Induction of inflammatory cytokine and cyclooxygenase-2 mRNA expression by secreted substances from oral streptococci

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[Received : October 16, 1995 ; Accepted : November 6, 1995]

Abstract : Induction of inflammatory cytokine and cyclooxygenase (COX)-2 mRNA expression in human peripheral blood mononuclear cells (PBMC) was studied with the culture supernatants of eight species of oral streptococci using reverse transcription-polymerase chain reaction. The mRNA levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in PBMC were increased after incubation with the culture supernatants of *Streptococcus anginosus* and *S.constellatus*. The level of IL-1β mRNA was increased significantly with the culture supernatants of *S.anginosus*, *S.constellatus* and *S.gordonii*. *S.anginosus* culture supernatant also demonstrated the induction of COX-2 mRNA expression and prostagrandin E_2 production in PBMC culture medium. These results suggest that *S.anginosus* and *S.constellatus* produce bioactive substances inducing the mRNA expression of inflammatory cytokines and COX-2 in immunocytes.

Key words : oral streptococci, bioactive substance, inflammatory cytokine, cyclooxygenase-2

Introduction

Cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, and prostagrandins (PGs) play an important role in inflammation. TNF-α induces the resorption of bone, and has been implicated in the destruction of connective tissues[1]. IL-1 is a very potent multifunctional cytokine that appears to be a central regulator of inflammatory and immune responses[2]. IL-6 plays a major role in the terminal differentiation of B cells to plasma cells, which are the predominant inflammatory cells in tissues[3]. Cyclooxygenase (COX)-2 is an inducible type of enzyme, which synthesizes PGs from arachidonic acid, and has a unique active profile[4]. These locally produced cytokines or PGs are believed to be responsible for the bone loss and connective tissue breakdown which occur in periodontitis[5]. These cytokines or PGs are secreted from immunocytes, fibroblasts and endotherial cells according to the inflammatory response induced by antigens, lipopolysaccharide, exotoxins or mitogens.

Oral streptococci are seen in normal flora of oral cavity and these bacteria have been regarded as little clinical signficance. However, some oral streptococci have been isolated from clinical sources. For example, *Streptococcus anginosus* have been isolated frequently from dental abscess[6], subgingival plaque[7] and subgingival

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samples of advanced periodontitis[8]. *S. constellatus* have been also isolated from subgingival plaque frequently. The pathological agents of these bacteria, however, have not been found clearly.

In this study, in order to detect bioactive substances from oral streptococci, we tested the induction of inflammatory cytokine and COX-2 mRNA expressions, and PGE_2 production of human peripheral blood mononuclear cells (PBMC) by culture supernatant of oral streptococci, and found that *S.anginosus* and *S.constellatus* can produce bioactive substances inducing the mRNA expression of inflammatory mediator of immunocytes.

Materials and methods

Bacterial strains

S.anginosus NCTC 8787, *S.intermedius* GAI 1157, *S.constellatus* ATCC 27823, *S.gordonii* ATCC 10558, *S.sanguis* ATCC 10556, *S.mitis* ATCC 9811, *S.salivarius* ATCC 9756 and *S. mutans* ATCC 25175 were used.

Bacterial culture and preparation of culture supernatants

Bacteria were cultured by the gas pack method in Todd Hewitt broth (BBL Microbiology Systems, Cockeysvill, MD., USA) at 37℃ for 24 h. After cultivation, the culture was centrifuged at 3,000 *g*, for 20 min, and the resultant supernatant was filterated with a membrane filter (0.45 μm). The bacterial growth was monitored by measurement of absorbance at 600 nm, and protein concentration of the filtrate was measured by BioRad protein assay kit (BioRad Laboratories, Richmond, CA., USA).

