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Human leukocyte histocompatibility antigen class II-induced cytokines from human gingival fibroblasts promote proliferation of human umbilical vein endothelial cells: potential association with enhanced angiogenesis in chronic periodontal inflammation

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Background and Objective: The role of human leukocyte histocompatibility antigen (HLA) class II molecules on non-antigen-presenting cells has been a matter of controversy. We previously reported that HLA-II molecules on human gingival fibroblasts (GF) do not present antigens, but transduce signals into the cells, resulting in the expression of several cytokines, such as interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), regulated upon activation, normal T-cell expressed and secreted (RANTES) and IL-8. However, the exact role of these cytokines, as well as other cytokines which are potentially secreted from GF, in the pathogenesis of chronic periodontal inflammation is not fully understood. The aim of this study was to observe the effects of HLA-II-induced cytokines on the proliferation of human umbilical vein endothelial cells (HUVEC).

Material and Methods: Antibody-based cytokine-microarray analyses were performed to detect potential cytokines associated with angiogenesis. Next, cytokine

productivity was confirmed by quantitative methods. Then, cell proliferation assay was performed to see whether these cytokines promoted the proliferation of HUVEC.

Results: Besides IL-6, MCP-1, RANTES and IL-8, growth-related gene product (GRO) was newly identified as an HLA-II-induced cytokine released from GF. This was confirmed by a quantitative method. Cell culture supernatant from HLA-II-stimulated GF cultures promoted the growth of HUVEC. Addition of anti-IL-8 neutralizing antibody, anti-CXC receptor (CXCR)1 antibody and anti-MCP-1 antibody inhibited the growth of HUVEC in a dose-dependent manner, while addition of anti-GROα antibody did not.

Conclusion: The HLA-II-induced IL-8, via CXCR1, as well as MCP-1 from GF, promotes endothelial cell proliferation, which is possibly associated with enhanced angiogenesis in chronic periodontal lesions.

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Although the precise role of human leukocyte histocompatibility antigen (HLA) class II molecules on non-antigen-presenting cells is not yet fully understood, gingival fibroblasts (GF) in inflamed periodontal tissues have been reported to express HLA-II molecules on their cell surface (1), suggesting that cell surface HLA-II molecules on GF may have some pathophysiological roles in periodontal pathology. We previously suggested that expression of HLA-II molecules on GF in periodontal lesions was associated with the establishment and maintenance of chronic periodontal inflammation. For example, ligation of HLA-DR, one of HLA-II molecules on cultured human GF with anti-HLA-DR monoclonal antibodies (L243) resulted in secretion of cytokine/chemokines, such as regulated upon activation, normal T-cell expressed and secreted (RANTES), monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6) and interleukin-8 (IL-8), and we suggested that these cytokines might further deteriorate the clinical course of inflammatory periodontal diseases (2). We also confirmed that formation of HLA-DR–antigenic peptide–T-cell receptor complex by using emetine-treated GF and an established Th_0 clone (memory T-lymphocyte cell line) sharing identical HLA-DR antigens with antigenic peptide derived from *Porphyromonas gingivalis* outer membrane protein also stimulated GF and resulted in secretion of these cytokines in a similar manner (2). Additionally, we reported that ligation of HLA-DR molecules with anti-DR antibodies on interferon-γ (IFN-γ)-stimulated GF resulted in the phosphorylation, hence, activation of c-jun N-terminal kinase (JNK), and that the JNK pathway was at least involved in the RANTES production, since inhibition of JNK phosphorylation completely abolished RANTES production (3). Furthermore, we recently reported that focal adhesion kinase (FAK), a submembrane kinase that plays important roles in cell adhesion and motility, structurally associated with HLA-DR molecules and mediated HLA-II-induced signals into the cells (4).

However, although production of several cytokines/chemokines was confirmed by conventional methods as described, it is possible that other biologically active molecules are secreted from GF upon stimulation via HLA-II molecules. In addition, there are several other HLA-II molecules than HLA-DR molecules expressed on human fibroblastic cells, such as HLA-DQ molecules. Whether stimulation via other HLA-II molecules on GF results in the activation of the cells in a similar way is largely unknown. Therefore, in this study, we first aimed to identify HLA-DR- and HLA-DQ-induced molecules in a comprehensive approach by using antibody-based cytokine array technique. Since we could detect several angiogenesis-associated molecules by this approach, we next determined whether HLA-II-induced GF activation was associated with angiogenesis by investigating the proliferation of human umbilical vein endothelial cells (HUVEC).

