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ORIGINAL ARTICLE

NO production in RAW264 cells stimulated with *Porphyromonas gingivalis* extracellular vesicles

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OBJECTIVE: This experiment was carried out in order to prove the inducible nitric oxide synthase (iNOS) expression and the nitric oxide (NO) production in mouse macrophage cells (RAW264) which were stimulated by vesicles released from *Porphyromonas gingivalis*, and discussed about the role of vesicles in advance periodontal diseases.

MATERIALS AND METHODS: Production of NO_2^- in RAW264 cells was investigated after 0, 1, 3, 6 and 12 h of stimulation with *P. gingivalis* vesicles. NO was analyzed by HPLC-based flow reactor system with Griess reagent. The cells stained by the enzyme-labeled antibody method, after being stimulated with vesicles for 12 h. The iNOS proteins, which were expressed in RAW264 cells after 12 h of stimulation with vesicles, were detected by western blot.

RESULTS: When stimulated with vesicles from W83 and from ATCC33277, the RAW264 cells produced NO, but cell proteins that came in contact with the vesicles were degraded by protease activities in vesicles. When stimulated with vesicles from gingipain-deficient mutant strain KDP136, the RAW264 cells produced NO, but the quality was about 60%, compared with the vesicles from ATCC33277.

CONCLUSION: The results suggest that vesicles are not only just a part of bacterial component, but also are a toxic complex of lipopolysaccharide and protease, and one of the putative virulence factor for periodontal diseases that continue inflammation and cause chronic conditions.

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Keywords: nitric oxide; iNOS; vesicles; *Porphyromonas gingivalis*

Introduction

Porphyromonas gingivalis is a gram-negative obligately anaerobic rod that produces black pigments. A lot of them are isolated from infected periodontal pockets (Slots *et al*, 1986; Dahlen, 1993; Holt and Ebersole, 2005). *P. gingivalis* is said to cause abscesses or cellulites when inoculated in experimental animals (Holt *et al*, 1988). It is considered to be a major pathogenic bacterium of periodontal disease and has a variety of virulence factors in both cell-associated and secretory forms such as fimbriae, vesicles, lipopolysaccharide (LPS) and capsules (Mayrand and Holt, 1988; Mayrand and Grenier, 1989; Cutler *et al*, 1991; Kawata *et al*, 1994; Reife *et al*, 1995; Holt *et al*, 1999). Being asaccharolytic, which is one of the main characteristics of the bacteria, they degrade proteins in oral cavity into peptide or amino acid as sources of energy and growth. Consequently, they produce various proteases and secrete them outside or on the surface (Holt *et al*, 1988).

Since vesicles are from outer membranes, their composition is similar to that of an outer membrane. They have proteins (showing protease activity), which are cell compounds, as well as LPS. Vesicles produced by batch-cultured *P. gingivalis* vary in size between 50 and 500 nm in diameter, with the majority being of the order of 50 nm (Grenier and Mayrand, 1987; Smalley *et al*, 1993). The release of vesicles depends on the strains and culture conditions. Although the role of vesicles produced by *P. gingivalis* in periodontitis has not yet been fully found out, they are called 'toxin carriers' because of their distinctive component. Having two pathogenic factors – LPS and protease activity, they degrade host proteins in order to obtain nutrition for growth and increase. Consequently, it is highly possible that they attacks a host's immune system and worsen localized infection.

On the other hand, nitric oxide (NO) is a gaseous inorganic radical. It is known to regulate a diverse array of physiological functions such as vascular smooth muscle relaxant action generated in endothelium, and neurotransmission, it acts as a mediator of infection defense and inflammation, and it controls immune reactions, apoptosis induction and carcinogenesis action (Akaike and Maeda, 2000; Ignarro, 2000). Inducible NO synthesis (iNOS) expression comes with infection and inflammation by various pathogens such as bacteria, fungus, virus and protozoa. According to a report,

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effector cells such as macrophage also produced NO for host infection defense (Nussler and Billiar, 1993; Fujisawa *et al*, 1995). In various microbial infections, NO was generated and iNOS expressed, stimulated with LPS, lipoteichoic acid, cytokine, etc., causing diversity of physiological phenomena such as infection defense and pathological conditions within hosts (Zembowicz and Vane, 1992; De Kimpe *et al*, 1995; Nathan, 1997). It is inferred that similar phenomena occur in the inflammation foci of periodontitis.

