RESEARCH COMMUNICATION

Toll-like Receptor 5 Agonist Inhibition of Growth of A549 Lung Cancer Cells in Vivo in a Myd88 Dependent Manner

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Abstract

The purpose of this study was to examine the effect of a Toll-like receptor 5 (TLR5) agonist, CBLB502, on the growth and radiosensitivity of A549 lung cancer cells in vivo. Expression of myeloid differentiation factor 88 (MyD88) or TLR5 was stably knocked down in human lung cancer cells (A549) using lentivirus expressing short hairpin RNA targeting human MyD88 or TLR5. Lack of MyD88 or TLR5 expression enhanced tumor growth in mouse xenografts of A549 lung cancer cells. CBLB502 inhibited the growth of A549 lung cancer cells, not A549-MyD88-KD cells in vivo in the murine xenograft model. Our results showed that the inhibition of A549 by CBLB502 in vivo was realized through regulating the expression of neutrophil recruiting cytokines and neutrophil infiltration. Finally, we found that activation of TLR5 signaling did not affect the radiosensitivity of tumors in vivo.

Keywords: TLR5 agonist - CBLB502 - tumor suppressor - lung cancer

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Introduction

Human innate immune system is the first line of defense against invading organisms. Apart from defending against invading microorganisms, the role of innate immune system in modulating tumorigenesis and tumor growth was recognized as early as in the 1890s (Thomas and Badini, 2011). Under the enlightenment of the report that some cancer patients who developed acute bacterial infection survived longer than those without infection, Dr William Coley began to treat people with sarcomas using the reparations derived from streptococcal cultures (Coley's toxins) to activate general systemic immunity, which provided complete response rates of approximately 15% in the 1890s (Bickels et al., 2002). The anti-tumor effect of innate immune activation has been continually reported since then (Ostberg et al., 2005; Xie et al., 2007; Tse et al., 2011).

Initially, typical innate immune response was regarded as a non-specific defense against foreign invaders. However, the following studies found that innate immune response depended on a variety of transmembrane or secreted pattern-recognition receptors (PRRs) to trigger a defensive response against foreign invaders (Kawai and Akira, 2010). Toll-like receptors (TLRs) are important members of PRRs. They could identify a variety of products characteristically associated with microbial species, such as peptidoglycan, bacterial lipoproteins, bacterial lipopolysaccharide, and bacterial flagellin (Armant and Fenton, 2002). Up to now, 11 members of Toll-like receptors (TLRs) and a series of their respective ligands have been identified in humans. MyD88-dependent pathway has been utilized by almost all the TLRs to activate NF-κB and AP-1 which subsequently drive the immune response (Kawai and Akira, 2006).

In the TLRs family, TLR5 is a specific receptor for bacteria flagellin (Rhee et al., 2005). Through MyD88, TLR5 can activate NFκB and subsequently initiate immune response to pathogen (Rhee et al., 2006). Apart from expressing on colonic epithelium, where the invading microorganisms most frequently reach, TLR5 is expressed in internal tissues including heart, brain, spleen, kidney, and testis, suggesting a wide role for TLR5 in the host defense (Gewirtz et al., 2001). Besides mediating the anti-infection immune system, TLR5 has been found to modulate tumor development and growth in a mouse xenograft model of human colon cancer (Rhee et al., 2008). CBLB502, a kind of polypeptide drug derived from Salmonella flagellin, can bind to TLR5 specifically and activate NF–kB signaling. Furthermore, it has been reported that CBLB502 has radioprotective activity in mouse and primate models (Burdelya et al., 2008).

In the current study, based on the report that TLR5 was expressed in alveolar epithelial cell, we investigated whether there was TLR5/MyD88/ NFκB pathway in lung adenocarcinoma cell line A549 and the role of TLR5 pathway in regulating lung adenocarcinoma growth in a mouse xenograft model. Furthermore, the effect of TLR5 signaling activation on the radiosensitivity of lung adenocarcinoma was investigated to assess the possibility

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of using TLR5 agonist as adjuvant for cancer radiotherapy because a previous study had reported the radioprotective activity of TLR5 signaling. Our data demonstrated that TLR5/MyD88/ NFϰB signaling pathway existed in A549 cells, and this pathway modulated the growth of A549 xenograft in vivo through regulating the expression of neutrophil recruiting cytokines and neutrophil infiltration. Finally, we found that the activation of TLR5 signaling did not affect the radiosensitivity of tumor in vivo.

