Toll-like receptor 4 signaling promotes the immunosuppressive cytokine production of human cervical cancer

Y.X. Cheng, X.Y. Qi, J.L. Huang, M. Hu, L.M. Zhou, B.S. Li, X.X. Xu

Department of Gynaecology and Obstetrics, Renming Hospital of Wuhan University, Hubei, Wuhan (China)

Summary

Objectives: To investigate the expression of TLR-4 (toll-like receptor) on human cervical cancer and find the biological function of the TLR-4 signal system. *Methods:* The immunohistochemistry method was performed to study the protein expression and distribution of TLR-4. The viability of HeLa cells was determined by cell viability assay. Cell proliferation was detected by FCM, ELISA and Western blot were used to observe the gene and protein expression of IL-6 and TGF-β1 in Hela cell lines. *Results:* TLR-4 was over-expressed in cervix cancer, and its activation by LPS promotes proliferation and anti-apoptosis in Hela cells in vitro. Moreover the cell line proliferation increased in a dose- and time-dependent manner. The production of IL-6 and TGF-β1 were promoted through the activation of the NF-κB signaling pathway.

Key words: Cervical cancer; TLR-4; IL-6; TGF-β1.

Introduction

Cervical cancer is one of the most frequent female malignant tumors worldwide, accounting for approximate 500,000 new cases and the deaths of 270,000 women every year. Despite great improvement in the treatment of cervical cancer, the 5-year survival rate is still lower for cervical cancer at IIb-IV Stage. Therefore, in sight into the molecular biological mechanisms underlying cervical cancer is important for diagnosis, prevention and treatment of cervical cancer.

Toll-like receptors (TLRs) are a family of pattern recognition receptors that are composed of 13 types which play a key role in innate immune responses and participate in regulation of adaptive immune responses. TLRs are mainly expressed in immune cells such as dendritic cells (DC), macrophages and B cells. Recently, TLRs have also been detected in many cancers, including stomach cancer [1], ovarian cancer [2], lung cancer [3], prostate cancer [4] and breast cancer [5]. There were some studies suggesting that the activation of the TLR signaling pathways may fuel the proliferation, anti-apoptosis, invasion and immune escape of tumors [6]. However, the role of TLR-4 in cervical cancer is poorly understood.

Many epidemiological studies have demonstrated that chronic infection and inflammation are important epigenetic and environmental factors promoting tumorigenesis. Progress has been made in the understanding of tumor immune evasion, which is facilitated by the production of inflammatory profile of cytokines and chemokines, including IL-6 and TGF-β1, the sources of which are to a certain extent tumor cells stimulated by ligand lipopolysacharide (LPS) (stimulating TLR-4) potentially through nuclear factor κB (NF-κB) pathway activation.

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Recently, TLR-4 has been found to be expressed in tumor cells, and whether human cervical cancer cells can express TLR-4 remains poorly understood. In this study, we attempted to detect the expression of TLR-4 on human cervical cancer tissue and to find the biological function of the TLR-4 signaling system which was activated by LPS.

Materials and Methods

Reagents

LPS, carboplatin, HTA125, PDTC and all other chemicals mentioned in the methods were purchased from Sigma Chemical Co. (St Louis, MO, USA). The mouse anti-TLR-4, mouse anti-NF-κB, mouse anti-IL-6, mouse anti-TGF-β1 and rabbit anti-β-actin were purchased from Abcam pl. (Cambridge, UK). FITC-conjugated anti-TLR-4 mAb and FITC-conjugated isotype IgG1 mAbs were from eBioscience (San Diego, CA, USA) and Serotec (Oxford, UK), respectively.

Study samples

A total of 68 patients were recruited from women who were scheduled to undergo cervical cancer radical surgery in our hospital between January 2008 and May 2011, while the control group comprised 39 women who underwent surgery for other diseases and had a normal cervix. The mean age of the patients was 43 ± 12 (range 25 to 68 years).

Cell line and culture

HeLa cells, a human cervical cancer cell line, were obtained from Wuhan University, and grown in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin (100 units ml^{-1}) in a 5% CO_2 incubator that was maintained at 37°C.