In this study, we performed experiments on iNOS expession and NO production in murine macrofage cells stimulated with the vesicles that were secreted by *P. gingivalis,* and discussed the role of vesicles in periodontal diseases.

Materials and methods

Bacterial strains and culture conditions

Porphyromonas gingivalis strain W83, ATCC33277 and KDP136 (a mutant of ATCC33277) were used. *P. gingivalis* ATCC33277 and KDP136 were kindly provided by Professor K. Nakayama, Nagasaki University, Japan. The bacterial strains were grown in tryptic soy broth (Difco Laboratories, Detroit, MI, USA) supplemented with 0.5 g L-cysteine (Wako Pure Chemical Industries, Ltd, Osaka, Japan), 5 mg hemin (Sigma-Aldrich Co., St Louis, MO, USA), 1 mg menadione (Wako) and 5 g yeast extract (Difco) per liter and cultured in an anaerobic cabinet (10% CO_2, 10% H_2, 80% N_2) at 37°C for 72 h.

Preparation of vesicles

Vesicles were prepared by the method of Grenier and Mayrand (Grenier and Mayrand, 1987). Two liters of bacterial cell cultures were removed by centrifugation (10 000 *g* at 4°C for 15 min). Then 264 g of ammonium sulfate (Wako Pure Chemical Industries, Ltd) was added to the culture supernatant (40% saturation) by stirring gently. After being left in ice overnight, the culture supernatant was centrifuged at 20 000 *g* for 40 min, the precipitate obtained was suspended in 30 ml of 50 mM Tris–HCl buffer, pH 9.5. The suspension was dialyzed against 6 liters of the same buffer at 4°C for 16 h. The vesicles were then collected by centrifugation (27 000 *g* at 4°C for 40 min) and the precipitant was resuspended in 9.6 mM Dulbecco's PBS(–), pH 7.3 (Nissui, Tokyo, Japan) and washed twice by centrifugation in the same condition. The vesicles obtained were suspended in PBS(–) and kept at −80°C until used. The heated vesicles were heated at 100°C for 10 min.

Cell culture and NO_2^- quantity

The cell culture conditions and NO_2^- detection were according to the method of Akaike *et al* (1997). RAW264 cells, a murine macrophage cell line, kindly provided by Professor T. Akaike, Kumamoto University, Japan. The cells were cultured in 24-well plates (Falcon, Lincoln Park, NJ, USA) with Dulbecco's minimal essential medium (DMEM) (Gibco-BRL, Gaithersburg, MD, USA). They were supplemented with 10% fetal bovine serum (FBS) and nonessential amino acids (NEAA) (Gibco) at 37°C in a 5% CO_2 incubator (5% CO_2/95% air, v/v). The cells at saturation density (1×10^6 cells per well) were stimulated with 50 μg per well vesicles, 100 U ml^{-1} (10 U per well) recombinant mouse IFN-γ (Genzyme, Cambridge, MA, USA) and 10 μg ml^{-1} (5 μg per well) and LPS (*E. coli* 026: B6) (Difco) for 0–12 h at 37°C in the CO_2 incubator. The culture medium was removed and the cells were washed one time with 1 ml Krebs–Ringer–phosphate buffer (KRP), pH 7.4. The culture plates were further incubated with 200 μl of KRP containing 1 mM L-arginine and 0.2% BSA (Sigma) at 37°C for 1 h in the incubator. The supernatants were centrifuged (12 000 *g* at 4°C for 10 min) and 10 μl of each aliquot was analyzed for NO_x (NO_2^- + NO_3^-) by using a high-performance liquid chromatography-based flow reactor with Griess reagent (NO_x ANALYZER ENO-20) (Eicom, Kyoto, Japan).

Enzyme-labeled antibody method

The cells were washed with PBS(–) after incubated and were fixed with 2% paraformaldehyde at 4°C for 60 min, and then washed with PBS(–) for 5 min three times at 4°C. The expression of iNOS proteins in RAW264 cells were confirmed by enzyme-labeled antibody method used by the Ultra Tek Polyvalent- HRP-DAB (Scy Tek Laboratories, Logan, UT, USA), according to the manufacture's protocol.

