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Acute phase protein induction by experimental inflammation in the salivary gland*

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BACKGROUND: The submandibular gland (SMG) is a major salivary gland, which plays an important role in maintenance the oral health. In this study, we intended to explore the role of the SMG's defense system of the animals in which experimental inflammation is induced.
METHODS: The levels of mRNAs for inflammation cytokines and acute phase proteins were detected by reverse transcriptase-polymerase chain reaction (RT-PCR).
RESULTS: The mRNAs for acute phase proteins were found to be increased in the SMG and extraorbital and intraorbital lacrimal gland (ELG and ILG) of rats at 24 h after subcutaneous injection of turpentine oil. The induction of mRNA for these inflammatory proteins by turpentine oil was preceded by a transient increase in the level of mRNAs for IL-1β, IL-6 and TNF-α at 6 h after subcutaneous injection of the oil. Such cytokine induction was similarly seen by lipopolysaccharide (LPS) injection, and involvement of Toll-like receptor 4 (TLR4) was strongly suggested from experiment using C3H/HeJ mice, a TLR4-deficient mutant strain.
CONCLUSION: The up-regulation of acute phase proteins and inflammation cytokines in the SMG, ELG and ILG by experimental inflammation suggests the existence of a strict defense system via the innate immune system in the SMG and other exocrine gland.
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Introduction

The salivary gland diseases are classified into many different kinds; these are salivary gland abnormal development, regressive change and metaplasia (including atrophy, degeneration, and metaplasia), abnormal secretion, sialolithiasis (ptyalolithiasis) mucous cyst, salivary gland hypertrophy, and sialadenitis. The sialadenitis is especially very common and classified further into acute and chronic non-specific inflammations, specific inflammation, viral inflammation, and sialadenitis by abnormal immunity; all of these diseases are accompanied with inflammation. It is generally known that acute phase proteins like C-reactive protein (CRP) increase more than a thousand-fold in severe inflammatory state (1). Induction of CRP expression in hepatocytes requires IL-6, which cytokine triggers the signaling pathway to activate NF-κB, a transcription factor (2, 3). The transcription factor thus activated binds to the promoter region of acute phase protein genes and activates the transcription to produce acute phase protein mRNAs.

On the other hand, the salivary gland is known to express kallikrein strongly and its substrate kininogens very weakly (4). Kininogens are acute phase proteins having a responsive element for IL-6 (5), and they may be related to salivary gland inflammations. In this study, we therefore examined if kininogens and other acute phase proteins, CRP and serum amyloid P-component (SAP), can be increased following the experimental inflammation and if such increment is preceded with increase of inflammation cytokines in the submandibular gland (SMG).

Sialadenitis is known to be caused partly by infection via excretory duct; some of them would be caused by infection of Gram-negative bacteria and others may not. In order to understand pathological or immunological response occurs in the salivary gland and other exocrine gland, we prepared the experimental model by injecting turpentine oil and lipopolysaccharide (LPS) and induced the systemic inflammation to examine the expression of inflammation cytokines as well as acute phase proteins in these tissues. Reverse transcriptase-polymerase chain

Table 1 Sequences of primers used for amplification of mRNAs for inflammatory cytokines, TLR4 and β-actin

mRNAs	*Primer sequences*	*Product size (bp)*
mIL-1β	Sense, 5′-GTTCATCTTTGAAGAAGAGCC-3′ Anti-sense, 5′-CTCTGCAGACTCAAACTCCAC-3′	418
mIL-6	Sense, 5′-CCAAACTGGATATAATCAGGAAAT-3′ Anti-sense, 5′-CTAGGTTTGCCGAGTAGATCTC-3′	337
mTNF-α	Sense, 5′-ATGAGCACAGAAAGCATGATC-3′ Anti-sense, 5′-TCCACTTGGTGGTTTGCTACG-3′	305
rIL-1β	Sense, 5′-CCTTCTTTTCCTTCATCTTTG-3′ Anti-sense, 5′-ACCGCTTTTCCATCTTCTTCT-3′	371
rIL-6	Sense, 5′-CTTGGGACTGATGTTGTTGAC-3′ Anti-sense, 5′-TCTGAATGACTCTGGCTTTGT-3′	389
TLR4	Sense, 5′-AGCAGAGGAGAAAGCATCTATGATGC-3′ Anti-sense, 5′-GGTTTAGGCCCCAGAGTTTTGTTCTCC-3′	524
β-actin	Sense, 5′-ACCCACACTGTGCCCATCTA-3′ Anti-sense, 5′-CGGAACCGCTCATTGCC-3′	289

reaction (RT-PCR) technique was employed especially because this method can detect very low level of mRNA expression and suitable for the analysis of cytokine expression.