Here we report that HLA-II-induced molecules, especially IL-8 and MCP-1, promote the proliferation of HUVEC, and suggest that the HLA-II-induced signaling event is not only associated with the recruitment of immune cells but may also promote angiogenesis in chronic periodontal lesions, thereby having an additional role in maintaining chronic inflammation.

Material and methods

Reagents

Mouse anti-HLA-DR monoclonal antibodies (L243; Leinco Technologies Inc., Ballwin, MO, USA) and anti-HLA-DQ monoclonal antibodies (1a3; Leinco Technologies Inc.) were used for ligation with HLA-DR and HLA-DQ molecules, respectively. Isotype-matched control mouse IgG_{2a} were obtained from Pharmingen (San Diego, CA, USA). Interferon-γ was obtained from Genzyme (Cambridge, MA, USA). As neutralizing antibodies against IL-8, growth-related gene product (GROα), CXCR1 chemokines and CXCR2 chemokines, monoclonal

anti-human CXCL8/IL-8 Antibody (mouse IgG_1), monoclonal anti-human CXCL1/GROα antibody (mouse IgG_{2b}), monoclonal anti-human CXCR1 (IL-8 receptor A) antibody (mouse IgG_{2a}), and monoclonal anti-human CXCR2 (IL-8 receptor B) antibody (mouse IgG_{2a}) were used (all from R&D Systems, Oxford, UK). As neutralizing antibody against MCP-1, monoclonal anti-human CCL2/MCP-1 antibody (mouse IgG_1) was used (also from R&D Systems).

Cells and cell culture

Human gingival fibroblasts were isolated from the gingival tissues of healthy volunteers during the extraction of an impacted third molar. Gingival tissues were plated in a 35 mm cell culture dish precoated with type I collagen and fibronectin. Outgrown cells from tissue explants were expanded and maintained as described previously (2). Briefly, the cells were cultured in a medium composed of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 10 mg/mL gentamicin, 0.1 mM non-essential amino acids, MEM vitamin solutions and 2 mM L-glutamine. When the cells reached confluence, they were passaged with a split ratio of 1:4. The cells were used between passages five and seven for all experiments.

Human umbilical vein endothelial cells (HUVEC) were originally purchased from Cambrex Bio Science Walkersville, Inc. (East Rutherford, NJ, USA), and were maintained in a Endothelial Basal Medium-2 (EBM-2; Lonza Biologics, Slough, UK) containing 2% FBS and Endothelial Cell Growth Supplement-2 (EGM-2 SingleQuots; Lonza Biologics).

Cytokine array analyses

To identify intracellular cytokines/chemokines induced in GF by the stimulation via HLA-DR and HLA-DQ molecules, GF were first cultured with 10% FBS in the presence of 200 U/mL of IFN-γ for 48 h. Then, GF were further cultured for 24 h with IFN-γ without serum to synchronize the cell cycle. Then, the cells were stimulated with 1 mg/mL of either L243 or 1a3 for 16 h. After 16 h, the cells were lysed with cell lysis buffer contained in a commercial cytokine array kit (Human Cytokine Antibody Array 5.1; RayBiotech, Atlanta, GA, USA) according to the manufacturer's instructions, followed by the measurement of protein concentration. The same amounts of cell lysates were subjected to cytokine array analyses.

Cytokine assay

The concentrations of IL-8 and GRO in the culture supernatants of the cells stimulated via HLA-DR molecules, or with 1 ng/mL of recombinant human tumor necrosis factor-α (TNF-α; Peprotech Inc., Le Perray-en-Yvelines, France), or with 1 or 100 ng/mL of *Escherichia coli* lipopolysaccharide (LPS; Sigma, St Louis, MO, USA) was measured using a commercial immunoassay kit (Quantikine Human CXCL8/IL-8, Quantikine Human CXCL1/GROα; R&D Systems) following the indicated time periods of cell culture.