Western blot

Sample preparation. The cells were washed by 1 ml PBS(–) after being incubated and then 100 μl M-PER^{TM} Mammalian Protein Extraction Reagent (Pierce Chemical Co., Rockford, IL, USA) was added to each plate well. The cell lysates were collected and centrifugated at 6000 *g* for 10 min to pellets the cell debris. The supernatants were transferred to a new clean tube for the analysis.

Electrophoresis. SDS-PAGE was performed in 7.5% gel, according to the methods of Laemmli (1970). The proteins were transferred from the gel to PVDF membrane using an electroblotting apparatus under the condition of the manufacture's protocol. The PVDF membrane was dried up overnight at 37°C.

Immunoblotting. The PVDF membrane was dipped in 100% methanol and washed with the washing buffer and incubated for 2 h with the blocking buffer containing 5% skim milk (Difco) and 2% BSA. After the blocking, the PVDF membrane was incubated at 4°C overnight with the blocking buffer containing 1st antibody (NOS2: C-11), (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Then the membrane was washed with the washing buffer four times for 10 min at room temperature. After washing, the PVDF membrane was incubated with the blocking buffer containing the 2nd antibody (anti-mouse IgG-HRP) (Santa Cruz Biotechnology) for 2 h at room temperature. The membrane was washed with the washing buffer four times for 10 min at room temperature.

Detection. The iNOS proteins were detected with the ECL Plus Western Blotting Detection System (Amersham Pharmacia Biotech) using X-ray film according to the manufacture's protocol.

Determination of proteolytic activity of vesicles
Proteolytic activity in the vesicles was measured according to the methods of Kadowaki *et al* (1994), using a synthetic substrate (final conc. 10 μM), carbobenzoxy-L-phenylalanyl-L-arginine 4-methyl-7-coumarylamide (Z-Phe-Arg-MCA), in 20 mM Tris–HCl buffer, pH 7.5, containing 5 mM $CaCl_2$ and 5 mM cysteine in a total volume of 1 ml. After incubation at 37°C for 15 min, the reaction was terminated by adding 500 ml of 17% acetic acid, and the released 7-amino-4-methylcoumarin was measured at 460 nm (excitation at 380 nm).

Statistical analysis
The data were expressed by the method of Bonferroni.

Results

Production of NO_2^- from RAW264 cells stimulated with vesicles and heated vesicles
Production of NO_2^- from RAW264 cells was investigated after 0, 1, 3, 6 and 12 h of stimulation with IFN-γ or LPS/IFN-γ: as a positive control (Figure 1a). In the cells stimulated with LPS/IFN-γ, the production of NO_2^- peaked after 6 h of incubation.

RAW264 cells stimulated with *P. gingivalis* W83 vesicles or vesicles/IFN-γNO_2^- was produced about 5 pmol after 12 h in vesicles-stimulated cells (Figure 1b). When stimulated with vesicles/IFN-γ, production reached its maximum at the 6 h mark at about 20 pmol, showing a quick increase. For another, RAW264 cells stimulated with *P. gingivalis* W83 heated vesicles or heated vesicles/IFN-γ (Figure 1c). There was little difference of NO_2^- production in stimulated cells between vesicles and heated vesicles. However, in those stimulated with heated vesicles/IFN-γ, the NO_2^- production did not peak in 6 h, indicating a slower increase by about 18 pmol in 12 h, compared with vesicles/IFN-γ.

The iNOS gene expression was observed continuously in RAW264 cells stimulated with vesicles or heated vesicles starting from 1 h after stimulation (data not shown).

Detection of iNOS protein in RAW264 cells stimulated with vesicles and heated vesicles
iNOS proteins in RAW264 cells after 12 h of stimulation were detected by enzyme-labeled antibody method.

