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Oxidized low-density lipoprotein increases interleukin-8 production in human gingival epithelial cell line Ca9-22

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Background and Objective: Recent epidemiological studies have shown a correlation between periodontitis and hyperlipidemia. We have found high levels of oxidized low-density lipoprotein (OxLDL) in the gingival crevicular fluid of dental patients. In the present study, we tried to examine the possible role of OxLDL in periodontal inflammation *in vitro*.

Material and Methods: Cells of the human gingival epithelial cell line Ca9-22 were cultured in media containing OxLDL, and the amounts of interleukin-8 (IL-8) and prostaglandin E_2 (PGE_2) produced were measured using ELISAs.

Results: Production of IL-8 by Ca9-22 cells was significantly increased when the cells were treated with OxLDL, but not with native LDL or acetylated LDL. Production of PGE_2 by Ca9-22 cells was enhanced by co-incubation with OxLDL and interleukin-1β (IL-1β). Scavenger receptor inhibitors, fucoidan and dextran sulfate, inhibited the OxLDL-induced IL-8 and PGE_2 production in the presence of IL-1β. The p^{38} MAPK inhibitors SB203580 and SB202190 and the ERK inhibitor PD98059 inhibited the OxLDL-induced IL-8 production. Among oxidized lipids and chemically modified LDL, 7-ketocholesterol enhanced IL-8 production.

Conclusion: This is the first report to show that OxLDL enhances IL-8 production in epithelial cells.

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Recently, attention has been focused on the relation between periodontitis and systemic diseases, including hyperlipidemia. In blood from periodontitis patients, concentrations of low-density lipoprotein (LDL), triglyceride (TG) and total cholesterol were elevated (1,2). Periodontal patients with impaired cholesterol metabolism had deeper periodontal pockets compared with those having normal metabolic status (3). These epidemiological reports suggest that the abnormality in lipid metabolism could be involved in the development of periodontitis; however, the mechanism responsible for this is yet to be clarified.

Low-density lipoprotein is a particle consisting of a large number of lipid molecules, including TG, cholesterol, cholesteryl ester and phospholipids, together with a single molecule of apolipoprotein B. Since polyunsaturated fatty acid moieties in these lipids are susceptible to oxidation reaction, they could produce oxidized lipids in

oxidative conditions. Once LDL is oxidized, a variety of oxidized lipids, such as malondialdehyde (MDA) and oxysterol, or oxidized phospholipids are formed. It was demonstrated that serum oxidized LDL (OxLDL) levels decreased significantly after periodontal treatment (4).

It is well known that OxLDL plays an important role in cardiovascular diseases. Macrophages contribute to the formation of atherosclerotic lesions by accumulating large amounts of cholesteryl ester through the uptake of OxLDL by a variety of mechanisms, including the scavenger receptor pathway (5,6). Furthermore, OxLDL has a number of effects on endothelial cell function, including activation of nuclear factor κB (NFκB; 7), induction of cytokine-mediated vascular cell adhesion molecule-1 (8) and production of interleukin-8 (IL-8; 9). There are, however, few reports on the effect of OxLDL on epithelial cells. Recently, we detected a significant amount of OxLDL in gingival crevicular fluid from healthy sites, which appeared to be higher than in peripheral plasma (10). In addition, amounts of OxLDL, IL-8 and interleukin-1β (IL-1β) in gingival crevicular fluid from deep sites (probing pocket depth ≥ 4 mm) seemed to be higher than those from shallow sites (probing pocket depth < 4 mm; data not shown), leading to the assumption that OxLDL might have a role in inflammatory responses in periodontitis. When OxLDL is present in periodontal tissue, the neighbouring epithelial tissue may be exposed rapidly.

Epithelial cells function as a mechanically protective barrier against invasion by pathogenic organisms. Moreover, increasing evidence indicates that gingival epithelial cells play an important role in the progression of periodontal disease through the production of inflammatory cytokines (11). Interactions between periodontal bacteria and oral epithelial cells lead to activation and expression of a variety of inflammatory mediators in epithelial cells (12). The level of IL-8 production was far greater than that of the other cytokines produced by epithelial cells (13).

