Abstract. - OBJECTIVE: We studied the involvement of the TLR4/NF-κB pathway in the growth of colon cancer using human colon cancer specimens, human colon cancer SW620 cell line, and nude mouse xenograft model.

MATERIALS AND METHODS: Tissue samples were surgically harvested. The human colon cancer SW620 cell line was pre-treated with the TLR4 inhibitor CRX-526 and stimulated with LPS. The nude mouse xenograft model was established by subcutaneous injection of SW620 cells with or without CRX-526, the TLR4 inhibitor. The study outcomes were mRNA and protein expressions of TLR4 and NF-κB in specimens of colon cancer and adjacent normal tissue, SW620 cell line, and xenografts. In addition, we studied production of interleukin (IL)-6 and IL-8 in culture supernatants of LPS-stimulated SW620 cells.

RESULTS: Both mRNA and protein expressions of TLR4 and NF-κB in colon cancer specimens were higher than those in the adjacent normal tissue. LPS up-regulated expression of TLR4 and NF-κB, and stimulated production of IL-6 and IL-8 in SW620 cells. These effects were attenuated by CRX-526. TLR4 inhibition was also effective in the nude mouse xenograft model, as tumor sizes were significantly smaller, and expressions of TLR4 and NF-κB significantly lower, in the mice treated with CRX-526.

CONCLUSIONS: The TLR4/NF-κB signaling pathway is activated in colon cancer, causing production of IL-6 and IL-8, and, thereby, tumor growth and metastasization. Inhibition of TLR4 attenuates up-regulation of NF-κB and inhibits tumor growth.

Key Words: Colon cancer, TLR4, NF-κB, IL-6, IL-8, CRX-526.

Introduction

Colorectal cancer (CRC) is one of the most common tumors and the second leading cause of cancer deaths. There is an increasing trend in the CRC incidence rates. While the quality of life and survival rates of patients with CRC improved due to advanced surgery and chemotherapy, most patients with advanced CRC poorly respond to comprehensive therapies at the time of initial diagnosis and have high mortality rates. Therefore, early diagnosis is crucial for more effective treatment of CRC. From the literature, it appears that intestinal and systemic non-specific inflammation contributes to the development of CRC. Specifically, chronic rectal inflammation is associated with malignant transformation of intestinal mucosa. Furthermore, inhibition of inflammation, such as by fish oil, induces apoptosis in colon cancer cells via inhibition of the release of inflammatory cytokines. In addition, chronic rectal inflammation leads to accumulation of free radicals and suppression of immune surveillance of tumors and may, thus, promote intraepithelial neoplasia. There are also reports that inflammation instigated through Toll-like receptors (TLRs) is involved in human cancers. TLRs are Pattern Recognition Receptors that recognize invading pathogens to enable immune responses by both innate and adaptive immunity. Currently, drugs targeting TLR signaling pathways are tested in clinical settings for inflammatory and autoimmune diseases, as well as for cancer. However, it was unclear whether TLRs are involved in the development of CRC, and what is the potential efficacy of TLR inhibition against these cancers.

TLR4 is the TLR receptor that is activated by bacterial lipopolysaccharide (LPS). Once activated, TLR4 stimulates downstream Mitogen-Associated Protein Kinases and Nuclear Factor (NF)-κB signaling pathways and up-regulates expression of genes encoding inflammatory cytokines. The involvement of TLR4 in colon cancer was documented in previous studies. For example, it was shown that activation of TLR4 signaling
pathway facilitates migration of colon cancer cells, and preserves tumor cells from immune surveillance and apoptosis. Furthermore, down-regulation of TLR4 expression by rapamycin inhibits TLR4/NF-κB signaling pathway and promotes cell death of colon cancer.

