RESEARCH ARTICLE

Toll-like Receptor 5 Agonism Protects Mice from Radiation Pneumonitis and Pulmonary Fibrosis

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Abstract

Radiation pneumonitis and pulmonary fibrosis are the main complications with radiotherapy for thoracic neoplasms, directly limiting the efficient dose in clinical application and currently there are few medicines that effectively function as radioprotectants. However, a TLR5 agonist, CBLB502, was confirmed to have protective efficacy against hematopoietic and gastrointestinal radiation syndromes in mice and primates. This study points to a new direction for protection against thoracic radiation-induced pulmonary syndromes and skin injury by CBLB502. We utilized the TUNEL assay, pathological analysis and immunohistochemistry to obtain evidence that CBLB502 could alleviate the occurrence of radiation pneumonitis and pulmonary fibrosis as well as radiation-induced skin injury. It may thus play a promising role in facilitating clinical radiotherapy of thoracic neoplasms.

Keywords: CBLB502 - radiation pneumonitis - pulmonary fibrosis - thoracic radiotherapy

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Introduction

Radiation therapy, known as one of the three main therapeutical methods for pulmonary cancer, has extensive application in current pulmonary neoplasms clinical treatment. However, radiation pneumonitis and subsequent pulmonary fibrosis are the major complications received thoracic radiation therapy and considerably influence the radiotherapeutic effectiveness (Mehta et al., 2005). Given that the pathogenesis of radiation pneumonitis and pulmonary fibrosis is still indefinite, it has reported (Kuwano et al., 1999) that the apoptosis of pulmonary epithelial cells and vascular endothelial cells induced by radiation is the initiating agent of radiation pulmonary pneumonitis and pulmonary fibrosis. Currently the methods for prevention and cure of radiation pulmonary injuries are relatively restricted and the only radioprotectors used clinically, amifostine, owing to its toxicity and worse selectivity, could not satisfy clinic emergency and new radioprotective compounds with better efficacy are extremely needed.

TLR5, a typical Toll-like receptor, interacting with its ligand, bacterial flagellin, subsequently activate the NF-κB by the MyD88-Dependent Pathway. Previous research (Burdelya et al., 2008) found that CBLB502 can dramatically protect mice and primates from hematopoietic, gastrointestinal radiation syndromes induced by total body irradiation without reducing the radiosensitivity of tumor cells. A further research found that the agonist of TLR5 has significantly suppression effect to intestinal tract epithelial and vascular endothelial apoptosis. On these backgrounds, recently it has reported (Burdelya et al., 2012) CBLB502 protects mice from dermatitis and mucositis caused by local radiation that provides implications for head-and-neck cancer radiotherapy. It was proved by our previous research (Zhou et al., 2012) that CBLB502 inhibited the growth of lung cancer tumor cell line A549 and hardly affect the radiosensitivity of tumors in vivo.

In this study, we explore whether CBLB502 has effectiveness in protecting mice from pneumonitis and subsequent pulmonary fibrosis developed as a consequence of single dose local irradiation of thoracic area and whether it could be used against the dermitis induced by radiation.

Materials and Methods

Animal grouping, treatment and γ-irradiation

Six-week-old male C57BL/6J mice weights in approximately 20g were purchased from the center of experimental animal, the Academy of Military Medical Sciences (Beijing, China). The mice were divided into five groups: untreated control (CON), irradiated-alone (20Gy), irradiated+CBLB502 0.05mg/kg (20Gy+L), irradiated+CBLB502 0.2mg/kg (20Gy+M) and irradiated+CBLB502 0.5mg/kg (20Gy+H) (each group n=40).30 minutes before irradiation, mice were injected subcutaneously (s.c.) with CBLB502 in medication administration groups and with equal-volume

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physiological saline in irradiated-alone group. For thoracic irradiation, each mouse was anesthetized by intraperitoneal (i.p.) application of 300μL 3‰ pentobarbital sodium for fixation. Cobalt-60 gamma radiation was administered as 20Gy single dose to the entire thorax (297.43cGy/min), while other parts shielded with lead bricks. Diet and water were supplied ad libitum postirradiation. Respectively mice were sacrificed at 24 hours, 1 month, 3 months and 5 months postirradiation for parameters analysis.

