Journal of
PERIODONTAL RESEARCH

J Periodont Res 2010; 45: 193–199
All rights reserved

© 2010 The Authors.
Journal compilation © 2010 Blackwell Munksgaard

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2009.01217.x

Proinflammatory effects of muramyldipeptide on human gingival fibroblasts

I. Hosokawa[1], Y. Hosokawa[1], K. Ozaki[2], H. Yumoto[1], H. Nakae[1], T. Matsuo[1]

[1]Department of Conservative Dentistry, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Tokushima, Japan and [2]The University of Tokushima, Faculty of Dentistry, School of Oral Health and Welfare, Oral Health Care Promotion, Tokushima, Tokushima, Japan

Hosokawa I, Hosokawa Y, Ozaki K, Yumoto H, Nakae H, Matsuo T. Proinflammatory effects of muramyldipeptide on human gingival fibroblasts. J Periodont Res 2010; 45: 193–199. © 2010 The Authors. Journal compilation © 2010 Blackwell Munksgaard

Background and Objective: Because human gingival fibroblasts (HGFs) are the predominant cells in periodontal tissues, we hypothesized that HGFs are contributed to receptors for components of bacteria. In this study, we focused on expression and function of nucleotide binding oligomerization domain 2 (NOD2) in HGFs, which is a mammalian cytosolic pathogen recognition molecule.

Material and Methods: Expression of NOD2 in HGFs was examined by reverse transcriptase-polymerase chain reaction (RT-PCR) and flow cytometry. Production of interleukin (IL)-6, IL-8, cc chemokine ligand2, cxc chemokine ligand10 (CXCL10) and CXCL11 from HGFs was examined by enzyme-linked immunosorbent assay (ELISA). We used RT-PCR and immunohistochemistry to detect the NOD2 expression in human gingival tissues.

Results: We found clear NOD2 expression in HGFs. Upon stimulation with NOD2 agonist, muramyldipeptide (MDP), production of proinflammatory cytokines was enhanced. Moreover, MDP-induced production of proinflammatory cytokines was inhibited in a different manner by mitogen-activated protein kinase inhibitors and phosphatidylinositol 3-kinase inhibitor. Furthermore, MDP enhanced CXCL10 and CXCL11 productions by tumor necrosis factor-α (TNF-α)- or interferon-γ (IFN-γ)-stimulated HGFs, although MDP alone did not induce these chemokines. TNF-α and IFN-γ increased NOD2 expression in HGFs. In addition, we detected NOD2 expression in mononuclear cells and HGFs in periodontally diseased tissues.

Conclusion: These findings indicate that MDP which induces production of cytokines and chemokines from HGFs is related to the pathogenesis of periodontal disease.

Dr Yoshitaka Hosokawa, Department of Conservative Dentistry, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima, Tokushima 770-8504, Japan
Tel: 011 81 886 33 7340
Fax: 011 81 886 33 7340
e-mail: hosokawa@dent.tokushima-u.ac.jp

Key words: Nucleotide binding oligomerization domain 2; human gingival fibroblast; periodontal disease; proinflammatory cytokine; chemokine

Accepted for publication February 4, 2009

The innate immune system detects the presence of microorganisms and some viruses using transmembrane and cytosolic receptors with specificities for distinct components of the infectious agents. Members of the toll-like receptor (TLR) family are well recognized for this function. A second family, known as the NOD/CATERPILLAR protein family, includes at least two proteins, nucleotide-binding oligomerization domain (NOD) 1 and NOD2, which recognize substructures present in bacterial peptidoglycans (1–3). The protein NOD2 has been shown to be involved in genetically determined human diseases; mutations in NOD2 have been linked to susceptibility to Crohn's disease (4,5) and to Blau's syndrome (6). Crohn's disease and Blau's syndrome are chronic diseases with autoinflammatory and autoimmune components. Although NOD2 was initially thought to be a receptor for bacterial lipopolysaccharide (LPS), more recent studies have shown that NOD2-expressing cells respond to peptidoglycan-derived structures, including muramyl dipeptide (MDP; 7,8).

Fibroblasts play important roles in maintaining connective tissues (9). Fibroblasts are not a homogeneous population within a single tissue, and modulate immune cell behavior by conditioning the local and cellular

microenvironment (9). Human gingival fibroblasts (HGFs) are the major cells of gingival connective tissue. In the studies on the innate immune responses of HGFs, the cells can produce various inflammatory cytokines, such as interleukin (IL)-1, IL-6 and IL-8, upon stimulation with LPS from periodontal pathogen (10,11). Moreover, the heterogeneous expression of CD14 in HGFs was reported (12). Furthermore, HGFs constitutively expressed TLR2 (13–15), TLR4 (13–16), and TLR6 (15), and produced various cytokines by interaction with their ligands (12,16), indicating that HGFs involved in immune responses. However, the role of NOD2 in HGFs is uncertain.