Preparation of PBMC

PBMC were isolated from heparinized peripheral blood of healthy donors by Ficoll Hypaque density gradient centrifugation. PBMC (2×10^6/ml) were suspended in RPMI 1640 medium (Nissui Corporation, Tokyo, Japan) suplemented with 2 mM L-glutamine, 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. PBMC were cultured with an aliquot (0.2 μg/ml) of each culture supernatant of eight species of oral streptococci.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepaired from PBMC (2×10^6 cells) after 4 h stimulation by acid guanidinium thiocyanate-phenol-chloroform method[9]. One μg of total RNA with 2.5 μM of oligo $(dT)_{8\text{-}12}$ (Pharmacia Biotech, Uppsala, Sweden) was heated at 70℃ for 10 min, followed by quick chilling, and then cDNA was synthesized in 1×RT buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl and 15 mM $MgCl_2$], supplemented with 10 mM dithiothreitol, 0.63 mM dNTP, 1 U of RNase inhibitor (Promega Corporation, Madison, USA) and 2.5 U of RTase (Gibco-BRL, Gaithersburg, USA) at 37℃ for 1 h. Finally, the mixture was heated at 100℃ for 5 min, followed by quick chilling. PCR was performed with an appropriate amount of the RT-products in 1×PCR buffer [100 mM Tris-HCl (pH 9.0), 500 mM KCl and 15 mM $MgCl_2$] containing 0.13 mM dNTP, 1 U of Taq DNA polymerase (Pharmacia) and 0.75 μM of each sense and antisense primer. PCR was carried out under the following amplification conditions ; 94℃ for 1 min, and 55℃ for 1 min. The reaction cycles of β-actin, TNF-α, IL-1β, IL-6 and COX-2 were 23, 23, 19, 19 and 23 respectively, to observe each quantifiable signal within the linear range of the amplification. The sequences of sense and antisense primers used are shown

Table 1. Sequence of oligonucleotide primers used for PCR.

mRNA		Sense and antisense primers
β-actin	:	5'-TGC TGG GCC GCT CTA GGC AC-3'
		5'-TGG CCT TAG GGT TCA GGG GG-3'
TNF-α	:	5'-GAG TGA CAA GCC TGT AGC CCA TGT TGT AGC A-3'
		5'-GCA ATG ATC CCA AAG TAG ACC TGC CCA GAC T-3'
IL-1β	:	5'-GAC CTG GAC CTC TGC CCT CTG-3'
		5'-AGG TAT TTT GTC ATT ACT TTC-3'
IL-6	:	5'-ATG AAC TCC TTC TCC ACA AGC G-3'
		5'-CTG GAC TGC AGG AAC TCC TT-3'
COX-2	:	5'-GTA CCC GGA CAG GAT TCT ATG G-3'
		5'-TCT GTC TTG AAA AAC TGA TGC GTG-3'

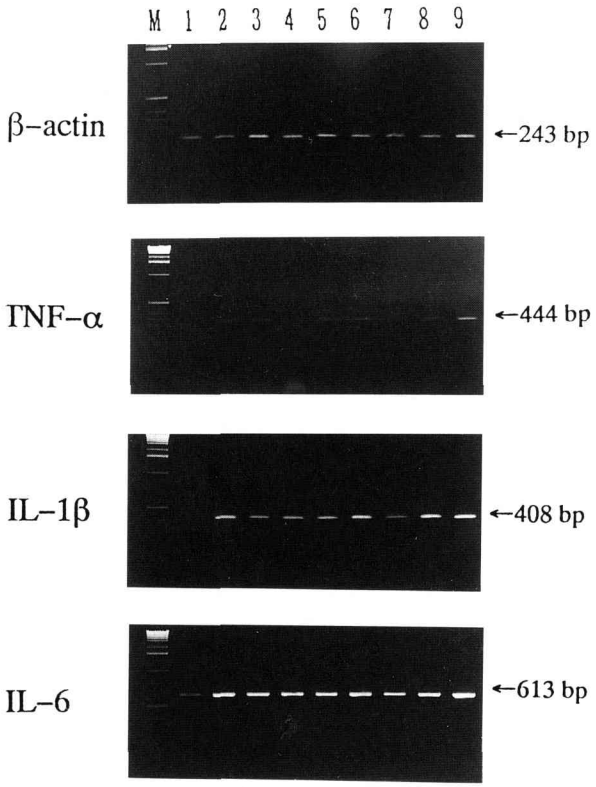

Fig.1. TNF-α, IL-1β and IL-6 mRNA expressions in human PBMC. Human PBMC were incubated with Todd Hewitt broth or with 0.2μg/ml of culture supernatant of oral streptococci at 37℃ for 4 h. Messenger RNA levels of cytokines were analysed by RT-PCR. Aliquots (10μl) of PCR products were run on 1.8 agarose gel containing ethidium bromide for qualitative comparision. Gel lanes indicate M; Maker (1 kb radder, Gibco-BRL), 1 ; Todd Hewitt broth, 2 ; *S.anginosus*, 3 ; *S.intermedius*, 4 ; *S.mutans*, 5 ; *S.mitis*, 6 ; *S.salivarius*, 7 ; *S.sanguis*, 8 ; *S.gordonii* and 9 ; *S.constellatus*.

Table 2. Relative amount of RT-PCR products of TNF-α, IL-1β and IL-6 mRNA in PBMC cultured with oral streptococcal culture supernatant.