Little is known about association between TLR4 and the differentiation degree of CRC. Also, the anti-tumor activity of TLR4 inhibitors was unclear. To address these questions, we examined mRNA and protein expression of TLR4 and NF-κB in specimens of colon cancer and normal tissues. We also evaluated the association between TLR4 and NF-κB expressions, and pathological and clinical features of CRC. In addition, we studied how cytokine production in a SW620 colon cancer cell line is up-regulated as a result of activation of TLR4/NF-κB signaling pathway. Finally, we tested the anti-inflammatory and anti-tumor effects of TLR4 inhibitor CRX-526 in SW620 cells and in nude mouse xenograft model.

Materials and Methods

Human Specimens

All work with human specimens was approved by the Human Ethics Committee of Hubei province, China. A total of 63 colon cancer tissue samples and 63 adjacent normal tissue specimens were surgically harvested in our Hospital from February 2011 to December 2013. The adjacent normal tissue specimens were ≥2 cm away from the cancer tissue and were pathologically confirmed to be free of cancer cell infiltration. All patients did not receive radiotherapy or chemotherapy. The patients comprised of 35 men and 28 women. Among the patients, 27 were found to have lymph node metastasis, while 36 were metastasis-free. Furthermore, 23, 17, 20, and 3 patients had, respectively, well, moderately, and poorly differentiated tumors, and other types of differentiation. According to the tumor stage, the tumors were staged at the Dukes stages A, B, C, and D in 18, 14, 22, and 9 patients.

Cell Culture

The human colon cancer SW620 cell line was cultured until 80% confluency. The cells were trypsinized and counted, and cell concentration was adjusted to 2 × 10^3/ml. The cells were seeded and cultured on culture plates, and synchronized in serum-free medium for another 24 hours. Then, the cells were washed with PBS (phosphate buffered saline), LPS (lipopolysaccharide) was added at different concentrations (0, 0.01, 0.5, and 1 mg/L), and cells were incubated for 0, 1, 3, 6, or 12 hours. Control cells were treated with RPMI 1640 instead of LPS.

In the TLR4 inhibition experiments, we utilized the inhibitor CRX-526 (Invitrogen, Carlsbad, CA, USA) at 5, 10, or 20 µg/ml, or 20 µg/ml of anti-TLR4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). These inhibitors were added simultaneously with LPS.

Animals

All animal work was approved by the Animal Ethics Committee of Hubei Province, China, and was performed in accordance with the “Guide for the Care and Use of Laboratory Animals” of the National Institutes of Health. Thirty 28-day-old male athymic nude mice were purchased from Beijing HFK Bioscience Co., Ltd (Beijing, China). The mice were kept under pathogen-free conditions and had free access to both food and water. The mice were then randomized into study and control groups (20 mice per group). Culture of 1 × 10^6 SW620 cells stimulated with 0.5 mg/L of LPS for 6 hours was subcutaneously injected at the upper part of the forelimb. Physical activities and eye movement of experimental mice were observed. Four weeks later, mice in the study group received injections of CRX-526 (50 µg) at multiple locations for 5 consecutive days. Animals in control group did not receive injections of CRX-526. Eight weeks later, the animals were euthanized under anesthesia, their tumors were removed, and minor (a) and major (b) diameters of the tumor were measured using a caliper. The tumor volume was calculated using a formula: (a^2 × b)/2. Part of tumor tissue was harvested for real-time PCR to quantify mRNA expressions of TLR4 and NF-κB, and for Western blot analysis to quantify the expressions at protein level.

Real-time PCR

Total RNA was extracted from the human colon cancer tissues and adjacent healthy tissues, SW620 cells, and xenografts of nude mice using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocols. Total RNA (1 µg) was reverse transcribed into cDNA using an RT kit (Toyobo, Kurashiki, Japan). The cDNA was diluted 1:10 and used as
template for real-time PCR. The primers were synthesized by Invitrogen and were as follows: TLR4 (upstream primer) 5’-AGCCACCTCTCTACCTTAAATTTGA-3’, (downstream primer) 3’-CCGAHTGTTAGAATAGGTAGTAAGAG-5’, and β-actin (endogenous control; upstream primer) 5’-CCAGAAGTTTTCAGCATACTCC-3’, (downstream primer) 3’-TGGCATGTAGCCGAGGCT-5’. The □□ CT method was used for relative quantification of mRNA expression.