In this study, we aimed to elucidate the role and expression of NOD2 in HGFs and periodontal tissues. At first, we examined the expression of NOD2 in HGFs and the capacity of the NOD2 agonist, MDP, to induce release of cytokines and chemokines by HGFs. Moreover, we investigated effects of proinflammatory cytokines on NOD2 expression in HGFs. Furthermore, NOD2 expression in periodontal diseased tissues was examined.

Material and methods

Gingival tissue biopsy and cell culture

Tissue samples were obtained at surgery from the inflamed gingiva of patients diagnosed with chronic periodontitis or from the gingiva of clinically healthy subjects. We collected samples after basic periodontal therapy such as scaling. Samples of gingival tissues were obtained from 17 chronic periodontitis patients (4 males and 13 females; average age 61.0 ± 9.8 years, average probing depth 6.33 ± 2.06 mm and average attachment loss 7.02 ± 2.26 mm) and five healthy control subjects (5 females; average age 31.2 ± 9.8 years, average probing depth 2.4 ± 0.54 mm and average attachment loss 2.7 ± 0.57 mm). All gingival biopsy sites in the chronic periodontitis group exhibited radiographic evidence of bone destruction, as well as having clinical probing depths > 4 mm, with sulcular bleeding on probing; otherwise, the patients were systemically healthy. Gingival biopsy sites in healthy control subjects did not exhibit radiographic evidence of bone destruction, as well as having clinical probing depths less than 3 mm without sulcular bleeding on probing. We used two clinically healthy gingival samples and nine chronic periodontitis samples for reverse transcriptase-polymerase chain reaction (RT-PCR), and two clinically healthy gingival samples and nine chronic periodontitis samples for immunohistochemical staining. We used HGFs isolated from three clinically healthy gingival samples obtained during a routine distal wedge surgical procedure. Gingival specimens were cut into small pieces and transferred to culture dishes. The HGFs that grew from the gingiva were cultured on uncoated 100 mm^2 plastic dishes in Dulbecco's modified Eagle's medium (DMEM; Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; JRH Bioscience, Lenexa, KA, USA) and antibiotics (penicillin G 100 units/ml and streptomycin 100 μg/ml) at 37°C in humidified air with 5% CO_2. Confluent cells were transferred and cultured for use in the present study. After three to four subcultures by trypsinization, cultures contained homogeneous, slim, spindle-shaped cells growing in characteristic swirls. The cells were used for experiments after five passages. Informed consent was obtained from all subjects participating in this study. The study was performed with the approval and compliance of the Tokushima University Ethical Committee.

Extraction of RNA and RT-PCR analysis

Total RNA was prepared from biopsied gingival tissue or HGFs using the RNeasy protect Mini Kit (Qiagen, Hilden, Germany). Single-stranded cDNA for a PCR template was synthesized from 48 ng of total RNA using a primer, oligo$(dT)_{12–18}$ (Invitrogen, Carlsbad, CA, USA), and superscript III reverse transcriptase (Invitrogen) under the conditions indicated by the manufacturer. Specific primers were designed from the cDNA sequences for NOD2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each cDNA was amplified by PCR using HotStarTaq DNA polymerase (Qiagen). The sequences of the primers were as follows: NOD2 forward, 5′-GGTTAACAGGACACGGTCA-GG-3′; NOD2 reverse, 5′-CTCAG-TCTCGCTTCCTAGTA-3′; GAPDH forward, 5′-TGAAGGTCGGAGTC-AACGGATTTGGT-3′; and GAPDH reverse, 5′-CATGTGGGCCATGAG-GTCCACCAC-3′. The conditions for PCR were one cycle at 95°C for 15 min, 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, and one cycle at 72°C for 10 min. The products were analyzed on a 1.5% agarose gel containing ethidium bromide.

