Toll-like receptor 9 agonist inhibits ERα-mediated transactivation by activating NF-κB in breast cancer cell lines

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Abstract. Primarily, Toll-like receptor 9 (TLR9) is a specific receptor for microbial DNA in human immune cells. TLR9 has been found to be a promising target in tumor immunotherapy but the direct effect of its activation on tumor cells remains unknown. In this study, we examined the effect of TLR9 ligation on estrogen receptor α (ERα)-mediated transactivation of breast cancer. Luciferase reporter gene assays, RNA interference of TLR9 and Chromatin immunoprecipitation were performed to measure the effect of TLR9 ligation on ERα-mediated transactivation of T47D and MCF-7 cells. Bromodeoxyuridine incorporation assay was used to examine the effect of TLR9 ligation on estrogen (E2)-induced proliferation of breast cancer cells. We also investigated the mechanism for the effect of TLR9 ligation on ERα-mediated transactivation. We found that ERα-mediated transcription via estrogen response element of human breast cancer cells line T47D was significantly suppressed when treated with 17β-estradiol in combination with TLR9 agonist CpG oligonucleotides; ChIP, chromatin immunoprecipitation; BrdU, bromodeoxyuridine

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Abbreviations: TLR9, Toll-like receptor 9; ERα, estrogen receptor α; NF-κB, nuclear factor κB; E2, estradiol; siRNA, small RNA interference; ERE, estrogen responsive element; CpG, CpG oligonucleotides; ChIP, chromatin immunoprecipitation; BrdU, bromodeoxyuridine

Key words: Toll-like receptor 9, estrogen receptor α, breast cancer, nuclear factor κB, estrogen

of decreased ERα-mediated transactivation. Taken together, our data suggest that TLR9 signal pathway, by activating NF-κB, negatively regulates ERα-mediated transactivation of breast cancer. Thus, TLR9 agonist inhibits the proliferation of breast cancer cells in response to estrogen.

Introduction

Toll-like receptors (TLRs) are evolutionarily well-conserved transmembrane proteins that are present in almost all multicellular organisms and recognize patterns specific for microbial components (1). TLR9, specifically recognizing unmethylated CpG oligonucleotides in vertebrates, is localized at endoplasmic reticulum, where it is translocated to the endosomal/lysosomal compartment for ligand recognition (2). On ligand binding, TLRs and their associated adaptors, such as MyD88 and TRIF, recruit intracellular signaling mediators that activate transcription factors, such as nuclear factor κB (NF-κB). The outcome of TLR activation is an immune reaction characterized by increased production of various proinflammatory cytokines and interleukins (3).

Estrogen is involved in the growth and differentiation of diverse tissues (4). As a potent mitogen, it accounts for at least 40% of female breast cancer (5). Effects of estrogen are largely exerted through the activation of estrogen receptor α (ERα), the classic estrogen receptor that belongs to the steroid/thyroid nuclear receptor superfamily of ligand regulated transcription factors (6). Binding of estrogen to ERα leads to transcription of a wide range of genes which stimulate proliferation of mammary cells and the increase in cell division and DNA synthesis elevates the risk for replication errors, which may result in the acquisition of detrimental mutations that disrupt normal cellular processes such as apoptosis, cellular proliferation, or DNA repair (7). Because of the pivotal role of the ERα axis in breast cancer development, targeting ERα or its ligands is a major strategy for breast cancer treatment (8).

Several previous studies have suggested that TLR9 is highly expressed in breast cancer cells (9,10). We wondered whether TLR9 has a role in regulating ERα-mediated transactivation of breast cancer directly. In this study, we demonstrated, for the first time, that TLR9 ligation can significantly inhibit response of breast cancer to estrogen by down-regulating ERα-mediated transactivation.
Materials and methods

Reagents and cell culture. 17β-estradiol and NF-κB inhibitor BAY 11-7082 were purchased from Calbiochem (La Jolla, CA). Human-specific TLR9 agonist CpG oligonucleotides (type C: 5-tcgtgcggattgtaatg-3) and non-CpG control (type C control: 5-tcgtgcggattgtaatg-3) was purchased from InvivoGen (San Diego, CA). T47D and MCF-7 cells were grown in DMEM without phenol red supplemented with 5% FCS treated with dextran-coated charcoal (DCC-FCS), 4.5 g/l D-glucose, non-essential amino acids (100 Amol/l each), 100 units/ml penicillin, 100 Ag/ml streptomycin and 2 mmol/l glutamine at 37°C in a 5% CO₂ atmosphere.

