Activation of Toll-like Receptor 5 on Breast Cancer Cells by Flagellin Suppresses Cell Proliferation and Tumor Growth

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Abstract

Increasing evidence showed that Toll-like receptors (TLR), key receptors in innate immunity, play a role in cancer progression and development but activation of different TLRs might exhibit the exact opposite outcome, antitumor or protumor effects. TLR function has been extensively studied in innate immune cells, so we investigated the role of TLR signaling in breast cancer epithelial cells. We found that TLR5 was highly expressed in breast carcinomas and that TLR5 signaling pathway is overly responsive in breast cancer cells. Interestingly, flagellin/TLR5 signaling in breast cancer cells inhibits cell proliferation and an anchorage-independent growth, a hallmark of tumorigenic transformation. In addition, the secretion of soluble factors induced by flagellin contributed to the growth-inhibitory activity in an autocrine fashion. The inhibitory activity was further confirmed in mouse xenografts of human breast cancer cells. These findings indicate that TLR5 activation by flagellin mediates innate immune response to elicit potent antitumor activity in breast cancer cells themselves, which may serve as a novel therapeutic target for human breast cancer therapy. Cancer Res; 71(7); 2466–75. ©2011 AACR.

Introduction

Toll-like receptors (TLR) are membrane-bound receptors that play key roles in both the innate and adaptive immune systems, particularly in inflammatory responses against pathogen infection (1–3). These receptors are primarily expressed on innate immune cells and recognize conserved pathogen-associated molecular patterns (4–7). TLRs can also recognize some endogenous ligands (8, 9). Activation of TLRs might play a role in cancer progression and development (10, 11); however, activation of different TLRs might display completely different results. Several studies have shown that activation of TLR4 signaling by LPS protects tumor cells from immune attack and thus promotes tumor growth (12–14). Trigerring of TLR9 on cancer cells has been shown to protect cancer cells against TRAIL (TNF-related apoptosis-inducing ligand)-induced apoptosis and promote tumor cell proliferation (15). In contrast, activation of TLR3 induced an anti-proliferative signaling in human breast cancer and melanoma (16, 17). Thus, the function and biological importance of TLRs expressed on various tumor cells seem complex.

Unlike other TLR family members, TLR5 is not expressed on mouse macrophages and conventional dendritic cells (DC). Instead, TLR5 expression is high in intestinal epithelial cells and lamina propria DCs (18, 19). It has been shown that TLR5 is highly expressed on gastric carcinoma cells (20, 21). Recent evidence has shown that activation of TLR5 by its ligand flagellin elicits potent antitumor activity and thus inhibits colon tumor growth in vivo (21). In contrast, Sfondrini and colleagues showed that early administration of flagellin at the time of implanting mouse mammary cells resulted in increased tumor growth (22). Thus, the different results of these studies reveal the unique responses and susceptibilities to flagellin stimulation in different cancer cells. To date, the specific function and detailed mechanism of TLR5 signaling pathways in breast cancer cells are still poorly understood.

Here, we report a comprehensive characterization of TLR5 expression and TLR5 signaling in human breast cancer cells themselves. We found that TLR5 was highly expressed in human breast carcinomas and TLR5 signaling was overly functional in human breast cancer cells. Remarkably, triggering of TLR5 signaling pathway by flagellin in breast cancer cells inhibits cell proliferation and elicits the potential antitumor activity in mouse xenografts of human breast cancer.

Materials and Methods

Mice and reagents

Six-week-old female athymic nude mice (Foxn1nu/Foxn1nu) were purchased from Harlan. Salmonella typhimurium flagellin was purchased from InvivoGen and used at the indicated concentrations. Antibodies against IκBα, phospho-IκBα,
STAT3, cyclin B1, cyclin D1, cyclin E2, and p21Waf1/Cip1 were subjected to a human cytokine array, as previously described (21, collected and equal amounts of the supernatants were sub-
from Ray Biotech. MCF-7 cells were treated with flagellin (0.1
Cytokine microarray analysis
RayBio Human Cytokine Antibody Array V was purchased from Ray Biotech. MCF-7 cells were treated with flagellin (0.1 μg/mL) or PBS (control) for 24 hours. Then, supernatants were collected and equal amounts of the supernatants were subjected to a human cytokine array, as previously described (21, 25, 26). The cytokine expression was determined by measuring the density of each spot, using TotalLab TL100 software.

