Bacterial Lipopolysaccharides Induce Steroid Sulfatase Expression and Cell Migration through IL-6 Pathway in Human Prostate Cancer Cells

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Abstract
Steroid sulfatase (STS) is responsible for the conversion of estrone sulfate to estrone that can stimulate growth in endocrine-dependent tumors such as prostate cancer. Although STS is considered as a therapeutic target for the estrogen-dependent diseases, cellular function of STS are still not clear. Previously, we found that tumor necrosis factor (TNF)-α significantly enhances steroid sulfatase expression in PC-3 human prostate cancer cells through PI3K/Akt-dependent pathways. Here, we studied whether bacterial lipopolysaccharides (LPS) which are known to induce TNF-α may increase STS expression. Treatment with LPS in PC-3 cells induced STS mRNA and protein in concentration- and time-dependent manners. Using luciferase reporter assay, we found that LPS enhanced STS promoter activity. Moreover, STS expression induced by LPS increased PC-3 tumor cell migration determined by wound healing assay. We investigated that LPS induced IL-6 expression and IL-6 increased STS expression. Taken together, these data strongly suggest that LPS induces STS expression through IL-6 pathway in human prostate cancer cells.

Key Words: Steroid sulfatase, Lipopolysaccharides, Interleukin-6, Tumor cell migration, PC-3

INTRODUCTION
Steroid sulfatase (STS) is responsible for the enzymatic hydrolysis of steroid sulfates such as estrone sulfate (E1S) and dehydroepiandrosterone sulfate (DHEAS) to their unconjugated, biologically active forms (Reed et al., 2005). DHEAS can be converted to 5-androstene-3β, 17β-diol, which has been shown to have weak estrogenic properties. However, due to the high concentration of this steroid, it can stimulate the growth of breast cancer cells in vitro and in vivo (Aidoo-Gyamfi et al., 2009). E1S is a steroid conjugate present in high concentrations in tissue and blood in women with breast cancer. E1S is considered an important source for the formation of active estrogens which is able to be produced by STS activity (Aidoo-Gyamfi et al., 2009).

Sulfated estrogens are unable to bind to estrogen receptors and thus they are inactive as hormones. In other words, sulfation changes the polarity of the hydrophobic steroid ring and then is used for hormone transport (Kriz et al., 2008). Furthermore, sulfated steroid conjugates play a role as a reservoir of active hormones or their precursors via STS action.

Metabolic activation of E1S has been suggested to play a major role in mammary carcinogenesis (Chang, 2011). STS is involved in the formation of active estrogens such as E1 or E2. A high proportion, approximately 40% of breast cancers are hormone-dependent and it is the E2 that is suggested to be important in the initiation, promotion and progression of this disease (Ahmed et al., 2002). Moreover, STS expression is increased in malignant breast tumors and has prognostic importance (Utsumi et al., 2000; Pasqualini, 2009).

Just like breast tumor, estrogens are locally synthesized by aromatase in human prostate cancer (Härkönen and Mäkelä, 2004). Various types of 17β-hydroxysteroid dehydrogenase (17β-HSD) also have been reported to be expressed at least in some human prostate cancers (Koh et al., 2002). These findings above all suggest that in situ estrogen production and metabolism are involved in human prostate cancer (Koh et al., 2002). However, in situ or intratumoral production of estrogens has remained relatively unknown compared to that of androgens (Nakamura et al., 2005).

Besides 17β-HSD type I and aromatase, STS belongs to the most important targets for potential endocrine therapy in humans (Suzuki et al., 2005). Inhibitors of steroid sulfatase are being developed as a novel therapy for hormone-dependent...
breast cancer in postmenopausal women (Stanway et al., 2007). It maintains the equilibrium between sulfated and unconjugated steroids whose effects in breast cancer development are, in many cases, completely different (Reed et al., 2005).

