Toll-like Receptor 4 Activation Increases Akt Phosphorylation in Colon Cancer Cells

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Abstract. *Background: Toll-like receptors (TLRs) are involved in innate immunity. Overexpression of TLRs has been implicated in various types of cancer including colorectal cancer (CRC). The phosphatidylinositol-3'-kinase (PI3K)/Akt signaling pathway is involved in CRC growth and progression. In this study, we determined whether TLR4 signaling and PI3K/Akt pathway activation occur in CRCs. Materials and Methods: Human CRCs and adjacent mucosa were evaluated for TLR4 expression. CRC cell lines were treated with lipopolysaccharide (LPS), endogenous TLR4 ligand, to assess Akt phosphorylation. Results: Human CRCs overexpressed TLR4 compared to matched normal mucosa. Additionally, TLR4 was expressed in CRC cells and LPS treatment increased Akt phosphorylation of TLR4-positive CRCs in a time-dependent manner. Conclusion: Our results identify TLR4 expression in human CRCs and activation of PI3K with LPS treatment. These findings suggest possible treatment strategies targeting TLR4 in CRC.*

The colonic mucosa is constantly exposed to different types of pathogens; the ability to recognize microorganisms and invasive microbes depends, in large part, on the family of Toll-like receptors (TLRs) (1, 2). TLRs recognize a variety of pathogen-associated molecular patterns (PAMPs) through evolutionary-conserved motifs (3). Binding of PAMPs on TLRs classically leads to activation of inflammation-associated genes. Whereas TLRs can recognize and elicit an appropriate inflammatory response when the PAMPs are recognized by immune cells, inappropriate activation or regulation may lead to chronic inflammatory conditions and diseases (1). TLRs are known to elicit appropriate immune activation; however, they are also implicated in various types of cancer such as lung (4), ovary (5), stomach (6) and the colon (7). Additionally, TLRs have been implicated in the pathogenesis of chronic gastrointestinal disorders such as celiac disease (8) and inflammatory bowel disease (9). Despite our current understanding of TLR activation in immune cells, little else is known of the involvement of TLRs in the progression of colorectal cancer (CRC).

The phosphatidylinositol-3'-kinase/Akt (PI3K/Akt) pathway is involved in the proliferation and progression of a number of types of human cancer. Our group and others have shown a causative role for this pathway in the growth and progression of CRC (10-13). Typically, this pathway is activated when growth factors bind receptor tyrosine kinases (RTKs), leading to activation of PI3K (14). This active kinase complex then phosphorylates phosphoinositides, ultimately, leading to Akt phosphorylation and activation (15, 16). Activation of Akt kinase then leads to modulation of cell growth and metabolism.

TLR signal transduction has been characterized in immune cells, but our knowledge of the expression and activation of TLRs in other cell types is minimal. Since the colon is exposed to a high bacterial load, we sought to evaluate the involvement of the PI3K/Akt pathway in the activation of TLRs in CRC cells. While others have demonstrated a role for TLR2 and TLR5, the natural ligands of which are peptidoglycan and bacterial flagellin respectively, we focused on TLR4 since the natural ligand is lipopolysaccharide (LPS), a bacterial component. Therefore, the purpose of this study was to evaluate the expression and function of TLR4 in CRCs and to determine whether there is involvement of the PI3K/Akt pathway.

Materials and Methods

CRC cell lines. Five CRC cell lines were used: HT29, HCT116, SW480, Caco-2 (all from ATCC, Manassas, VA, USA), and KM20 (from Dr. Isaiah Fidler, University of Texas MD Anderson Cancer Center, Houston, TX, USA). All cells were cultured as previously described in a 5% CO_2 chamber in complete media containing 10% fetal bovine serum (17).

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Table I. *Colon cancer tissue specimens.*

Stage	Age (years)	Gender	Location
I	54	F	Cecum
I	77	F	Sigmoid colon
II	51	M	Transverse colon
II	54	M	Transverse colon
III	48	F	Cecum
III	50	M	Sigmoid colon
IV	72	F	Transverse colon
IV	63	F	Cecum
IV	63	M	Sigmoid colon

LPS preparation and treatment. LPS 055:B5 variant (Sigma-Aldrich, St. Louis, MO, USA) was prepared in phosphate-buffered saline (PBS) to 1 mg/ml stock, aliquoted, and frozen at –20°C until ready for use. For experiments, cells were plated into 60 mm dishes at a density of approximately 1×10^6 cells/dish and allowed to adhere and grow until approximately 80% confluent. Cells were then washed three times in PBS to remove residual complete media and incubated overnight in serum-free media. The next day, cells were treated at different timepoints with LPS (final concentration=1 μg/ml). After treatment cells were washed with PBS to remove LPS and cell lysates were immediately prepared using a Triton®-X-based Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA) supplemented with protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) and 1 mM phenylmethyl sulfonyl fluoride (PMSF; Sigma-Aldrich).