Cell-cycle analysis
Fluorescence-activated cell sorting (FACS) was performed for cell-cycle analysis. Flagellin-treated or nontreated cells in different days were detached with trypsin and stained with FITC BrdU Flow Kit (BD Pharmingen). Flow cytometry was performed by using a FACS Calibur system, and data were analyzed with Flow Jo software (Tree Star).

Soft agar colony formation
Breast cancer cells (5 × 10^3) were plated in 0.35% low melting point agarose/growth medium onto 6-well plates with a 0.6% agarose underlay. About 1.5 mL growth medium with or without flagellin at indifferent concentrations was added on the top of agarose, and the medium was changed every 3 to 4 days and incubated at 37°C in a humidified incubator for 14 days. At the end of the treatment, the cells were washed with PBS and incubated in a solution of 0.005% crystal violet and 10% formalin for 10 minutes and then rinsed with water. The integrated density of the colonies on each plate was determined using TotalLab TL100 software.

Xenograft model of human breast cancer
To establish breast cancer xenografts in nude mice, MDA-MB-468 cells (5 × 10^3) were mixed with Matrigel and injected under aseptic conditions into the mammary fat pads of nude mice (n = 6 for each group). For MCF-7 xenografts, ovariecto-
mized mice were first implanted subcutaneously with 60-day estradiol-releasing pellets (1.7 mg per pellet from Innovative Research) before 24 hours of cell injection. Then, MCF-7 cells (2 × 10^7) were mixed with Matrigel and injected into both fourth mammary fat pads of ovariectomized athymic nude mice. For peritumoral flagellin treatment, 10 days after injecting cells into the mammary fat pads of nude mice, we administered flagellin solution (1.0 μg in 50 μl) around the tumor site (1 injection/every 2 days for 3–4 weeks). For intravenous flagellin administration, 1 month later, MDA-MB-468 xenografts were fully established and each mouse was injected with regulated flagellin solution (2 μg in 100 μl) through the tail vein (1 injection/every 7 days for 6 weeks). The control group was injected with saline solution by the same method. The tumor was monitored and evaluated every 2 and 7 days for peritumoral and intravenous flagellin administration, respectively. Tumors were measured in 2 dimensions, and volume was calculated according to the formula: V = 0.5(length × width^2).

Results
Expression of TLR5 in breast cancer
Although TLRs are mainly expressed and activated on innate immune cells such as macrophages and DCs, TLRs may be functional in other cell types. To investigate the expression and potential function of TLR5 in breast cancer epithelial cells, we performed immunohistochemical staining on tissue arrays containing normal breast tissues and breast carcinomas (n = 75) with different subtypes, stages, and
grades. We found that TLR5 was expressed in normal breast duct epithelium but not in the fibrofatty tissue of normal breast samples (Fig. 1A). Of all the breast cancer tissues tested, 60 tumor samples (80%) were positive for TLR5 and the majority of TLR5-positive cases were high-grade ductal carcinomas (Fig. 1A and Supplementary Table S1). Specifically, TLR5 was highly expressed in most of invasive ductal carcinomas (IDC) and moderately expressed in medullary carcinomas and invasive lobular carcinoma (Fig. 1A). The staining was localized in the cytoplasm and on the cell surface, which was similar to the localization of TLR4 in ovarian cancer cells (14). However, no staining of TLR5 was observed in breast mucinous adenocarcinomas (Supplementary Table S1).

