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Hepatocytes produce tumor necrosis factor-α and interleukin-6 in response to *Porphyromonas gingivalis*

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Takano M, Sugano N, Mochizuki S, Koshi RN, Narukawa TS, Sawamoto Y, Ito K. Hepatocytes produce tumor necrosis factor-α and interleukin-6 in response to Porphyromonas gingivalis. *J Periodont Res; 2012; 47: 89–94. © 2011 John Wiley & Sons A/S*

Background and Objective: The liver plays a major role in clearing systemic bacterial infections. In addition, inflammatory cytokines produced in the liver play a critical role in systemic cytokine levels. The aim of this study was to investigate the production of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) by hepatocytes in response to periodontal pathogens.

Material and Methods: The mouse hepatic carcinoma cell line Hepa-1.6 and the mouse macrophage-like cell line RAW 264 were co-cultured in Transwell insert plates. Cells were stimulated with bacterial extracts prepared from *Porphyromonas gingivalis* and the induction of TNF-α and IL-6 was measured using real-time PCR and ELISA.

Results: After stimulation with bacteria, the induction of TNF-α and IL-6 was observed in RAW 264 cells and Hepa-1.6 cells. Significant reduction of TNF-α mRNA expression in Hepa-1.6 cells was observed after treatment with antibody to TNF-α.

Conclusion: The results obtained in the present study show that *P. gingivalis* extract induces TNF-α and IL-6 in an *in vitro* liver model and that macrophage-derived TNF-α mediates the induction of TNF-α in hepatocytes.

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Periodontitis is a chronic inflammatory disease characterized by periodontal pocket formation, loss of connective tissue attachment and alveolar bone resorption, which results in tooth loss. Current evidence suggests that periodontal disease is also associated with systemic diseases. Inflammatory cytokines are central to the initiation of the immune response to periodontopathic bacteria, and local cytokine production in response to periodontal infection may affect the systemic environment (1,2). In particular, periodontitis is regarded as having a close relationship with diabetes mellitus (3). A large number of studies have provided evidence that type 1 and type 2 diabetes increase the risk and severity of periodontal disease (4–9). Previous studies suggest that elevated levels of circulating tumor necrosis factor-α (TNF-α) may contribute to insulin resistance in patients with type 2 diabetes (10). Cross-sectional studies performed in humans have shown that elevated levels of circulating TNF-α are associated with insulin resistance and diabetes mellitus (11,12). A longitudinal human study has also shown that elevated plasma TNF-α levels result in poorer glycemic control (13). TNF-α inhibits the action of insulin in animal and *in vitro* models (14–17). The overexpression of TNF-α in diabetes is thought to contribute to several complications of diabetes, including retinopathy, nephropathy, neuropathy and diabetes-enhanced periodontal

disease (18–21). Interleukin-6 (IL-6) is among the first cytokines implicated as a predictor or pathogenetic marker of insulin resistance and cardiovascular diseases (22).

TNF-α and IL-6 are produced by various cell types, including mast cells, macrophages, neutrophils, eosinophils and epithelial cells, in response to inflammation (23,24). In addition, inflammatory cytokines produced in the liver, mainly synthesized by Kupffer cells, play a critical role in systemic cytokine levels (25–27). Previously, we found that diabetic mice showed a greater increase of TNF-α mRNA and *Il6* mRNA expression in their livers after inoculation with *Porphyromonas gingivalis* compared with normal (nondiabetic) mice (28). Furthermore, we noted that anti-TNF-α IgG suppressed the expression of TNF-α mRNA and IL-6 mRNA in the livers of diabetic mice after inoculation with *P. gingivalis* (29). However, little is known about cytokine expression in the liver.

Therefore, this study focused on the liver to investigate the production of TNF-α and IL-6 in response to a periodontal pathogen. We examined the effects of *P. gingivalis* on TNF-α and IL-6 expression in a model system consisting of a co-culture of hepatocytes and macrophages.

