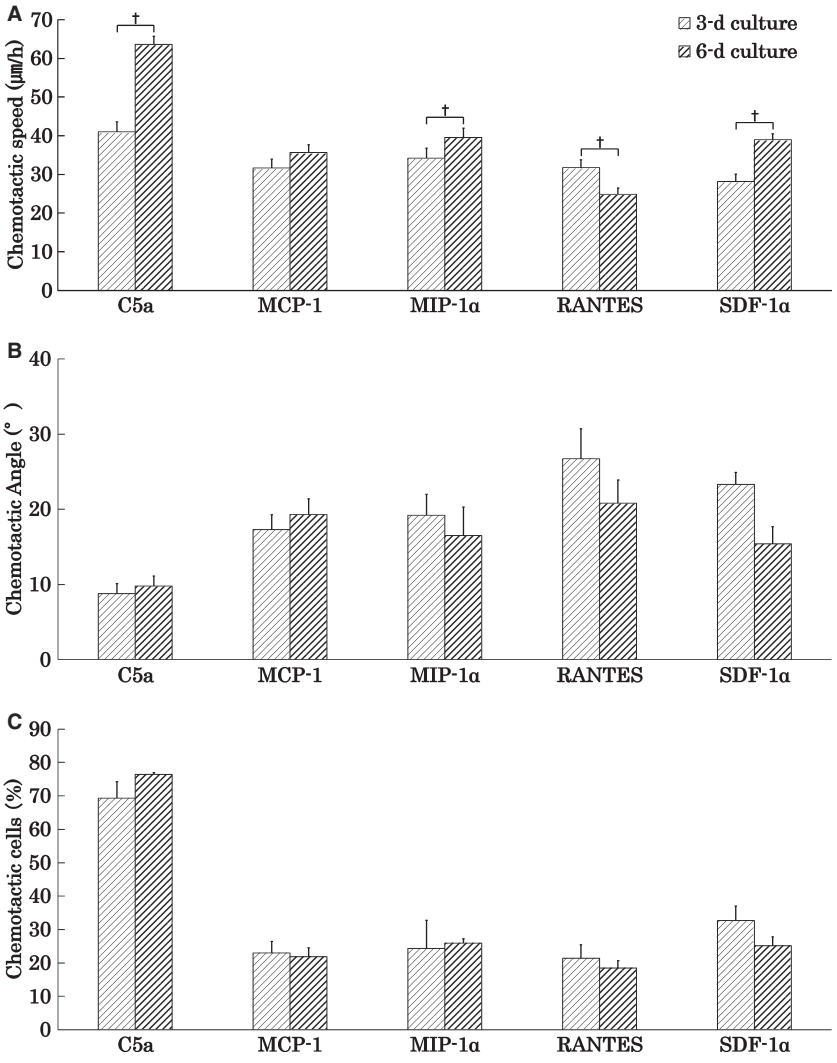

Fig. 2. Differences in the chemotaxis of osteoclasts, cultured for different periods of time, toward various chemotactic factors. Chemotaxis of rat osteoclasts, cultured for 3 and 6 d, toward the indicated factors was determined by assaying (A) the chemotactic speed and (B) the chemotactic angle (°) using the EZ-TAXIScan™. The percentage of chemotactic cells in each population was then determined (C). Cells cultured for 6 d migrated significantly faster toward complement activation product 5a (C5a) and stromal cell derived factor-1 alpha (SDF-1α) than did cells cultured for 3 d (A). Cells cultured for 3 d migrated significantly faster toward regulated on activation, normal T-cell expressed and secreted (RANTES) than did cells cultured for 6 d (A). No significant differences were observed in the chemotactic angle and percentage of chemotactic cells between the two groups (B, C). Data are expressed as mean ± SE. $^{\dagger}p < 0.05$.

concentration in which 1 μM H-8135 was diluted, had no significant inhibitory effect on the chemotactic response toward C5a (Tables 3 and 4).