Plasmids. pcDNA3.1/His-Myc-TLR9 was constructed as described (11). Human pSG5-ERα (12) and the 3xERE-TATA-Luc reporter construct (13) were generous gifts from Ingemar Pongratz (Karolinska Institute).

Luciferase reporter activity assays. 3xERE-TATA-Luc reporter construct was used as described (15). Human pSG5-ERα (12) and the 3xERE-TATA-Luc (Invitrogen). Luciferase activity was measured on a Monolight 2000 luminometer using the dual luciferase reporter assay kit (Promega, Madison). The experiments were performed three times in triplicate. The Student's t-test was used to determine the statistical differences between various experimental and control groups. P<0.05 was considered significant. Shown are means ± SD.

RNA isolation and real-time PCR. Cells were grown for 48 h in phenol red-free DMEM supplemented with 5% DCC-FCS serum and treated with 17β-estradiol and CpG oligonucleotides at indicated concentration. DNA was collected with TRIzol (Invitrogen), as previously described (14). cDNA was synthesized with 1 μg of total RNA using first-strand cDNA synthesis kit-RevertAid H Minus (Toyobo) at 99°C and 42°C for 5 and 20 min, respectively. Quantitation of inducible expression of pS2 and PR was performed using SYBR master mix (Toyobo) on an iCycler fitted with an optical assembly unit (Bio-Rad). The relative concentration of pS2 and PR mRNA was calculated, as previously described (15).

TLR9 RNA interference assay. For stable silencing TLR9 in T47D cells, the cells were transfected with TLR9-RNAi or Neo plasmid using Lipofectamine reagent (Invitrogen), as previously described. Chromatin fractions were immunoprecipitated with 1 μg of Erα, MCC-20 (Sant Cruz Biotechnology) antibody, and the immune complexes were recovered using protein A/G Sepharose (Calbiochem) and processed, as described. The immunoprecipitated DNA was amplified by real-time PCR. The primer-specific primers of pS2 and PR have been described (15).

Western blot analysis. A BCA protein assay reagent kit (Pierce) was used to measure protein concentration. Samples containing equal amounts of protein were prepared, separated by 12% SDS-PAGE and transferred to Protran nitrocellulose membranes. The blots were probed with antibodies specific for TLR9 (Cell Signaling), and anti-phospho-NF-κB p65 (Ozyme), with appropriate horseradish peroxidase-conjugated antibodies as secondary antibodies (Cell Signaling). Supersignal West Femto Maximum Sensitivity substrate (Pierce) was used for the chemiluminescent visualization of membrane-bound proteins. The same blots were stripped and rebotted, using anti-actin antibody (Sigma), to show equal loading.

Stable transfection of TLR9 into MCF-7 breast cancer cells. The pcDNA3.1/His-Myc-TLR9 and Neo plasmid were stably transfected into the MCF-7 cells using Lipofectamine reagent (Invitrogen) for 48 h. The cells were then screened under 0.8 mg/ml G418 (Merck, Darmstadt, Germany) for 3 weeks. MCF-7 overexpressing TLR9 was subcloned as MCF-7-TLR9. The expression of TLR9 was verified by Western blot analysis with anti-TLR9 antibody (Cell Signaling).

BrdU incorporation assay. The protocol was performed, as previously described (18). Briefly, T47D or MCF-7 cells were seeded in the absence of E2 for 48 h and then stimulated with 10 nM 17β-estradiol combined with 10 μM CpG oligonucleotides for 24 h. BrdU (Calbiochem) at a final concentration of 10 μmol/l was added 2 h prior to harvesting. Then cells were fixed, permeabilized, incubated with FITC-conjugated antibody and subjected to fluorescence-activated cell sorting (FACS) analysis.

Statistical analysis. The results are given as mean ± SD. Student's t-test was used to calculate differences between the various study groups. The difference was considered statistically significant at P<0.05.