Material and methods

Cell culture

The mouse hepatic carcinoma cell line Hepa-1.6 and the mouse macrophage-like cell line RAW 264 were obtained from a commercial source (Riken Cell Bank, Tsukuba Science City, Japan). Hepa-1.6 cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; Gibco BRL, Rockville, MD, USA) containing 10% fetal bovine serum (Hananesco Bio Corp., Tokyo, Japan) and 1% penicillin–streptomycin solution (Sigma Chemical, St Louis, MO, USA). RAW 264 cells were cultured in minimal essential medium alpha (MEM α; Kohjin Bio Corp., Saitama, Japan) containing 10% fetal bovine serum and 1% penicillin–streptomycin solution. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Bacterial extracts

P. gingivalis FDI 381 was used in all experiments. Bacteria were maintained on Brucella HK agar (Kyokuto Pharmaceutical, Tokyo, Japan) supplemented with 10% horse blood under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) at 37°C. Growth in liquid medium was monitored at an optical density of 550 nm. The cells were harvested by centrifugation at 10,000 *g* for 15 min at 4°C. Bacterial extracts were prepared by sonication at 20 W for 5 min; the insoluble debris was subsequently removed by centrifugation at 10,000 *g* for 30 min at 4°C. The supernatant was then filter-sterilized and stored at −80°C until used. The protein concentration of the extract (162 μg/mL) was determined using the Bradford protein assay (Bio-Rad, Hercules, CA, USA).

Co-culture system

Cell suspensions were quantified using a hemocytometer. Hepa-1.6 cells were seeded at 8.5×10^5 cells/well into a six-well multiple well plate, and RAW 264 cells were seeded at 3.75×10^5 cells/well onto six-well Transwell insert plates (4.67 cm^2, 0.4 μm pore size; Corning CoStar Corp., Cambridge, MA, USA) (30,31). After replacing all media with DMEM, the Transwell insert on which RAW 264 cells had been cultured was added to multiple plate wells preloaded with Hepa-1–6 cells. Some experiments were performed in single-cell culture using Transwell plates and normal plates.

Cells were co-cultured with 3 μg/mL of bacterial extracts (32) prepared from *P. gingivalis* FDC 381 at 37°C in a humidified environment of 5% CO_2.

Real-time PCR

After 24 h of incubation, the medium was replaced with fresh medium containing bacterial extract or fresh medium alone (control), and the cells were grown for an additional 1, 3, 6, 12 or 24 h at 37°C in a humidified atmosphere of 5% CO_2. We also examined the effects of a rat anti-murine TNF-α mAb (BioSource International, Inc., Camarillo, CA, USA) or rat IgG (BioSource International, Inc.) control on this *in vitro* liver model, after 6 h of incubation.

Total RNA was extracted from the cells using an RNeasy Mini kit (Qiagen, Valencia, CA, USA). Complementary DNA was synthesized using a Ready-To-Go T-Primed First-Strand kit (Amersham Biosciences, Tokyo, Japan). The primer and probe sets for TNF-α and IL-6 were obtained from Applied Biosystems (Foster City, CA, USA). Real-time PCR was performed on an ABI PRISM 7700 Sequence Detector (Applied Biosystems) using the following parameters: 50°C for 2 min, 95°C for 10 min, followed by 40 - cycles of denaturation at 95°C for 15 s and primer extension at 60°C for 1 min. The expression level of each gene was first normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same sample, and the relative differences between the control and treatment groups were calculated and expressed as relative increases, with the control set at 100%. The data are shown as the mean ± standard deviation of six independent experiments. Each experiment was run in duplicate.

ELISA

The concentrations of TNF-α and IL-6 in each culture supernatant were determined by ELISA using commercially available ELISA kits (BioSource International, Inc., Camarillo, CA, USA), according to the manufacturer's instructions.

Statistical analysis

Data were analyzed for significance using a one-way analysis of variance with Bonferroni post-test correction for multiple comparisons. The Student's *t*-test was used to determine the statistical significance of differences between control and test groups.