Western blot analysis. Cell lysates were prepared for Western blot analysis as described elsewhere (18). Blots were probed with phosphorylated and total Akt (Cell Signaling Technology), exposed to HRP-conjugated secondary antibodies, and imaged using enhanced chemiluminescence (ECL; GE Lifesciences, Indianapolis, IN, USA) on autoradiography film.

Tissue preparation and immunohistochemistry. CRCs (n=9) were examined from our tissue bank containing cancer samples and associated normal mucosa from UTMB patients who had undergone resection (17). Demographic information about each patient is listed in Table I. CRCs were staged based on clinical presentation and pathology reports. Stages are based on the American Joint Committee on Cancer (AJCC) Tumor-Node-Metastasis (TNM) staging (19). Stage I is defined as cancer cells invading the mucosa with or without involvement of the muscularis propria; stage II cancer contains cells that have invaded into the subserosa but without lymph node involvement; stage III cancer has cells that are found in lymph nodes but not distant metastases; and any distant metastasis is diagnosed as stage IV. Cancer tissue and normal mucosa (approximately 6 cm from the tumor) were formalin fixed and paraffin-embedded. Sections (5 μm) were de-paraffinized in xylene and rehydrated in descending ethanol series then stained using the Dako EnVision kit (Dako Corp., Carpinteria, CA, USA). Sections were incubated overnight at 4°C with monoclonal TLR4 antibody (Imgenex Corp., San Diego, CA, USA), then incubated in HRP-conjugated mouse secondary antibody and treated with DAB substrate for staining. Slides were counterstained with hematoxylin and observed using light microscopy.

RNA isolation, cDNA synthesis and reverse transcription PCR. Total RNA was isolated using the RNeasy Kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). First-strand cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and PCR performed using β-actin primers and commercially available TLR4 primers from Invivogen (San Diego, CA, USA). Quantitative real-time PCR was performed as described (22) using primers to TLR4 based on the published gene sequence (NCBI Accession no. NM_138554.2). Briefly, Applied Biosystems assays-on-demand 20× assay mixes of primers and TaqMan MGB probes (FAMTM dye-labeled) for our target gene (human *TLR4*) and pre-developed 18S rRNA (VICTM-dye labeled probe) TaqMan® assay reagent (P/N 4319413E) for endogenous control were used. Duplicate CT values were analyzed using the comparative CT (ΔΔCT) method as described by the manufacturer.

Results

Human CRCs express TLR4. We first examined CRCs and matched normal mucosa from the same patient to determine whether TLR4 is expressed. We examined tumors at different stages with representative staining of stage I and III cancer and matched mucosa as shown in Figure 1A. TLR4 expression was noted in all of the tumors regardless of stage as compared with the normal mucosa, which exhibited minimal to no staining. The stromal compartments of both normal and cancer our tissues expressed TLR4, which was expected since TLR4 is noted primarily in immune cells. These findings demonstrate up regulation of TLR4 expression in cancer tissues as compared with the matched normal mucosa.

We next examined whether TLR4 expression is present in human CRC cell lines Caco-2, HCT116, HT29, SW480 and KM20 using RT-PCR. As shown in Figure 1B (top panel) SW480, HT29 and KM20 cells demonstrated abundant *TLR4* mRNA expression, whereas Caco-2 and HCT116 cells showed significantly less expression; assessment of β-actin run in parallel lanes demonstrated relatively equal loading. Additionally, as shown in Figure 1B (bottom panel), after quantification of *TLR4* mRNA levels from our RNA samples using quantitative RT-PCR, significant increases in TLR4 expression were noted in HT29, SW480 and KM20 cells with Caco-2 and HCT116 cells showing only minimal expression. These results confirm that TLR4 is expressed in CRC cell lines as well as resected tissues, suggesting that TLR4 may play a role in CRC tumorigenesis.

TLR4 is expressed in CRC arising from chronic inflammation. Since we noted increased TLR4 expression in spontaneous cancer, we next determined whether TLR4 is expressed in cancer arising from inflammation. Samples

were obtained from a patient with a chronic history of ulcerative colitis and a diagnosis of metastatic poorly differentiated adenocarcinoma (Figure 2). TLR4 expression was noted in the primary tumor arising from chronic inflammation; this staining was noted to be both cytoplasmic and nuclear (Figure 2A). A section of invasive tumor demonstrated TLR4 staining in the invasive components (Figure 2B). The mucosa adjacent to the primary tumor demonstrated some TLR4 staining in the stromal compartment (Figure 2C). However, mucosa 5 cm from the primary tumor demonstrated only minimal TLR4 expression (Figure 2D).