Turpentine oil, one of most common agents to induce the inflammation model in animals, has long been used in the field of experimental pharmacology and immunology (6, 7). On the other hand, LPS has recently become known well as this compound induces the innate immune system via Toll-like receptor 4 (TLR4). In the present study therefore, we used turpentine oil and LPS to induce the experimental inflammation in rats and mice and examine the expression of inflammation related proteins by RT-PCR.

Materials and methods

Wistar rats, C3H/HeN and C3H/HeJ mice (all animals were males and 8–10-week old) were used in the present study. All animals were purchased from SLC (Shizuoka, Japan).

In the rat experiment, turpentine oil (5 ml/kg body weight) was injected subcutaneously into the back at the level of the lower thoracic and opera lumber vertebrae whereas LPS [originated from *Escherichia coli*, Serotype 0111B4; Sigma–Aldrich (St. Louis, MO)] was injected intraperitoneally (0.001% in sterile saline (0.9% NaCl); 0.1 μg/kg body weight). Rats were killed at 0, 6 and 24 h after injection.

In the mouse experiment, LPS was dissolved in saline at a concentration of 0.5 μg/ml and injected intraperitoneally at a volume of 1 ml/100 g body weight. Mice were killed at 6 h after injection. The mice of normal controls were injected with saline intraperitoneally and killed at 0 and 6 h after injection.

Total RNA was isolated from the liver, SMG, parotid gland (PG), extraorbital lacrimal gland (ELG), and intraorbital lacrimal gland (ILG) by use of Tri-Reagent™ following the standard procedure described (8, 9). The mRNAs for β-actin, IL-6, IL-1β, TNF-α and H-, L- and T-kininogens, SAP, and CRP were reverse transcribed and cDNAs formed were amplified by using the SuperScript one-step™ RT-PCR system. The RT-PCR amplification was performed by heating at 45°C for 30 min and at 94°C for 2 min, followed by 32–35 PCR cycles, each consisting of denaturation at 94°C for 15 s, primer annealing at 55°C for 30 s, and

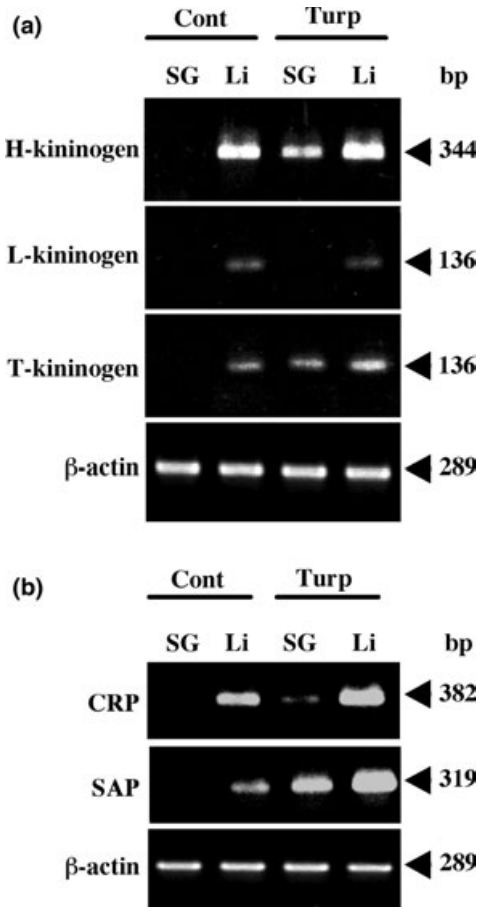

Figure 1 Induction of kininogens and acute phase proteins, CRP and SAP in the submandibular gland and liver of rats injected with turpentine oil. Rats were killed after 24 h, and total RNA from the salivary gland and liver tissues were prepared. The RT-PCR reaction was carried out as described in the text. (a) Kininogens and (b) acute phase proteins. SG, submandibular gland; Li, liver; Cont, control; Turp, turpentine oil.