Flow cytometric analyses

Following the required time in culture, cells were washed twice with ice-cold phosphate-buffered saline (PBS). The HGFs were harvested by incubation with trypsin and EDTA (Sigma). Most of cells rounded up following this treatment and could be removed by gentle agitation. Any cells that failed to detach were removed with gentle scraping. Cells were washed twice with ice-cold PBS and incubated (20 min on ice) in PBS containing 1% BSA. Cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences, San Jose, CA, USA), and then incubated with mouse anti-human NOD2 antibody (Cayman, Ann Arbor, MI, USA) or isotype control antibody on ice for 30 min. After washing twice with PBS containing 1% BSA, the cells were incubated with the fluorescein isothiocyanate-conjugated rabbit anti-mouse $F(ab')_2$ fragments (DAKO, Kyoto, Japan) for 30 min on ice. After washing twice with PBS containing 1% BSA, cells were immediately analyzed by flow cytometry (Epics XL-MCL; Coulter, Hialeah, FL, USA). Cells were gated using forward- vs. side-scatter to remove any dead cells and cellular debris and thus give a uniform population of HGFs. For each sample, 10,000 cells were analyzed.

Cytokine release by HGFs

The HGFs were stimulated with MDP (MurNAc-L-Ala-D-iso-Gln, MDP; Sigma), tumor necrosis factor-α (TNF-α; Peprotech, Rocky Hill, NJ, USA) and interferon-γ (IFN-γ; Peprotech) for 24 h. Supernatants from the cells were collected, and the concentrations of IL-6, IL-8, cc chemokine ligand2, cxc chemokine ligand10 (CXCL10) and CXCL11 were measured in triplicate by enzyme-linked immunosorbent assay (ELISA). Duoset ELISA Development System (R&D Systems, Minneapolis, MN, USA) was used for the determination of all cytokines used in this study. All assays were performed according to the manufacturer's instructions, and cytokine levels were determined using a standard curve prepared for each assay. In selected experiments, HGFs were cultured for 1 h in the presence or absence of SB203580 (0.2–20 μM; Santa Cruz Biotechnology, Santa Cruz, CA, USA), PD98059 (0.2–20 μM; Calbiochem, La Jolla, CA, USA), SP600125 (0.2–20 μM; Sigma) and LY294002 (0.2–20 μM; Caxman) prior to their incubation with the various stimulants.

Immunohistochemistry

Gingival tissue samples were immediately embedded in the OCT compound (Miles Laboratories Inc., Elkhart, IN, USA) and quenched and stored in liquid nitrogen. The specimens were cut into 6-μm-thick sections using a cryostat (SFS, Bright Instrumental Co., Huntingdon, UK) and collected on poly-L-lysine-coated slides. Expression of NOD2 was analyzed with a specific antibody, mouse anti-human NOD2 antibody (Cayman). An isotype-matched control antibody was used as a negative control. The sections were reacted with specific antibodies overnight at 4°C. After being washed with PBS, the sections were incubated with biotinylated anti-mouse and rabbit immunoglobulins (Universal Ab; DAKO) for 20 min at room temperature and washed with PBS to remove any unreacted antibodies. The sections were then treated with peroxidase-conjugated streptavidin (DAKO) for 10 min, and washed and reacted with DAB (3,3-diaminobenzidine tetrahydrochrolide; DAKO) in the presence of 3% H_2O_2 to develop color. The sections were counterstained with hematoxylin and mounted with glycerol.

Statistical analysis

Statistical significance was analyzed with one-way ANOVA; P values < 0.05 were considered significant. We used StatView software (SAS Institute Inc, Cary, NC, USA) to perform the statistical analysis.

Results

Expression of NOD2 in HGFs

Initially, we examined NOD2 expression in HGFs. The RT-PCR analysis revealed that NOD2 mRNA is constitutively expressed by HGFs (Fig. 1A). Moreover, we investigated NOD2 protein expression in HGFs. Flow cytometric analysis showed that NOD2 is expressed in non-stimulated HGFs (Fig. 1B).

MDP induces proinflammatory cytokine production by HGFs

Next, we examined the capacity of the NOD2 agonist, MDP, to induce the production of the proinflammatory cytokines IL-8, IL-6 and CCL2 by HGFs. Figure 2 shows that MDP induced IL-6, IL-8 and CCL2 production in a dose-dependent manner.

Mitogen-activated protein kinase (MAPK) inhibitors and phosphatidylinositol 3-kinase (PI3K) inhibitor modulate MDP-induced IL-6, IL-8 and CCL2 production by HGFs to a different extent

It has been reported that MDP activated MAPKs and PI3K signal transduction pathways (17). Therefore, we used several signal transduction inhibitors to determine whether p38 MAPK, mitogen-activated protein kinase kinase (MEK), c-Jun NH_2-terminal kinase (JNK) and PI3K are required for the production of IL-6, IL-8 and CCL2 by HGFs in response to MDP (Fig. 3). SB203580 (p38