Immunohistochemistry

All tissues were formalin fixed, paraffin embedded, and then cut into 5 μm sections on glass slides. Following deparaffinization in xylene and rehydration in alcohol, sections were treated

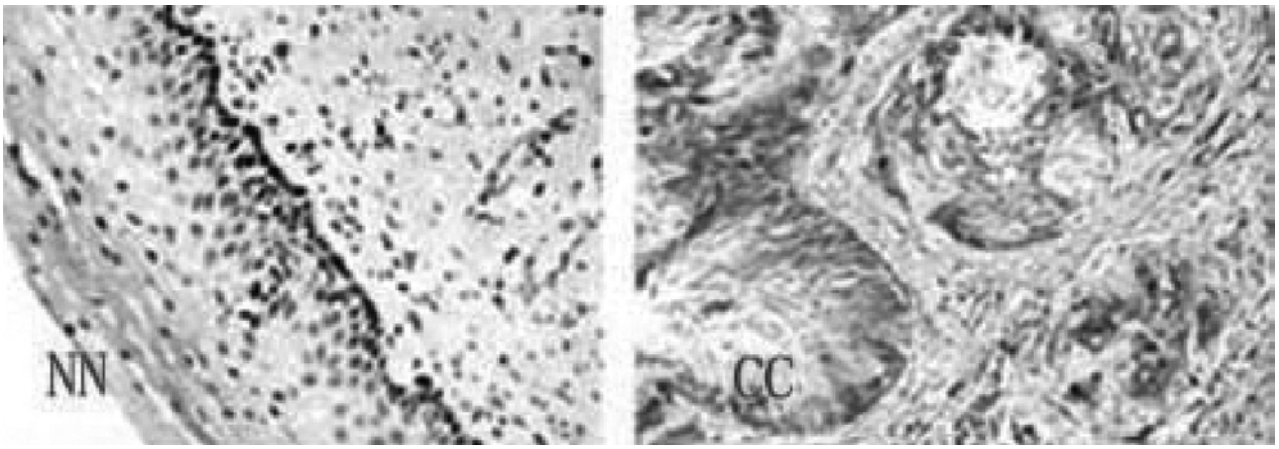

Figure 1. — Expression of TLR-4 in cervical cancer samples. Brown color displays positive expression. A: Negative control (isotype-matched control) B: Expression of TLR-4 protein in carcinoma tissue (SP × 200).
Legend 1. — Differential response of Hela cells cells to ligation of TLR-4 with LPS. Monolayers of Hela cells, exposed to different periods in the presence of increasing amounts of LPS. Cell viability was determined using CellTiter 96 AQueous One Solution Cell Proliferation Assay.

with 3% H_2O_2 for 10 min to inactivate endogenous peroxidase, then immersed in 10 mM citrate buffer (pH 6.0) and boiled for 10 min in a microwave oven to retrieve masked antigens. Slides were allowed to cool for 30 min, and then were pre-incubated with blocking solution containing normal goat serum for 15 min. The staining was performed with a streptavidin-biotin-peroxidase technique using mouse anti-human TLR-4 (Abcam plc, Cambridge, UK). After incubation with primary antibodies against TLR-4, overnight at +4°C, slides were exposed to biotinylated goat anti-mouse secondary antibodies (DakoCytomation) for TLR-4 staining for 30 min. Then peroxidase-labelled streptavidin was added to the slides and applied for 30 min at 37°C. Peroxidase activity was detected by exposure of slides to 3,3'-diaminobenzidine (DAB). The slides were then counterstained with hematoxylin, dehydrated, hyalined, and sealed with neutral resin. For a negative control, PBS was used as a substitute for the primary antibody.

Cell viability assay

Cell viability was evaluated using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay according to the manufacturer's instructions. The values from the treated cells were compared with the values generated from the untreated cells and reported as percent viability. Each experiment was done at least thrice. In order to assess the role of LPS, cells cultured in six-well plates (1×10^6 cells per well) were exposed to various concentrations of LPS (0, 1, 5 and 10 $\mu g.ml^{-1}$) for different time periods (12, 24 and 48 h) at 37°C in humidified 5% CO_2 conditions. Cell-free supernatants were collected by centrifugation at 400 g for 10 min and frozen at -70°C or analyzed immediately.

