PROCEEDING

Induction of calprotectin mRNAs by lipopolysaccharide in the salivary gland of mice

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Abstract : Calprotectin is a major cytosolic calcium-binding protein of leukocytes which belongs to the S100 protein family. S100A8 and S100A9, major types of calprotectin are heterodimeric complexes being composed of light- and heavy-chain subunits. The calprotectin levels in the plasma, feces, synovial fluid, gingival crevicular fluid, dental calculus and saliva change when the host animal suffers from several inflammatory diseases. Members of Toll-like receptor (TLR) family are pattern-recognition receptors for lipopolysaccharide (LPS) and other pathogens. Here we examined if the biological role of TLR receptor is reflected to the calprotectin expression in the salivary gland. Time course study by using real-time RT-PCR detected higher levels of S100A8 and S100A9 mRNA at 1.5-3 h after injection of LPS in both the submandibular gland (SMG) and parotid gland (PG) of C3H/HeN mice but not in the same tissues of C3H/HeJ, a TLR-4 mutant strain, indicating that this induction is mediated via the TLR-4. These results indicate that, an inflammatory marker, calprotectin, is expressed in the mouse salivary gland and that LPS stimulated its synthesis. Calprotectin (S100A8/A9) showed minimum expression in all cellular segments in the SMG except excretory duct cells, which showed strong signal at the cytoplasm. LPS induced their expressions in the granular convoluted tubular cells and striated duct cells. In the PG, these proteins were expressed very weakly in both duct and acinar cells with a little stronger staining for the former cells. LPS injection induced calprotectin (S100A8/A9) in both duct and acinar cells especially in the former cells. J. Med. Invest. 56 Suppl. : 287-289, December, 2009

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INTRODUCTION

Calprotectin was originally discovered as an antimicrobial protein that was present in the cytoplasm of neutrophil granulocytes. Calprotectin belongs to the S100 protein family consisting of members, S100A2, S100A3, S100A4, S100A6, S100A7, S100A8, S100A9, S100A10, S100A11, S100A12, S100A15, S100B, and S100P proteins, all of which are characteristic by the presence of two Ca^2 binding sites of the EF-hand type (1). One species of calprotectin, a complex of S100A8 and S100A9, which are also designated as migration inhibitory factor or myeloid-related protein-8 (MRP8) and MRP14, or calgranulin

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A and B, respectively, is identified to stimulate the production of proinflammatory cytokines. The calprotectin levels in the plasma, feces, synovial fluid, gingival crevicular fluid, dental calculus and saliva change when the host animal suffers from several inflammatory diseases (2). Thus the physiological role of S100A8/A9 complex is that it has an antimicrobial activity against *Capnocitophaga sputigena, Candida* strains, *Escherichia coli, Staphilococcus aureus* and *Staphilococcus epidermidis* (3). It is likely that calprotectin plays an important role in a defence mechanism by protecting mucosal tissue against microbes.

Salivary proteins with antimicrobial activity, like lysozyme or interleukins, are known to be major components of the oral defense (4, 5). In the present study, therefore, we investigated whether S100A8/A9 is expressed in the two salivary glands, submandibular gland (SMG) and parotid gland (PG), and if their levels in these tissues are affected by inflammation.

MATERIAL AND METHODS

LPS-Hyporesponsive C3H/HeJ mice (a TLR-4 mutant mouse strain), and their wild type C3H/HeN mice (LPS-responsive), were purchased from CLEA Japan, Inc., and Japan SLC, Inc. *E.coli* LPS was purchased from Sigma-Aldrich (St. Louis, MO).