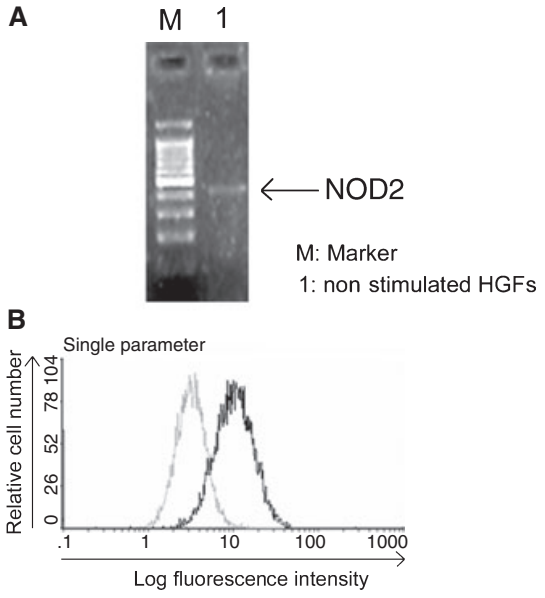

Fig. 1. Expression of NOD2 in HGFs. (A) Total RNA was prepared from non-stimulated HGFs. The expression of NOD2 mRNA in non-stimulated HGFs was analyzed by RT-PCR, as described in the Material and methods section. (B) Flow cytometric analysis of NOD2 expression in non-stimulated HGFs. The black line represents NOD2 specific fluorescence and the gray line represents the background level of fluorescence caused by isotype-matched antibody.

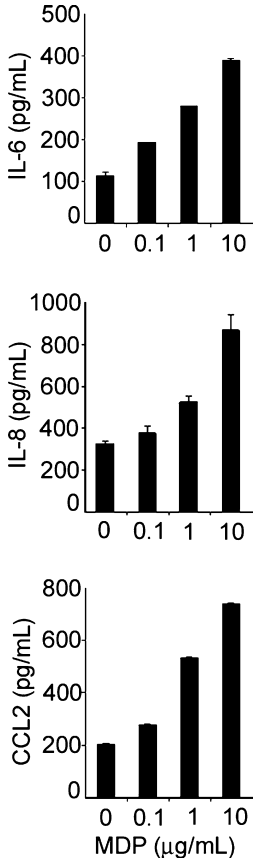

Fig. 2. MDP induced production of IL-6, IL-8 and CCL2 by HGFs. The HGFs were treated with MDP (0.1, 1 or 10 μg/ml), and the supernatants were collected after 24 h. The expression levels of IL-6, IL-8 and CCL2 in the supernatants were measured using ELISA. Data are representative of three different HGF samples from three different donors. The results were calculated as the mean and SD of one representative experiment performed in triplicate. Error bars show the SD of the values.