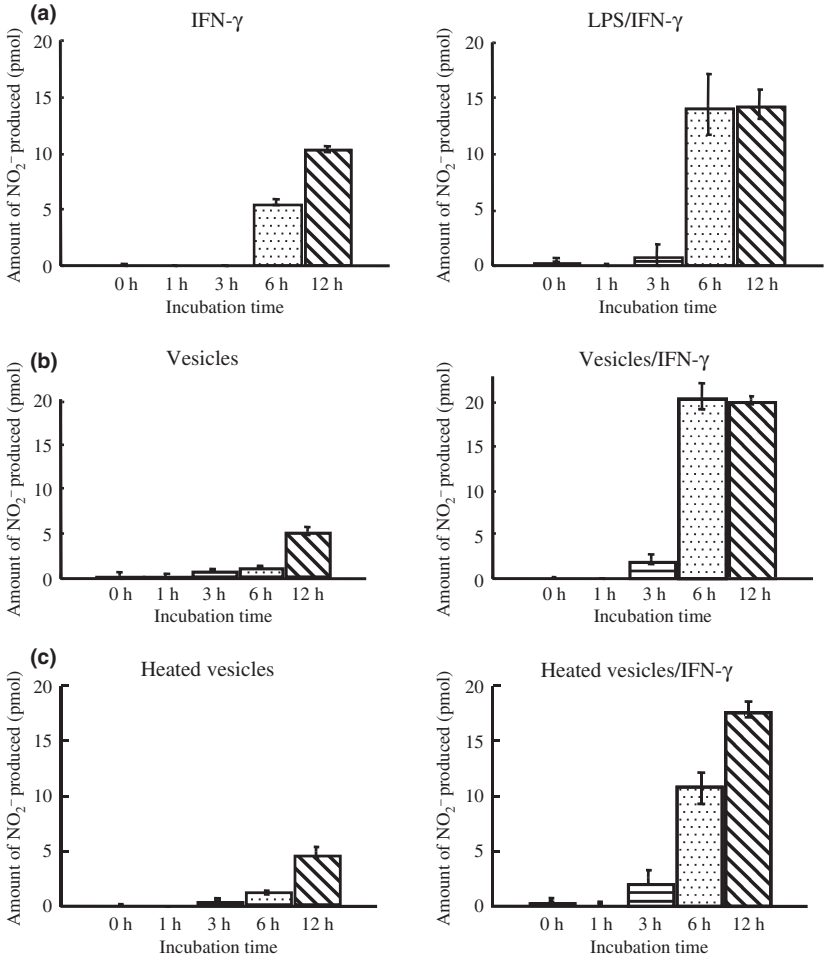

Figure 1 NO_2^- production from RAW264 cells were induced by IFN-γ (left), LPS/ IFN-γ (right). The cells at a density of 1×10^6 cells per well were incubated with IFN-γ, LPS/-IFN-γ. NO_2^- production from the stimulated cells was examined 0, 1, 3, 6 and 12 h stimulation, respectively. The supernatant of the reaction mixture was then subjected to the HPLC flow reactor analysis. The amount of NO in the culture supernatant was measured to assess NO_2^- production. NO_2^- production from RAW264 cells stimulated with *P. gingivalis* W83 vesicles (left), *P. gingivalis* W83 vesicles/IFN-γ (right) was showed at Fig. 1b. The cells at a density of 1×10^6 cells per well were incubated with 50 μg vesicles, 50 μg vesicles/IFN-γ. NO_2^- production from the stimulated cells was examined 0, 1, 3, 6 and 12 h stimulation, respectively. (**a**) NO_2^- production from RAW264 cells stimulated with IFN-γ (left), LPS/IFN-γ (right). (**b**) NO_2^- production from RAW264 cells stimulated with vesicles (left), vesicles/IFN-γ (right). (**c**) NO_2^- production from RAW264 cells stimulated with heated vesicles (left), heated vesicles/IFN-γ (right)