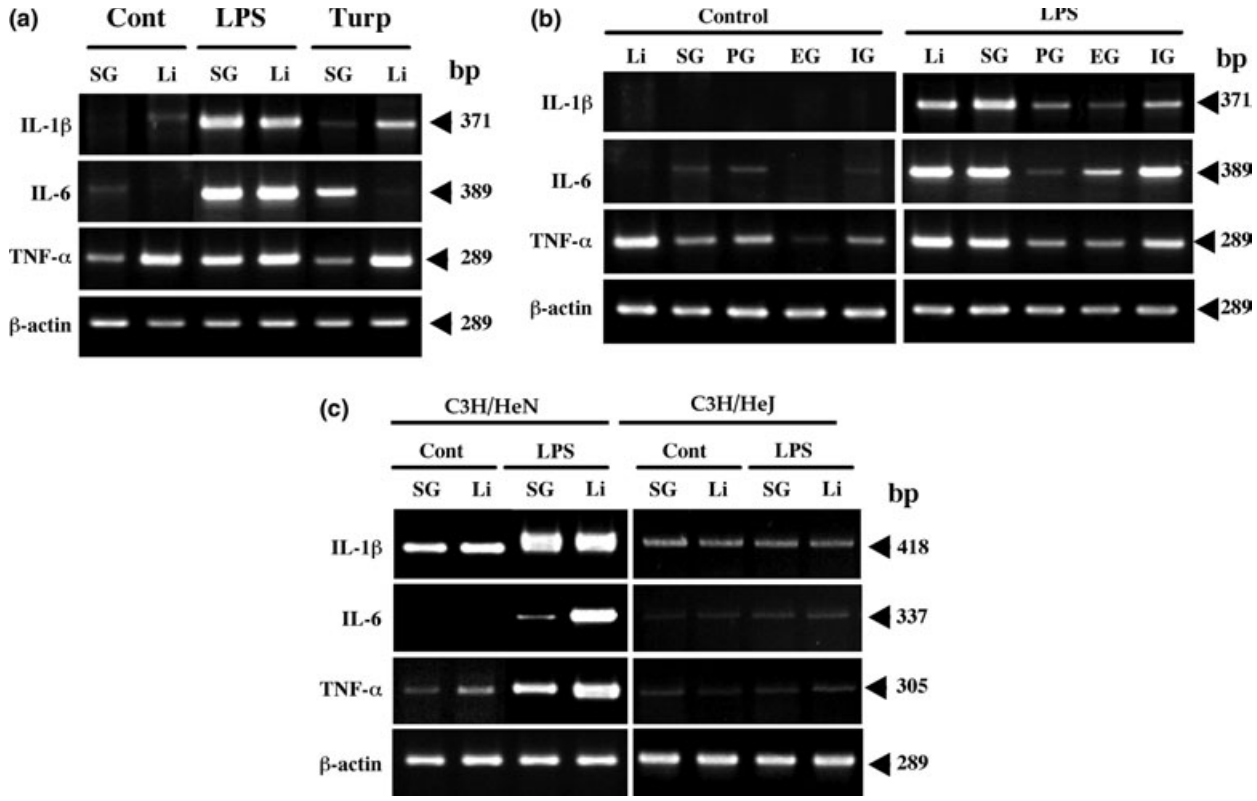

Figure 2 Induction of inflammation cytokines in the exocrine glands of rats and mice. (a) Induction of IL-1β, IL-6, and TNF-α in the submandibular gland and liver of rats at 6 h after injection of turpentine oil and LPS. (b) Induction of IL-1β, IL-6 and TNF-α in the submandibular gland, liver, parotid gland, extraorbital and intraorbital lacrimal gland of rats at 6 h after injection of LPS. (c) Induction of IL-1β, IL-6 and TNF-α in the submandibular gland of C3H/HeN and C3H/HeJ mice at 6 h after injection of LPS. Cont, control; Turp, turpentine oil; SG, submandibular gland; Li, liver; PG, paritid gland; EG, extraorbital lacrimal gland; IG, intraorbital lacrimal gland.

extension at 72°C for 1.5 min. All RT-PCR products were resolved by electrophoresis in 3% agarose gel ($NUSi_{eve}$: $S_{EA}K_{EM} = 3{:}1$) following a standard procedure. The primer sequences for H-, L- and T-kininogens, SAP, CRP, and TNF-α were described previously (8), whereas those sequences for other mRNAs are listed in Table 1.

Results

In control Wistar rats, mRNAs for H-, L- and T-kininogens were barely expressed in the SMG while they were expressed in the liver. By injection of turpentine oil, H- and T-kininogen mRNAs were induced in the SMG, but not that for L-kininogen. These kininogens appeared to be a little increased in the liver. The other acute phase proteins, CRP and SAP were also scarcely detected in the SMG but again they were strongly induced at 24 h after injection of turpentine oil (Fig. 1a, b). In other exocrine gland such as ELG and ILG, the mRNAs for H- and L-kininogens, SAP, and CRP were also strongly induced at 24 h after injection of turpentine oil (data not shown).

On the other hand, in control rats, the mRNAs for IL-1β and IL-6, the inflammation cytokines, were not or scarcely detected in the SMG and liver. At 6 h after injection of turpentine oil, however, TNF-α was detected in the SMG (Fig. 2a). At 12 and 24 h, the level was decreased again indicating that the increase was transient. Such changes were also seen in the ELG and ILG as well (data not shown).