Results

TNF-α and IL-6 induction

First of all, we investigated the effect of *P. gingivalis* extracts on a co-culture of Hepa-1.6 cells and RAW 264 cells. The induction of TNF-α mRNA and IL-6 mRNA in Hepa-1.6 cells and RAW 264 cells was examined by real-time PCR. After treatment with *P. gingivalis* extracts, the expression of TNF-α mRNA in RAW 264 cells showed significant increases at 1, 3, 6 and 12 h post-treatment (Fig. 1A). In Hepa-1.6 cells, significant inductions of TNF-α mRNA were observed at 3, 6 and 12 h post-treatment (Fig. 1B). Similar results were obtained for expression of IL-6 mRNA in both cell types (Fig. 1C and 1D). The concentration of TNF-α in the co-culture supernatant was significantly increased at 1, 3, 6, 12 and 24 h poststimulation (Fig. 2A). The concentration of TNF-α was also increased in control cells; however, no induction of TNF-α in control cells was observed in single-cell culture using a Transwell plate and a normal plate (Fig. 2B and 2C). Significant increases of IL-6 secretion were also found at 3, 6, 12 and 24 h poststimulation (Fig. 2D).

Effect of anti-TNF-α IgG on TNF-α and IL-6 induction

We investigated the effect of anti-TNF-α IgG on the levels of TNF-α and IL-6 mRNAs 6 h after the administration of *P. gingivalis* extracts. Expression of TNF-α and IL-6 mRNAs in RAW 264 cells after treatment with anti-TNF-α IgG was not significantly changed compared to treatment with control antibody (Figs. 3A and 4A). In contrast, significant reduction of expression of TNF-α mRNA in Hepa-1.6 cells was observed after treatment with anti-TNF-α IgG (Fig. 3B). The expression of IL-6 mRNA in Hepa-1.6 cells was significantly reduced after treatment with 5 μg/mL of anti-TNF-α IgG, but not after treatment with 2.5 μg/mL of anti-TNF-α IgG (Fig. 4B). A significant decrease in the secretion of TNF-α was observed, 6 h post-stimulation in Hepa-1.6 cells, after treatment with anti-TNF-α IgG (Fig. 5A). In contrast, there was no significant change in the

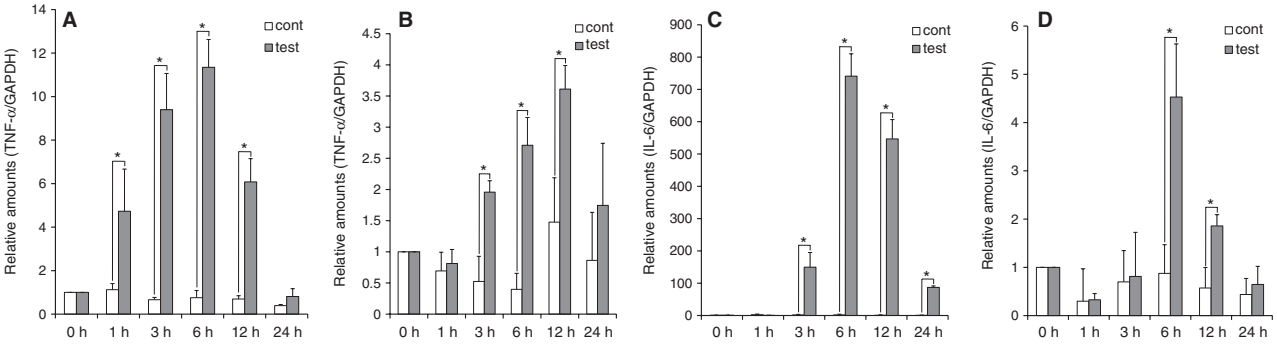

Fig. 1. Time course of expression of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) mRNAs in the RAW 264/Hepa-1.6 co-culture. Cells were incubated with medium that contained *Porphyromonas gingivalis* extracts (3 μg/mL) for 0, 1, 3, 6, 12 or 24 h (Test) or medium alone (Control). Real-time PCR was used to monitor the levels of TNF-α mRNA and IL-6 mRNA, which were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH); the relative differences between the control and experimental groups are expressed as relative increases. (A) Expression of TNF-α mRNA in RAW 264 cells. (B) Expression of TNF-α mRNA in Hepa-1.6 cells. (C) Expression of IL-6 mRNA in RAW 264 cells. (D) Expression of IL-6 mRNA in Hepa-1.6 cells. The data are shown as the mean ± standard deviation of six independent experiments. Each experiment was run in duplicate. $*p < 0.05$ vs. control.