Interleukin-8, which belongs to the CXC-chemokine subfamily, is a pro-inflammatory cytokine that participates in the cascade leading to clinical and histological inflammation. For example, IL-8 plays a role in chronic arthritis, where an excessive amount of IL-8 is found in the synovial fluid (14). Jin *et al.* (15) reported that IL-8 levels in gingival crevicular fluid decreased significantly after non-surgical periodontal therapy. The expression of IL-8 in gingiva may be an efficient way for establishing a chemotactic gradient that is able to induce leukocyte migration towards the sites of bacterial infection (16).

The human gingival epithelial cell line Ca9-22, derived from gingival carcinoma (17,18), produces epidermal growth factor receptor extensively (19). The Ca9-22 cells have been used as an *in vitro* culture model of oral epithelial cells in periodontitis-related studies (20–27).

In the present study, we found effects of OxLDL on IL-8 production in Ca9-22 human gingival epithelial cells *in vitro*. We studied the factors involved in the OxLDL-induced IL-8 production in Ca9-22 cells, in order to investigate a possible mechanism leading to periodontal disease.

Material and methods

Preparation of LDL and OxLDL

Low-density lipoprotein was prepared from the plasma of healthy subjects by potassium bromide density gradient ultracentrifugation as reported previously (28). This study was approved by the ethical committee of Showa University School of Pharmacy, Tokyo, Japan. Phosphate-buffered saline (PBS) containing 0.25 mM EDTA was used throughout the preparation procedure to prevent divalent cation-mediated oxidation of LDL. The fraction with a density of $d = 1.019–1.063$ g/cm^3 was collected as the LDL fraction and was then dialyzed against PBS containing 0.25 mM EDTA to remove potassium bromide. Oxidized LDL was prepared as follows. First, an aliquot of LDL stock solution was desalted by gel filtration using the Econo-Pac® 10 DG column (Bio-Rad, Tokyo, Japan) to eliminate EDTA. Then, 2 mg/mL LDL in PBS was incubated with 50 μM $CuSO_4$ at 37°C for 24 h, and the reaction was stopped by the addition of 0.25 mM EDTA and then was cooled on ice (29). Acetylated LDL (AcLDL), as an artificially modified LDL, was prepared as described by Basu *et al.* (30). Modification of LDL with MDA and acrolein was performed by incubating 2 mg/mL native LDL with 2 mM MDA and acrolein at 37°C for 24 h and then dialyzing against PBS containing 0.25 mM EDTA.

Chemical reagents

The scavenger receptor inhibitors, fucoidan (Sigma, St Louis, MO, USA) and dextran sulfate (WaKo Pure Chemical Industries, Osaka, Japan), were dissolved in PBS and stored at −20°C. The MAPK inhibitors, SB203580, SB202190, PD98059 (EMD Chemical Inc., San Diego, CA, USA) and SP600125 (Sigma), were dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C. 7-Ketocholesterol and 25-hydroxycholesterol were purchased from Sigma, dissolved in ethanol and stored at −20°C. 1-Palmitoyl-2-linoleoyl-phosphatidylcholine (PC) and synthetic oxidized PCs, 1-palmitoyl-5-oxovaleroyl-PC (5-oxovaleroyl-PC) and 1-palmitoyl-9-oxononanoyl-PC (9-oxononanoyl-PC; 5), were kind gifts from Drs Y. Soda and T. Kishimoto of Alfresa Pharma Corp. (Osaka, Japan).