Chronic inflammation has been linked to the development of cancer. As a potent inflammatory stimulus, lipopolysaccharides (LPS), a cell wall constituent of Gram negative bacteria, induce the release of a wide range of cytokines and growth factors from immune cells (Wilson et al., 2002; Li et al., 2012). In addition, LPS can alter cytokine levels by stimulating inflammatory cells in the tumor microenvironment to induce tumor cell proliferation, promote angiogenesis, and facilitate invasion and metastasis (Schartter et al., 2010). Recently, some reports have demonstrated that LPS can exert direct effects on tumor cells in vivo and in vitro (Wang et al., 2003). Although recently explored roles of STS in many pathophysiological processes in cancers prompted efforts for preparation of efficient STS modulators, the cellular function of STS is still not clear.

Induction of STS expression may require mRNA or protein synthesis. However, which factors can directly affect the expression of STS has not been examined in detail in human prostate cancer. As shown in former studies, LPS is known to stimulate STS expression in monocytes and macrophages (Guha and Mackman, 2001). Macrophage-borne STS converts DHEAS to DHEA. It has been shown that DHEA, through receptor-mediated mechanisms similar of steroid hormones, represents an important regulator of interleukin-2 (IL-2) production and the TH1 immune response (Daynes et al., 1990).

Inflammatory cytokines such as IL-6 and IL-1β regulate proliferation of breast cancer cells through estrogen production by steroid-catalyzing enzymes including STS and aromatase (Honma et al., 2002). IL-6 enhances the activity of steroid catalyzing enzymes (Purohit et al., 1996; Reed and Purohit, 1997).

LPS released from gram-negative bacteria binds to LPS binding protein (LBP), and then the LPS-LBP complex binds to CD14 on the surface of neutrophils, monocytes and macrophages. These complexes may stimulate cytokine expression including IL-1β and IL-6 (Okada et al., 1999).

In these studies, we explored the effect of LPS on STS expression in PC-3 human prostate cancer cells to elucidate whether LPS is able to regulate transcription of the STS gene. We also studied LPS-induced STS expression enhances tumor cell migration. As IL-6 is an important contributor to the effects of LPS in inflammation (Wright et al., 1993), evidence for the involvement of IL-6 pathway in STS expression by LPS has also been determined.

**MATERIALS AND METHODS**

**Reagents**

Rabbit polyclonal antibody for STS was purchased from Abcam (Cambridge, UK). Antibodies against GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bacterial lipopolysaccharides were purchased from Sigma-Aldrich (St. Louis, MO, USA). Enhanced chemiluminescence (ECL) detecting reagent was from Thermo Scientific (Rockford, IL, USA). Dual-Luciferase Reporter Assay kit was purchased from Promega (Madison, WI, USA). Ex taq polymerase was obtained from TaKaRa Bio (Shiga, Japan). Other chemicals and reagents were of the highest quality commercially available.

**Cell culture**

Human prostate cancer PC-3 cells and HeLa cells were obtained from Korean Society Cell Bank (KCLB). Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. After incubation, the cells were harvested by scraping and solubilized in 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 1% nonidet P-40, 1 mM PMSF, 1 μg/ml aprotonin, and 1 μg/ml leupeptin. Cells were centrifuged at 1,000 x g for 4 min at 4°C and the pellets were resuspended and stored in -70°C.

**Transient transfection of siRNA**

Cells were harvested at a density of 5x10⁵ cells per 6-well plate. Transfection was carried out using the Neon™ transfection system (Invitrogen, Carlsbad, CA), and 1x10⁶ cells were transfected with 50 nM STS siRNA (Thermo Scientific, Rockford, IL, USA). Following microporation, cells were cultured in 100-mm dishes with antibiotic-free RPMI for 24 h.

