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Growth inhibition and apoptosis by an active component of OK-432, a streptococcal agent, via Toll-like receptor 4 in human head and neck cancer cell lines

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SUMMARY

Toll-like receptor 4 (TLR4) plays a significant role in cancer therapy as receptors of bacteria-derived immunotherapeutic agents such as OK-432, a streptococcal immunotherapeutic agent. In addition, recent reports demonstrated that TLRs, including TLR4, are also expressed in cancer cells as well as in immunocompetent cells. It is a problem in cancer therapy that the immunoadjuvant may activate survival signals such as nuclear factor (NF)-κB or mitogen-activated protein kinases (MAPKs) in cancer cells via TLRs. In the current study, we investigated responsiveness of human head and neck cancer cell lines against TLR4 ligands, OK-PSA, an active component of OK-432, and a lipopolysaccharide (LPS).

Stimulation with LPS or OK-PSA resulted in the activation of NF-κB in these cell lines expressing TLR4 and MD-2 that is a significant coreceptor for TLR4 signaling. Interestingly, OK-PSA induced cell-growth inhibition, while LPS enhanced the proliferation of the cancer cells. OK-PSA induced NF-κB activation more slowly than that induced by LPS. In addition, phosphorylation of p38 MAPK by OK-PSA was only slight compared with that by LPS. OK-PSA also induced apoptosis of the cancer cells mediated by the activation of caspase 1, 3 and 8 in a p53-independent manner.

These findings strongly suggest that active components of OK-432 may elicit anti-cancer effects via enhancing host immunity as well as via directly inducing the growth inhibition and apoptosis of head and neck cancer cells through TLR4 signal.

Introduction

OK-432, which is a penicillin-killed and lyophilized preparation of a low-virulence strain of *Streptococcus pyogenes* (group A),[1] has been successfully used as an immunotherapeutic agent against many types of malignancies, including head and neck cancer (HNC).[2–8] It has been reported that OK-432 elicits anti-tumor effects by stimulating immunocompetent cells such as macrophages, T cells, and natural killer (NK) cells, and by inducing helper T-cell 1 (Th1) type cytokines, including interleukin-2 (IL-2), IL-12, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ).[9–12] We have isolated an active component [a lipoteichoic acid (LTA)-related molecule: OK-PSA] by affinity chromatography of a butanol extract of OK-432 on cyanogen bromide-activated Sepharose 4B bound by the mouse monoclonal antibody that recognizes an IFN-γ-inducing component of OK-432.[13,14] We have reported that OK-PSA is a far more potent inducer of Th1-type cytokines in human peripheral blood mononuclear cells (PBMCs) than the original OK-432, and that it engages in a marked antitumor activity in tumor-bearing mice.[13,15–20] Furthermore, we showed that Toll-like receptor (TLR) 4 signaling is involved in regulating OK-432/OK-PSA-induced antitumor immunity in tumor-bearing mice[21] as well as in oral cancer patients.[22]

TLRs are transmembrane proteins that belong to a newly recognized family of vertebrate pattern recognition receptors within the innate immune system.[23,24] Subsequent to ligand binding, TLRs initiate signaling through the sequential recruitment of myeloid differentiation protein 88 (MyD88), IL-1R-associated kinase, and TNFR-associated factor 6, which in turn activate downstream mediators such as mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB).[25,26] Among the family of ten identified human TLRs, TLR2 recognizes peptidoglycan (PGN),[27] lipoprotein,[28] etc., TLR4 recognizes lipopolysaccharide (LPS),[29] and

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TLR9 recognizes bacterial unmethylated CpG-DNAs.[30] MD-2 acts as a significant coreceptor in the TLR4 signaling.[31]

Recent report demonstrated that TLRs, including TLR4 and MD-2, are expressed in cancer cells[32] as well as in immunocompetent cells, however, the role of the TLR ligands in cancer cells remains uncertain. In cancer therapy using OK-432, it is a problem that the immunopotentiator may activate survival signals such as NF-κB and MAP kinases in the carcinoma cells via the TLR4/MD-2 signal. In the current study, we conducted in vitro experiments to investigate the direct effects of TLR4/MD-2 ligands, OK-PSA and LPS, in human HNC cell lines.

Materials and methods

Preparation of OK-PSA

OK-PSA was prepared from OK-432 (Chugai Pharmaceutical Co. Ltd., Tokyo, Japan) as described previously.[13] The OK-PSA preparation was tested for LPS contamination using the Endospecy ES-50M set (Seikagaku Kogyo, Tokyo, Japan), according to the manufacturer's recommended protocol.[33]

Cells and culture

As the human HNC cell lines, we used seven squamous cell carcinoma (SCC) cell lines, BHY, B88, HI, HNT, HSC-2, Ho-1-u-1, and KB, and five salivary gland carcinoma (SGC) cell lines, HSY, HSG, AZA1, AZA3, and TYS. BHY, B88, HI, HNT, KB, HSY, HSG, AZA1, AZA3, and TYS were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc., Grand Island, NY) with 10% heat-inactivated fetal bovine serum (FBS) (Bio-Whittaker, Walkersville, MD). HSC-2 and Ho-1-u-1 were cultured in RPMI 1640 with 10% FBS.