TUNEL staining of apoptotic cells

The mice were sacrificed by cervical dislocation 24 hours postirradiation and the lungs were obtained. Briefly, lungs were fixed in 4% formalin, followed by overnight fixation, embedding in paraffin, sectioned at 3 μm, then deparaffinized in xylene solution, and hydrated using graded ethanol. Sections were then placed in an antigen retrieval solution. The solution was heated at 100°C for 30 min in a microwave oven and cooled at room temperature for 10 min. After rinsing with deionized water, an endogenous peroxide blocking solution of 0.3% hydrogen peroxide was applied for 15 min at room temperature. The sections were then incubated in 10% goat serum in phosphate-buffered saline (PBS) for 10 min at room temperature to reduce nonspecific antibody binding. Terminal deoxynucleotidyl transferase (TdT) reactive fluid was added and the course reacted in moist circumstance 37°C for 1 hour. Labeled with HRP-Digitab and stained with DAB, the sections were taken pictures with Olympus BX61 microscope. The mean density of random visual fields was semiquantitatively analyzed with software Image Pro plus 6.0.

Histological analysis

For histological analysis, the left lungs and skin around the neck were fixed in 4% formalin, embedded in paraffin, and sectioned at an average thickness of 3 μm. The partial mounted sections were subjected to hematoxylin and eosin staining. The rest sections of lungs were first treated to remove paraffin and rehydrated. Nonspecific sites were blocked with PBS-10% solcoseryl for 30 min at room temperature, and sections were incubated with primary antibody overnight at 4°C. Anti-surfactant proteins B (SP-B) and anti-laminin (LN) rabbit polyclonal antibodies were at a dilution of 1:200 used for primary antibodies. The next day, after washes, a poly-HRP-conjugated goat anti-rabbit secondary antibody was applied at a dilution of 1:300 for 2h at room temperature. The sections was stained by DAB and taken pictures with Olympus BX61 microscope. The mean density of random visual fields was semiquantitatively analyzed with software Image Pro plus 6.0. At least 10 pictures of each group were counted.

Statistical analysis

Quantitative data are given as mean values ± SD or as indicated. For analysis of differences between the groups, ANOVA followed by the appropriate post hoc test for individual comparisons between the groups was performed. All tests were two-tailed. $P<0.05$ was considered statistically significant.

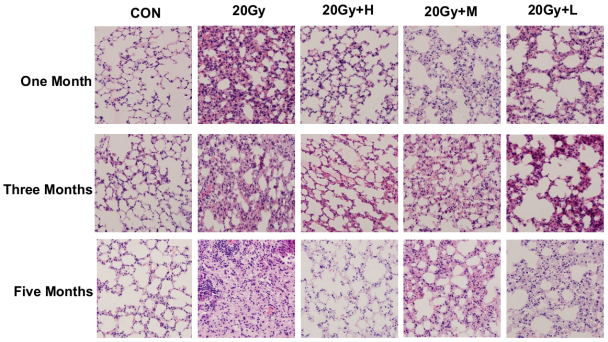

Figure 1. The Effect of CBLB502 on the Development of Inflammatory Cells Infiltrate and Fibrous Degeneration Postirradiation. The mice were sacrificed respectively after 1 month (upper row), 3 months(middle row) and 5 months (lower row). The lungs embedded in paraffin was cut into sections and subjected to H&E staining. The pictures showed pathological change of each group during the development of radiation pneumonitis and fibrosis (400X)

Results

CBLB502 moderates the development of inflammatory cells infiltrate and fibrous degeneration postirradiation