**DNA isolation, reverse transcription and RT-PCR**

After transfection, total RNA was extracted using Ribospin™ (GeneALL, Seoul, Korea). Total RNA (500 ng) was transcribed at 37°C for 1 h in a volume of 20 μl containing 5x RT buffer, 10 mM dNTPs, 40 units of RNase inhibitor, 200 units M-MLV reverse transcriptase and 100 pmole of oligo-dT primer. Subsequently, 0.8 μl of the reaction mixture from each samples was amplified with 10 pmole of each oligonucleotide primers, 0.2 mM dNTPs, 1.5 mM MgCl₂ and 1.25 units of Ex taq polymerase in a final volume of 25 μl. PCR was performed as follows: One cycle of 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 10 sec, annealing at 58°C for 15 sec, and extension at 72°C for 15 sec. The number of amplification cycles was optimized in preliminary experiments to ensure that the PCR did not reach a plateau. PCR products were subjected to a 2% (w/v) agarose gel electrophoresis, and analyzed by ChemiDoc XRS (Bio-Rad, Hercules, CA, USA). The primers used for human STS were 5’- CCT CCT ACT GTT CTT CCT GTG GG-3’ (sense) and 5’- GGT CGA TAT TGG CAG GCC TGA TA-3’ (antisense). The primers used for human IL-6 were 5’-CCA CCT TCA ACT CTG TTG TTG CAG TGG GTA GTA-3’ (sense) and 5’-CAG GGG TGA TTC CTG CAT CT-3’ (antisense).

**Western blot analysis**

Following transfection, whole cell lysates were prepared and protein concentration was determined using BCA Protein Assay Reagents. Cellular extracts (20 μg) were separated on 10% SDS-PAGE at 100 V and transferred onto 0.45 μm PVDF membrane. Nonspecific binding was blocked with 5% nonfat milk in TBS-T for 1 h at room temperature. Primary antibody was used at a 1:1000 dilution. Secondary antibody was used in a 1:5000 dilution. The incubation of primary antibodies was done at 4°C for overnight incubation. Secondary antibodies were done at 4°C for 2 h. Proteins were visualized by an ECL method and the band intensity was analyzed by Chemidoc XRS densitometer system and quantified by Quantity One software (Bio-Rad).

**Luciferase assay**

Cells (1.5x10⁴ cells/well) were transfected with 0.4 μg of STS luciferase reporter plasmid according to the manufac-
In PC-3 cells, the mRNA and protein expression of STS were measured consecutively using the Dual Luciferase Assay System (Promega) with Synergy H1 hybrid microplate reader (Biotek, Winooski, VT, USA).

**Wound healing assay**

Cells (1×10⁵ cells/well) were cultured in 6-well cell culture plates. Cells were washed with PBS and treated with mitomycin C (25 μg/ml) for 30 min. One mm wide scratch was made across the cell layer using a sterile pipette tip. Plates were photographed after indicated time.

**Statistical analysis**

Statistical analysis was performed by using one-way analysis of variance, followed by Dunnett’s Multiple Comparison test using Graph-Pad Prism Software (GraphPad Software Inc., San Diego, CA) when appropriate. The difference was considered statistically significant at p<0.05.

**RESULTS**

**Effects of LPS on STS expression in prostate cancer cells**

To elucidate whether LPS regulate STS gene expression in PC-3 cells, the mRNA and protein expression of STS were measured by RT-PCR and Western blot, respectively. PC-3 cells were grown for 24 h in the presence of increasing concentrations of LPS (0, 0.1, 1 or 10 μg/ml). As shown in Fig. 1A, when cells were treated with LPS, STS mRNA expression was significantly enhanced in a concentration-dependent manner. In agreement with increasing mRNA levels, levels of STS protein were also significantly induced by LPS (Fig. 1A). We compared the time courses for the induction of STS mRNA and protein expression in LPS-treated cells (Fig. 1B). At 1 μg/ml of LPS, the mRNA level of STS was ~2.2-fold higher than that of control. Similarly, we found ~2.3-fold induction of STS expression after 24 h treated with LPS (1 μg/ml). When cells were treated with LPS (10 μg/ml), the induction of STS was shown after 6 h of treatment. Induction of STS mRNA was plateaued after 48 h treatment (Fig. 1B).