Materials and Methods

Cell lines

The A549 cell line was obtained from the Beijing Xiehe Cell Culture Center and maintained in DMEM plus 10% fetal bovine serum (FBS). The HEK293 cells were stored by our laboratory. 293FT cells were bought from Invitrogen.

Generating TLR5 or MyD88 knocked down A549 cells

Lentivirus expressing small hairpin RNA targeting TLR5 (named LV-TLR5) or MyD88 (named LV-MyD88) were constructed. An empty pSicoR lentivirus vector was used as a negative control and named LV-NC. A549 cells were infected by LV-TLR5, LV-MyD88 or LV-NC. These lentivirus encode GFP protein for tracking the infected cells. Stable infected cells were isolated selected by fluorescence-activated cell sorting using GFP (detected by flow cytometry) as the marker (named TLR5-KD cells and MyD88-KD cells, respectively). LV-NC-infected and GFP-positive A549 cells were also selected and used as negative control A549 cells (named as WT cells)

Reverse transcription-PCR (RT-PCR)

Total cellular RNA was extracted with TrizolTM (Gibco, USA) according to the manufacturer's protocol, and complimentary first-strand DNA was generated using the reverse transcription system kit (promega, US according to the manufacturer's protocol. Oligonucleotide primer sequences used were as follows: MyD88 sense 5'-ACACAAGCGGACCCCACTG-3' and antisense 5'-AGCTCCAGCAGCACGTCGT-3', TLR5 sense 5'-TCAAACCCCTTCAGAGAA-3' and TLR5 antisense 5'-TTGGAGTTGAGGCTTAGTCCCC-3', ENA-78 sense 5'-GCCCGTGTCCCCGGTCCTTCGAG-3' and antisense 5'-CTGGATCAAGACAAATTTCCTTC-3', IL-8 sense 5'-CTGCGCCAACACAGAAATTA-3' and antisense 5'-ATTGCATCTGGCAACCCTAC-3', MIP-3α sense 5'-GCAAGCAACTTTGACTGCTG-3' and antisense 5'-TGGGCTATGTCCAATTCCAT-3', and β-actin sense 5'-AGCGAGCATCCCCCAAAGTT-3' and antisense 5'-GGGCACGAAGGCTCATCATT-3'. PCR was done by the PCR Mix kit (Tiangen, China) according to the manufacturer's protocol.

Luciferase Assay

Cells (1.5×10^4) were seeded in a 24-well plate and transfected with 0.5 μg of the NF-ϰB promoter-driven luciferase plasmid (a very generous gift from Professor XM Yang) and 0.5 μg of renilla plasmid. Transfected cells were treated with CBLB502, a polypeptide drug derived from Salmonella flagellin that binds to Toll-like receptor 5 (TLR5) and activates nuclear factor-kappaB, 24 hours after transfection. The luciferase activity in cells was measured by Dual-Lucy Assay Kit (promega, US) 4 hours after CBLB502 treatment.

Tumor growth in nude mice and CBLB502 treatment

Male athymic nu/nu mice weighing 16–18 g were obtained from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences. A549 cells (5×10^6) infected by LV-TLR5, LV-MyD88 or LV-NC were suspended in physiological saline and subcutaneously inoculated into the subcutaneous tissue at the back of the mice shoulder. Six days after inoculation, tumor diameters were measured every 4 days, and each tumor volume in mm^3 was calculated by the following formula: $V = 0.5\times D\times d^2$ (V, volume; D, longitudinal diameter; d, latitudinal diameter). In CBLB502 treatment group, the CBLB502 in physiological saline was injected around tumor every 2 days at the dose of 10 μg/kg. The mice in the control group were given the same volume of physiological saline with the same route of administration. At the end of the experiment, the mice were sacrificed, necropsy was performed, and the tumors were weighed. All experimental protocols and animal handling practices adopted in this study were designed for the maximum well-being of the animals, conformed to the Chinese National Research Council guidelines and were approved by the Subcommittee on Research Animal Care and Laboratory Animal Resources of the Beijing Institute of Radiation Medicine (2010-0346).

Irradiation of tumor in nude mice

As described above, A549 cells (5×10^6) were injected into subcutaneous tissue at the back of the mice shoulder and the volume of tumor was measured. At day 7 and day 21 after inoculation, CBLB502 solution or same volume of physiological saline was administrated to mice as described above. Thirty minutes after administration, the mice were anesthetized and the tumors were locally irradiated with or without a dose of 4-Gy γ ray.