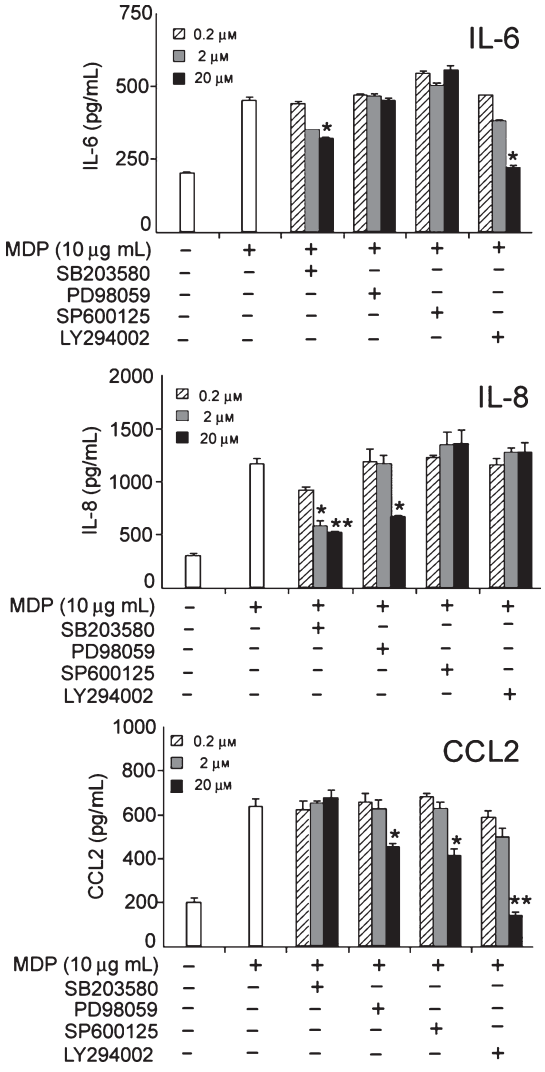

Fig. 3. Effects of MAPK inhibitors and PI3K inhibitor on MDP-stimulated IL-6, IL-8 and CCL2 production by HGFs. The HGFs were pre-incubated with SB203580 (0.2, 2 or 20 μM), PD98059 (0.2, 2 or 20 μM), SP600125 (0.2, 2 or 20 μM) or LY294002 (0.2, 2 or 20 μM) for 1 h and then incubated with MDP (10 μg/ml). After 24 h incubation, the supernatants were collected. The expression levels of IL-6, IL-8 and CCL2 in the supernatants were measured using ELISA. Data are representative of three different HGF samples from three different donors. The results were calculated as the mean and SD of one representative experiment performed in triplicate. Error bars show the SD of the values. $*p < 0.05$, $**p < 0.01$ significantly different from MDP-stimulated HGFs without inhibitors.

MAPK inhibitor) and LY294002 (PI3K inhibitor) inhibited IL-6 production induced by MDP. In contrast, IL-8 production was inhibited by SB203589 and PD98059 (MEK inhibitor). Meanwhile, MDP-induced CCL2 production was down-regulated by PD98059, SP600125 (JNK inhibitor) and LY294002.

MDP enhanced TNF-α- or IFN-γ-induced CXCL10 and CXCL11 production by HGFs

Recently, it has been reported that T helper 1 (Th1) cells are related to bone resorption in periodontal diseased sites (18). Therefore, we examined the production by HGFs of CXCL10 and CXCL11, which are involved in Th1 cell migration. MDP alone did not induce CXCL10 and CXCL11 production. However, MDP synergistically enhanced IFN-γ- or TNF-α-induced CXCL10 or CXCL11 production by HGFs (Fig. 4).

IFN-γ and TNF-α enhanced NOD2 expression in HGFs

We hypothesized that IFN-γ and TNF-α might augment NOD2 expression in HGFs because MDP could not induce CXCL10 and CXCL11 without TNF-α or IFN-γ. The RT-PCR analysis revealed that TNF-α and IFN-γ treatment enhanced NOD2 mRNA

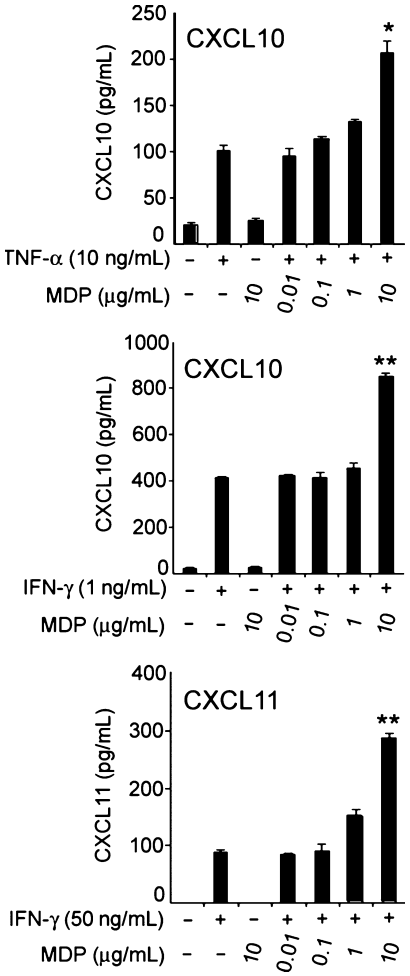

Fig. 4. MDP induced CXCL10 and CXCL11 production by TNF-α- or IFN-γ-stimulated HGFs. The HGFs were treated with MDP (0.1, 1 or 10 μg/ml) with or without IFN-γ (1 or 50 ng/ml) or TNF-α (10 ng/ml), and the supernatants were collected after 24 h. The expression levels of CXCL10 and CXCL11 in the supernatants were measured with ELISA. Data are representative of three different HGF samples from three different donors. The results were calculated as the mean and SD of one representative experiment performed in triplicate. Error bars show the SD of the values. $*p < 0.05$, $**p < 0.01$ significantly different from the HGFs stimulated with IFN-γ or TNF-α without MDP.