Cell culture and treatment

A human gingival epithelial cell line, Ca9-22 cells, and a human oral epithelial cell line, squamous cell carcinoma of the floor of the mouth, HO-1-u-1 cells, were purchased from RIKEN BioResource Center (Ibaraki, Japan). The Ca9-22 cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) with 10% fetal bovine serum (FBS) supplemented with 1% antibiotics (50 U/mL penicillin and 50 μg/mL streptomycin). The HO-1-u-1 cells were cultured in RPMI 1640 medium with 10% FBS supplemented with 1% antibiotics (50 U/mL penicillin and 50 μg/mL

streptomycin). Primary human oral keratinocytes from gingival mucosa tissue (ScienCell, Carlsbad, CA, USA) were cultured in keratinocyte medium (ScienCell) on poly-L-lysine-coated plates (Iwaki brand, Asahi Glass Co., Chiba, Japan). Cells were maintained at 37°C in an atmosphere of air containing 5% CO_2 and were passaged at 80% confluency, and experiments were done between passages 2 and 4. Cells ($5 \times 10^4/cm^2$) were cultured for 24 h in a medium containing 10% FBS. Then that medium was replaced with a medium supplemented with 1% FBS with either LDL, AcLDL or OxLDL. During the 24 h incubation, cells were treated with or without 10 ng/mL of recombinant human IL-1β (PeproTech EC Ltd, London, UK) for the last 5 h. To examine the effect of various inhibitors on OxLDL-induced response, cells were treated with either an inhibitor of OxLDL–scavenger receptor binding or an inhibitor of MAPK for 30 min before exposure to 50 or 200 μg/mL OxLDL and throughout the incubation period of the experiment (24 h). To test the effect of oxidized phospholipids on the cells, the PC, 5-oxovaleroyl-PC and 9-oxononanoyl-PC were resuspended in PBS and added to the cell culture medium at 20 μM. For oxysterols, 2.5 μL of ethanol or an ethanol solution containing 7-ketocholesterol or 25-hydroxycholesterol were added to 5 mL of the cell culture medium.

Measurement of cytokines and PGE_2

Interleukin-1β, IL-8, monocyte chemotactic protein-1 (MCP-1; R&D Systems Inc., Minneapolis, MN, USA) and prostaglandin E_2 (PGE_2; Cayman, Ann Arbor, MI, USA) released in media were measured using ELISA kits. After Ca9-22 cells were incubated in various conditions, supernatants recovered from each well were frozen at −20°C until analysis. All data are presented as means ± SD in picograms per milliliter of triplicate cell cultures.

Estimation of cytotoxicity

The cytotoxicity of LDL, AcLDL and OxLDL for Ca9-22 cells was evaluated by the release of a cytoplasmic enzyme, lactate dehydrogenase (LDH), into culture supernatants. Levels of LDH in the samples were measured using an LDH detection kit (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's instructions.

Statistical analysis

Data are expressed as means ± SD of triplicate assays in a single experiment. Reproducibility was confirmed by repeating similar experiments several times. Results were analyzed using one-way ANOVA, using STATVIEW™ version 5.0 (SAS Institute Inc., Cary, NC, USA) for Windows XP. Dunnett's test was used in the *post hoc* comparisons of specific groups. Statistical significance for all comparisons was assigned at $p < 0.05$.

Results

Effect of OxLDL on the IL-8 production in human gingival epithelial cell line Ca9-22

When Ca9-22 cells were incubated with OxLDL for 24 h, release of IL-8 into the culture media increased significantly (Fig. 1A). The increase in IL-8 production was dose dependent. Production increased more than 20-fold with 200 μg/mL OxLDL. Neither