3-(45-(Dimethylthiazol-2-thiazoyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay

The effects of byproducts from GF stimulated via HLA-DR molecules on the proliferation of HUVEC were evaluated by MTT assay as described previously (5). Briefly, HUVEC were grown in 24-well cell culture plates at a concentration of 10,000 cells per well. The cells were maintained in a basal medium containing 2% FBS and EGM-2 for 24 h. Then, the cells were cultured in the same medium without FBS for 24 h. After serum starvation, the cells were cultured for another 72 h with or without GF culture supernatant obtained by the method described in 'Cytokine array analyses' section, except that these culture supernatants were obtained following repeated washes of the cells with phosphate-buffered saline to avoid contamination of residual IFN-γ. After 72 h, 500 μg/mL of Thiazolyl Blue Tetrazolium Bromide (Sigma) was added to the cell culture and incubated for 4 h (6), followed by the solubilization of formazan with 0.04 N HCl in isopropanol (both from Nacalai Tesque, Kyoto, Japan) (7). One-sixth of each supernatant was transferred to 96-well plates, and the absorbance at 570 nm was read by plate reader (Bio-Rad, Hercules, CA, USA). In some experiments, the cells were co-incubated with an indicated concentration of neutralizing antibodies against IL-8, GROα, CXCR1, CXCR2 and MCP-1.

Statistical analyses

For statistical analysis to compare the cytokine productivity of GF stimulated via either HLA-DR or -DQ, Spearman's rank test was performed. To compare HUVEC proliferation between culture conditions, ANOVA and Scheffe's *F* test were performed using SPSS software (version 13.0; SPSS, Chicago, IL, USA).

Results

Ligation of HLA-DR molecules and -DQ molecules on GF with L243 or 1a3 shows similar production patterns of intracellular cytokines/chemokines

We first investigated whether there was any difference in cytokine/chemokine production from GF when the cells were stimulated via either HLA-DR or -DQ molecules. Ligation of HLA-DR molecules with L243 or HLA-DQ molecules with 1a3 resulted in the intracellular productions of several cytokines/chemokines (Fig. 1). Interestingly, very a similar production pattern was observed between HLA-DR- and -DQ-stimulated cells when the cells were stimulated either with L243 or 1a3 ($r = 0.708$, $p = 0.000$, Spearman's rank test). We also performed same experiments using GF isolated from different donors. Even in that case, the result was very similar ($r = 0.785$, $p = 0.000$). We therefore concluded that GF produce cytokines/chemokines in a similar way via HLA-II-mediated stimulation regardless the particular HLA-class II molecule.

We previously reported that GF produced RANTES, IL-6, IL-8 and

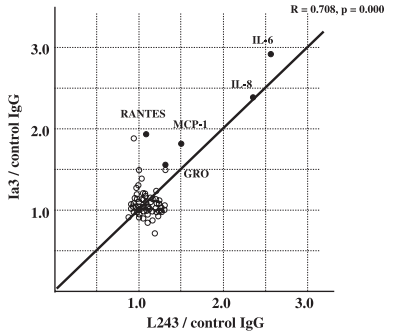

Fig. 1. The HLA-DR- and -DQ-stimulated cytokine/chemokine production patterns show a similar profile. To see the difference between HLA-DR- and -DQ-stimulated cytokine/chemokine production in GF, antibody-based cytokine array analyses were performed. The data are expressed as the ratio of L243- or 1a3-induced cytokine/chemokine production against isotype-matched control IgG-stimulated production. Both HLA-DR- and -DQ-induced cytokine/chemokine production showed very similar patterns ($r = 0.078$, $p = 0.00$, Spearman's rank test).

MCP-1 when HLA-DR molecules on GF were ligated with L243. These results were confirmed by the present cytokine array analyses. In addition to these molecules, however, GROα, another chemokine, was found to be produced in the present experiments.