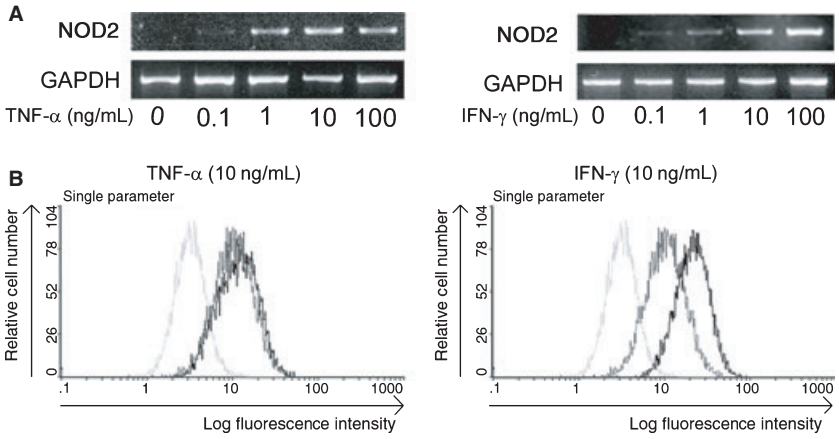

Fig. 5. TNF-αγ and IFN-γ enhanced NOD2 expression by HGFs. (A) The HGFs were treated with TNF-α (0.1, 1, 10 or 100 ng/ml) or IFN-γ (0.1, 1, 10 or 100 ng/ml), and total RNA was prepared after 4 h stimulation. The expressions of NOD2 and GAPDH mRNA in HGFs were analyzed by RT-PCR, as described in the Material and methods section. (B) The HGFs were treated with TNF-α (10 ng/ml) or IFN-γ (10 ng/ml), and the cells were collected after 24 h. The expression levels of NOD2 in HGFs were measured using flow cytometry. The data are representative of three different HGF samples from three different donors. The black line represents TNF-α- or IFN-γ-treated HGFs, the dark gray line represents non-stimulated HGFs and the light gray line represents the background level of fluorescence caused by isotype-matched antibody.

expression in HGFs in a dose-dependent manner (Fig. 5A). Moreover, flow cytometric analysis revealed that NOD2 expression in HGFs was increased by TNF-α or IFN-γ stimulation (Fig. 5B).

Expression of NOD2 in human periodontal tissues

Finally, we examined NOD2 expression in periodontal tissues. The RT-PCR analysis revealed that NOD2 mRNA expression was very strong in inflamed gingiva compared with healthy periodontal tissues (Fig. 6A). Immunohistochemical study revealed that NOD2 expression was very weak in healthy periodontal tissues. In contrast, NOD2 expression was detected in mononuclear cells and fibroblasts near inflammatory cell infiltration in diseased periodontal tissues (Fig. 6B).

Discussion

HGFs are the dominant cells in gingival tissue and come into contact with invading oral bacteria. In the host–bacteria interaction in periodontal tissues, recognition of bacteria by host cells via the innate immune system may play an important role, especially in the case of non-myeloid cells such as HGFs. In the present study, we found that HGFs expressed NOD2. It has been reported that peptidoglycan activates HGFs via TLR2 (13). In this report, we revealed that MDP, the monomer of the peptidoglycan subunit, activated HGFs, and that HGFs constitutively expressed NOD2. Recently, Inohara *et al.* (8) reported that MDP was recognized by NOD2 but not by TLR2. These reports explain that HGFs recognize peptidoglycan-related protein via TLR2 and NOD2 in periodontal tissues.

Recently, Uehara & Takada (19) reported that HGFs expressed NOD2 and that MDP induced IL-6, IL-8 and monocyte chemoattactant protein-1 (MCP-1) production from HGFs. Their report agrees with ours. Moreover, they reported that nuclear factor-κB (NF-κB) inhibition suppressed IL-8 production from MDP-stimulated HGFs. We showed that p38 MAPK and ERK pathways are related to IL-8 production by MDP-stimulated HGFs. It is known that p38 MAPK and ERK can modify NF-κB activation in some types of cells (20,21). Further investigation might be necessary to clarify signal transduction pathways in MDP-stimulated HGFs.

It has been reported that MDP activated p38 MAPK, ERK, JNK and PI3K pathways (17). However, it is uncertain which signal transduction pathways control cytokine production

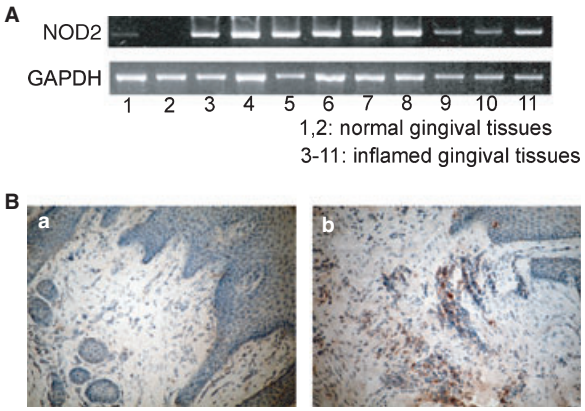

Fig. 6. Expression of NOD2 in periodontal tissues. (A) Total RNA was prepared from two clinically healthy gingival samples (pocket depth 2 mm) and nine periodontally diseased gingival samples (pocket depth 4–10 mm). The expressions of NOD2 and GAPDH mRNAs in periodontal tissues were analyzed by RT-PCR, as described in the Material and methods section. (B) Immunohistochemical staining of human healthy periodontal tissue (Ba) and diseased periodontal tissues (Bb) with anti-NOD2 antibody. Original magnification for each photograph was ×200.