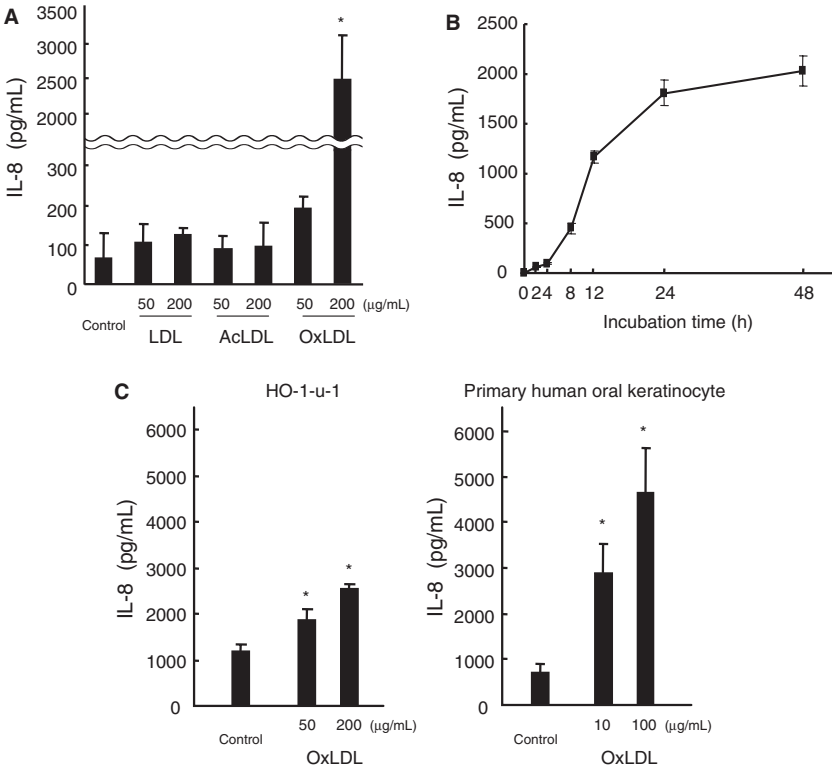

Fig. 1. Effects of oxidized low-density lipoprotein (OxLDL) on the interleukin-8 (IL-8) production in Ca9-22 cells, HO-1-u-1 cells and primary human oral keratinocytes. Cells were seeded at a density of 7.5×10^4 cells per well in a 24-well culture plate. (A) After incubation for 24 h, cells were treated with 50 or 200 μg/mL of LDL, acetylated LDL (AcLDL) or OxLDL for 24 h in the presence 1% fetal bovine serum (FBS). (B) Cells were incubated with 200 μg/mL OxLDL for up to 48 h. (C) The oral epithelial cell line, HO-1-u 1 cells, squamous cell carcinoma of floor of the mouth, were treated with 50 or 200 μg/mL OxLDL for 24 h in the presence 1% FBS. Primary human oral keratinocytes from gingival mucosa tissue were treated with 10 or 100 μg/mL OxLDL for 24 h. Interleukin-8 in culture supernatants was determined using ELISA. Data are expressed as the means ± SD of triplicate assays in a single experiment. *Statistically significant ($p < 0.05$) compared with control culture (medium alone).

native LDL nor AcLDL affected IL-8 release from Ca9-22 cells. When cells were incubated with OxLDL (200 μg/mL) for up to 48 h, IL-8 was produced in a time-dependent manner (Fig. 1B). Interleukin-8 is a major chemokine responsible for recruiting neutrophils in inflammatory sites; however, OxLDL did not change the amount of MCP-1 released in the medium, which is another chemokine that recruits monocytes (data not shown). Another oral epithelial cell line, HO-1-u-1 cells, squamous cell carcinoma of floor of the mouth, and primary human oral keratinocytes from gingival mucosa tissue enhanced IL-8 production by OxLDL (Fig. 1C).

Effect of OxLDL on cytotoxicity

Exposure of the cells for 24 h to various concentrations of LDL or AcLDL had no effect on LDH release (data not shown), and a little cytotoxicity (6.4%) was observed only at 200 μg/mL OxLDL in Ca9-22 cells (Fig. 2A). When cells were incubated with 200 μg/mL OxLDL, we observed no LDH release by 8 h and very little release by 12 h (Fig. 2B). Maximal cytotoxicity was estimated by adding 1% Triton X-100 to the cells for 5 min and measuring the LDH activity recovered in the medium, and was assumed to be 100%.