We then analyzed the expression of TLR5 in breast cancer cell lines including MCF-7, MDA-MB-468, SKBR3, T47D, MDA-MB-231, and MDA-MB-435 cells. Immunofluorescence confirmed the expression of TLR5 in these 6 cell lines (Fig. 1B). Similarly to our observation on the tissue sections, TLR5 was distributed both inside the cell and on the cell surface in MCF-7, MDA-MB-468, SKBR3, and T47D cells. However, TLR5 was localized only in the cytoplasm in MDA-MB-231 and MDA-MB-435 cells (Fig. 1C). The expression of MyD88, which is a critical adaptor protein in TLR5 signaling pathway, was also detected in these breast cancer cell lines by Western blotting (Supplementary Fig. S1).

**Activation of TLR5 signaling pathway by flagellin in breast cancer cells**

We next asked whether TLR5 was also functional to activate TLR signaling pathway in breast cancer cells. To address this question, the 6 breast cancer cell lines were transfected with a construct containing a luciferase reporter under the control of NF-κB response element and stimulated with the TLR5 natural ligand flagellin in different concentrations. As shown in Figure 2A, the NF-κB luciferase reporter activities in MCF-7, MDA-MB-468, SKBR3, and T47D cells were increased in a dose-dependent manner after challenging with flagellin. However, the other 2 cell lines, MDA-MB-231 and MDA-MB-435, seemed to be unresponsive to flagellin stimulation.
To verify that the activation of NF-κB following flagellin incubation was due to TLR5 activation, we first preincubated MCF-7 cells with an anti-TLR5 blocking antibody in different concentrations and then challenged the cells with flagellin. We found that the flagellin-induced NF-κB luciferase reporter activity can be suppressed by anti-TLR5 blocking antibody in a dose-dependent manner (Fig. 2B). Then, we further established an MCF-7 tumor cell line stably expressing TLR5 short hairpin RNA (shRNA; Supplementary Fig. S2). The phosphorylation levels of IκB, ERK, and JNK after flagellin stimulation were increased in MCF-7 wild-type cells but not in TLR5 knocked down cells (Fig. 2C). We next asked whether flagellin/TLR5 signaling pathway in MCF-7 cells can activate the expression of cytokines and/or chemokines in canonical TLR downstream signaling. Interestingly, we found that upon flagellin stimulation, there was a striking induction of TNF-α, interleukin (IL)-1β, IL-6, and IL-8 mRNA in MCF-7 cells but not in TLR5 knocked down cells (Fig. 2D). Thus, these data collectively suggested that flagellin activated TLR5-dependent signaling pathway in breast cancer cells. To further analyze the profile of cytokines and chemokines induced by flagellin in breast cancer cells, the flagellin-stimulated tumor cell supernatant was used for cytokine microarray. Flagellin substantially increased the production of several chemokines including epithelial cell–derived neutrophil–activating peptide-78 (ENA-78), macrophage inflammatory protein (MIP)-3α, monocyte chemotactic protein-1 (MCP-1), macrophage-derived chemokine (MDC), IL-6, Gro-α, and osteoprotegerin (Fig. 2E). Interestingly, the induced chemokines upon flagellin stimulation are mostly involved in leukocyte recruitment (27).
Thus, activation of TLR5 by flagellin initiated TLR signaling pathway transduction and induced the secretion of several cytokines and chemokines in breast cancer cells.

**Flagellin inhibits tumor cell proliferation and colony formation in vitro**

To investigate the role of flagellin/TLR5 signaling on tumor cell proliferation, a BrdUrd (bromodeoxyuridine) incorporation assay was performed. MCF-7 cells were treated with flagellin (0.1 μg/mL) for periods up to 3 days and the number of BrdUrd-incorporated cells was determined daily by flow cytometry. As shown in Figure 3A, the percentage of BrdUrd-positive cells was decreased from 46% to 32% after 1 day of flagellin treatment, suggesting that flagellin inhibited cell proliferation. Moreover, after 2 and 3 days of treatment, the percentage of BrdUrd-positive cells was further decreased and maintained up to 13% of the cells. However, we could not detect the population of cells in sub-G₀/G₁ phase (data not shown), indicating that flagellin did not induce apoptosis.