by MDP-stimulated cells. In this report, we elucidated that MDP induced IL-6, IL-8 and CCL2, and the production of those cytokines was regulated differently by the MAPKs and PI3K signal transduction pathways. IL-6 production was inhibited by p38 MAPK inhibitor and PI3K inhibitor in this report. Turner *et al.* (22) reported that IL-6 production from TNF-α-stimulated human cardiac fibroblasts was inhibited by p38 MAPK inhibitor and PI3K inhibitor, though MEK inhibitor did not affect IL-6 production. Their report is in total agreement with ours. We demonstrated that p38 MAPK and MEK inhibitor down-regulated IL-8 production by MDP-stimulated HGFs. Wang *et al.* (23) reported that IL-8 production by thrombin-stimulated dermal fibroblasts was inhibited by p38 MAPK and MEK inhibitor, though PI3K and Jak/STAT (Janus kinase-signal transducer and activator of transcription) inhibitor did not influence IL-8 production. Their report is also similar to ours. Moreover, we elucidated that CCL2 production by MDP-stimulated HGFs was inhibited by MEK, JNK and PI3K inhibitors. Amin MA *et al.* reported that JNK and PI3K inhibitor down-regulated CCL2 production by IL-18-stimulated synovial fibroblasts (24). In contrast, p38 MAPK inhibitor did not affect CCL2 production. Their results agree with ours. Therefore, our report and previous reports show that MDP-induced production of cytokines in HGFs uses the same signal transduction pathway.

In this report, we showed that HGFs did not secret CXCL10 and CXCL11 upon stimulation with MDP alone. However, MDP stimulation did induce CXCL10 and CXCL11 production by IFN-γ- or TNF-α-treated HGFs. Kawai *et al.* (18) recently reported that Th1-type cells induced bone resorption in a rat periodontal disease model compared with Th2-type cells. Therefore, this phenomenon might explain that MDP is related to periodontal tissue destruction, including bone resorption, in inflammatory conditions because MDP stimulation induced Th1 cell migration in periodontally diseased sites to enhance Th1-type chemokine production from HGFs.

Immunohistochemical study showed that mononuclear cells and fibroblasts mainly expressed NOD2 protein in diseased periodontal tissues. It has been reported that macrophages expressed NOD2, and that its expression was enhanced by treatment with TNF-α and IFN-γ (25). Therefore, TNF-α and IFN-γ are involved in the innate immune response in periodontal tissues to enhance NOD2 expression in HGFs and macrophages.

RT-PCR study using human gingival tissues showed that NOD2 mRNA expressions in inflamed tissues were strong compared with healthy periodontal tissues. However, the average ages of healthy patients and periodontal diseased patients were different in this study. Further investigation of the effects of age on NOD2 expression is needed.

We found that HGFs constitutively expressed NOD2, and that the NOD2 agonist, MDP, induced production of proinflammatory cytokines by HGFs. Furthermore, MDP synergistically enhanced production of Th1-type chemokines by IFN-γ- or TNF-α-stimulated HGFs, and these cytokines increased NOD2 expression in HGFs. These results show that MDP is related to the pathogenesis of periodontal disease to induce cytokines and chemokines by HGFs, and NOD2 might be related to the pathogenic mechanism of periodontal disease. Further study of NOD2 in periodontal disease is needed to elucidate the pathogenesis of periodontitis.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (B) (19791616) from the Japan Society for the Promotion of Science.

References

1. Chamaillard M, Hashimoto M, Horie Y *et al.* An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat Immunol* 2003;**4:**702–707.
2. Girardin SE, Boneca IG, Carneiro LA *et al.* Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* 2003;**300:**1584–1587.
3. Girardin SE, Travassos LH, Herve M *et al.* Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. *J Biol Chem* 2003;**278:**41702–41708.
4. Hugot JP, Chamaillard M, Zouali H *et al.* Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 2001;**411:**599–603.
5. Ogura Y, Bonen DK, Inohara N *et al.* A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 2001;**411:**603–606.
6. Miceli-Richard C, Lesage S, Rybojad M *et al.* CARD15 mutations in Blau syndrome. *Nat Genet* 2001;**29:**19–20.