Western Blot Analysis

Proteins from human colon cancer tissues and adjacent healthy tissues, SW620 cells, and xenografts were extracted using a protein extraction kit (Thermo, Rockford, IL, USA) according to the manufacturer’s protocols. Protein concentration was quantified using bicinchonic acid (BCA) protein assay (Thermo). After denaturation, lysates (30 µg protein/well) were subjected to SDS-PAGE electrophoresis and electroblotted at constant current of 300 mA. Primary antibodies (anti-TLR4 and anti-NF-κB, 1:500 dilution; anti-β-actin, 1:1000 dilution; all antibodies from Santa Cruz) were added and incubated with membranes overnight at 4ºC. After this, membranes were stained with an horseradish peroxidase (HRP)-labeled secondary antibody (goat anti-rabbit IgG antibody at 1:5000 dilution; Thermo) for 1 hour at room temperature. Then, chemiluminescent detection (ECL-Plus, Amersham, Exeter, UK) and chemoluminiscence-sensitive film were used to visualize protein bands.

Immunofluorescence

The cells were grown on coverslips in 6-well tissue culture plates. At confluency, the cells were rinsed 3 × 5 min with PBS, followed by an overnight incubation with an anti-TLR4 antibody. The cells were washed with PBS and incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG antibody at 1:5000 dilution; Thermo) for 1 hour at room temperature. Then, chemiluminescent detection and chemoluminiscence-sensitive film were used to visualize protein bands.

ELISA

The IL-6 and IL-8 ELISA kits (Shanghai Yanjibio, Shanghai, China) were used according to manufacturer’s protocols.

Statistical Analysis

The data were analyzed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Quantitative data were presented as mean ± SEM from at least three independent experiments. The differences were tested using the Student’s t test or one-way ANOVA test with Dunnett’s analysis. The difference at the p value of < 0.05 was considered statistically significant.

Results

Up-Regulation of TLR4 mRNA and Protein Expression in Colon Cancer Tissue Specimens

As shown in Figure 1, TLR4 (both mRNA and protein) expression was significantly higher in colon cancer tissues compared with adjacent normal tissue. We next tested how TLR4 expression correlated with the degree of differentiation and stages of colon cancer. For this, we compared the levels of TLR4 mRNA expression between patients with or without lymph node metastasis. We did not find a significant difference between patients in these two groups (Figure 1A). However, when the patients were divided according to the degree of differentiation and Dukes’ classification of colon cancer, we observed that TLR4 mRNA expression was higher in the poorly differentiated tumors compared with the well- or moderately differentiated tumors (Figures 1B and C). TLR4 expression was also different throughout the stages of the tumor. Thus, expression of both mRNA and protein in Dukes A tumor was significantly lower than that in other stages (Figure 1E). However, there was no statistically difference in TLR4 expression among Dukes B, -C and -D tumors.

NF-κB p65 subunit mRNA and protein expression in colon cancer tissue specimens

NF-κB is an important downstream target of TLR4 signaling pathway. We next tested expression of one of the subunits of NF-κB, p65. We observed that both mRNA and protein levels of NF-κB p65 were highly expressed in colon cancer tissue, as compared with adjacent normal tissues. The difference in expression levels between cancer and normal tissue was statistically significant (Figures 2A–C). Further, similar to TLR4 expression in the colon cancer specimens, expression of NF-κB in patients with lymph node metastasis was significantly higher than in those without metastasization (Figure 2A). In addition, NF-κB expression levels in well-differentiated tumors were significantly lower than in poorly or moderately differentiated tumors (Figure 2B).
The TLR4/NF-κB signaling pathway mediates the growth of colon cancer

Decreased levels of NF-κB mRNA and protein were found in the tumors with the Dukes A stage (Figures 2C and F). There was no significantly difference of NF-κB expression among other stages of colon cancer, except for the Dukes A tumor (Figures 2C and F).