In vivo fluorescence imaging

In vivo GFP fluorescence imaging was acquired by illuminating the animal with a Xenon 150-W lamp. The reemitted fluorescence was collected through a long-pass filter on a Hamamatsu C5810 3-chip color charge-coupled device camera (Hamamatsu Photonics Systems). High-resolution image acquisition was accomplished by using an EPSON PC. Images were processed for contrast and brightness with Adobe Photoshop 4.0.1J software (Adobe). A fluorescence stereomicroscope (SZX7; Olympus) was also used to visualize GFP-positive tissues.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were treated with anti-Gr-1 (Santa Cruz USA), -CD31 (Santa Cruz USA), or -CD68 (Santa Cruz USA) antibody at a dilution of 1:100 for 1 hour and stained with the LSAB+System-HRP kit (DakoCytomation) according to the manufacturer's instructions.

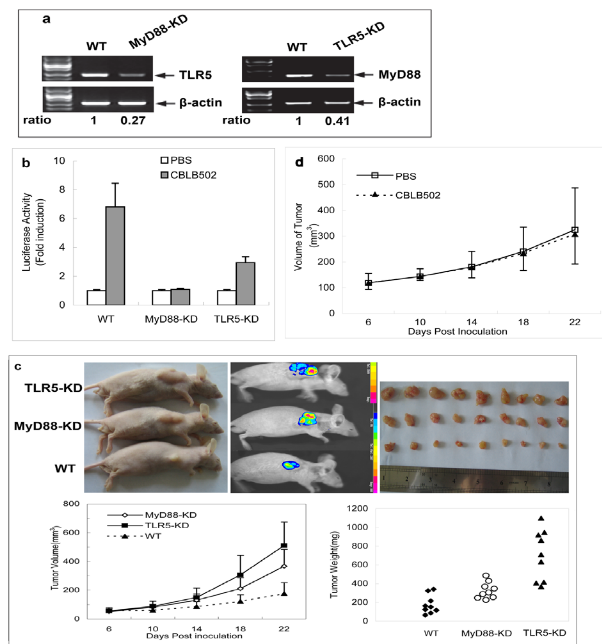

Figure 1. Blocking of TLR5/MyD88-dependent Signaling Enhanced Tumor Growth in A549 Xenografts Model. (a) The expression of TLR5 mRNA (right) and MyD88 mRNA (left) in TLR5-KD cells, MyD88-KD cells and WT cells, detected by RT-PCT. The expression of β-actin mRNA was monitored as the normalizing control. The ratios of TLR5/β-actin and MyD88/β-actin were calculated using densitometry. (b) TLR5-KD cells, MyD88-KD cells or WT cells were transfected with NF-ϰB promoter-driven luciferase plasmid, and subsequently tranfected cells were treated with 10 nM of CBLB502 or PBS. After 44 h, the cells were lysed and the luciferase activity was determined. Values of luciferase activity are mean±S.D. of three independent experiments in 24-wells plate. All values are expressed as n-fold increased activities compared with WT cells treated with PBS. (c) TLR5-KD, MyD88-KD or WT tumor were inoculated into mice. Tumor xenografts (upper right) and its fluorescence imaging (upper middle) in vivo and tumor masses ex vivo (upper left) were shown 22 days after inoculation. 6 days after inoculation, tumor volume was measured every 4 days (lower right), and the weight of tumors resected from nude mice at day 26 post-inoculation was recorded (lower left). There was a statistically significant difference in the growth curve between TLR5-KDand WT orMyD88-KD and WT ($p<0.05$)

Statistical analysis

A 2-tailed, unpaired Student t test was used for all statistical analyses.

Results

CBLB502 activated TLR5 signaling in A549 cells and inhibited tumor growth in vivo

TLR5 was reported to express in colon cancer cells besides epithelial cells, macrophages, or dendritic cells. To determine whether TLR5 was expressed in lung adenocarcinoma cells, the mRNA of TLR5 in A549 cells was detected using RT-PCR. TLR5 was expressed in A549 cells. Based on the finding that TLR5 engagement by bacterial flagellin mediates MyD88/IRAK/TRAF6-dependent signaling, resulting in the activation of transcription factor NF-ϰB in colonic epithelial cells, we presumed that the same path way existed in A549 cells. To confirm the presumption, a luciferase assay was performed after A549 cells were treated with NF-ϰB promoter-driven luciferase plasmid and CBLB502. CBLB502 activated the transcription of NF-ϰB in a dose-dependent manner in A549 cells. As it was reported that the agonists of TLRs possessed antitumor effect, the effect of TLR5 activation by CBLB502 on the in vivo growth of A549 xenograft was subsequently assessed. A549 cells were inoculated into nude mice and the CBLB502 or PBS was administrated around tumor. Compared with PBS treatment, CBLB502 administration significantly inhibited the tumor growth in vivo.