7. Girardin SE, Boneca IG, Viala J *et al*. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem* 2003;**278:**8869–8872.
8. Inohara N, Ogura Y, Fontalba A *et al*. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J Biol Chem* 2003;**278:**5509–5512.
9. Buckley CD, Pilling D, Lord JM, Akbar AN, Scheel-Toellner D, Salmon M. Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. *Trends Immunol* 2001;**22:**199–204.
10. Takada H, Mihara J, Morisaki I, Hamada S. Induction of interleukin-1 and -6 in human gingival fibroblast cultures stimulated with Bacteroides lipopolysaccharides. *Infect Immun* 1991;**59:**295–301.
11. Tamura M, Tokuda M, Nagaoka S, Takada H. Lipopolysaccharides of *Bacteroides intermedius* (*Prevotella intermedia*) and *Bacteroides* (*Porphyromonas*) *gingivalis* induce interleukin-8 gene expression in human gingival fibroblast cultures. *Infect Immun* 1992;**60:**4932–4937.
12. Sugawara S, Sugiyama A, Nemoto E, Rikiishi H, Takada H. Heterogeneous expression and release of CD14 by human gingival fibroblasts. characterization and CD14-mediated interleukin-8 secretion in response to lipopolysaccharide. *Infect Immun* 1998;**66:**3043–3049.
13. Hatakeyama J, Tamai R, Sugiyama A, Akashi S, Sugawara S, Takada H. Contrasting responses of human gingival and periodontal ligament fibroblasts to bacterial cell-surface components through the CD14/Toll-like receptor system. *Oral Microbiol Immunol* 2003;**18:**14–23.
14. Wang PL, Ohura K, Fujii T *et al*. DNA microarray analysis of human gingival fibroblasts from healthy and inflammatory gingival tissues. *Biochem Biophys Res Commun* 2003;**305:**970–973.
15. Okusawa T, Fujita M, Nakamura J *et al*. Relationship between structures and biological activities of mycoplasmal diacylated lipopeptides and their recognition by toll-like receptors 2 and 6. *Infect Immun* 2004;**72:**1657–1665.
16. Tamai R, Sakuta T, Matsushita K *et al*. Human gingival CD14(+) fibroblasts primed with gamma interferon increase production of interleukin-8 in response to lipopolysaccharide through up-regulation of membrane CD14 and MyD88 mRNA expression. *Infect Immun* 2002;**70:**1272–1278.
17. Kanneganti TD, Lamkanfi M, Nunez G. Intracellular NOD-like receptors in host defense and disease. *Immunity* 2007;**27:**549–559.
18. Kawai T, Eisen-Lev R, Seki M, Eastcott JW, Wilson ME, Taubman MA. Requirement of B7 costimulation for Th1-mediated inflammatory bone resorption in experimental periodontal disease. *J Immunol* 2000;**164:**2102–2109.
19. Uehara A, Takada H. Functional TLRs and NODs in human gingival fibroblasts. *J Dent Res* 2007;**86:**249–254.
20. Ryan S, McNicholas WT, Taylor CT. A critical role for p38 map kinase in NF-kappaB signaling during intermittent hypoxia/reoxygenation. *Biochem Biophys Res Commun* 2007;**355:**728–733.
21. Hoshi S, Goto M, Kanaya N, Nomoto K, Tanaka H. Regulation of vascular smooth cell proliferation by nuclear factor -kappaB and its inhibitor, I-kappaB. *J Biol Chem* 2000;**275:**883–889.
22. Turner NA, Mughal RS, Warburton P, O'Regan DJ, Ball SG, Porter KE. Mechanism of TNFalpha-induced IL-1alpha, IL-1beta and IL-6 expression in human cardiac fibroblasts: effects of statins and thiazolidinediones. *Cardiovasc Res* 2007;**76:**81–90.
23. Wang L, Luo J, Fu Y, He S. Induction of interleukin-8 secretion and activation of ERK1/2, p38 MAPK signaling pathways by thrombin in dermal fibroblasts. *Int J Biochem Cell Biol* 2006;**38:**1571–1583.
24. Amin MA, Mansfield PJ, Pakozdi A *et al*. Interleukin-18 induces angiogenic factors in rheumatoid arthritis synovial tissue fibroblasts via distinct signaling pathways. *Arthritis Rheum* 2007;**56:**1787–1797.
25. Takahashi Y, Isuzugawa K, Murase Y *et al*. Up-regulation of NOD1 and NOD2 through TLR4 and TNF-alpha in LPS-treated murine macrophages. *J Vet Med Sci* 2006;**68:**471–478.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.