Species	Relative amount of PCR products		
	TNF-α	IL-1β	IL-6
control	1.0	1.0	1.0
S. anginosus	5.1	6.0	5.1
S. intermedius	2.5	2.6	2.4
S. mutans	1.8	2.8	2.2
S. mitis	1.1	2.4	2.6
S. salivarius	2.0	2.9	2.7
S. sanguis	1.1	2.2	2.4
S. gordonii	1.3	4.3	2.8
S. constellatus	3.3	4.5	4.3

The amount of PCR products (Fig.1) were measured with a LKB 2202 Ultroscan Laser densitometer. Relative amounts were indicated sample PCR products standardized by β-actin PCR products.

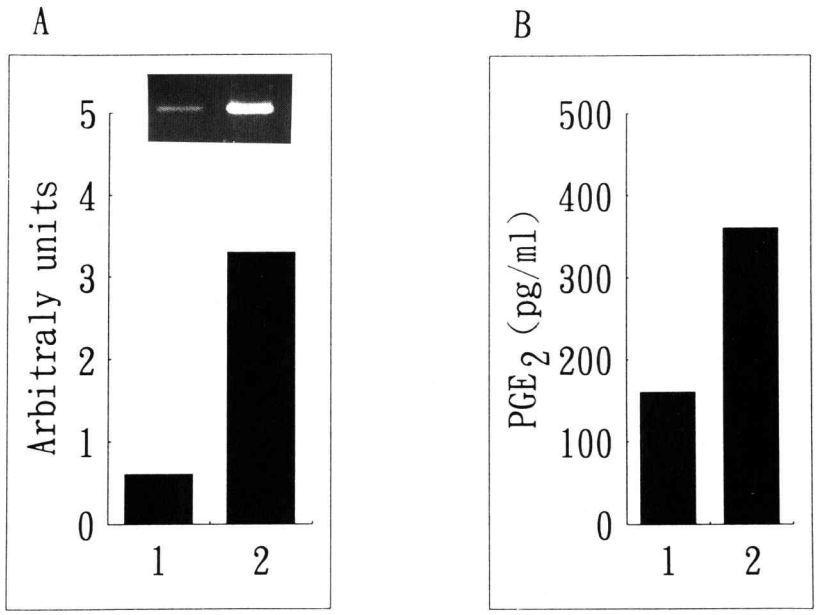

Fig.2. COX-2 mRNA expression and PGE_2 production in PBMC stimulated with the culture supernatant of *S.anginosus*. A) PBMC were incubated with Todd Hewitt broth (1) or the culture supernatant of *S.anginosus* (2) for 4 h, and then total RNA was prepaired. mRNA levels were analysed by RT-PCR.
B) PBMC were incubated with Todd Hewitt broth (1) or the culture supernatant of *S.anginosus* (2) for 24 h, and then aliquots of the medium from the PBMC were assayed for PGE_2 by ELISA.

in Table 1. Each sample of 10 μl was analysed on 1.8% agarose gels containing 0.5 μg/ml ethidium bromide in 0.5× Tris-borate-EDTA buffer (pH 8.2). The products were processed by Polaroid 665 films and band intensity was measured by a

2202 Ultroscan Laser Densitometer (Pharmacia LKB Biotechnology, Uppsala, Sweden).

PGE_2 determination

PBMC were stimulated with *S.anginosus* culture supernatant for 24 h. PGE_2 in the culture medium was measured using an ELISA kit (Cayman Chemical Campany, MI., USA)

Results

Effects of culture supernatant of oral streptococci on the TNF-α, IL-1β and IL-6 mRNA expression in PBMC

Eight species of oral streptococci were cultured in Todd Hewitt broth and the culture supernatants were obtained from the cultures at logarithmic growth phase. PBMC were incubated with 0.2 μg/ml of the culture supernatants. After 4 h, the steady state levels of mRNA of TNF-α, IL-1β and IL-6 in PBMC were semiquantitated by RT-PCR analysis. As shown in Fig.1, under unstimulated conditions, PCR products of TNF-α, IL-β and IL-6 were extremely low. However, incubation with culture supernatant from *S.anginosus* increased 5-fold of TNF-α and IL-6 expression, and 6-fold of IL-1β mRNA expression. These amounts were the highest in each product. Messenger RNA expressions were increased 3-fold in TNF-α, 4.5-fold in IL-1β, and 4.3-fold in IL-6 by *S. constellatus* culture supernatant (Table 2). Addition of the culture supernatant from *S. gordonii* also increased 4.3-fold in IL-1β mRNA expression, whereas it had almost no effect on the expression of TNF-α. The culture supernatants from *S.intermedius, S. mutans, S.mitis, S.salivarius* and *S.sanguis* were less effective. About two to three-fold moderate increase in IL-1β and IL-6 message was observed. These results indicate that oral streptococci, especially *S. anginosus* and *S.constellatus,* may produce soluble substances stimulating expression inflammatory cytokines, TNF-α, IL-1β and IL-6, in human PBMC.