We next studied the induction of IL-1β, TNF-α and IL-6 mRNAs in the SMG and liver of both rats and mice (Fig. 2b, c).

In rats, IL-1β, Il-6 and TNF-α were strongly induced in all of exocrine gland examined although there was a little variation (Fig. 2b). Similarly, IL-1β and TNF-α were strongly induced in the SMG of C3H/HeN mice (normal mice). In all of these tissues, all cytokine examined increased at 3–6 h and the level was decreased at 12 and 24 h. Mice injected with saline at 6 and 24 h did not show any change. In TLR4-deficient mutant C3H/HeJ mice, injection of LPS did not induce all these cytokines in the SMG as well as in the liver (Fig. 2c). These facts clearly indicate that TLR4 is involved in the cytokine induction in the SMG.

In order to confirm above fact, we examined by RT-PCR if TLR4 mRNA is expressed in the SMG. The results indicated that mRNA for TLR4 was strongly expressed in the SMG and weakly in PG (Fig. 3).

Discussion

Kininogens are precursors for kinins, which have strong biological activities during inflammation (10). CRP and SAP are acute phase proteins, which have opsonin

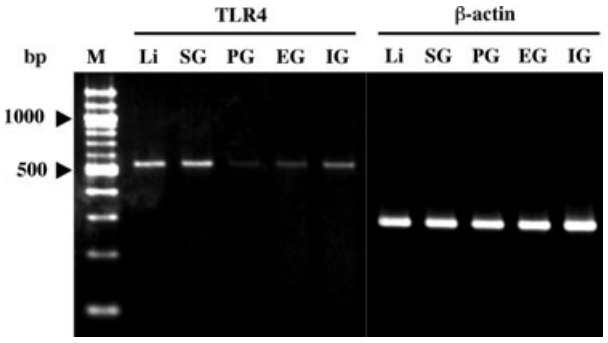

Figure 3 RT-PCR analysis to verify the presence of TLR4 mRNA. The total RNA was perpared from indicated tissues of rats. Other experimental conditions are described in the text. Li, liver; SG, submandibular gland; PG, parotid gland; EG, extraorbital lacrimal gland; IG, intraorbital lacrimal gland; M, 100 bp DNA Ladder markers.

activity and play an important role during micro-organism infection (1). In the present study, we found that mRNAs for acute phase proteins, H- and T-kininogens and CRP and SAP, were induced in the salivary and lacrimal glands in response to experimental inflammation by turpentine oil or LPS. This induction was observed at 24 h after injection and preceded by a transient increase of IL-1β, IL-6 and TNF-α, the inflammation cytokines at 6 h. Therefore, an increase of inflammation cytokines may have induced the transcription of acute phase proteins.

The LPS is selected and studied if inflammation cytokines can be induced by this pathogen in the SMG; the results indicated that IL-1β and two other cytokines (TNF-α and IL-6) were induced by LPS in this gland. Since LPS belongs to PAMPs (pathogen-associated molecular patterns) and therefore serves its action through TLR4, an LPS receptor, we examined if the cytokine induction in the SMG is also conducted via TLR4. For this purpose, TLR4-mutant mice expressing non-functional TLR4 were utilized to examine if LPS can induce the cytokines shown above. In the SMG of C3H/HeJ mice, a mutant strain of C3H/HeN, LPS induced none of cytokines examined, suggesting that TLR4 be involved in the cytokine induction in the SMG. The signaling pathway of TLR4 is known to activate innate immune system (11, 12). The present results suggest that such a defense system as well as the system to induce acute phase proteins exist in the salivary gland.

Using both rats and mice as experimental animals, present study focussed on changes of inflammation cytokines and acute phase proteins induced by turpentine oil and LPS in the salivary gland and other exocrine glands. The changes in the level of mRNA for these proteins were analysed by RT-PCR as this technique is sensitive enough to detect the changes.

The present study provided a model system for analysing the cellular event of the salivary gland and other exocrine glands during the systemic inflammation. Although it may not reflect exactly the same pathological condition seen in the sialolithiasis, the result presented here would provide some idea how inflammation cytokines and acute phase proteins are induced in the salivary gland and other exocrine glands.

IL-1β, one of inflammation cytokines, induced by LPS in the SMG is found to be secreted into saliva although acute phase proteins (CRP, SAP and kininogens) are not (Yao in preparation and Ref. 13). The secreted IL-1β may play an important role in the oral cavity acting on the oral mucosal (Yao in preparation) whereas acute phase protein may function within the gland or in the circulation. These issues are now investigating from the point of immunological function of the salivary gland.

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