Inhibition of TLR5-dependent signaling promoted tumor growth in vivo

The fact that TLR5 activation could delay the tumor growth in vivo indicated that suppression of TLR5-dependent signaling might enhance the tumor growth in vivo. To detect whether suppression of TLR5-dependent signaling could alter tumorigenesis and tumor growth, lentivirus expressing small hairpin RNA targeting TLR5 or MyD88 were constructed and infected into A549 cells. TLR5-KD cells, MyD88-KD cells and WT cells were obtained after selecting flow cytometry (see Materials and methods). The silencing effects of lentivirus were examined by RT-PCR. As shown in Figure 1a, compared with LV-NC, LV-TLR5 and LV-MyD88 significantly inhibited the endogenous expression of TLR5 and MyD88 in A549 cells respectively. Furthermore, luciferase assay showed that the transcriptional activation of NF-ϰB induced by CBLB502 was significantly suppressed in LV-TLR5- or LV-MyD88-infected A549 cells compared with LV-NC-infected A549 cells (Figure 1b), which further confirmed the silencing effect of LV-TLR5 and LV-MyD88.

Next, we used a mouse xenograft model of human lung adenocarcinoma, in which TLR5-KD cells, MyD88-KD cells or WT cells were subcutaneously injected into nude mice, followed by measuring tumor growth. As shown in Figure 1c, tumor volume, size and weight were similar to tumors from TLR5-KD cells and LV-MyD88-A549 cells. However, compared with tumors from WT cells, tumor volume, size and weight were significantly larger in tumors from both TLR5-KD cells and MyD88-KD cells. More importantly, tumor growth delay resulting from CBLB502 administration did not appear in tumors from MyD88-KD cells (Figure 1d).

Knockdown of TLR5 or MyD88 suppressed the expression neutrophil recruiting cytokines

It was reported that TLR5 pathway could mediate the expression of IL-8 and MIP3α, both of which play important roles in inflammatory cell recruitment during inflammation, and in non-transformed human colonic epithelial cells. In order to investigate the mechanism by which TLR5 pathways regulate the development of tumor in vivo, the differential expression of cytokines/chemokines in tumor xenografts was detected by RT-PCR. As shown in Figure 2, the expression of IL-8, which played a paradoxical role in tumorigenesis, was suppressed after TLR5 knockdown. The expression of MIP-3α, which was

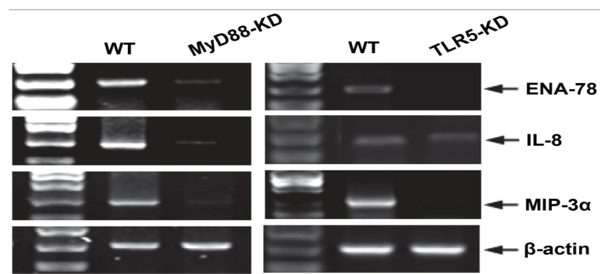

Figure 2. The Expression of Neutrophil Recruiting Cytokines Reduced in Tumor Xenografts from TLR5-KD Cells or MyD88-KD Cells. The expressions of ENA-78 mRNA (lane 1), IL-8 mRNA (lane 2) and MIP-3αmRNA (lane 3) in tumor xenografts from TLR5-KD cells, MyD88-KD cells or WT cells were detected by RT-PCT after the total RNA of tumor xenografts was extracted. The expression of β-actin mRNA was monitored as the normalizing control

found to possess anti-tumor effect, was also inhibited after TLR5 knockdown. Furthermore, the expression of ENA-78, a chemokine for neutrophils, decreased in tumors from TLR5-KD cells compared with those from WT cells. The similar results were also observed after MyD88 inhibition.