The SMG and PG tissues dissected from the animal at 1.5, 3, 6, 12, 24 hours after injection of LPS were homogenized in TRI-Reagent and mRNAs were extracted. RT-PCR (SuperScript™ One Step RT-PCR., Invitrogen) was conducted using the following primer pairs : 5‘-GGAATCACCATGCCCTCTA-3‘ (sense) and 5‘-GCTGTCTTTGTGAGATGCCA-3‘ (antisense) for S100A8, and 5‘-TCATCGACACCTTCCATCAA-3‘ (sense) and 5‘-GATCAACTTTGCCATCAGCA-3‘ (antisense) for S100A9. β-Actin was used as a positive control of the RT-PCR reaction. cDNAs amplified were resolved by electrophoresis in 3% agarose gel following the standard procedure. In addition, real-time PCR was carried out using primer sets : 5‘-GCAAATCACCATGCCCTCTAC-3‘ (sense) and 5‘-GCCACACCCACTTTTATCACC-3‘ (antisense) for S100A8, 5‘-CGACACCTTCCATCAATACTC-3‘ (sense) and 5‘-GAGGGCTTCATTTCTCTTCTC-3‘ (antisense) for S100A9, and these mRNA levels were quantified. One Step PrimeScript™ was purchased from TaKaRa (Shiga, Japan).

The immunohistological localization of S100A8 and S100A9 were examined in the SMG and PG of mouse at 6 hour after LPS injection; tissue secions were cut at 7 μm thickness, mounted on APS-coated slides, and dried at 37°C overnight. These sections were stored at -20°C until used for immunostaining. Anti-mouse S100A8 and S100A9 antibody were purchased from R&D Systems. After immunostaining, the sections were reacted with PBS containing propidium iodide and RNase at room temperature for nuclear staining. The stained sections were examined under a fluorescence microscope (Nikon, Tokyo, Japan).

RESULTS AND DISCUSSION

Calprotectin expression in the salivary gland may be modified by TLR signaling pathway. To clarify the mechanism of calprotectin elevation in the SMG and PG, we examined mRNA and protein expression after LPS injection in C3H/HeN and C3H/HeJ mice.

The induction of calprotectin mRNAs by LPS at 3 h were analyzed in C3H/HeN and C3H/HeJ mice by real-time PCR. The expressions of these inflammatory mediators by LPS between C3H/HeN and C3H/HeJ mice were compared, which showed that S100A8 and A9 mRNAs were highly expressed in both salivary gland tissues of wild type mice. In the mutant mice which are lacking the functional TLR-4 receptor, these mRNAs were only slightly expressed in both glandular tissues. Time course of changes in calprotectin, S100A8 and S100A9 mRNA levels in the SMG and PG of C3H/HeN mice, showed their elevated expression at 1.5-3 h after LPS injection. On the basis of these results we speculated that calprotectin was induced by TLR-4/NF-κB pathway in the salivary gland.

Immunohistochemical localization of S100A8 and S100A9 in the salivary gland was examined before and after stimulation with LPS. We found that these antigens became strongly expressed in the granular convoluted tubular cells and the striated duct cells after LPS injection. These results suggest that an inflammatory marker, calprotectin, was expressed in the mouse salivary gland and that LPS stimulated its synthesis. Calprotectin (S100A8/A9) showed minimum expression in all components of the SMG cells except for the excretory duct cells which showed a strong signal at the cytoplasm. LPS induced their expressions in the granular convoluted

tubular cells and the striated duct cells in the SMG. In the PG, these proteins were expressed very weakly in both duct and acinar cells with a little stronger staining for the former cells. LPS injection induced calprotectin (S100A8/A9) in both duct and acinar cells, especially in the former cells.

CONCLUSION

Calprotectin (S100A8/A9) mRNAs were expressed weakly in the salivary gland. LPS induced S100A8/A9 mRNAs and proteins strongly in both the SMG and PG most likely *via* TLR4. In the SMG, only excretory duct cells expressed these proteins while both duct and acinar cells expressed them weakly in the PG under normal condition. LPS-induced S100A8/A9 were localized mainly in the granular convoluted tubular cells and striated duct cells in the SMG and in duct cells in the PG although appreciable expression was recognized also in the acinar cells in the latter gland.

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