**Effects of LPS on activation of STS promoter in cervical cancer cells**

To examine whether induction of STS expression by LPS is related to activation of promoter region of STS, luciferase assay was performed. HeLa cells were selected because it has higher transfection efficiency of STS reporter plasmid. HeLa cells were transfected with STS reporter plasmid (pGL3-STS2 luc). After transfection, cells were treated with LPS (10 μg/ml) for 24 h and luciferase activity was determined. As shown in Fig. 2, the results of luciferase assay indicated ~1.5-fold induction in STS transcription by LPS.

**Effects of LPS-induced STS expression on tumor cell migration in prostate cancer cells**

To elucidate whether LPS-induced STS expression causes PC-3 cell migration and invasion, we determined the effect of LPS on tumor cell migration using a wound healing assay. To determine the role of STS on tumor cell migration, cells were treated with STS siRNA (50 nM) for 48 h to knockdown STS expression selectively. Only 22.2% of the injury line was removed by STS knockdown although almost all of the injury line was closed by scrambled control. When STS knock-downed cells were treated with LPS (10 μg/ml), 74.0% of the injury line was recovered by LPS (Fig. 3). These results show that STS expression induced by LPS may cause PC-3 cell migration.

**Fig. 1, LPS induce STS mRNA and protein expression in PC-3 cells.** (A) Concentration-dependency. PC-3 cells were treated with various concentrations of LPS (0, 0.1, 1 or 10 μg/ml) for 24 h. The protein and mRNA level of STS was determined using RT-PCR and Western blot. Total RNA was isolated and human STS genes were amplified with specific primers. The PCR products were separated on a 2% agarose gel. Expression of GAPDH mRNA was determined as a RNA control. Total cellular lysates were prepared for Western blot analysis using antibody against STS. GAPDH was used as a loading control. (B) Time-dependency. Cells were treated with 10 μg/ml LPS for 0, 6, 12, 24 or 48 h. The protein and mRNA level of STS was determined using RT-PCR and Western blot.

**Fig. 2, LPS induce STS promoter activity in HeLa cells.** HeLa cells were transiently transfected with pGL3-STS2 luc and pRL-CMV plasmids. At 24 h after transfection, cells were incubated at 37°C with 10 μg/ml of LPS for 24 h. Luciferase activity was measured and expressed as the mean ± S.D. of four separate experiments. *p<0.05 compared with untreated control cells. The relative firefly luciferase activity, normalized by the renilla luciferase activity, is shown.
IL-6 involvement in LPS-induced STS expression in prostate cancer cells

To elucidate how LPS induce STS expression, we studied whether LPS-induced STS expression correlates with the IL-6 pathway. LPS enhance the release of inflammatory cytokines including IL-6. Previous report showed that various cytokines such IL-6 and IL-1β enhance the activity of STS (Honma et al., 2002). We determined that LPS induced IL-6 and STS mRNA expression in PC-3 cells in concentration-dependent manner (Fig. 4A). To confirm that IL-6 induces STS expression, the mRNA and protein levels of STS were measured by RT-PCR and Western blot. PC-3 cells were grown for 16 h in the presence of increasing concentrations of IL-6 (0, 10, 20, 40 or 80 ng/ml). IL-6 increased levels of STS mRNA and protein in a concentration-dependent manner (Fig. 4B). These data indicated that released IL-6 by LPS treatment may transduce intracellular signal to induce STS expression.

DISCUSSION

STS has an important role in regulating estrogen biosynthesis that is required for tumor growth and survival within hormone-dependent cancers such as prostate and breast cancers. STS is the only well-known enzyme in human cancer cells that is capable to desulfate E1S and DHEAS as an important step in the conversion of these precursors to active forms (Nussbaumer and Billich, 2005). STS expression is increased in malignant breast tumors and has prognostic importance. STS immunoreactivity was strongly correlated with tumor size (Suzuki et al., 2003). Research into the control and inhibition of this enzyme has been stimulated by its important role in supporting the growth of hormone-dependent tumors of the breast and prostate. STS is also known to be involved in many physiological functions, such as regulating part of the immune response and some aspects of cognitive function (Reed, 1995; Reed and Purohit, 1997).