Effect of OxLDL on PGE_2 production

We also measured the amount of another proinflammatory factor, PGE_2, in the culture medium and found that none of these treatments increased PGE_2 release from Ca9-22 cells (Fig. 3A). However, OxLDL may have a priming effect on the cells, since treatment with IL-1β for the last 5 h within the 24 h incubation with 50 μg/mL OxLDL resulted in significantly increased PGE_2 production compared with the treatment of the cells with IL-1β alone without the OxLDL pretreatment (Fig. 3B). Oxidized LDL alone does not induce IL-1β production from Ca9-22 cells (data not shown); hence, it is likely that PGE_2 production may be caused when multiple inflammatory stimuli are present in local sites.

Scavenger receptors are involved in OxLDL-induced IL-8 and PGE_2 production

To test whether the OxLDL-induced IL-8 production was mediated by scavenger receptors, Ca9-22 cells were treated with fucoidan or dextran sulfate, which are competitors for OxLDL binding to scavenger receptors, before the addition of OxLDL. As shown in Fig. 4A, the addition of either fucoidan or dextran sulfate to the cells reduced the production of IL-8 induced by OxLDL. In addition, we observed that fucoidan and dextran sulfate inhibited OxLDL-induced PGE_2 production in the presence of IL-1β (Fig. 4B).

Possible mediation of the OxLDL-induced IL-8 production by p38 MAPK and ERK

It has been demonstrated that MAPK is important for the IL-8 production in gingival epithelial cells (31–33). We investigated whether MAPK activation is involved in OxLDL-induced IL-8 production in human epithelial cells. When Ca9-22 cells were treated with p38 MAPK inhibitors (SB203580 and SB202190) and an ERK inhibitor

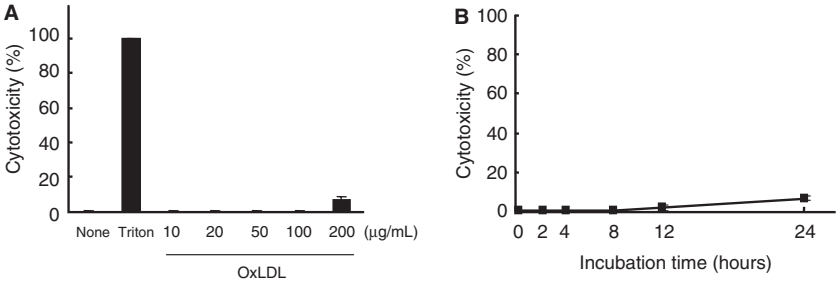

Fig. 2. Effects of OxLDL on the cytoxicity in Ca9-22 cells. Oxidized LDL caused little cytotoxic effect in the conditions investigated. Cells were seeded at a density of 2.5×10^4 cells per well in a 96-well culture plate and incubated for 24 h. Subsequently, cells were treated with various concentrations of OxLDL for 24 h (A) or incubated with 200 μg/mL OxLDL for up to 24 h (B). The lactose dehydrogenase (LDH) activity in the culture medium was measured using an LDH detection kit. As positive and negative controls, cells were incubated with or without 1% Triton X-100, respectively. Data are expressed as the means ± SD of triplicate assays in a single experiment.