Results

TLR9 agonist inhibits ERα-induced ERE transcriptional activity. ERα-mediated transcriptional activity is the most important mechanism by which estrogen exerts its carcinogenic effect (19). To investigate the effect of TLR9 ligation on ERα-mediated signal pathway, we firstly examined TLR9 expression in two estrogen responsive breast cancer cell lines, T47D and MCF-7 cells. As shown in Fig. 1A, we found that TLR9 was abundant in T47D cells but only slightly expressed in MCF-7 cells. In order to study the effect of TLR9 ligation on ERα-mediated signal pathway, T47D cells were grown in phenol red-free DMEM with 5% DCC-FCS for two days and then treated with 17β-estradiol in combination with CpG...
oligonucleotides. The result of ERE-specific reporter gene assay (Fig. 1B) indicates that CpG oligonucleotides significantly suppressed ERα-mediated transcription. Furthermore, we measured two endogenous target genes transcriptionally activated by ERα with real-time PCR. We found that both pS2 and PR were significantly down-regulated due to TLR9 ligation (Fig. 1C and D). In addition, to confirm that the effect of CpG oligonucleotides on ERα-mediated transactivation was exactly triggered by TLR9 activation, we established a subclone of T47D cells, of which the TLR9 expression was effectively silenced as shown by Western blot analysis (Fig. 2A). In this subclone of T47D cells, we found that CpG stimulation failed to yield significant effect on ERα-mediated transactivation, which validates the indispensable involvement of TLR9 in the inhibition of ERα-mediated transactivation by CpG oligonucleotides (Fig. 2B). These results demonstrate that TLR9 agonist CpG oligonucleotides can inhibit ERα-induced transactivation.

**TLR9 agonist inhibits recruitment of ERα to estrogen-responsive promoter.** To disclose the mechanism of the inhibitory effect of TLR9 agonist on ERα-mediated transactivation, we examined the amount of ERα recruited to estrogen-responsive promoter of pS2 and PR with chromatin immunoprecipitation. We found that 17β-estradiol alone resulted in a dramatic increase of ERα binding with estrogen responsive promoter. When added together with CpG oligonucleotides, ERα bound to the estrogen-responsive promoter of pS2 and PR were significantly decreased (Fig. 3A and B). Those results imply that the inhibitory effect of CpG oligonucleotides on ERα-mediated transactivation may proceeds through suppressing the binding of ERα with its responsive promoter sequences.

**TLR9 agonist down-regulates ERα-mediated transactivation by activating NF-κB.** To further clarify the mechanism underlying reduced amount of ERα recruited to estrogen responsive promoter on TLR9 ligation, we examined the effect of CpG oligonucleotides on total amount of ERα. However, we did not observe any effect of CpG oligonucleotides on ERα protein level (data not shown). Because it has been determined that there might be a reciprocal antagonism between NF-κB and ERα (20), we used BAY 11-7082, a specific inhibitor of NF-κB by inhibiting IκBα activation, to test whether the effect of TLR9 agonist on ERα-induced transactivation was caused by activating NF-κB. We found that CpG oligonucleotides was capable of activating NF-κB within 30 min and BAY 11-7082 could almost completely block the activation of NF-κB (Fig. 4A). More importantly, we found that BAY 11-7082 could abolished the inhibitory effect of CpG oligonucleotides on ERα-mediated transactivation (Fig. 4B). These data suggest that inhibition of TLR9 agonist on ERα-mediated transactivation may be associated with NF-κB activation.

**Inhibitory effect of TLR9 agonist on ERα-mediated transactivation is not restricted to T47D cells.** Because TLR9 was...
only mildly expressed in MCF-7 cells, we first established an MCF-7 subclone stably overexpressing TLR9 as indicated by Western blot analysis (Fig. 5A). Then we examined the effect of TLR9 ligation on ERα-mediated transcriptional activity. We found that consistent with the result of T47D cells, CpG oligonucleotides significantly inhibited ERα-mediated transactivity in MCF-7 cells (Fig. 5B). Once NF-κB activity was blocked with BAY 11-7082, no more significant difference of ERα-mediated transactivation was seen between MCF-7 cells treated with E2 in combination with CpG oligonucleotides and those treated with E2 alone (Fig. 5B). Thus, consistent with the results of T47D cells, TLR9 agonist could also inhibit ERα-mediated transactivation in another breast cancer cell line MCF-7 by activating NF-κB.

TLR9 agonist inhibits E2-induced proliferation of breast cancer cells. To evaluate the biological significance of TLR9 for breast cancer as a consequence of inhibiting ERα-mediated transcriptional activity, we finally examined the effect of TLR9 agonist on E2-induced proliferation of breast cancer cells using BrdU incorporation assay. We found that the relative proportion of proliferative cells were significantly decreased in T47D cells treated with E2 and CpG oligonucleotides compared with E2 alone (Fig. 6A). For MCF-7 cells, though the mean...
proliferative percent of cells in CpG group was lower than non-CpG control, the difference is not statistically significant. However, the proliferation ability was significantly decreased in all subclones of MCF-7 cells forced to overexpress TLR9. It seems that the inhibition of CpG on E2-induced proliferation is proportionate to the over-expression level of TLR9 in MCF-7 cells (Fig. 6B and C). This result suggests that TLR9 ligation may negatively regulate the proliferation of breast cancer in response to estrogen.