The inhibition of TLR5 pathway blocked the neutrophil infiltration but did not affect macrophage infiltration and angiogenesis in tumors

The essential roles of TLR5 pathway in innate immune responses have been well established. Moreover, it has been reported that innate immune activation could enhance tumor immunotherapy. Neutrophils, an essential effector cell of the innate immune system, have been proved to possess potent antitumor activity. Further, depleting neutrophils could enhance tumor growth in vivo. In the present study, we found that knockdown of TLR5 or MyD88 could inhibit the express of ENA-78 and IL-8, both of which served as chemical signals attracting neutrophils at the site of inflammation. Given these findings, we investigated whether the block of TLR5 pathway could inhibit the neutrophil infiltration in tumor xenografts. To test this, immunohistochemistry was performed with tumor xenografts using antibody against Gr-1 which was the neutrophil specific marker. There were significantly fewer Gr-1-positive cells in TLR5- or MyD88-knock down tumors than untreated tumors.

Apart from ENA-78 and IL-8, the expression of MIP-1α, a chemotatic factor for macrophage which was another important component of the human innate immune system, was also regulated by TLR5. Therefore, the infiltration of macrophage in tumor xenografts was also detected by immunohistochemistry, using antibody recognizing the macrophage-specific antigens CD68. There was no significant difference in macrophage infiltration between TLR5- or MyD88-knock down tumors and untreated tumors.

Angiogenesis performs a critical role in the development of cancer. Circulatory vessels are required by tumor to supply oxygen and nutrients and to remove metabolic wastes. To examine whether the angiogenesis is affected by TLR5 blocking, the vascularity in xenografts was further detected by immunohistochemistry using an antibody against CD31, the specific marker of vascular endothelial cell. There was no difference in micro-vessel formation between TLR5- or MyD88-knock down group and control group, indicating that the suppression of TLR5 pathway did not affect tumor angiogenesis in xenografts. These data indicated that the neutrophil infiltration, rather than macrophage infiltration or angiogenesis, could be a key component for TLR5-associated anti-tumor activity.

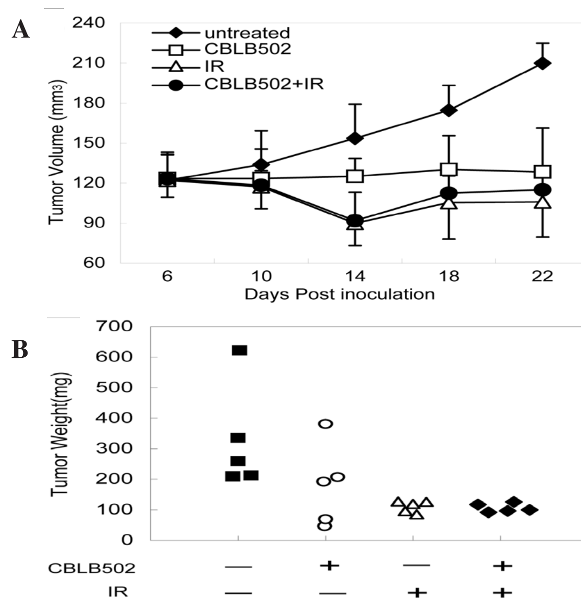

Figure 3. CBLB502 Did Not Affect the Radiosensitivity of Tumors in Vivo. Mice bearing A549 tumor were stratified into groups of 4 and treated with CBLB502 or PBS with or without irradiation as described in methods. (a) 6 days after inoculation, tumor volume was measured every 4 days. (b) The weight of tumors resected from mice at day 26 post-inoculation was recorded

The activation of TLR5 signaling did not affect the radiosensitivity of tumor in vivo

The radioprotective activity of TLR5 signaling has been well established, indicating that TLR5 agonist may be a valuable adjuvant for cancer radiotherapy. A radioprotective adjuvant for cancer radiotherapy should protect healthy tissues from the adverse side effects of radiotherapy without having radioprotective effect on the tumors. Therefore, we subsequently assessed the effect of TLR5 signaling on the radiosensitivity of tumor in vivo using a model of experimental radiotherapy. As shown in Digure 3, 4-Gy irradiation or CBLB502 administration significantly inhibited tumor growth in vivo compared with untreated control, and there was no difference in tumor volume and weight between the group with 4-Gy irradiation alone and the group with 4-Gy irradiation plus CBLB502 administration, showing that CBLB502 had no radioprotective role in tumor in vivo, although CBLB502 and radiation had no synergy effect on inhibiting tumor growth.