Stimulation of TLR4 with LPS increased phosphorylated Akt. Previously, we demonstrated a significant role for PI3K/Akt pathway in CRC progression and metastases (10, 11, 17). We next determined whether PI3K activation occurred after treating cells with LPS which acts through TLR4 (Figure 3). Serum-starved TLR4-positive HT29, SW480 and KM20 cells were treated over a time course with either LPS (1 μg/ml) or vehicle. Protein was extracted and Western blots were assessed for expression of phosphorylated Akt. In HT29 cells, the maximal activation of phosphorylated Akt was within 10 min of LPS stimulation, whereas Akt activation was maximal by 30 min in SW480 and KM20 cells. To control for protein loading, the blots were stripped and reprobed for total Akt as well as β-Actin. Additionally, we treated HCT116 and Caco-2 cells with LPS and phosphorylated Akt expression was virtually undetectable following treatment (data not shown). These results demonstrate LPS-mediated Akt phosphorylation, suggesting that bacterial components act through TLR4, which activates PI3K/Akt and may contribute to CRC formation or progression.

Discussion

There is a clear link between inflammation and the development of cancer in a multitude of organs. For example, chronic inflammatory bowel disease is associated with increased incidence of CRC compared with the normal population (20, 21). This link between inflammation and cancer suggests that the mechanisms contributing to inflammation may also be critical for tumor formation. In our current study, we demonstrate increased expression of TLR4 in primary human CRC of various stages and also in tumors arising from inflammation. TLR4 expression was also noted in human CRC cell lines and, furthermore, LPS, the natural ligand for TLR4, increased Akt phosphorylation in CRC cell lines in a time-dependent fashion. These findings suggest a role for TLR4 in colorectal tumorigenesis and further suggest that Akt activation, following stimulation, may play a contributory role in this process as well.

Our findings show that increased TLR4 expression in CRC is consistent with other reports suggesting a correlation between TLR4 and CRC progression. A recent study by Earl *et al*. (22) demonstrated that in a mouse model of liver metastases, *TLR4* knockdown decreased liver metastasis and tumor burden, particularly in animals known to be more sensitive to LPS. Additionally, Fukata *et al*. (7) utilized a murine a model of inflammation-mediated colon cancer to show that TLR4 is important in the pathogenesis of colitis-associated cancer. Furthermore this study demonstrated that TLR4 expression and activation was necessary for dysplasia and, in *TLR4* knockout mice, dysplasia and polyp formation were absent. Our findings demonstrate increased TLR4 expression in both sporadic CRC and cancer derived from chronic inflammation, which is consistent with a potential role for TLR4 in CRC formation and/or progression.

We and others have shown that PI3K acts through its downstream effector protein Akt to contribute to CRC proliferation and metastasis. We have previously shown that the PI3K/Akt pathway is aberrantly regulated in CRC in a stage-dependent manner and that Akt components, in combination with PTEN suppression, were important in establishing CRC liver metastases (10, 17). Numerous studies have also implicated the PI3K/Akt pathway in inflammatory-mediated disease processes (23-25). Our current study showed that LPS activated Akt in CRC cell lines that express TLR4. The finding that LPS can activate Akt in epithelial-derived cells is intriguing and suggests that the PI3K/Akt pathway may be implicated in CRC through inflammatory-mediated pathways. Current paradigms suggest that TLRs on immune cells may actually have anticancer properties but that aberrant TLR4 expression can promote certain types of cancer (7, 26, 27). In this regard, our current study would suggest that TLR4 expression may contribute to colon cancer progression. Furthermore, the PI3K/Akt pathway may be a downstream mediator of TLR4 activation and subsequent effects on tumorigenesis.