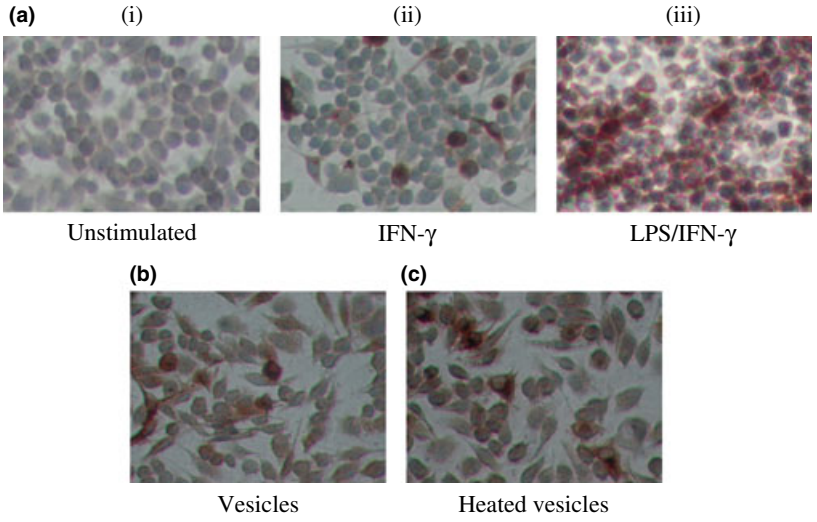

Figure 2 The expression of iNOS protein in RAW264 cells was detected by enzyme-labeled antibody method using an iNOS mouse monoclonal IgG (1:200 dilution). The cells were stimulated with vesicles, heated vesicles for 12 h, and bound antibody was detected with HRP-DAB. The heated vesicles were heat-treated at 100°C for 10 min. (**a**) (i) unstimulated cells (control), (ii) The cells were stimulated with IFN-γ, (iii) The cells were stimulated with LPS and IFN-γ. (**b**) The cells were stimulated with *P. gingivalis* W83 vesicles. (**c**) The cells were stimulated with *P. gingivalis* W83 heated vesicles

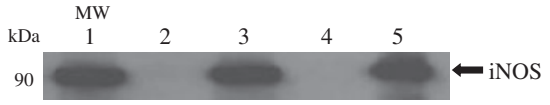

Figure 3 Western blot analysis for expression of iNOS protein in RAW264 cells stimulated with *P. gingivalis* W83 vesicles/IFN-γ, *P. gingivalis* W83 heated vesicles/IFN-γ for 12 h. Lane 1, Molecular weight marker; Lane 2, unstimulated (control); Lane 3, LPS/IFN-γ (positive control) and Lane 4, vesicles/IFN-γ, Lane5: heated vesicles/IFN-γ

More cells stimulated with LPS/IFN-γ were pigmentized than those stimulated with only IFN-γ (Figure 2a). The cells stimulated with *P. gingivalis* W83 vesicles were stained (Figure 2b). iNOS protein in the cells stimulated with *P. gingivalis* W83 heated vesicles were detected as well as stimulated with vesicles (Figure 2c).

Western blot of iNOS protein in RAW264 cells stimulated with vesicles and heated vesicles

The iNOS proteins, which were expressed in RAW264 cells stimulated with *P. gingivalis* W83 vesicles or heated vesicles for 12 h, were also detected by western blot (Figure 3). A band of iNOS protein was found in the cells stimulated with heated vesicles, but none were detected in vesicles. Although iNOS proteins were found by the enzyme-labeled antibody method, iNOS protein could not been detected by the western blot method despite the fact that NO_2^- was produced and the iNOS gene was expressed there. Considering that protease activity might have disappeared when the vesicles were heated and that the cells were affected somehow by the vesicles where protease activity still remained, the following experiments were performed in order to determine these facts.

Total proteins, LPS and protease activity of ATCC33277-vesicles and KDP136-vesicles

In order to investigate the above, experiments were conducted by using a mutant strain KDP136, which has deficient trypsin-like cysteine protease (gingipain) activity generated by *P. gingivalis* ATCC33277. Vesicles were collected from ATCC33277 and KDP136 respectively in the same method in order to measure and compare total proteins, LPS and protease activity mg^{-1} protein (Table 1). Total proteins mg^{-1} vesicles, LPS and protease activity mg^{-1} ATCC33277-vesicles were about 1.5, 1.6 and 5 times (in AMC release per minute) as much as that of KDP136 respectively.

Table 1 Comparative property of total proteins, LPS and protease activity of ATCC33277-vesicles and KDP136-vesicles were showed at Table 1. *P.gingivalis* ATCC33277 or KDP136 were cultured in anaerobic condition respectively, and vesicles were prepared from culture supernatant. Total protein of vesicles was collected from 1 l of culture supernatant. LPS mg^{-1} protein (EU/mg protein) was determined by Endospecy (Biochemicals Co.). The protease activity was determined by Z-Phe-Arg-MCA as a substrate

vesicles	*mg protein (/L)*	*LPS EU (EU/mg protein)*	*protease activity (nM AMC/mg protein/min)*
ATCC 33277 (wild type)	107	1.32	0.88
KDP136 (mutant)	72.6	0.81	0.18

Generation of NO_2^- from RAW264 cells stimulated with ATCC33277-vesicles and KDP136-vesicles

The same LPS volume, 0.3 EU/10^6 cells, was added to the above vesicles respectively. The total protein of ATCC33277-vesicles was smaller than KDP136-vesicles by about 40%. The amount of NO_2^- production after being stimulated with ATCC33277-vesicles for 12 h was about 1.7 times as much as KDP136-vesicles (Figure 4).