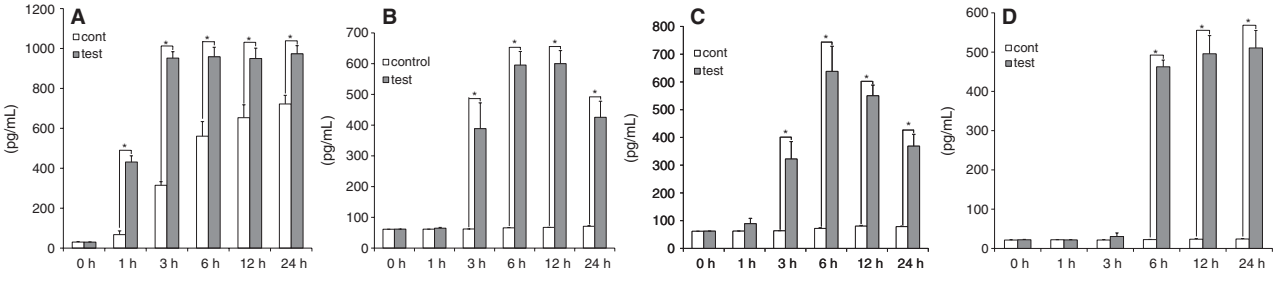

Fig. 2. Effect of *Porphyromonas gingivalis* extracts on tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) secretion. Cells were incubated with medium that contained bacterial extracts (3 μg/mL) for 0, 1, 3, 6, 12 or 24 h (Test), or with medium alone (Control), in the RAW 264/Hepa-1.6 co-culture. Culture supernatants were assayed for TNF-α (A) and IL-6 (D) using ELISA kits. RAW 264 cells were also incubated in a normal plate (B) and in a Transwell plate (C). The data are shown as the mean ± standard deviation of six independent experiments. Each experiment was run in duplicate. $*p < 0.05$ vs. control.