Discussion

Like gastrointestinal tract, lungs open to the exterior, which makes lungs vulnerable to attack by exogenous pathogens, including various bacteria. Even under physiological condition, there exist many bacteria in

almost all the parts of respiratory tract except pulmonary alveoli. In fact, bacterial pneumonia is one of the most common infectious diseases. Based on the finding that activation of TLR5/MyD88 signaling could inhibit the growth of colon cancer (Rhee et al., 2008) and the facts that TLR5 was highly expressed in human lung tissue by airway epithelial cells, neutrophils, and alveolar macrophages, this study further investigated the effects of CBLB502, a derivant of flagellin, and TLR5/ MyD88 signaling on the growth of lung cancer in vivo. We proved the existence of TLR5/MyD88/NFϰB in lung adenocarcinoma cell line A549 derived from bronchial epithelial cells. The activation of TLR5 could suppress the viability of A549 cells in vivo and delay the tumor growth in vivo. On the contrary, the inhibition of TLR5 signaling enhanced the tumor growth in vivo. Further, we found that TLR5 signaling regulated tumor growth through affecting leukocyte recruiting cytokines, including IL-8, MIP-3α, and ENA-78, and subsequently changing the neutrophil infiltration in tumor. Finally, we confirmed that the activation of TLR5 signaling did not affect the radiosensitivity of tumor in vivo.

In the past several years, TLR signaling has been extensively investigated. More evidence indicated that TLR signaling could enhance the anti-cancer immunity and the molecules involved in this pathway were often used as targets for therapy against cancer, although there were reports that expression of TLR could lead to tumor progression (Wolska et al., 2009). Many TLR agonists were proved to be helpful for the treatments of various kinds of cancer (Salaun et al., 2006; Zhang et al., 2009; Cheng and Xu, 2011; Lin et al., 2011; Zhang et al., 2011), and TLR5 agonist was reported to result in significant tumor regression (Rhee et al., 2008). Here, we further proved that TLR5 agonist could also inhibit the growth of A549 xenograft model in vivo.

Although the activation of various TLRs are anticancer and many TLR-specific agonists are being tested currently (Kanzler et al., 2007), the effectiveness of single TLR agonists is constrained due to the variable cellular TLR expression. MyD88 is the common key downstream molecule of many TLR signaling except for TLR3, thus it is considered as a more potent target for tumor therapy, compared with single TLR agonists. MyD88-expressing vector could inhibit local and systemic growth of multiple tumor types in vivo through enhancing adaptive immune responses (Hartman et al., 2010). In addition, the activation of MyD88 could enhance the mouse and human dendritic cells for antitumor efficacy (Narayanan et al., 2011). In the current study, the inhibition of MyD88 promoted the tumor progression. In the previous study, infiltrating inflammatory cells, such as neutrophils and macrophages, could destroy cancer cells by rapid cytolysis (Hicks et al., 2006). Furthermore, neutrophils also showed antituomor effects in several types of cancer models (Challacombe et al., 2006; Nozawa et al., 2006). Neutrophils could kill tumor cells through producing some cytotoxic mediators, including reactive oxygen species, proteases, membrane-perforating agents, and several cytokines, such as TNF-α, IL-1β, and IFNs (Di et al., 2001). Our results indicated that CBLB502 could induce the secretion of neutrophil recruiting cytokines through MyD88, subsequently promote the infiltration of neutrophils, and finally result in the inhibition of tumor growth in vivo. The angiogenesis is important for the growth of both primary and metastatic tumor. Agents blocking the specific processes essential for tumor vascular development are one of the most promising approaches for tumor therapy. In this study, our result demonstrated that the inhibition of tumor growth induced by CBLB502 was not involved in suppressing angiogenesis.

Recent study has shown that the activation of TLR5 by CBLB502 possesses radioprotective activity in mouse and primate models (Burdelya et al., 2008). More importantly, CBLB502 has no radioprotective effect on the tumors when it is administrated to tumor-bearing mouse treated with radiotherapy. These results suggest that combining CBLB502 with radiotherapy could provide protection for healthy tissues without affecting radiation-induced cell death. In accordance with this previous study, our result indicated that the activation of TLR5 signaling did not interfere with the radiosensitivity of A549 xenograft in vivo.

The role of TLR5 signaling in response to infection has been well characterized (Khoo et al., 2011). In the current study, we showed that the activation of TLR5/Myd88 signaling could upregulate the secretion of cytokine which regulates the neutrophil infiltration in tumor xenografts. The infiltrating neutrophil finally inhibited the growth of tumor xenografts. These results implied that microflora in the respiratory tract, which could initiate the innate immune, might be helpful to tumor regression.

Acknowledgements

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