Gingival fibroblasts secrete angiogenesis-associated CXC chemokines, IL-8 and GROα, by stimulation via HLA-II

Since GRO and IL-8 have often been associated with angiogenic activity, we confirmed by quantitative methods that GF secreted these chemokines into culture medium. The results are shown in Fig. 2. As expected, GF secreted both IL-8 and GROα into culture medium in a time-dependent manner. The amount of IL-8 secreted was about three times higher than that of GROα. In addition, the amount of IL-8 produced from GF stimulated via HLA-DR molecules was greater than that from GF stimulated with *E. coli* LPS or TNF-α, while the amount of GROα produced from GF stimulated via HLA-DR molecules was very similar to that from the cells stimulated with 1 ng/mL of LPS.

Culture supernatants of GF stimulated via HLA-II molecules promote HUVEC growth

To determine whether HLA-II-mediated GF stimulation was associated with enhanced endothelial cell proliferation, we then observed whether culture supernatants of GF stimulated via HLA-DR molecules promoted HUVEC growth by MTT assay. We first confirmed the validity of MTT assay by constructing a standard curve with a known number of HUVEC, as shown in Fig. 3A. The absorbance at 570 nm increased proportionally as the number of the cells increased. Based on this experiment, we considered that the assay was valid.

We observed the effects of GF culture supernatants on the proliferation of HUVEC when the GF were stimulated via HLA-II. As shown in Fig. 3B, HUVEC growth was significantly promoted when HUVEC was cultured in a medium containing supernatants of GF stimulated with L243 compared with the cells cultured in GF culture medium stimulated with control IgG or without any stimulation ($p < 0.05$, ANOVA and Scheffe's F test).

Anti-interleukin-8 neutralizing antibody and anti-CXCR1 neutralizing antibody, but not anti-GRO neutralizing antibody, suppressed GF culture supernatant-induced HUVEC proliferation

We wondered whether IL-8 and/or GRO contained in the culture supernatant of GF stimulated via HLA-II molecules were associated with the enhanced proliferation of HUVEC. We first determined the effects of neutralizing antibody against IL-8 and GROα on the proliferation of HUVEC, as shown in Fig. 4. Anti-IL-8 (Fig. 4A), but not anti-GROα (Fig. 4B), suppressed HUVEC growth in a dose-dependent manner. The inhibition of HUVEC growth by anti-IL-8 neutralizing antibody was statistically significant ($p = 0.05$, ANOVA and Scheffe's F test).

It is suggested that IL-8 is the ligand against both CXCR1 and CXCR2, while GRO belongs to the CXCR2 family. To see whether enhanced HUVEC growth by culture supernatant of GF stimulated via HLA-II was predominantly mediated by CXCR1, we next observed the effects of anti-CXCR1 neutralizing antibodies, as shown in Fig. 5. As indicated, anti-CXCR1 antibody suppressed HUVEC growth in a dose-dependent manner (Fig. 5A). In contrast, neutralizing antibody to CXCR2 had no effect on HUVEC growth (Fig. 5B). Thus, we considered that upregulation of HUVEC growth by GF culture supernatant was partly mediated by IL-8 via CXCR1.

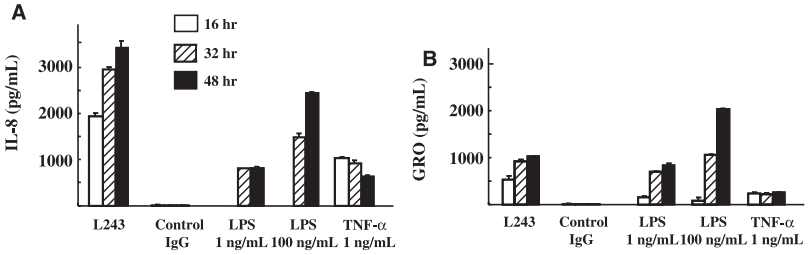

Fig. 2. Interleukin-8 and GROα production from GF stimulated via HLA-DR, and its comparison with LPS or TNF-α stimulation. The GF secreted both IL-8 and GROα in a time-dependent manner when cell surface HLA-DR was stimulate with L243. The HLA-II-induced IL-8 production was greater than that stimulated by 100 ng/mL of *E. coli* LPS in GF (A), while GRO production from HLA-II-stimulated GF was almost identical to that from GF stimulated with 1 ng/mL of LPS (B).