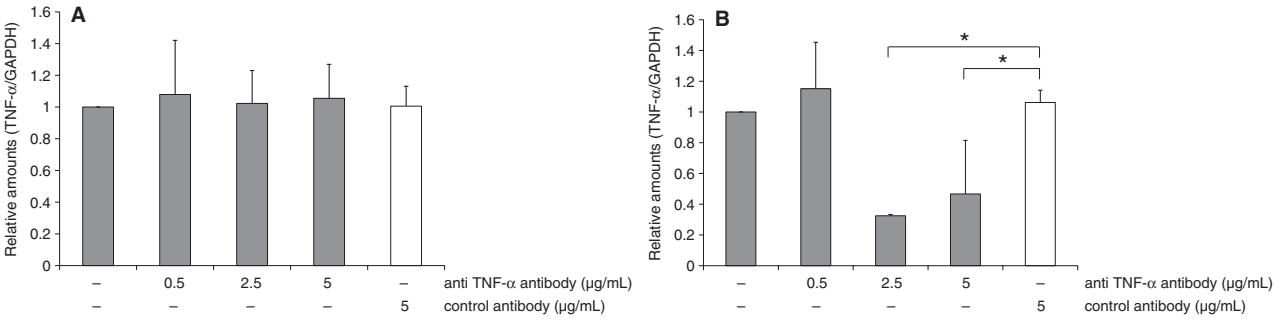

Fig. 3. The inhibitory effect of treatment with anti-tumor necrosis factor-α (TNF-α) IgG on TNF-α mRNA expression in the RAW 264/Hepa-1.6 co-culture model. Cells were incubated for 6 h in medium that contained *Porphyromonas gingivalis* extracts (3 μg/mL) and anti-TNF-α IgG. Real-time PCR was used to monitor the levels of TNF-α mRNA, which were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH); the relative differences between the control and experimental groups are expressed as relative increases. (A) TNF-α mRNA expression in RAW 264 cells treated with 0, 0.5, 2.5 or 5 μg/mL of anti-TNF-α IgG, compared with the IgG control antibody. (B) Expression of TNF-α mRNA in Hepa-1.6 cells treated with 0, 0.5, 2.5 or 5 μg/mL of anti-TNF-α IgG compared with the IgG control antibody. The data are shown as the mean ± standard deviation of six independent experiments. Each experiment was run in duplicate. $*p < 0.05$ vs. control antibody.

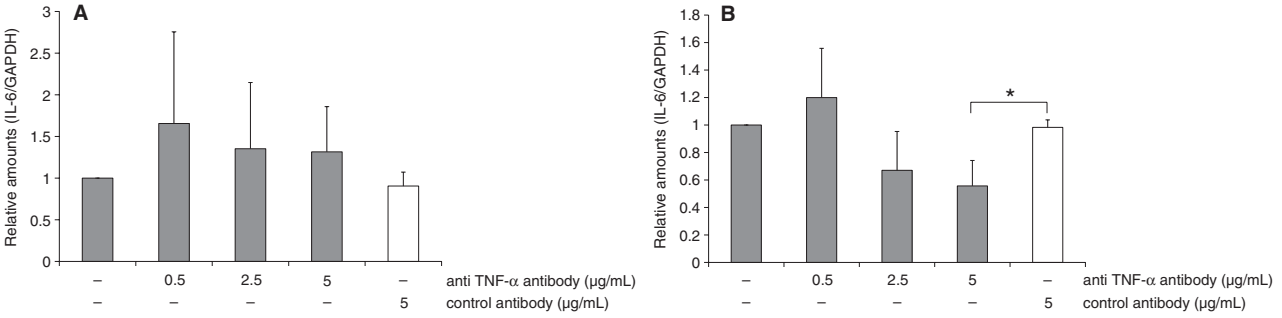

Fig. 4. The inhibitory effect of treatment with anti-tumor necrosis factor-α (TNF-α) IgG on expression of interleukin-6 (IL-6) mRNA in the RAW 264/Hepa-1.6 co-culture model. Cells were incubated for 6 h with medium that contained *Porphyromonas gingivalis* extracts (3 μg/mL) and anti-TNF-α IgG. Real-time PCR was used to monitor the levels of IL-6 mRNA, which were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH); the relative differences between the control and experimental groups are expressed as relative increases. (A) Expression of IL-6 mRNA in RAW 264 cells treated with 0, 0.5, 2.5 or 5 μg/mL of anti-TNF-α IgG compared with the IgG control antibody. (B) Expression of IL-6 mRNA in Hepa-1.6 cells treated with 0, 0.5, 2.5 or 5 μg/mL of anti-TNF-α IgG compared with the IgG control antibody. The data are shown as the mean ± standard deviation of six independent experiments. Each experiment was run in duplicate. $*p < 0.05$ vs. control antibody.

secretion of IL-6 after treatment with anti-TNF-α IgG (Fig. 5B).

Discussion

The liver is an exceptional organ in terms of its metabolic, synthetic and detoxifying functions. Besides its various metabolic functions, the liver is also a central immunological organ. The liver plays a major role in clearing systemic bacterial infections (33–36). Rapid clearance of bacteria from peripheral blood is often attributed to fixed liver macrophages (i.e. Kupffer cells), which line the hepatic sinusoids (33,34). Kupffer cells are found in the greatest numbers in the periportal area, where they constitute the first macrophage population to come into contact with bacteria, endotoxins and microbial debris derived from the bloodstream (37,38). In lipopolysaccharide (LPS)-induced liver injury, activated Kupffer cells produce IL-1, IL-6 and TNF-α (39,40). Furthermore, hepatocytes and hepatoma cells produce inflammatory cytokines in response to LPS and cytokines or in response to conditioned medium from activated Kupffer cells (41,42). In this study, we showed significant induction of TNF-α and IL-6 in response to *P. gingivalis* extracts using a co-culture system of Hepa-1.6 cells and RAW 264 cells, using hepatocyte and Kupffer cells as an *in vitro* model of the liver. Our previous study showed a significant increase in the expression of TNF-α and IL-6 in the livers of normal and diabetic mice after *P. gingivalis* infection (28). These results indicate that *P. gingivalis* infection in periodontitis patients may induce the expression of TNF-α and IL-6 in the liver. In this co-culture study, substantially greater amounts of TNF-α were released in control supernatant. In contrast, no induction was observed in single-cell culture. The expression of TNF-α mRNA by cells in the co-culture Transwell plate was about twice as high as the expression of TNF-α mRNA by cells in the normal plate