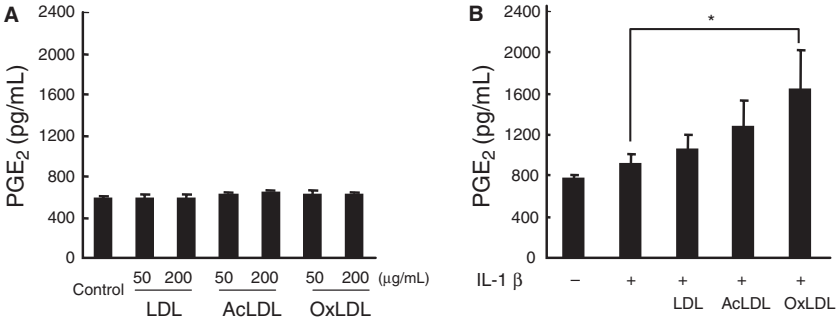

Fig. 3. Effects of OxLDL on the prostaglandin E_2 (PGE_2) production in Ca9-22 cells. (A) Cells were treated with 50 or 200 μg/mL of LDL, AcLDL or OxLDL for 24 h in the presence 1% FBS. Prostaglandin E_2 was determined in culture supernatants using ELISA. (B) Cells were treated with 50 μg/mL LDL, AcLDL or OxLDL for 24 h, during which incubation at 19 h, interleukin-1β (IL-1β) was added and co-incubated for the following 5 h. Data are expressed as the means ± SD of triplicate assays in a single experiment. *Statistically significant ($p < 0.05$) compared with control culture (IL-1β alone).

(PD98059), the OxLDL-induced IL-8 production was inhibited (Fig. 5).

Oxysterols enhanced IL-8 production in human epithelial cells

To investigate which component of OxLDL is responsible for the enhanced IL-8 production, we studied several chemically modified LDLs and lipid peroxidation products. In preliminary experiments, we tested several appropriate concentrations of these compounds. The concentration ranges used were 1–20 μM for PC, 5-oxovaleroyl-PC and 9-oxononanoyl-PC, 50–200 μg/mL for MDA-LDL and acrolein-modified LDL (acrolein-LDL), and 5–100 μM for oxysterols based on several previous reports (34–36). These experiments concluded that only the highest concentration of 7-ketocholesterol induced the production of IL-8. We then decided to use the maximal doses of these compounds, as shown in Fig. 6. Since fucoidan and dextran sulfate reduced IL-8 production, chemically modified LDLs which are known to bind to scavenger receptors were tested; MDA-LDL and acrolein-LDL did not induce IL-8 production. It is suggested that the interaction of negatively charged ligand with scavenger receptors *per se* is not the active stimulant. It is well known that some of the OxLDL biological activities are due to oxidized lipids generated in OxLDL. Therefore, we tested the effect of synthetic phosphatidylcholine (PC) and oxidized PC (OxPC) on IL-8 production from Ca9-22 cells, and found that they did not induce IL-8 production. Oxysterols are another group of known bioactive lipids, in addition to oxidized phospholipids. We found that treatment of the cells with 7-ketocholesterol but not 25-hydroxycholesterol increased the production of IL-8 more than twofold.

Discussion

The importance of OxLDL in atherogenesis is well recognized. A number of studies have shown that OxLDL has a variety of stimulatory activities, such as increasing IL-8 expression by vascular cells, endothelial cells, smooth muscle cells and macrophages (9,37,38). Little is known, however, about the effects of OxLDL on epithelial cells. We demonstrated in this study that OxLDL enhanced production of the chemotactic cytokine IL-8 in Ca9-22 cells, which are a human oral epithelial cell line. Another oral epithelial cell line, HO-1-u-1 cells, squamous cell carcinoma of floor of the mouth, demonstrated enhanced IL-8 production in response to OxLDL. This is the first study to show that OxLDL stimulates gingival epithelial cells. It is a novel idea that lipoprotein metabolites could have a role in the inflammatory reaction in mucous membrane tissues and not only in blood vessels.

We recently found that gingival crevicular fluid contained a high concentration of OxLDL (10). The inflammatory responses of gingival epithelial cells to OxLDL *in vitro* suggest that local exposure to oxidized products, such as OxLDL, could promote inflammatory reactions in periodontal tissue.

In our study, the other major chemokine, MCP-1, was not increased by OxLDL (data not shown), suggesting that the effect of OxLDL on gingival epithelial cells was different from that on endothelial cells. Oral epithelial cells provide a physical barrier against invading pathogens and play an important role in the innate host defenses. The induction of IL-8 in gingival epithelial cells in response to OxLDL may promote periodontitis by recruiting leukocytes. Since our results were derived from experiments using a human gingival epithelial cell line, further studies are needed to clarify how the presence of OxLDL may affect gingival fibroblasts or periodontal ligament cells.