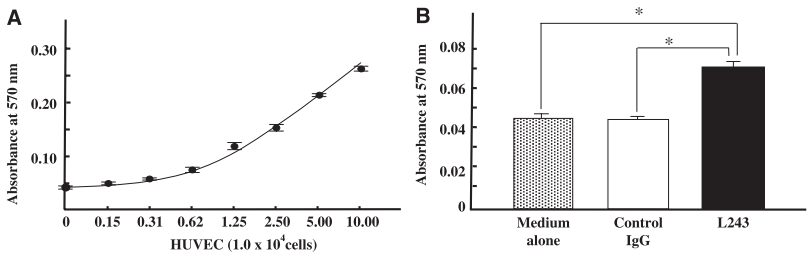

Fig. 3. The effects of GF culture supernatants on the proliferation of HUVEC. (A) The MTT assay was standardized by using a known number of HUVEC cells. (B) We then determined the effects of GF culture supernatants on the proliferation of HUVEC by MTT assay. The GF culture supernatants with control IgG or medium alone showed little growth promoting effects on HUVEC, while GF supernatant obtained from the cells stimulated with L243 significantly promoted HUVEC growth (*$p < 0.05$, ANOVA and Scheffe's *F* test).

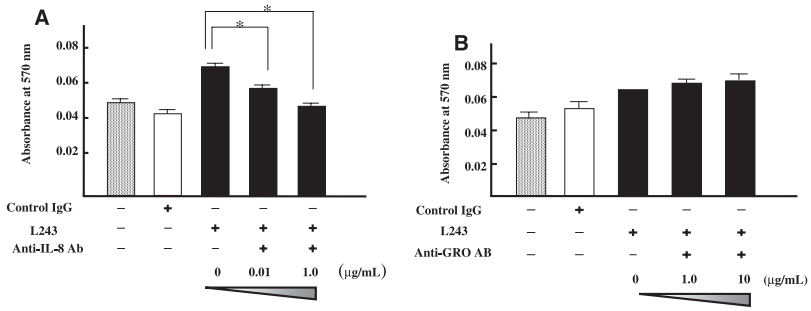

Fig. 4. The effects of IL-8 (A) and GRO neutralizing antibodies (B) on the proliferation of HUVEC. Anti-IL-8 neutralizing antibody suppressed L243-stimulated GF culture supernatant-induced HUVEC growth in a dose-dependent manner (A; *$p < 0.05$, ANOVA and Scheffe's *F* test), while anti-GRO antibody had no effect (B).

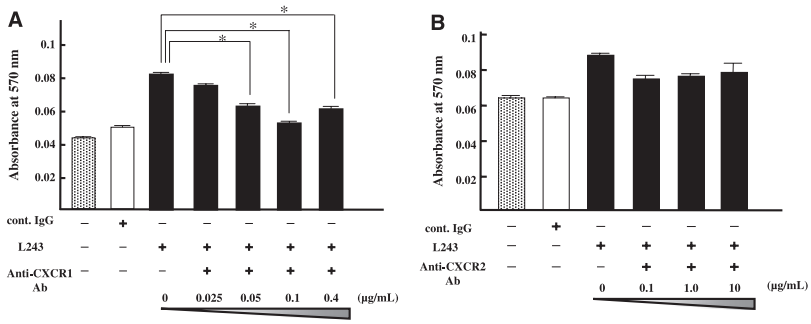

Fig. 5. The effects of anti-CXCR1 and anti-CXCR2 antibodies on the proliferation of HUVEC. Anti-CXCR1 antibody suppressed L243-stimulated GF culture supernatant-induced HUVEC growth in a dose-dependent manner (A; *$p < 0.05$, ANOVA and Scheffe's *F* test), while anti-CXCR2 antibody had no effect (B).