To further characterize the effect of flagellin on tumor cell proliferation, a colony formation assay was performed in MCF-7 cells after challenging with flagellin with increased concentrations. This experiment was designed to assay the ability of cells to grow unattached to a surface and therefore suspended in agar. Thus, it could be used to measure the tumorigenicity of tumor cells. As shown in Figure 3B, the colony formation ability of MCF-7 was significantly decreased by flagellin in a dose-dependent manner. This phenomenon was TLR5 dependent because knocking down TLR5 in the cells could block the suppressive activity of flagellin (Fig. 3C). Flagellin also inhibited colony formation of MDA-MB-468, SKBR3, and T47D cells in higher concentration (10 μg/mL; Fig. 3D). However, for the other 2 flagellin nonresponsive cells MDA-MB-231 and MDA-MB-435, no significant decrease of colony number was observed even in the presence of flagellin with the highest concentration. Thus, the activation of TLR5 by flagellin inhibited proliferation and colony formation of breast cancer cells *in vitro*.

**Soluble factors mediate the antiproliferation effect of flagellin**

To gain more insights into the molecular mechanism in the antiproliferation effect of flagellin on breast cancer cells, we analyzed the expression level of various proteins involved in cell-cycle regulation upon flagellin treatment. We found that the levels of cyclin B1, cyclin D1, and cyclin E2 were significantly decreased after flagellin treatment. A marginal increase of the CDK inhibitor p27 was also observed at early stage of flagellin treatment (Fig. 4A). To analyze whether treatment of MCF-7 cells with flagellin results in the secretion of soluble factors that might contribute in an autocrine fashion to the growth inhibitory activity, cells were treated for 4 hours with flagellin, washed, and replenished with fresh medium for another 6 hours. Then, the medium was collected as conditioned medium. We found that conditioned medium from MCF-7 cells treated with flagellin decreased the colony size in...
comparison with the untreated control or the conditioned medium from flagellin-treated TLR5 knocked down cells (Fig. 4B). Thus, at least, in part, flagellin-induced soluble factors mediate the antiproliferation effect on breast cancer cells. To further investigate the molecular mechanisms in activation of cytokine signaling by flagellin-induced soluble factors in breast cancer cells, the phosphorylation levels of STATs were analyzed by Western blotting after flagellin treatment. As expected, the phosphorylation of STAT1 and STAT3 was induced by flagellin in later stage, suggesting that cytokine-induced signaling pathways were activated in breast cancer cells (Fig. 4C). Thus, these data indicate that the soluble factors induced by flagellin in an autocrine fashion are involved in an antigrowth effect of flagellin on breast cancer cells.

**Activation of TLR5 by flagellin inhibits tumor growth in vivo**

To determine the effect of flagellin on tumor growth in vivo, we first used MCF-7 breast cancer cell xenograft mouse model. After the palpable tumors were established, the mice were injected with or without flagellin solution (1.0 μg in 50 μL) around the tumor site and administered every 2 days. As shown in Figure 5A, tumors in the control group increased from 52 ± 13 to 428 ± 178 mm³ whereas tumors in the flagellin-treated group increased only from 48 ± 12 to 162 ± 35 mm³. The average weight of tumors from the control group was 425 ± 11 mg, whereas the average weight in flagellin-treated group was only 168 ± 5 mg (Fig. 5A), suggesting that flagellin retarded tumor growth in the xenograft mouse breast tumor model. Flagellin had no significant effect on the body weight of mice, indicating low toxicity of flagellin at the test dosage and conditions (data not shown). However, in the mouse inoculated with TLR5 shRNA–expressing MCF-7 cells, although the cells maintained similar growth rate in vivo compared with wild-type cells, flagellin failed to inhibit tumor growth in vivo (Supplementary Fig. S3), indicating that TLR5-dependent antitumor activity is involved in this flagellin response.