We have measured the OxLDL present in human gingival crevicular fluid. It is noted that the OxLDL concentration in gingival crevicular fluid appeared to be more than 17-fold higher than that in peripheral blood (10). Up to now, we have tested 60 gingival crevicular fluid samples and found the mean OxLDL concentration to be 38.8 U/mL (10 and Y. Sakiyama, R. Kato, S. Inoue, K. Suzuki, H. Itabe and M. Yamamoto, unpublished data), which is estimated to be equivalent to 13.2 μg/mL (39). In this study, the Ca9-22 cells were exposed to 50–200 μg/mL OxLDL, a concentration range which has frequently been used in experiments on other types of cells, including endothelial cells and macrophages (8,9,28,40). The sixty gingival crevicular fluid samples we analyzed

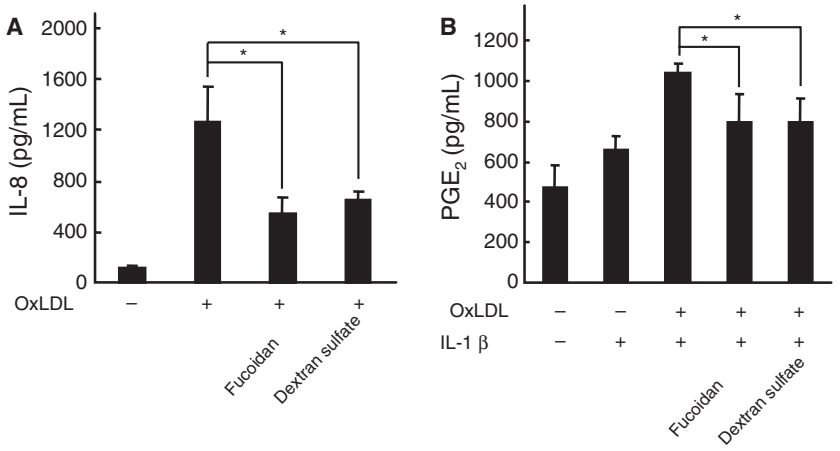

Fig. 4. Blockage of OxLDL-induced IL-8 and PGE_2 production by scavenger receptor inhibitors. (A) The Ca9-22 cells were stimulated with 200 μg/mL OxLDL for 24 h with or without 30 min pretreatment with fucoidan (50 μg/mL) or dextran sulfate (50 μg/mL). (B) Cells were pretreated either with or without fucoidan (50 μg/mL) or dextran sulfate (50 μg/mL) for 30 min, and then the cells were incubated with 50 μg/mL OxLDL for 24 h. After 19 h of incubation with OxLDL, IL-1β (10 ng/mL) was added and co-incubated for the following 5 h. Interleukin-8 and PGE_2 in culture supernatants were determined using ELISA. Data are expressed as the means ± SD of triplicate assays in a single experiment. *Statistically significant ($p < 0.05$) compared with OxLDL treatment alone.

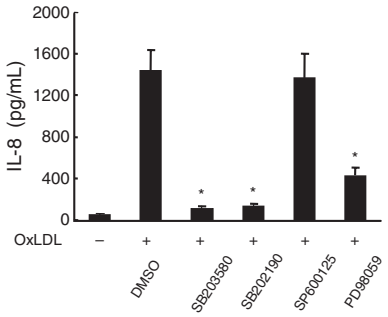

Fig. 5. Effect of p^{38} MAP kinase inhibitors, JNK inhibitor and ERK inhibitor on Ox-LDL-induced IL-8 production. Cells were pretreated either with or without the p38 MAPK inhibitors SB203580 or SB202190, the JNK inhibitor SP600125 or the ERK inhibitor PD98059 (10 μM) for 30 min, and then incubated with 200 μg/mL OxLDL for 24 h. Interleukin-8 in culture supernatants was determined using ELISA. Data are expressed as the means ± SD of triplicate assays in a single experiment. *Statistically significant ($p < 0.05$) compared with control cultures (OxLDL and DMSO).