Detection of iNOS protein in RAW264 cells stimulated with ATCC33277-vesicles and KDP136-vesicles

The RAW264 cells stained by the enzyme-labeled antibody method, after being stimulated with ATCC33277-vesicles and KDP136-vesicles respectively for 12 h, are shown at Figure 5. There was a difference in the number of cells expressing iNOS positive cells, with ATCC33277-vesicles at 19.8% and KDP136-vesicles at 10.6%.

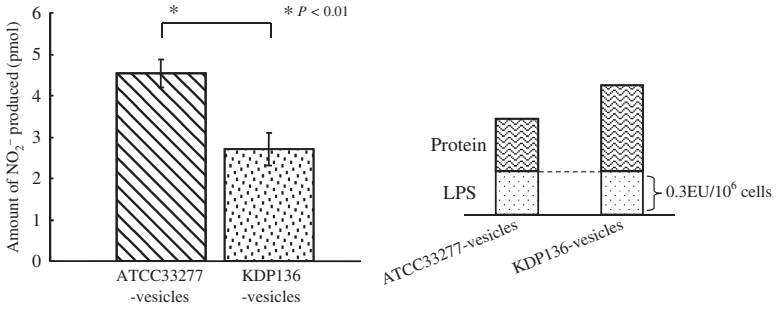

Figure 4 NO_2^- production from RAW264 cells stimulated with ATCC33277-vesicles and KDP136-vesicles. The cells were stimulated with ATCC33277-vesicles, KDP136-vesicles, and NO_2^- production was followed for 12 h (left). Same unit of LPS (0.3 EU/10^6 cells) of vesicles were added to the cells (right)

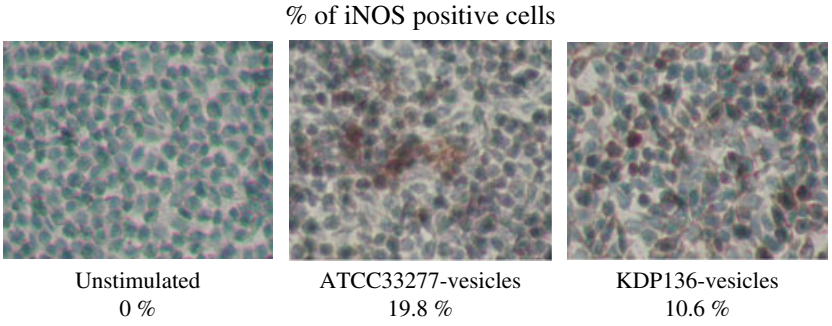

Figure 5 The expression of iNOS protein in RAW264 cells was detected by enzyme-labeled antibody method. The RAW264 cells were stimulated with ATCC33277-vesicles, KDP136-vesicles for 12 h. iNOS positive cells were counted. Details are the same in Figure 2. Unstimulated cells (control): 0%-(left). The cells stimulated with ATCC33277-vesicles: 19.8% (center). The cells stimulated with KDP136 -vesicles: 10.6% (right)

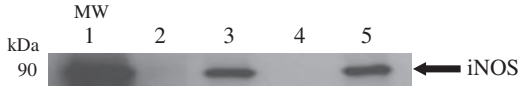

Figure 6 Western blot analysis for expression of iNOS protein in RAW264 cells stimulated with ATCC33277-vesicles/IFN-γ, KDP136-vesicles/IFN-γ for 12 h. Lane 1, Molecular weight marker; Lane 2, unstimulated (control); Lane 3, LPS/IFN-γ (positive control); Lane 4, ATCC33277-vesicles/IFN-γ; Lane 5, KDP136-vesicles/IFN-γ

Western blot of iNOS protein in RAW264 cells stimulated with ATCC33277-vesicles and KDP136-vesicles

The expression of iNOS protein in RAW264 cells, after being stimulated with ATCC33277-vesicles and KDP136-vesicles for 12 h with the same LPS volume (0.3 EU/10^6 cells), was detected by western blot (Figure 6). The iNOS protein was evident in the cells stimulated with KDP136-vesicles, whereas the band was not detected in from ATCC3327-vesicles as W83-vesicles.