Discussion

Periodontal disease, a highly morbid chronic inflammatory disease associated with bone resorption, is one of the most frequent causes of tooth loss in adults. Alveolar bone maintains a dynamic equilibrium through bone formation by osteoblasts and bone resorption by OCs. Pathological bone loss in periodontitis tissue is caused by an imbalance in this dynamic equilibrium with increased bone resorption by OCs. Although our understanding of the differentiation, cell fusion and bone-resorptive capacity of OCs has increased since the discovery of RANKL in 1998 (13,15,16,19), much remains unknown regarding OC activation in relation to the pathology of periodontal disease. Understanding this pathological mechanism is crucial for the development of potential new treatments for periodontitis. Periodontitis tissue and gingival crevicular fluid express various chemoattractants that increase the migration of inflammatory cells into the periodontitis tissue as part of the immune response (3). During long-term inflammation, inflammatory cytokines released by inflammatory cells enhance bone resorption by activating OCs (20).

The most widely used method for measuring chemotaxis of cells, such as OCs, *in vitro* is the Boyden Chamber method (15). However, maintaining a concentration gradient of the chemoattractant and quantifying the chemotactic response are difficult with this method. In the present study the EZ-TAXIScan™method was used to determine the chemotactic responses of cultured rat OCs. This method has several advantages for measuring chemotaxis compared with the widely used Boyden Chamber method and other methods. In addition, because OCs are strongly adherent cells, trypsin/EDTA or collagenase are usually used to harvest them (21). These reagents, however, damage cells and decrease cell activity. In the present study, we used the UpCell® culture dish (18), which allows cell harvesting without such reagents. Thus, the OCs used in this study could be considered as low reagent-impaired OCs.

In this study, MCP-1, MIP-1α, RANTES, SDF-1α and C5a, whose expression in periodontitis tissue and gingival crevicular fluid has been confirmed, were used as chemoattractants. MCP-1 is a major chemotactic factor for cells and recruits macrophages to periodontitis-affected tissue (22). MCP-1 also increases OC differentiation and chemotactic responses (23,24). Both MIP-1α and RANTES bind to the chemokine receptor, CCR1, which is expressed on pre-OCs (25,26) and

Table 3. Chemotaxis inhibitory effect of H-8135 on complement activation product 5a (C5a) in osteoclasts cultured for 3 d

Variable	C5a1 μM	H-8135 1 μM	H-8135 100 nM	H-8135 10 nM	H-8135 1 nM	C5a 1 μM + TFA 0.0001%
Speed (μm/h)	58.6 ± 2.0[a]	46.6 ± 2.1[b]	50.9 ± 1.3	52.9 ± 2.0	55.1 ± 1.4[a]	58.0 ± 1.5[a]
Angle (°)	17.7 ± 2.5	26.5 ± 2.7[c]	14.9 ± 1.6[d]	15.8 ± 2.4[d]	19.3 ± 1.8	19.9 ± 3.1
Rate (%)	71.7 ± 4.0	61.9 ± 4.3	75.4 ± 0.8	75.2 ± 0.6	76.5 ± 0.3	69.9 ± 1.2

Data are expressed as means ± SE, [a]$p < 0.05$ vs. [b], [c]$p < 0.05$ vs. [d].
MCP-1, monocyte chemotactic protein-1; MIP-1α, macrophage inflammatory protein-1 alpha; RANTES, regulated on activation, normal T-cell expressed and secreted; SDF-1α, stromal cell derived factor-1 alpha; TFA, trifluoroacetic acid.

Table 4. Chemotaxis inhibitory effect of H-8135 on complement activation product 5a (C5a) in osteoclasts cultured for 6 d

Variable	C5a100 nM	H-8135 1 μM	H-8135 100 nM	H-8135 10 nM	H-8135 1 nM	C5a 100 nM + TFA 0.0001%
Speed (μm/h)	70.4 ± 3.6[a]	54.6 ± 2.2[b]	60.7 ± 2.6	64.0 ± 2.6	69.0 ± 3.0[b]	71.0 ± 4.3[b]
Angle (°)	12.5 ± 1.7	10.7 ± 1.5	14.3 ± 1.5	14.6 ± 2.0	10.7 ± 1.0	15.9 ± 2.7
Rate (%)	68.4 ± 1.0	65.6 ± 3.7	62.5 ± 2.2	58.9 ± 0.4	69.9 ± 1.9	54.8 ± 2.8