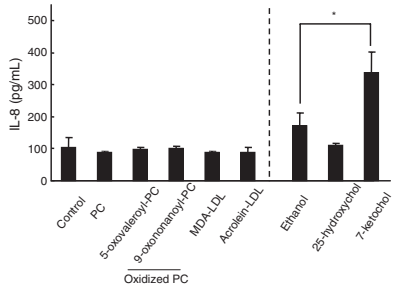

Fig. 6. 7-Ketocholesterol enhanced IL-8 production in Ca9-22 cells. Cells were incubated with 1-palmitoyl-2-linoleoyl-phosphatidylcholine (PC), two oxidized PCs (5-oxovaleroyl-PC, or 9-oxononanoyl-PC; 20 μM), MDA-LDL or acrolein-LDL (200 μg/mL), 25-hydroxycholesterol or 7-ketocholesterol (100 μM) for 24 h. Data are expressed as the means ± SD of triplicate assays in a single experiment. *Statistically significant ($p < 0.05$) compared with control cultures (medium or ethanol alone).

included some from healthy subjects and some from periodontal disease sites, which showed higher OxLDL concentrations.

It has been shown that PGE_2 is a potent stimulator of bone resorption (41,42) and is associated with attachment loss (43). Since PGE_2 is an important mediator of inflammation, local PGE_2 production may modulate periodontal inflammation. In gingival epithelial cells, fetal bovine serum stimulation generated PGE_2 via cyclooxygenase-2 mRNA induction (44). It is noteworthy that OxLDL alone did not increase PGE_2 or IL-1β, suggesting that OxLDL does not directly cause alveolar bone resorption. However, combined treatment with IL-1β and OxLDL significantly increased PGE_2 production. Thus, OxLDL may prime Ca9-22 cells to produce PGE_2.

The OxLDL-induced IL-8 and PGE_2 production by oral epithelial cells is likely to be mediated by some scavenger receptors, since fucoidan and dextran sulfate blocked the action of OxLDL. Since the chemically modified LDLs, AcLDL, MDA-LDL and acrolein-LDL, did not induce IL-8 production, lipid oxidation products rather than modified apolipoprotein B might have stimulated the Ca9-22 cells. It has been demonstrated that lipid oxidation products, such as oxidized phospholipids and oxidized cholesterol generated in OxLDL stimulate endothelial cells, macrophages and other cells. One of the scavenger receptors, CD36, recognized a series of oxidized PC molecules (45). However, two major oxidized PCs, 5-oxovaleroyl-PC and 9-oxononanoyl-PC, had no effect on Ca9-22 cells. We demonstrated that 7-ketocholesterol enhanced IL-8 production in Ca9-22 cells. Oxysterols, including 7-ketocholesterol, are one of the biologically active components in OxLDL, and they are detected in the nanomolar range in normocholesterolemic human serum (46,47). It was previously described that oxysterols are able to induce IL-8 production in monocytes/macrophages (33,48).

Consumption of a high-cholesterol diet augmented the effects of lipopolysaccharide and proteases on the production of proinflammatory cytokines and increased the level of the oxidative stress marker, mitochondrial 8-hydroxy deoxyguanosine, in periodontal tissues of rats (49,50). Bacterial pathogen-induced periodontitis also enhanced oxidative stress by potentiating inflammatory responses in the periodontal tissues of rats (49). The inflammatory response of periodontitis in turn produces oxidative stress, resulting in oxidative modification of the LDL in the periodontal milieu that progresses inflammation further in the periodontal tissue.

In conclusion, OxLDL enhanced IL-8 production in gingival epithelial cells, which may contribute to the inflammatory reaction in periodontitis.

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