**LPS Up-Regulates Expression of TLR4 mRNA and Protein in SW620 Cells**

The above in vivo data revealed increased expression of TLR4 and NF-κB, both mRNA and protein, in the colon cancer. Therefore, we tested how the expression of TLR4 is stimulated by LPS in the colon cancer SW620 cells. As shown in Figure 3, expression of TLR4 (both mRNA and protein) was up-regulated by LPS in these cells in a time- and dose-dependent manner. Specifically, the changes reached the peak after 6 hours exposure, at dose of 0.5 mg/L. Immunofluorescence experiments showed the localization of TLR4 mainly being in the cytoplasm and membrane, and confirmed up-regulated TLR4 expression after LPS exposure (Figure 3E).

**TLR4 Inhibition Attenuates the LPS-Induced Up-Regulation of NF-κB p65 in SW620 Cells**

Next, we tested the effects of the TLR4 inhibitor CRX-526 on LPS-induced up-regulation of NF-κB p65 (mRNA and protein) in the colon
cancer SW620 cells. Prior to LPS exposure, cells were pre-treated for 1 hour with different concentrations of TLR4 inhibitor CRX-526 or with TLR4 neutralizing antibody (20 mg/ml). After 6 hours stimulation with LPS, cells were harvested for the analyses. The low dose of 5 µg/ml of CRX-526 was not able to attenuate LPS-induced up-regulation of NF-κB p65 mRNA in SW620 cells (Figure 4A). However, higher doses (10 and 20 µg/ml) of CRX-526 did attenuate this up-regulation, similarly to TLR4 neutralizing antibody (Figure 4A). In the absence of LPS, NF-κB p65 mRNA expression did not differ between control cells and cells treated with 20 µg/ml CRX-526 (Figure 4A). Based on the above findings, we chose the dose of 20 µg/ml of CRX-526 as the dose for Western blot analysis. In line with mRNA data, we observed that CRX-526 attenuated the LPS-upregulated NF-κB p65 at the protein level (Figure 4B).

**TLR4 Inhibition Down-Regulates the LPS-Induced Cytokine Production in SW620 Cells**

Inflammatory cytokines IL-6 and IL-8 are downstream products of the TLR4/NF-κB sig-

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**Figure 2.** Changes of NF-κB mRNA and protein expressions in tissues of human colon cancer. **A,** mRNA expression in colon cancer tissue specimens from patients with and without lymph node metastasis. N: adjacent normal tissue; T: colon cancer tissue; NL: colon cancer tissue without lymph node metastasis; L: colon cancer tissue with lymph node metastasis; *p* < 0.05 vs. N. *p* < 0.05 vs. NL. **B,** mRNA expression in colon cancer tissue specimens with different differentiation degrees. N: adjacent normal tissue; H: well-differentiated colon cancer tissue; M: moderately-differentiated colon cancer tissue; L: poorly-differentiated colon cancer tissue; *p* < 0.05 vs. N. *p* < 0.05 vs. H. **C,** mRNA expression in colon cancer tissue specimens at different Dukes stages. N: adjacent normal tissue; *p* < 0.05 vs. N. *p* < 0.05 vs. Dukes' A stage. **D,** Protein expression in colon cancer tissue specimens of patients with or without lymph node metastasis. N: adjacent normal tissue; T: colon cancer tissue; NL: colon cancer tissue without lymph node metastasis; L: colon cancer tissue with lymph node metastasis; N: adjacent normal tissue; *p* < 0.05 vs. N. *p* < 0.05 vs. Dukes' A stage. **E,** Protein expression in colon cancer tissue specimens from patients with different differentiation degrees. N: adjacent normal tissue; H: well-differentiated colon cancer tissue; M: moderately-differentiated colon cancer tissue; L: poorly-differentiated colon cancer tissue; *p* < 0.05 vs. N. **F,** Protein expression in colon cancer tissue specimens at different Dukes' stages. N: adjacent normal tissue; *p* < 0.05 vs. N. *p* < 0.05 vs. H. *p* < 0.05 vs. Dukes' A stage. The shown blot is representative of three patients.
The TLR4/NF-κB signaling pathway mediates the growth of colon cancer. Here, we tested the effects of TLR4 inhibition on production of these cytokines in SW620 cells stimulated with LPS. We observed that both CRX-526 and inhibition of TLR4 by specific antibody partially blocked the LPS-induced secretion of IL-6 and IL-8 in colon cancer cells (Figures 5A and 5B).