Effect of *S.anginosus* culture supernatant on the COX-2 mRNA expression and PGE_2 production of PBMC

The COX-2 mRNA expression in PBMC treated with *S. anginosus* culture supernatant, which was the most inducible of 8 species oral streptococcal culture supernatants for inflammatory cytokines mRNA expressions, was induced significantly about 5.5 fold (Fig.2A). And the amounts of PGE_2 in PBMC culture medium treated with Todd Hewitt broth and *S. anginosus* culture supernatant were 160 pg/ml and 360 pg/ml, respectively (Fig.2B).

Discussion

In this study, we examined the activity of the culture supernatant of oral streptococci to stimulate the expression of inflammatory cytokine mRNA, and the induction of the COX-2 mRNA expression in human PBMC and PGE_2 production in the culture medium by *S.anginosus* culture supernatant. We observed the induction of TNF-α and IL-6 mRNA expressions in PBMC with *S. anginosus* and *S. constellatus* culture supernatants, and the expression of the IL-1β mRNA in PBMC was increased by *S. anginosus, S.constellatus* and *S.gordonii* culture supernatants. Furthermore, *S. anginosus* culture supernatant induced COX-2 mRNA expression in PBMC, and PGE_2 production in PBMC culture medium. The results suggest that bioactive substances, which can induce inflammatory cytokines

and COX-2 mRNA expression in PBMC, will be produced from *S.anginosus* and *S. constellatus*.

We observed that *S. anginosus*, *S. constellatus* and *S.gordonii* were isolated frequently from subgingival samples of periodontitis (unpublished data). *S. anginosus* and *S.constellatus* may produce inflammatory bioactive substances around gingivitis, whereas the substances of *S. anginosus* and *S.constellatus* might be associate with periodontitis or dental abscess. It was also reported that these organisms were isolated from subgingival plaque of advanced periodontitis[8] or dental abscess[6]. Immunoncytes such as T cell, B cell and macrophage are present in the tissue of gingiva. Many products, such as cytokines and lipid mediators, which have been implicates bone resorption[10], are released by bioactive substances from these stimulated immunocytes. Recently, some researchers reported that oral streptococci produced bioactive substances[11-13]. They suggested that these substances will cause some inflammatory diseases. We, here, speculate two roles of *S.anginosus* and *S.constellatus* bioactive substances as follows : the substances may affect immunocytes to produce inflammatory cytokines or PGs, resulting in inflammation of gingival tissues indirectly, then the substances may work synergistically with lipopolysaccharide or extracellular products of *Porphylomonas gingivalis*[14] or *Actinobacillus actinomysetemcomitans*[15], which induce inflammatory cytokines secretion from monocytes or fibroblasts. Especially, *S.constellatus*, together with these periodontal bacteria[16], are often isolated from dental pocket. The bacterial bioactive substances of *S.anginosus* and *S.constellatus* might enchance the virulence of these periodontal bacteria. These oral streptococcal bioactive substances may be involved in progression of inflammation in gingival region.

Acknowledgement

This work was supported by grant (No. 05807171 for M. K. and 05771482 for M. S.) from the Ministry of Education and Science of Japan.

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口腔レンサ球菌の産生物質による炎症性サイトカインおよびシクロオキシゲナーゼ－2 mRNA 発現の誘導

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抄　　録：口腔レンサ球菌（8菌種）の培養上清によるヒト末梢血単核球（PBMC）中の炎症性サイトカイン，およびシクロオキシゲナーゼ（COX）-2 mRNA 発現の誘導について，reverse transcription-polymerase chain reaction を用いて検討した。腫瘍壊死因子（TNF）-αとインターロイキン（IL）-6 mRNA は，*Streptococcus anginosus, S. constellatus* 培養上清により上昇した。IL-1β mRNA は，*S. anginosus, S. constellatus* および *S. gordonii* 培養上清により顕著に上昇した。*S. anginosus* 培養上清は，PBMC 中の COX-2 mRNA の誘導と培養上清中へのプロスタグランジン E_2 の産生を示した。これらのことは，*S. anginosus* および *S. constellatus* は免疫細胞中に炎症性サイトカインや COX-2 mRNA 発現を誘導する活性物質を産生することを示唆する。