In conclusion, our study provides additional compelling evidence to suggest the presence and role of TLRs in cancer progression. Specifically, we identified TLR4 expression in human CRC and cancer cell lines. Furthermore, our findings link Akt activation with stimulation of TLR4, thus suggesting that the PI3K/Akt pathway contributes to inflammation-mediated CRC progression. These results have important clinical implications for CRC prevention or treatment strategies based on inhibition of TLR4 signaling.
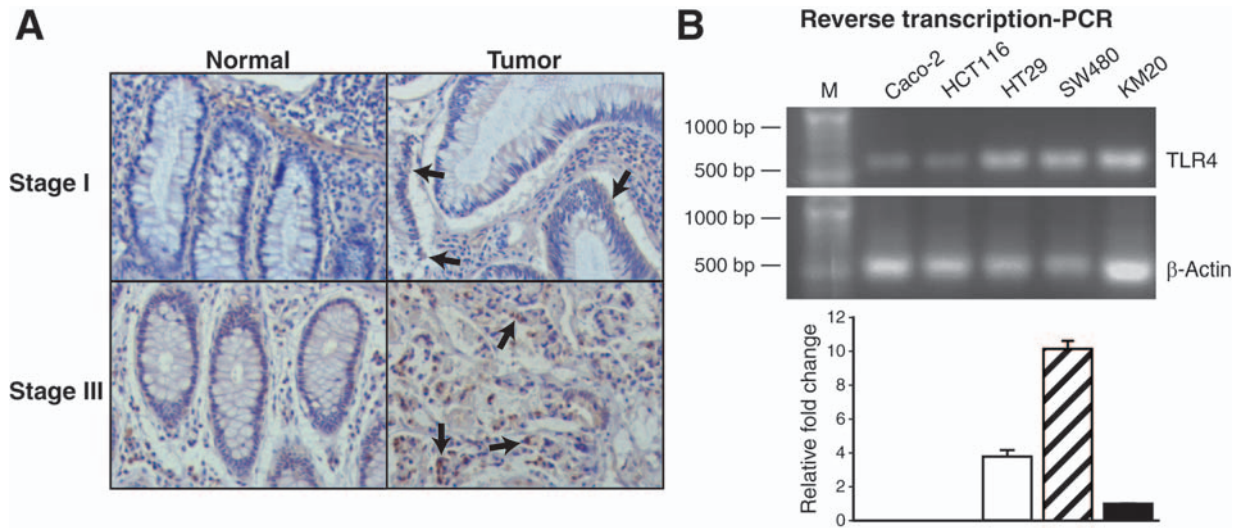
Acknowledgements

Figure 1. *Colon cancer expression of TLR4 compared to normal mucosa. A, Colon cancer tissues from patients (n=9) were obtained and evaluated for expression of TLR4. Samples were taken from carcinomas comprising all four stages of disease and from uninvolved normal mucosa (6 cm from cancer margins). Representative immunohistochemical sections from stage I and stage III tumors show elevated TLR4 expression (1A, top right and bottom right panels) relative to uninvolved normal mucosa. B, In order to evaluate whether CRCs express TLR4, reverse transcription PCR was performed as described in Materials and Methods with primers for human TLR4; β-actin primers were used as a control. C, Real-time quantitative PCR was performed on CRCs to determine relative TLR4 expressions in CRCs; results were normalized to 18S.*
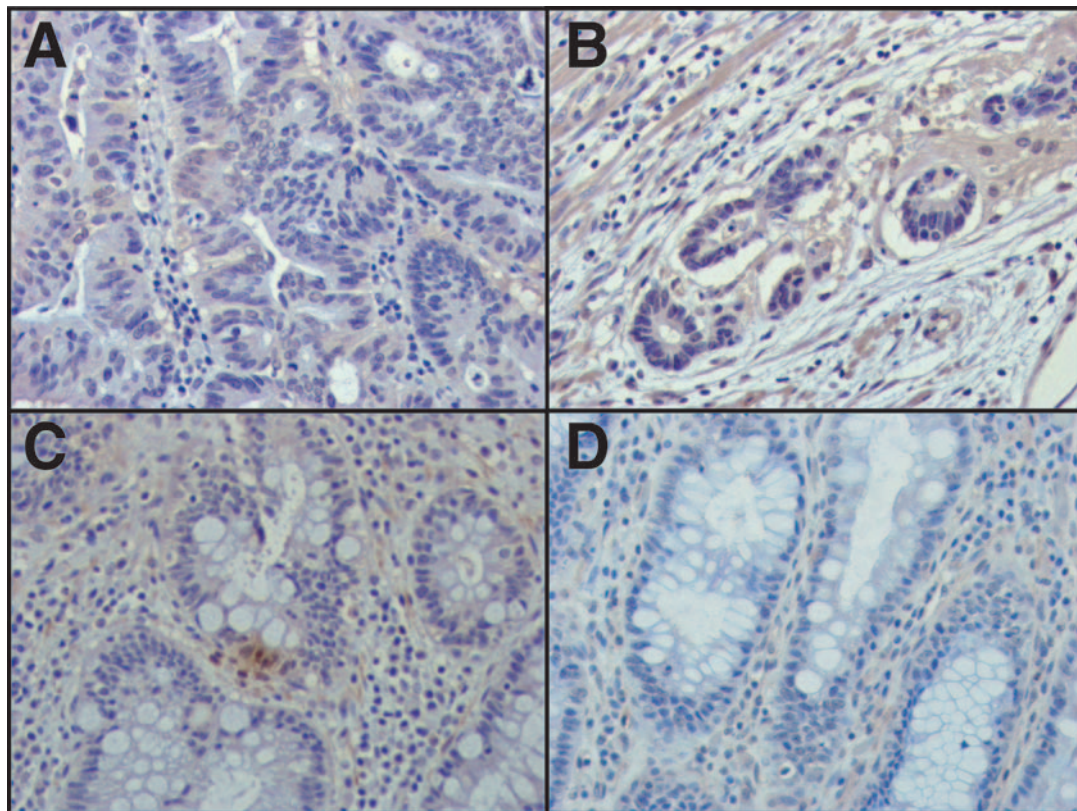