**The TLR4 Inhibitor CRX-526 Induces Tumor Regression and Reduces Expression of TLR4 and NF-κB p65 in a Tumor Xenograft Model**

We next verified that CRX-526 was capable of inhibiting tumor growth in the nude mouse xenograft model. Human colon SW-620 cells were injected into the flanks of 6-week-old female BALB/c nu/nu mice. Mice were treated with CRX-526 or LPS, and tumor growth was monitored. The results showed a significant reduction in tumor size in mice treated with CRX-526 compared to controls.

**Figure 3.** LPS up-regulates TLR4 mRNA and protein expression in colon cancer cells. **A,** mRNA expression before and after 6 hours of treatment with different concentrations of LPS (0, 0.01, 0.1, 0.5, and 1 mg/L). *p < 0.05 vs. 0 mg/L LPS. **B,** mRNA expression before and after treatment with 0.5 mg/L LPS at different time points (0, 1, 3, 6, and 12 hours). *p < 0.05 vs. LPS at 0 hours. **C,** Protein expression before and after 6 hours of treatment with LPS at different concentrations as in panel A. *p < 0.05 vs. 0 mg/L LPS. **D,** Protein expression before and after treatment with 0.5 mg/L LPS at different time points. *p < 0.05 vs. LPS at 0 hours. **E,** Immunofluorescence analysis of protein expression before and after treatment with 0.5 mg/L LPS at different time points (scale = 20 µm). The images are representative of three independent experiments.
stimulated with 0.5 mg/L of LPS for 6 hours were injected subcutaneously under the forelimb of nude mice. The tumors became visible starting from the 3rd day after the injection and reached on the 7th day an average diameter of > 0.5 cm. The tumors appeared with no swelling or ulceration, and were movable with clear borders. There were no deaths.

The tumor-bearing mice were randomized into two, control and treatment, groups. Tumor volumes were similar in both groups. Then, CRX-526 (50 µg per injection, at multiple locations for 5 consecutive days) was given to the mice in the treatment group. After 4 weeks of treatment, the average tumor volume in CRX-526 group was significantly smaller than that in control group (Figure 6A). In addition, both mRNA and protein levels of TLR4 and NF-κB were lower in the mice treated with CRX-526 (Figures 6B-E).


**Discussion**

Colorectal cancer has become the second main cause of cancer deaths.\(^1\) Thus, there are about 41,000 new cases of colorectal cancer each year and 16,000 deaths annually in the UK,\(^1\) highlighting the need for early diagnostics and more effective therapies. In the present study, using tissue specimens, colon cancer SW620 cell line, and nude mouse xenograft model, we demonstrate that expression of TLR4 and NF-κB is up-regulated in colon cancer. Overexpression of these signaling proteins promotes secretion of cytokines, leading to accelerated proliferation of tumor cells and metastasization. We found that NF-κB p65 expression was markedly down-regulated by TLR4 inhibition, which resulted in suppression of tumor growth. This implicates that