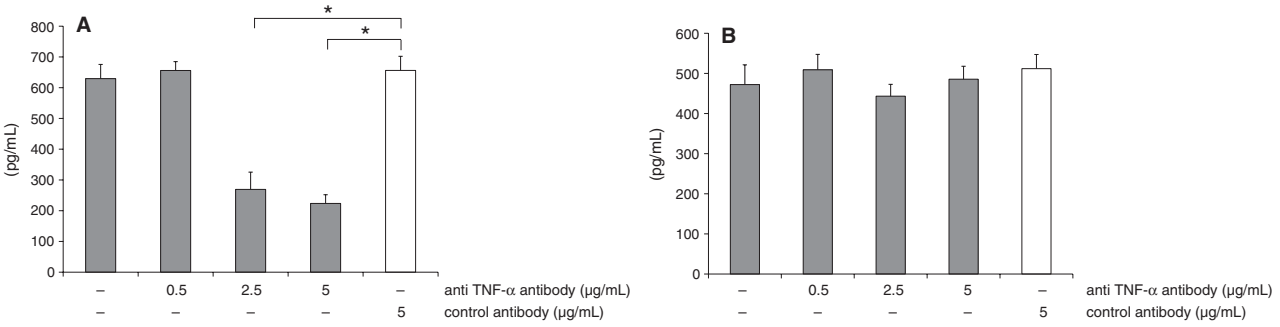

Fig. 5. The inhibitory effect of treatment with anti-tumor necrosis factor-α (TNF-α) IgG on TNF-α and interleukin-6 (IL-6) secretion. Cells were incubated for 6 h in medium that contained *Porphyromonas gingivalis* extracts (3 μg/mL) and anti-TNF-α IgG. Culture supernatants were assayed for TNF-α (A) and IL-6 (B) using ELISA kits. The data are shown as the mean ± standard deviation of six independent experiments. Each experiment was run in duplicate. $*p < 0.05$ vs. control antibody.

(data not shown), indicating that cell-to-cell interactions in the Transwell plate may have a stimulatory effect on TNF-α induction.

Our study also showed significant reductions of TNF-α mRNA expression in Hepa-1.6 cells after treatment with anti-TNF-α IgG. In contrast, no significant change was observed in RAW 264 cells. In our previous *in vivo* study, treatment with anti-TNF-α IgG markedly improved the host response to *P. gingivalis* and was associated with reduced serum TNF-α, fasting blood glucose levels, and TNF-α expression in the liver. The results obtained in the present study indicate that macrophage-derived TNF-α mediates the induction of TNF-α in hepatocytes. Therefore, anti-inflammatory pharmacological strategies that reduce the elevated levels of TNF-α might improve the host response to *P. gingivalis* infection. In this study, neutralization of TNF-α did not inhibit the *P. gingivalis*-stimulated induction of IL-6 by hepatocytes. A previous study showed that LPS directly stimulates hepatocyte IL-6 synthesis through a cytokine-independent mechanism (43). Thus, these observations suggest that *P. gingivalis* extracts stimulate the hepatic cells to produce IL-6 through TNF-α-independent mechanisms.

In conclusion, this study indicated that *P. gingivalis* infection may induce inflammatory cytokines in the liver, which, in turn, modulate liver metabolism and diabetes mellitus. Further studies are required to clarify this relationship.

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