Figure 2. *TLR4 expression was elevated in cancer from a patient with inflammatory bowel disease. A, Immunohistochemistry (IHC) of a representative cancer sample from a patient with ulcerative colitis and stained for TLR4 (x200). The brown staining represents positive TLR4 expression. B, IHC of a portion of invasive cancer from the same patient demonstrating TLR4 staining in the cancerous glandular structures. C, IHC from a portion of mucosa adjacent to the tumor proper from the same patient showing some TLR4 expression in the stroma. D, IHC of mucosa taken 5 cm from the tumor margin showing minimal TLR4 staining.*
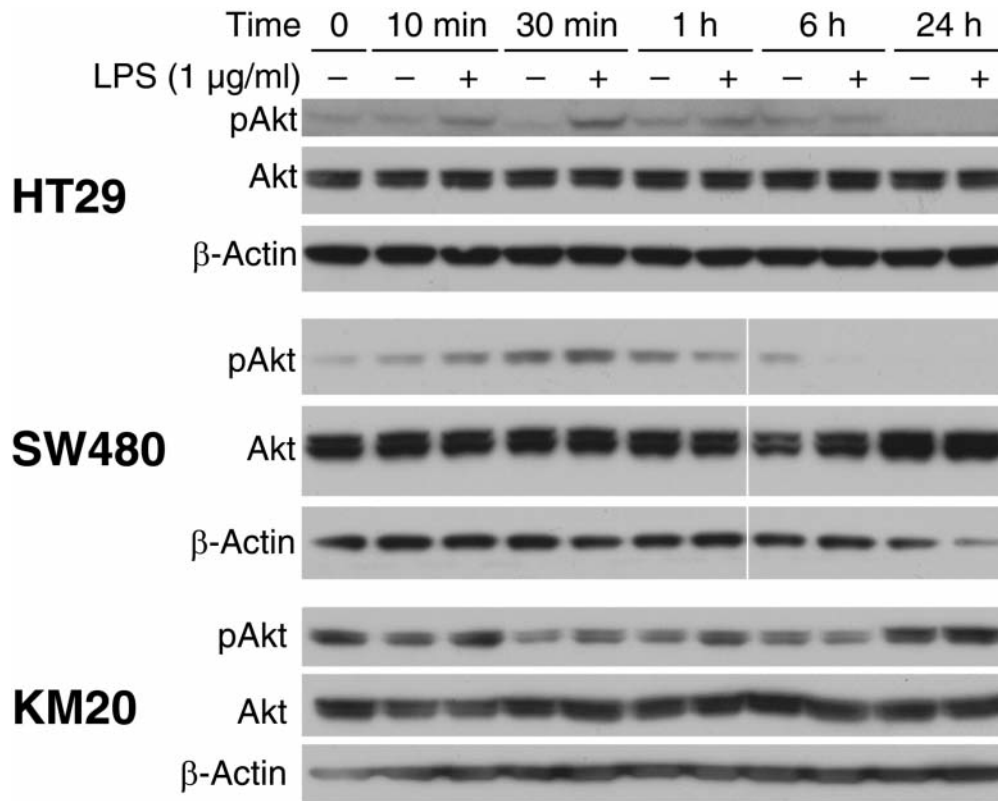
Figure 3. *Activation of TLR4 by LPS activate Akt phosphorylation. Western blot analysis of CRCs treated at the indicated timepoints with LPS (1 μg/ml) or vehicle. Lysates were prepared as described in Materials and Methods to determine Akt phosphorylation; β-Actin served as a loading control.*

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