![Figure 6](image)

**Figure 6.** CRX-526 induces tumor progression and down-regulates expression of both TLR4 and NF-κB p65 in the tumor xenograft model. **A,** CRX-526 inhibits tumor progression in the nude mice. \(* p < 0.05\) vs. control group. **B,** CRX-526 down-regulates mRNA expressions of TLR4 and NF-κB p65 in the tumor xenograft tissue. \(* p < 0.05\) vs. control group. **C,** CRX-526 down-regulates protein expression of TLR4 in the tumor xenograft tissue. The shown blot is representative of three independent experiments. **D,** CRX-526 down-regulates protein expression of NF-κB p65 in the tumor xenograft tissue. The shown blot is representative of three independent experiments. **E,** CRX-526 down-regulates protein expressions of TLR4 and NF-κB p65 in the tumor xenograft tissue. \(* p < 0.05\) vs. control group.
the TLR4/NF-κB signaling pathway may be considered as a potential target for prevention and treatment of colon cancer.

Activation of the TLR4/NF-κB signaling pathway is an important inflammatory response and is closely associated with colorectal tumorigenesis. Chronic nonspecific inflammatory bowel disease of the colon is the most common cause of colorectal cancer11. The mechanism underlying chronic inflammation-associated tumorigenesis has not been fully clarified but may be associated with genetic mutations caused by inflammatory environment. Inflammatory cytokines, such as IL-6 and IL-8, are produced by cancer and healthy cells in the ongoing inflammation process, which induces robust genotoxic response to promote DNA mutations in intestinal mucosa via reactive oxygen species. DNA damage and tumor-initiating mutations caused by inflammatory products are implicated in tumorigenesis, activation of anti-apoptotic machinery to prevent tumor cells from apoptosis, and promotion of tumor growth, migration, invasion and metastasis1,12-14.

We further found significant differences in TLR4 expression between tumor and healthy tissues, which further underscored potential role of TLR4 in the tumorigenesis of colon cancer. Furthermore, our study showed differentially expressed TLR4 in the tissues from different tumor stages, as well as differences between tumors with or without lymph node metastasis, suggesting that TLR4 expression is associated with tumor growth, metastasis, and prognosis of CRC patients. Our findings are consistent with other reports that showed TLR4 overexpression in stromal fibroblasts was associated with poor prognosis of CRC patients15.

NF-κB, a downstream target of TLR4 signaling cascade, is the major mediator of inflammation and cancer16-18. Our study found that up-regulation of expression of NF-κB subunit, p65, in colon cancer SW620 cell line promotes secretion of inflammatory cytokines IL-6 and IL-8. Our findings are in line with previous reports on association between NF-κB and malignant transformation of inflammatory cells19. Elevated serum levels of IL-6 and IL-8 correlate with development of colon cancer20,21. Thereby, inflammatory cytokines induced by the TLR4/NF-κB signaling pathway contribute to immune tolerance and inflammatory response involved in inflammation-associated neoplasia.

Reports by others highlighted the involvement of NF-κB in cancer development using the inhibitor pyrrolidine dithiocarbamate22. In our study, we undertook a different approach and utilized CRX-526, the TLR4 inhibitor, to test the effects on TLR4 and NF-κB in colon cancer cells and in xenograft model. In our studies, CRX-526 effectively inhibited tumor growth. The mechanism underlying anti-tumor effect of CRX-526 is similar to its inhibitory effect on development of advanced diabetic nephropathy by reducing inflammatory responses23.

Conclusions

We demonstrate that TLR4/NF-κB signaling pathway is activated in colon cancer, and that inhibition of TLR4 effectively inhibits tumor growth and associated inflammatory responses. Thereby, our study provides an experimental rationale for future clinical studies on the anti-cancer effects of TLR4 inhibition.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

The TLR4/NF-κB signaling pathway mediates the growth of colon cancer