Discussion

In this study, it is shown that mouse macrophage RAW264 cells induce iNOS and product NO when the cells were stimulated with *P. gingivalis*-derived vesicles, a periodontal pathogen. When NO_2^- production was compared between *P. gingivalis* W83 vesicles/IFN-γ and *P. gingivalis* W83 heated vesicles/IFN-γ, a difference was found 6 hrs after the start of stimulation. It was considered that the difference in production quantity was related to the protease component that lost its activity when vesicles were heat-treated. Considering that vesicle is a mixture of LPS and cell protein (protease), as in many other reports, it is also evident from our study that the production of NO_2^- is further improved due to the coexistence of LPS and IFN-γ. It is possible that even in clinical cases gingival inflammation would be aggravated by vesicles which were released from bacterium, stimulating macrophage in an early stage and accelerating the NO production, together with various immune cells producting IFN-γ.

Being asaccharolytic, *P. gingivalis* degrades proteins in oral cavity into peptide or amino acid as sources of energy and growth. Therefore, trypsin-like proteinase produced by the bacteria is an important factor for its growth and increase, and it is considered to cause various periodontal diseases, initiating protein degradation and damage to host cells (Grenier and Mayrand, 1993; Pike *et al*, 1994; Baba *et al*, 2001, 2002). Kaminishi *et al* (1993) explained that the protease secreted by *P. gingivalis* expresses inflammation in localized portions and seriously damages peripheral blood circulation, acting as a pathogenic factor (Hirota, 1998).

Protease is produced by bacteria after the start and progress of an infection, and is considered a pathogenic agent to cause direct damage to living organisms and worsen disease to a serious state (Maeda, 1995; Travis *et al*, 1995; Akaike *et al*, 2001). Gingipains are the major trypsin-like cysteine proteinases produced by *P. gingivalis* that occupies more than 85% of the total proteinase activity (Abe *et al*, 1998; Kadowaki *et al*, 2000). The enzymes are classified into two, by peptide binding site, as Arg-gingipain (Rgp) and Lys-gingipain (Kgp) (Kadowaki *et al*, 1994; Abe *et al*, 1998). Their structural and various properties, including virulence, have been gradually found out to, (1) degrade an extracellular matrix such as human collagen (types I and IV), fibronectin and laminin (Lantz *et al*, 1991; Kadowaki *et al*, 1994; Abe *et al*, 1998), (2) destroy the human

immunoglobulins (IgG, IgA) and complement factors (C3, C5), degrade and inactivate cytokine (interleukin-6, 8, TNF-α) and damage immunodefense mechanisms and inhibit neutrophil mycophagous activity (Wingrove *et al*, 1992; Calkins *et al*, 1998; Banbula *et al*, 1999).

In our experiment, the expression of iNOS protein was examined by using the enzyme-labeled antibody method and the western blot. The effect of protease activity was detected by the western blot. However, no degradation of the cells by protease was visible by the enzyme-labeled antibody method. Regarding the former, because the cell proteins were collected from the mixed solution of proteins and vesicles, it was considered that protease activity remained in vesicles might have degraded protein. While it is not shown in the results section, SDS-PAGE was conducted on the collected proteins, in which a degradation of cell proteins to lower molecules was observed.

In order to determine if the phenomenon was caused by a protease component, another experiment by western blot was conducted, using KDP136 (*rgpA*, *rgpB*, *kgp*-deficient triple mutant strain) from *P. gingivalis* ATCC33277 (wild-type strain), with Rgp and Kgp deficiency. As a result, the cells were not degraded and a protein band was detected in the mutant strains which did not have any protease activity. Subsequently, it is presumed that even after time passes after the release from bacteria, vesicles still possess protease activity to degrade cell proteins and affect the host cells.

Since vesicles are minute in size with 50 nm, they are able to invade cells. Therefore it is considered that bacteria discharges vesicles like missiles as a method for survival and to create an environment for growth and increase. Furthermore, when vesicles remain in the periodontal pocket, NO will be produced through iNOS induction by host cells such as macrophage, involving complicated physiological responses in the host. This suggests that vesicles are a toxin-enzyme complex having endotoxin LPS and protease activity, and affect host cells harmfully and continuously for a long time. It is inferred that a large quantity of NO is also produced by iNOS induction in the inflammation foci of peridontitis as a host's physiological reaction, causing diversity of physiological phenomena.

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