Data are expressed as mean ± SE, [a]$p < 0.05$ vs. [b].
MCP-1, monocyte chemotactic protein-1; MIP-1α, macrophage inflammatory protein-1 alpha; RANTES, regulated on activation, normal T-cell expressed and secreted; SDF-1α, stromal cell derived factor-1 alpha; TFA, trifluoroacetic acid.

increases differentiation (27,28). These chemoattractants induce a chemotactic response in bone marrow cells and RAW264.7 cells in a concentration-dependent manner (28). SDF-1α is expressed in periodontitis tissue and increases differentiation and chemotactic responses by binding to the chemokine receptor, CXCR4, which is expressed on the OC surface (14). C5a is an intermediate product of the complement pathway and a potent cellular chemotactic factor (29). C5a is involved in chronic inflammation, such as in rheumatoid arthritis, and is involved in periodontitis through its effect on neutrophils (11–13). Furthermore, activation of the C5a complement pathway plays an important role in OC differentiation from bone marrow cells (30).

We measured the chemotactic response of OCs toward the above chemoattractants using an EZ-TAXI-Scan™and confirmed their chemotaxis to these chemoattractants in a concentration-dependent manner. We confirmed that a peak chemoattractant concentration exists for the OC chemotactic response and that the chemotactic response was diminished or absent if the chemoattractant was not at that peak concentration. In this study, the 6-d group of OCs showed the highest chemotactic response toward C5a (Tables 1 and 2, Fig. 2). This finding is consistent with the findings of Ignatius *et al*., who reported that expression of the C5a receptor increases with OC differentiation (31). Yu *et al*., however, reported that expression of the SDF-1α receptor, CXCR4, decreases, whereas expression of the RANTES receptor, CCR1, increases, with OC differentiation (28,32). In the present study, a higher speed of chemotaxis was induced by SDF-1α in the 6-d group and by RANTES in the 3-d group. Thus, the chemotactic response of OCs toward SDF-1α and RANTES in this study (Tables 1 and 2) probably did not reflect expression of their receptor. The chemotactic speed and direct migration of OCs toward C5a, as well as the percentage of chemotactic cells, were significantly higher than those induced by other chemokines, for both the 3- and 6-d groups of OCs. Moreover, the speed of chemotaxis towards C5a was significantly higher for the 6-d group than for the 3-d group, indicating that the chemotactic response of OCs toward C5a increases with OC differentiation (Fig. 2).

Porphyromonas gingivalis produces the Arg-specific protease gingipain, resulting in increased production of C5a (33,34). In our study, 100 nM C5a induced the highest chemotactic response in the 6-d group of OCs. This concentration of C5a is within the range at which C5a is reported to inhibit neutrophil immune activation (10–100 nM) (35–37). This finding suggests that the *P. gingivalis*-induced increase in C5a plays a very important role in the progression of periodontitis by suppressing the immune response and increasing OC chemotaxis.

H-8135 is a selective C5a receptor antagonist and it combines with the C5a receptor to compete with C5a (38–40). In this experiment, H-8135 significantly inhibited OC chemotaxis toward C5a in both 3- and 6-d cultures. Thus, C5a antagonists may inhibit OC recruitment and thus inhibit bone resorption by OCs. Furthermore, C5a plays a role not only in periodontal disease but also in metabolic bone diseases such as rheumatoid arthritis (41,42). In addition, C5a and its receptor have attracted attention as a therapeutic target for neurodegeneration (43). The increased focus on C5a antagonists in recent years has led to

the development of antagonists with various molecular structures (44). These C5a antagonists may suppress the recruitment of OCs to periodontitis tissue and may decrease pathologic bone resorption in periodontitis.

Conclusion

In this study, we quantitatively measured and characterized the chemotactic response of OCs toward chemoattractants that are expressed in periodontitis tissue using the EZ-TAXIScan™. The chemoattractants expressed in periodontitis tissue induced a chemotactic response in cultured rat OCs in a concentration-dependent manner. The most potent chemoattractant for cultured rat OCs in this study, based on chemotactic speed, directed migration and the percentage of chemotactic cells, was C5a. These data suggest that C5a plays a very important role in the pathogenic bone resorption in periodontitis. Therefore, C5a inhibitors may function as potent novel therapeutic or preventive agents for periodontitis. More research regarding the role of C5a in periodontitis and the potential effect of C5a inhibitors on periodontitis is needed.

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