Flow cytometry (FCM) assay

Cells were cultured in 6-well plates for 24 hours. Nonadherent cells were removed by gentle washing. Medium was removed and pretreated with or without carboplatin (80 $\mu g/ml^{-1}$) for 12 h and HeLa cells ($4 \times l0^5$/m1) were exposed to the accompaniment of 10 mg/ml^{-1} LPS for 24 h at 37°C in humidified 5% CO_2. The expression of TLR-4 on Hela cells was determined using direct immunofluorescent staining. Briefly, 20 µl of FITC-conjugated anti-TLR-4 mAbs was added to 100 µl of cell suspension (1×10^6 cells per ml) and incubated at 4°C for 30 min. FITC-conjugated isotype IgG_1 mAbs were used as negative controls. Cell-surface expression of TLR-4 was analyzed on a FACS scan flow cytometer to detect the log of the mean channel fluorescence intensity with an acquisition of FL1. A minimum of 10,000 events were collected and analyzed on Cell Quest software.

ELISA analysis

Concentrations of the IL-6 and TGF-β1 were evaluated by ELISA analysis by collecting different treatment group supernatants according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Western blot analysis

Protein (20 µg) was denatured in sample buffer [2.5% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.15 mol/l Tris (pH6.8), and 0.01% bromophenol blue] and subjected to 12% SDS-PAGE as previously described [17]. Membranes were probed with the following primary antibodies specific for: mouse anti-NF-κB (Abcam; 1:1,000), mouse anti-IL-6 (Abcam; 1:1,000), mouse anti-TGF-β1 (Abcam; 1:1,000) and mouse anti-β-actin (Abcam; 1:10,000). Proteins were visualized using enhanced chemiluminescence (Pierce Biotechnology).

Results

TLR-4 in cervix cancer

To determine whether TLR-4 is expressed in cervix cancer, we evaluated TLR-4 expression in cervix cancer tissues by immunohistochemistry. As shown in Figure 1, positive staining for TLR-4 was easily detected in the cytoplasm and membrane of tumor cells and its expression was 68.7% in tumor tissue, which was significantly higher than that in the neighboring nondysplastic tissue.

TLR-4 activation by LPS in cervix cancer

Once the overexpression of TLR-4 in cervix cancer was confirmed, we evaluated the biological function of this receptor. Recent evidence suggests that TLRs may

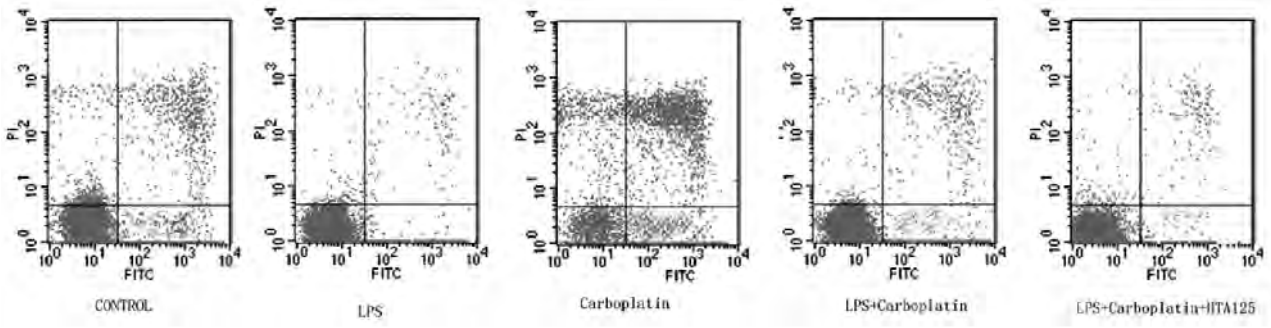

Figure 2. — LPS affects carboplatin induced Hela cell line apoptosis. Apoptosis detected by Annexin V/FITC staining.
Legend 2. — HeLa cells pretreated with HTA125 (10 μg/ml^{-1}) before incubation with 10 μg/ml^{-1} LPS for 24 h, were partially rescued from proliferation promotion induced by LPS. Cell viability was determined using CellTiter 96 AQueous One Solution Cell Proliferation Assay.