To gain more evidence with other breast cancer cells, we established MDA-MB-468 xenograft tumor model, in which the tumors are estrogen independent (28). Flagellin also significantly retarded tumor growth and decreased tumor weight when administrated with flagellin around the tumor sites (Fig. 5B). To access the antitumor activity of flagellin in mice with established larger tumor burden, we further allowed MDA-MB-468 tumor to grow up to 80 to 100 mm³. Then, we intravenously administrated flagellin at 2 μg per mouse and injected every week. We found that the systemic administration of flagellin also inhibited tumor growth and decreased tumor weight (Fig. 5C). Because systemic administration of flagellin might also elicit apoptotic signaling and systemic inflammatory responses in mice (29), we further analyzed the potential toxicity of flagellin by intravenous administration. Similar to a previous report, systemic administration of flagellin induced serum production of IL-6 in early time points. However, the level of IL-6 returned to baseline level 24 hours later (Supplementary Fig. S4A). Moreover, histologic examination of the livers and spleens revealed no obvious inflammation, necrosis, or fibrosis between control and treatment.
groups (Supplementary Fig. S4). Body weights, as well as spleen, lung, and heart weights, were unaffected in flagellin-treated groups (Supplementary Fig. S4; data not shown). Thus, these data collectively indicate that flagellin exhibits minimal to toxic effects in normal animals when systemically administered by intravenous injection at the test dosage.

**TLR5 activation by flagellin leads to increased tumor necrosis and neutrophil infiltration in vivo**

To further analyze the mechanisms for TLR5/flagellin signaling pathway–mediated tumor suppression in vivo, the histopathology of xenograft tumor was then investigated by hematoxylin and eosin (H&E) stain. As shown in Figure 6A, examination of tumor sections revealed that tumor necrosis and leukocytes infiltration were increased in the flagellin-treated group. Moreover, immunohistochemical staining for cell proliferation marker PCNA in tumor sections indicated that although PCNA was noticeably higher in MCF-7 cell-injected mice, the expression of this protein was inhibited by treatment of flagellin (Fig. 6B). To determine the infiltrated cell type in flagellin-treated tumors, we performed immunohistochemistry with tumor xenografts by using antibodies against neutrophil- and macrophage-specific marks (Gr-1 and F4/80, respectively). There were no obvious F4/80-positive cells in tumor sections either from control or flagellin-treated groups (Supplementary Fig. S5). However, Gr-1–positive cells
were significantly increased around necrotic areas of tumor sections from flagellin-treated groups (Fig. 6B), indicating that TLR5 activation by flagellin leads to increased neutrophil infiltration in vivo. As neutrophils have been shown to exhibit potent antitumor activity in tumor microenvironment (21, 30, 31), these data indicate that the increase of neutrophil infiltration could be an important mechanism for TLR5/flagellin-induced antitumor activity in vivo.

Discussion

Recent evidence suggests that TLRs, key receptors in innate immunity, play a role in tumorigenesis but different TLRs exhibit either antitumor or protumor activities (11). In this study, we investigated the expression and function of TLR5 in breast cancer epithelial cells. We found that TLR5 was specifically expressed in the ductal epithelium of normal breast tissues, implicating a role of TLRs in inflammatory responses of the mammary tissue against pathogen infection (32). Interestingly, we also found that most of human breast cancer samples expressed TLR5 and there was an enhanced expression of TLR5 in some subtypes of breast carcinomas. Because the endogenous ligands for TLR5 in tumors are not known, we used flagellin, the natural ligand of TLR5, to examine the activity of TLR5 signaling in breast cancer cells. Similar to other reports in immune sentinel cells and fibroblasts (33, 34), activation of TLR5 by flagellin in breast cancer cells induced the secretion of proinflammatory cytokines and chemokines. It is worthwhile to note that not every breast cancer cell line we tested was responsive for flagellin stimulation and there was no simple correlation between TLR5 expression and flagellin sensitivity in these breast cancer cell lines. Because we also observed the differences in subcellular localization of TLR5 between flagellin-sensitive and nonsensitive cell lines, the surface localization of TLR5 in flagellin-sensitive cells might explain why these cells are highly responsive for flagellin stimulation, which is similar to other TLRs (35).