Monocyte chemoattractant protein-1, CC chemokine, produced from GF stimulated via HLA-II molecules, is also associated with HUVEC growth

We previously reported that ligation of HLA-DR molecules on cultured human GF with L243 resulted in secretion of not only CXC chemokine IL-8 but also CC chemokine MCP-1 and RANTES. Since MCP-1 is also suggested to promote HUVEC growth, we investigated whether MCP-1 in culture supernatants of GF was also associated with HUVEC growth by using neutralizing antibody to MCP-1. The results are shown in Fig. 6. As expected, anti-MCP-1 neutralizing antibody suppressed HUVEC growth induced by culture supernatants of GF stimulated with L243 in a dose-dependent manner. Therefore, we concluded that HUVEC cell growth induced by HLA-II-stimulated GF culture supernatants was predominantly mediated by both IL-8 and MCP-1.

Discussion

There is increasing evidence that HLA-II molecules are not merely antigen-presenting molecules but act as signal

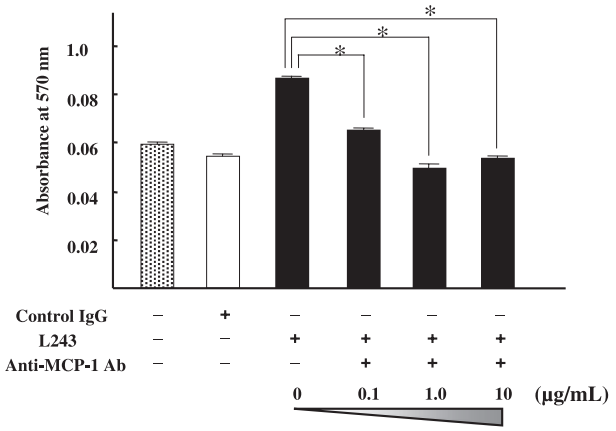

Fig. 6. The effects of MCP-1 neutralizing antibodies on the proliferation of HUVEC. Anti-MCP-1 neutralizing antibody suppressed L243-stimulated GF culture supernatant-induced HUVEC growth in a dose-dependent manner (*$p < 0.05$, ANOVA and Scheffe's F test).

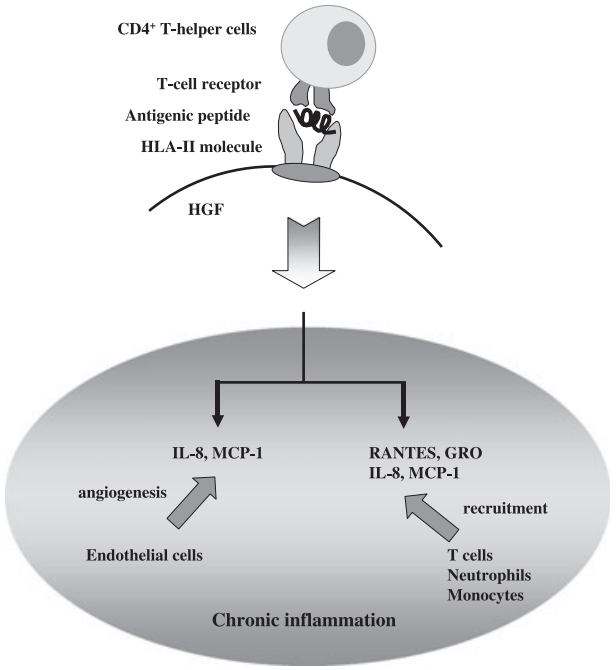

Fig. 7. Schematic presentation of the role of HLA-II-mediated signals in gingival fibroblasts. Our hypothesis concerning the pathophysiological role of HLA-II molecules in periodontal lesions is summarized. Formation of HLA-II-antigenic peptide-T cell receptor (TCR) complex results in the secretion of several cytokines/chemokines. RANTES, GRO, IL-8 and MCP-1 all act to recruit immune cells into the lesion, while IL-8 and MCP-1 may also promote angiogenesis, both of which could contribute to the deterioration of chronic inflammatory conditions.