RNA extraction and semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA were extracted from the human HNC cell lines and from human PBMCs by using ISOGEN RNA extracting mixture (Nippon Gene, Toyama, Japan), according to the manufacturer's recommendations. Expression of mRNAs for TLR4, MD-2, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was detected by semiquantitative RT-PCR. One microgram of total RNA was reverse-transcribed using Moloney murine leukemia virus reverse-transcriptase (Life Technologies, Inc.) and random primers (Life Technologies, Inc.) in a volume of 20 μl at 42 °C for 60 min. Two microliter of the reverse-transcribed mixture was subjected to PCR (10 mM Tris–HCl [pH 8.3]; 50 mM KCl; 1.5 mM MgCl2; 0.01% gelatin; 20 mM each dATP, dGTP, dTTP, and dCTP, each at 20 mM), 0.5 U Taq polymerase (TaKaRa Bio Inc., Otsu, Japan), and 0.25 pmol of each primer in a total volume of 20 μl. The primer sequences used in the present study are described in Table 1. We used 25 cycles of PCR amplification for GAPDH, 28 cycles for TLR4, and 30 cycles for MD-2 with each cycle consisting of 94 °C for 60 s, 55 °C for 90 s, and 72 °C for 150 s, with an initial denaturation step of 94 °C for 5 min and a final elongation step of 72 °C for 5 min. PCR was carried out in a DNA Thermal Cycler (TaKaRa Bio Inc.). Amplified complementary DNA was electrophoresed through 1.5% agarose gels containing 100 ng/ml ethidium bromide. After electrophoresis, gels were illuminated with ultraviolet light, viewed, and photographed (Polaroid type 667 film; Polaroid Corp., Cambridge, MA). Densitometric analysis for the RT-PCR band patterns was done using NIH Image 1.59 software (National Institutes of Health, Bethesda, MD). The relative density (RD) of each specific RT-PCR band was expressed as a ratio to the density of GAPDH, with negative expression defined as RD less than 0.1, marginal expression defined as RD equal to or more than 0.1 but less than 0.5, and positive expression defined as RD equal to or greater than 0.5.

Real-time quantitative RT-PCR

Expression levels of mRNAs forTLR4, MD-2 and GAPDH in the total RNAs from HNC cell lines were quantitatively analyzed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems Japan, Tokyo) by using TaqMan® Universal PCR Master Mix (Applied Biosystems) and Assays-on-Demand™ Gene Expression Products (Applied Biosystems) according to the manufacturer's recommendations. Using 96-well plates, 50 μl PCR reactions were prepared containing 25 μl of the 2× TaqMan Universal PCR Master Mix and 2.5 μl of the 20× TaqMan Assays-on-Demand™ Gene Expression Products. Thermal cycler conditions were as follows: at 95 °C for 10 min, and 40 cycles at 95 °C for 5 s and 60 °C for 30 s. An analysis of relative gene expression data was performed, using the $2^{-\Delta\Delta CT}$ method on an Sequence Detection System Software (Applied Biosystems). The fold change in studied gene expression, normalized to endogenous control, was calculated using: $RQ = 2^{-\Delta\Delta CT}$. The relative expression levels of TLR4 and MD-2 mRNAs were expressed as a fold-increase in GAPDH mRNA expression.

Transient transfection and luciferase assay

Human HNC cells were seeded at 1×10^5 cells per well into each well of a 6-well dish (Corning, Inc., Vernon, NY). These cells were transiently transfected with 0.4 μg of NF-κB reporter plasmid using Effectene Transfection Reagent (QIAGEN, Valencia, CA), and then these cells were treated with OK-PSA (10 μg/ml) or LPS (10 ng/ml, derived from *Escherichia coli* 055:B55; Sigma Chemical Co.). After 0.5, 1, 2, 3, or 5 h cultivation at 37 °C, the cells were harvested and lysed in 100 μl of Luciferase Cell Culture Lysis Reagent (Promega, Madison, WI). Luciferase activity was measured using 20 μl of lysate and 100 μl of Luciferase Assay Substrate (Promega). The luminescence was quantified in relative light units (RLU) by a luminometer (Bio-Orbit, Turku, Finland). Relative luciferase activity was obtained by dividing the RLU value of each untreated sample, and positive reaction was defined as relative luciferase activity equal to or more than 2.

Cell growth assay: 3-(3,4-dimethyl-thiazol-2-yl)-2,5-dihenyl tatrazolium bromide (MTT) assay

The cancer cells were seeded on flat-bottomed 96-well plates (Falcon, Oxnard, CA) at 3.0×10^3 cells per well in DMEM or

Table 1
Oligonucleotide primers used for cytokine mRNA amplification.

	Upstream primer	Downstream primer	PCR product size (bp)
TLR4	TGGATACGTTTCCTTATAAG	GAAATGGAGGCACCCCTTC	506
MD-2	GAATTCATGTTGCCATTTCTGTTT	GAATTCTAATTTGAATTAGGTTGG	493
GAPDH	GAAATCCCAGCACCATCTTCCAGG	GTGGTGGACCTCATGGCCCACCATG	781