Discussion

Infection and the resulting inflammation, are important regulators of tumor progression (21). For example, chemokines and cytokines derived from the immune and inflammatory cells can dramatically affect cancer cell behavior by shaping the host microenvironment. Since TLR9 was recognized as the receptor for unmethylated CpG DNA of most bacteria and DNA viruses, many TLR9-based approaches have been reported to enhance anti-tumor immunity, some of which have even entered Phase III trials (22). However, the direct effect of TLR9 on tumor cells largely remains unknown. Our study, for the first time, investigated the effect of TLR9 on estrogen responsiveness of breast cancer.

Using two estrogen responsive breast cancer cell lines as a model, we found that TLR9 may act as a negative regulator in ERα-mediated transactivtity. We pinned down the molecular mechanism of that effect to the downstream NF-κB activation of TLR9 signal pathway. Consequently we also observed that TLR9 activation may suppress E2-induced proliferation of breast cancer cells.

CpG binds to the TLR9 that resides in the endoplasmic reticulum and recruits MyD88 to initiate signaling (23-25). Adaptor recruitment is followed by phosphorylation of members of IL-1R-associated kinase (IRAK) family and TNFR-associated factor 6 (TRAF6), which further leads to the activation of NF-κB, the major endpoint of the TLR9 signal pathway. Consequently, we also observed that TLR9 activation may suppress E2-induced proliferation of breast cancer cells.

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signaling (26). The activation of I-kBα and its degradation thereafter is an indispensable step for NF-κB activation (27), so we used BAY 11-7082, an inhibitor of I-kBα phosphorylation, to block TLR9-induced NF-κB activation and determined that NF-κB activation is essential in TLR9-mediated negative regulation of estrogen responsiveness of breast cancer cells. In support of our observation, many studies have demonstrated that there might exist a reciprocal antagonism between ERα-mediated signaling pathway and NF-κB activity (20,28-31). This interaction is believed to take place by direct protein-protein interactions (30-32), inhibition of DNA binding (33,34), induction of IκB expression (35), or coactivator sharing (28,29). As a transcriptional nuclear factor activated directly by binding with E2, ERα is the major receptor for estrogen in estrogen-induced carcinogenesis of breast cancer. Our study indicates that TLR9, by activating NF-κB, might negatively regulate ERα-induced transactivation of breast cancer, which provides a novel biological example of the antagonism between NF-κB and ERα. Actually, we speculate that ERα transactivation also likely inhibits the expression of NF-κB regulated genes. So the extent of stimulation of both the ERα and TLR9 (or other TLRs that activate NF-κB) pathway is important for the final biological response. The balance between both pathway is important and the resulting gene expression profile determines whether cells proliferate, are capable of invasion or undergo apoptosis.

Apart from its adjuvant effect in tumor immunotherapy, TLR9 agonist was also recently found to promote tumor invasion in breast cancer, glioblastoma and prostate cancer (10,36). Given the higher expression of TLR9 in invasive breast cancer cells, we speculate that TLR9 may have different biological effect during the different phases of breast cancer development. Not until estrogen-independent stage, might breast cancer evolve to fully up-regulate TLR9 expression and utilize its invasive effect due to its negative effect on estrogen responsiveness of breast cancer at the early hormone-dependent stage according to our study. Or as a multifunctional molecule, the final effect of TLR9 may vary according to specific cellular context. Actually, it has been observed that incubation of breast cancer cells with CpG leads to a decreased viability (10), which is consistent with our data gained with a different method to measure viable cell number.

This adds new evidence to our conclusions that TLR9 might be a novel mechanism in negatively regulating ERα-mediated transactivation of breast cancer, which needs to be further confirmed in primary cancer cells.

In summary, our finding is the first to suggest that TLR9 activation may suppress ERα-mediated transactivation of breast cancer by activating NF-κB. Although the detailed mechanism of this regulation on estrogen responsiveness still needs further exploration, our study implies that TLR9 has its own biologically regulatory effect apart from its adjuvant role in immunotherapy. Considering its direct inhibitory effect on breast cancer and adjuvant effect in tumor immunotherapy, TLR9 may have the potential in the treatment of hormone-related cancers.

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References