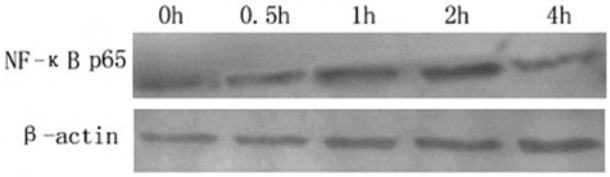

Figure 3. — TLR-4 ligation to LPS activates the NF-κB signaling pathway. HeLa cells stimulated with 110 μg/ml^{-1} LPS for for 0.5, 1, 2 and 4 h. The nuclear localization sequence of NF-κB p65 was assessed by Western blot.
Legend 3. — Enhancement of IL-6 and TGF-β1 secretion in Hela cells by LPS samulation which was mediated by NF-κB signaling pathway. The cytokine secretion was evaluated by ELISA analysis.

involved in proliferation and anti-apoptosis of many tumors. We determined whether TLR-4 has similar effects on cervix cancer. After treatment with increasing concentrations of LPS (lipopolysaccharide-ligand of TLR-4) (0,1, 5 and 10 μg.ml^{-1}) for different time periods (12, 24 and 48 h), viability of HeLa cells was determined by cell viability assay. Cell proliferation significantly increased in Hela cells treated with LPS than cells without LPS treatment in a dose- and time-dependent manner (Figure 2). Thus, TLR-4 upon recognition of its ligand lipopolysacharide (LPS) can promote proliferation in cervix cancer.

Carboplatin, a commonly used oral treatment of a variety of tumors, can induce apoptosis in HeLa cells and we tested if TLR-4 activation by LPS could decrease the carboplatin-induced apoptosis in HeLa cells. HeLa cells (4×10^5/ml) were pretreated with or without carboplatin 80 μg/ml^{-1} for 12 h before subsequent stimulation with 10 μg/ml^{-1} LPS for 24 h. As expected, the presence of LPS significantly reduced apoptosis induced by carboplatin in HeLa cells than absence of LPS. The result suggested that TLR activation by LPS promoted anti-apoptosis in cervix cancer (Figure 3).

TLR-4 inhibitor can inhibit LPS mediated promoting proliferation and rescue carboplatin-induced apoptosis

Then we further tested whether such responses to LPS in HeLa cells is special for TLR-4 activation. HTA125 (Functional Grade Purified anti-human Toll-like receptor 4) is known to function as an inhibitor of TLR-4. HeLa cells were pretreated with HTA125 (10 μg/ml^{-1}) before incubation with 10 μg/ml^{-1} LPS for 24 h. Here we demonstrate that the inhibition of TLR-4 reduced LPS-mediated proliferation of HeLa cell to normal levels (Figure 4). Furthermore, on the basis of the carboplatin mentioned above, we added HTA125 (10 μg/ml^{-1}) before incubation with LPS for 24 h. The inhibition of TLR-4 resumed apoptosis of HeLa cell induced by carboplatin (Figure 3).

TLR-4 upon recognition of its LPS can stimulate the activation of the NF-κB signaling pathway and promote the production of IL-6 and TGF-β1 in human cervix carcinoma cells

Some studies have confirmed that the TLR-4 upon recognition of its LPS can induce the production of inhibitory cytokines, inflammatory factors, proteinases, and other small molecules in the immune system [7]. Thus, our next objective was to evaluate whether TLR-4 ligation by LPS would have a similar effect in human cervix carcinoma cells.

As IL-6 and TGF-β1 have been reported to be involved in proliferation and anti-apoptosis of tumor cells, we incubated Hela cells in the presence or absence of 10 μg/ml^{-1} LPS for 24 h, and cytokine secretion was evaluated by ELISA analysis. As expected, the secreted levels of IL-6 and TGF-β1 were demonstrated to be significantly increased by LPS treatment (Figure 5). A similar result turned up with the inhibition of TLR-4 (10 μg/ml^{-1} HTA125); the levels of IL-6 and TGF-β1 were all down-regulated (Figure 5).

TLR-4 ligation to LPS activates the NF-κB signaling pathway in immune cells, which results in cytokine secretion, proliferation and anti-apoptosis of immune cells. Thus, we observed the activation status of NF-κB in human cervix carcinoma. HeLa cells were stimulated with 10 μg/ml^{-1} LPS for for 0.5, 1, 2 and 4 h. The nuclear localization sequence of NF-κB p65-active form of NF-κB was assessed by Western blot analysis. Stimulation of Hela cells with 10 μg/ml^{-1} LPS for 0.5 h increased NF-κB activity (Figure 6).