transducing molecules into the cells by forming a complex with antigenic peptide/T-cell receptor (8,9). We previously reported that HLA-II molecules on GF did not present antigens but induced secretion of IL-6, IL-8, MCP-1 and RANTES upon stimulation via HLA-DR with L243 antibodies as well as by forming a complex with antigenic peptide/T-cell receptor (2). In the present study, we first performed comprehensive analyses on HLA-II-mediated cytokine/chemokine production by using cytokine array analyses. The results indicated that stimulation of GF via HLA-DR or -DQ resulted in a production of similar cytokines regardless of the antibodies used in our experiments. We recently reported that HLA-II molecules might structurally associate with focal adhesion kinases (FAK) and suggested that FAK transduces signals into the cells, since inhibitors of FAK phosphorylation abolished HLA-DR-induced cytokine production (4). In addition, HLA-class I molecules also appeared to associate with FAK in endothelial cells, based on previous studies by others (10,11). These results may indicate that HLA-II molecules on gingival fibroblasts, including not only HLA-DR but also -DQ molecules, structurally associate with FAK, and mediate HLA-II-induced signals in a similar manner. Interestingly, besides IL-6, IL-8, MCP-1 and RANTES, we found, for the first time, that GROα production was also enhanced upon stimulation of GF via HLA-DR and -DQ molecules.

It has been suggested that IL-8 and GROα, both of which belong to the CXC chemokine family, not only act as chemokines against immune cells but also act as angiogenic factors (12). Since angiogenesis plays an important role in maintaining chronic inflammation, and enhanced neovascularization is reported in chronic periodontal lesions (13), we tested the possibility that culture supernatants of GF stimulated via HLA-II contained molecules enhancing endothelial cell proliferation. The results indicated that culture supernatants of GF stimulated with L243, but not with control IgG, promoted the proliferation of HUVEC. In addition, neutralizing the action of IL-8 with a specific antibody inhibited GF supernatant-induced proliferation of HUVEC in a dose-dependent manner. It is reported that IL-8 binds to both CXCR1 and CXCR2, while GROα binds to CXCR2 (14). Since neutralizing the action of GROα with a specific antibody had no effect on the proliferation of HUVEC induced by the culture supernatant of GF stimulated via HLA-DR, we speculated that IL-8, via CXCR1 receptors, dominantly acted as a growth factor for HUVEC. Therefore, we also observed the effects of neutralizing antibody against

CXCR1 and CXCR2. As expected, addition of anti-CXCR1 antibodies into HUVEC culture also inhibited the growth of HUVEC, while anti-CXCR2 antibody did not. Therefore, we considered that HUVEC proliferation induced by GF culture supernatant stimulated via HLA-II molecules was induced by IL-8 via CXCR1. This may be partly explained by the fact that the amount of IL-8 produced from GF was much greater than that of GROα when HLA-II molecules on GF were stimulated with L243 and 1a3 (Figs 1 and 2). Additionally, it is also reported in several previous studies that HUVEC expression of CXCR2 is far less than that of CXCR1 (15,16). We previously reported that ligation of HLA-DR molecules on cultured human GF with L243 resulted in secretion of not only the CXC chemokine IL-8 but also the CC chemokine MCP-1 and RANTES (2). Since MCP-1 is also suggested to promote HUVEC growth (17,18), we investigated whether MCP-1 in culture supernatants of GF stimulated via HLA-II was associated with HUVEC growth by using neutralizing antibody to MCP-1, and confirmed that the CC chemokine MCP-1 also promoted HUVEC growth, since neutralizing the action of MCP-1 with a specific antibody suppressed HUVEC growth in a dose-dependent manner.

In conclusion, this study demonstrated that ligation of HLA-II molecules on GF resulted in the secretion not only of chemotactic factors against immune cells but also of growth promoting molecules of endothelial cells. Interleukin-8, via CXCR1, and MCP-1 may play important roles in HLA-II-induced HUVEC proliferation. These chemokines produced from fibroblasts via HLA-II molecules, together with cytokines/chemokines from immune cells such as neutrophils, monocytes and T-lymphocytes, would further deteriorate the clinical course of inflammatory periodontal disease. Our hypothesis concerning the pathophysiological role of HLA class II molecules on gingival fibroblasts in periodontal lesions is summarized in Fig. 7. The HLA-II-mediated cascades may enhance endothelial cell growth as well as recruiting other inflammatory cells, which progresses the disease to the chronic conditions such as seen in chronic inflammatory periodontal diseases.

Acknowledgements

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