The STS pathway is responsible for the production of various steroids with estrogenic properties, namely 5-androstenediol (Adiol), from DHEAS and subsequent reduction of DHEA by 17β-HSD1 (Purohit et al., 2011; Purohit and Foster, 2012). Adiol, although an androgen, can bind to the ER and has been shown to stimulate the proliferation of a number of ER-positive breast cancer cells in an ER-dependent manner (Phan et al., 2011). Unrestricted production of Adiol can occur via the STS pathway and may promote tumor progression.

In prostate cancer cells, STS expression and their possible roles associated with in situ estrogen metabolism are significant. Although increased level of STS was often observed in prostate and breast cancer cells, the direct stimulator of STS expression was not established.

LPS which are known to induce TNF-α stimulates STS expression in monocytes and macrophages (Schmidt et al., 2000). LPS is viewed as critical stimuli in inflammation-associated cancer and promotes tumor metastasis (Lu et al., 2006;
Finzi et al., 2009). There were significantly increased lung metastases in animals that received an equivalent LPS injection. Triggering of TLR4 by LPS, induced tumor promotion by the induction of proliferation, activation of NF-κB, p65 binding to DNA, and resistance to cytotoxicity accompanied by the increased production of proinflammatory cytokines (IL-6 and IL-8), VEGF, and GM-CSF (Bao et al., 2011). These factors are known to promote the development of myeloid-derived suppressor cells as well as tumor progression.

In this study, we demonstrated whether human STS gene expression might be controlled by LPS. Our data clearly show that LPS are able to induce STS mRNA expression in time- and concentration-dependent manners. To elucidate LPS directly induce STS by activating STS promoter, we performed luciferase assay. LPS treatment showed ~1.5 fold higher luciferase activity than control.

LPS stimulate the lung adenocarcinoma A549 cell proliferation especially 24-hour treatment. The ratio of cells in G2/M cell cycle treated with LPS (100 ng/ml) was significant higher than non-treated groups (Hattar et al., 2012). LPS induce macrophages to migrate from inflamed tissues to lymph nodes using the adhesion molecule Mac-1. LPS promote prostat glandins concomitant to eliciting macrophage migration (Tajima et al., 2008). LPS enhanced the cell adhesion and migration of MDPC-23 cells, a process that requires TLR4, suggesting that TLR4 may play a pivotal role for tooth repair and regeneration (Park et al., 2011). To elucidate whether LPS induces tumor cell migration in PC-3 cells, we performed wound healing assay. Suppression of migration by STS knockdown was recovered by LPS. These results indicated that LPS-induced STS expression increased tumor cell migration.

Innate immune responses triggered by LPS involve the coordinated production of a multitude of inflammatory mediators, especially IL-6 (Sawa et al., 2008; Greenhill et al., 2011). We studied that LPS-induced STS expression are mediated by IL-6 pathway. We confirmed that LPS induced IL-6 expression and IL-6 enhanced STS expression. Because multiple TLR ligands like LPS induce an IL-6 transcriptional response via STAT3 signal pathway, LPS mediated STS expression may be caused by TLR signal pathway (Mäkelä et al., 2009; Walton et al., 2009). The detailed mechanisms of LPS-induced STS expression need to be studied.

In summary, our present study suggests an important mechanism and function of STS expression in PC-3 cells. Our results imply that LPS induces STS expression and tumor cell migration through IL-6 pathway. Future studies need to be conducted to identify the effect of LPS-induced STS expression and to confirm whether LPS induces STS expression and tumor cell migration in other cancer cells and in vivo.

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