In fibroblasts and DCs, TLR5/flagellin signaling pathway has been shown to promote cell proliferation and prevent apoptosis by inducing p27 degradation, which can be antagonized
by type I IFNs (33, 34). In contrast, our results show that activation of TLR5 by flagellin in breast cancer cells inhibits cell proliferation and anchorage-independent growth. As cell-specific TLR5 signaling might account for our different results, we also observed significant decrease of cell cyclin proteins with marginal increase of p27 levels in breast cancer cells upon flagellin treatment. We found that flagellin-induced soluble factors exhibited suppressive activity on tumor cell proliferation, suggesting that the effect of flagellin on cancer cell proliferation is not primarily mediated by the direct intracellular mechanism of TLR5 as previously reported in fibroblasts and DCs (33, 34). In these flagellin-induced soluble factors, several factors such as MIP-3α and IL-6 have been shown to inhibit proliferation of various types of cells including breast cancer cells (36, 37). However, in other scenarios, these factors have also been reported to promote cell proliferation and tumor growth (38, 39). The different results are dependent upon different cell types and different combinations with other factors in tumor microenvironment (40). Moreover, we observed flagellin-induced phosphorylation of STAT1 and STAT3 in breast cancer cells at late stages, further suggesting that cytokine-induced signaling pathways were activated in breast cancer cells after flagellin treatment. As a key mediator of cytokine-induced gene expression, STAT1 is activated by many cytokines including type I and type II IFNs (41). While we failed to detect the secretion of type I and type II IFNs upon flagellin treatment for 24 hours (data not shown), the phosphorylation of STAT1 suggests that other factors may be involved in cytokine-induced signaling pathway upon flagellin treatment and initiate a unique TLR5-signaling pathway in breast cancer cells to inhibit cell growth.

As a receptor highly expressed in intestinal epithelial cells, including colon cancer cells, TLR5 has been shown to mediate antitumor activity in a mouse xenograft model of human colon cancer (21). Consistent with this study, our data show that administration of flagellin in vivo retards tumor growth in the xenograft mouse model of human breast cancer. As we observed in vitro, flagellin induced secretion of endogenous cytokines and chemokines including MIP-3α, ENA-78, GRO-α, and MDC. These factors have been shown to attract monocytes, neutrophils, and lymphocytes to the site of infection (23–27). In agreement with the report by Rhee and colleagues, which showed that infiltration of neutrophils induced by flagellin exert potent antitumor responses in a mouse xenograft model of human colon cancer, we observed increased tumor necrosis and leukocyte infiltration upon flagellin treatment either by peritumoral or intravenous injection. Interestingly, a recent report also observed that flagellin induced expression of several chemokines to recruit neutrophils and monocytes in vitro in prostate cancer cells (42). Thus, these data suggest that the flagellin-induced proinflammatory factors further recruit neutrophils at the tumor site to modulate the growth of breast tumors.

Dynamic interaction between tumor and tumor microenvironment is essential for tumor growth, angiogenesis, and metastasis (43). Activation of TLRs on tumor cells has been shown to modulate tumor microenvironment and elicit pro- or antitumor activities. Manipulating TLR signaling to enhance its antitumor effects in tumor microenvironment has been investigated and reported by several studies (44, 45). In this study, we identified that TLR5 was highly expressed and activated in breast carcinomas and cancer cells. Furthermore, we showed that activation of TLR5 by flagellin in breast cancer cells modulate the production of proinflammatory cytokines to elicit a potent antitumor activity in breast cancer, which may serve as a novel therapeutic target for human breast cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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