Finally, to confirm whether IL-6 and TGF-β1 secretion induced by LPS was mediated by the NF-κB signaling pathway. Hela cells were incubated with the NF-κB specific inhibitor (PDTC) (10 μg/ml^{-1}) for 1 h with LPS of 10 μg/ml^{-1} for 24 h. PDTC significantly inhibited the secreted levels of IL-6 and TGF-β1 induced by LPS (Figure 5). These results indicated that TLR-4 ligation by LPS activated the NF-κB signaling pathway leading to proliferation and anti-apoptosis of HeLa cells.

Discussion

It has been well established that a variety of microbe-induced inflammatory processes promote tumorigenesis but the mechanisms of the phenomenon are poorly understood. Substantial evidence has identified TLR-4 as a crucial component of the immune system that plays an important role in regulating tumor cell proliferation, anti-apoptosis, angiogenesis, invasion, metastasis and immune escape. Kelly *et al*. [2] confirmed that the activation of the TLR-4 signaling pathway promoted an inflammed environment, improved tumor cell survival ability and chemotherapy resistance. However, whether human cervical carcinoma cells can express TLR-4 and what the biological function of TLR-4 on cervical carcinoma is remains to be fully understood.

Up to date, there have been increasing studies about the effect and corresponding mechanism of the TLR-4 signal pathway on tumorigenesis. Chronic inflammation is a high risk factor for the development of cancer. Progression of tumor is often associated with a generalized immunosuppression of the host. Tumors evade immune surveillance by multiple mechanisms, including the production of factors such as transforming growth factors (TGF-β1) and interleukin-6 (IL-6), which inhibit DC activation and impair tumor-specific T cell immunity. The TLR-4 signaling pathway involves the activation of NF-κB, which leads to the production of inflammatory cytokines. LPS-ligand for TLR-4, which has been shown to induce NF- B activation, cytokines/chemokine production, and inflammation [8], is expressed by the cells of the innate immune system. He *et al*. [9] has found that TLR-4 ligation promoted production of immunosuppressive cytokine TGF-β, vascular endothelial growth factor (VEGF), proangiogenic chemokine IL-8, and NF-κB was activated and contributed to apoptosis resistance of human lung cancer cells induced by LPS in human lung cancer cells.

In this study, we have described a specific inflammation and innate host defense mechanism, that could regulate the release of proinflammatory cytokines, leading to tumorigenesis. We found, for the first time, that TLR-4 expressed in cervical cancer tissue is significantly higher surrounding normal cervical tissue, suggesting that TLR-4 might contribute to immune escape in cervical cancer. At the same time, we ascertain that the activation of TLR-4 by LPS can accelerate the growth of cervical cancer cells by promoting proliferation and anti-apoptosis - mainly associated with the process where NF-κB mediates IL-6 and TGF-β1 secretion.

In conclusion, our study revealed that TLR-4 was over-expressed in cervical cancer. On the other hand, TLR-4 activation by LPS could promote proliferation and anti-apoptosis in cervix cancer. In addition, functional activity of TLR-4 was demonstrated by stimulation of LPS and subsequent activation of NF-κB, and release of the proinflammatory IL-6 and TGF-β1. Thus, TLR-4 might be a new marker for cervical cancer.

Conclusion

We investigated the expression of TLR-4 on human cervical cancer alls and found the biological function of the TLR-4 signal system. We have reported for the first time that TLR-4 expressed in cervical cancer tissue is significantly higher than in surrounding normal cervical tissue. At the same time, we ascertain that the activation of TLR-4 by LPS can accelerate the growth of cervical cancer cells by promoting proliferation and anti-apoptosis – mainly associated with the process where NF-κB mediates IL-6 and TGF-β1 secretion. Therefore, we conclude that TLR-4 might be a new biological marker for cervical cancer.

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Address reprint requests to:
X.X. XU, M.D.
Department of Gynaecology and Obstetrics
Renmin Hospital of Wuhan University
Jiefang Road 238, Wuhan
Hubei (China) 430060
e-mail: chengmin.jjjj@yahoo.com.cn