The mice subjected to single flushing dose of thorax irradiation apparently displayed a phase effect in the development of radiation pneumonitis and pulmonary fibrosis. The lungs of each group were obtained at 1 month, 3 months and 5 months respectively for pathological analysis (Figure 1). Sacrificed at 1 month postirradiation, the irradiated-alone group showed significantly acute inflammation compared with untreated control: it represented a dose-dependent leakage of inflammatory cells into the alveolar space, a thickening of the alveolar septa and fibroplasia, while the untreated control group had clear structure without abnormal alterations. The CBLB502-combined radiation groups, particularly 20Gy+H, had comparatively integrity pulmonary alveoli structure, less infiltration of inflammatory cells and unapparent alveolar septa thickening. However, at 3 months after radiation, the alveolar septa showed a further widening and an edema of the interstitium resulting in partial vanishing of alveoli structure in the irradiated-alone group. 20Gy+L appeared slight symptom of pneumonitis and 20Gy+H was much more approaching CON, which indicated an obvious drug concentration dependent. It was remarkable that CBLB502 took a role in overcoming the damage of 20Gy radiation while the irradiated-alone group showed severe distortion of structure and total fibrous obliteration at the 5th month.

CBLB502 inhibits the radiation-induced apoptosis of pulmonary cells

In the purpose of observing the effectiveness that CBLB502 displayed on the apoptotic cells, we sacrificed mice and dislodged lungs 24 hours after 20Gy single dose thoracic irradiation. It was obvious that CBLB502 effectively suppressed the apoptosis of pulmonary cells. The irradiated-alone mice presented a notable amount of positive cells while the untreated-control mice showed rare numbers. CBLB502 administration 30min before

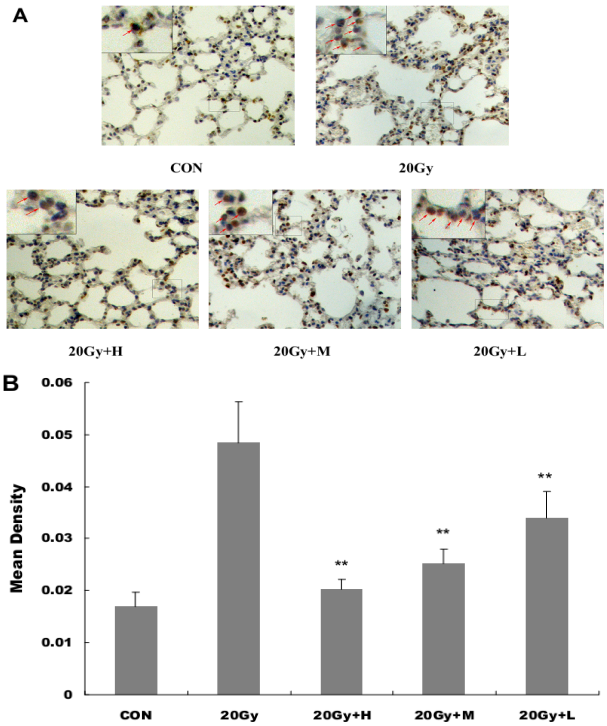

Figure 2. The Effect of CBLB502 on Radiation-Induced Apoptosis of Pulmonary Cells. C57BL/6J mice were sacrificed 24 h after 20 Gy thoracic radiation. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assayed the apoptotic cells which were stained in a color of brown. Arrows points show the positive cells (A, 200X). Semiquantitatively analyzing the mean density of apoptotic cells presents in B. **Significant difference between untreated control group and CBLB502-combined radiation groups ($p<0.01$)

radiation resulted in protective effect from lesion of radiation, especially the pulmonary cells of high dose (20Gy+H) group much approaching CON showed less stained, which indicated more efficacious function against apoptosis (Figure 2A). Semiquantitative analysis of mean Density showed significant difference between irradiated-alone group and CBLB502-combined radiation groups which also displayed in a concentration-dependent manner (Figure 2B).

CBLB502 influences the expression of laminin and surfactant proteins B postradiation

The expression alteration of Laminin (LN) and surfactant proteins B (SP-B) was thought to be the formation hallmark of pulmonary fibrosis. Thus, we estimated LN and SP-B expression levels of mice lungs by immunohistochemisty 3 months and 5 months postirradiation. Under the microscope, irradiated-alone group highly expressed LN at the 5th month by which time CBLB502-combined groups represented less changes. For SP-B, irradiated-alone group showed lower expression and no alteration were observed in CBLB502-combined groups. Semiquantitatively, LN expression was much higher in irradiated-alone group compared with the CON. Significant differences of LN were observed between irradiated-alone group and CBLB502-combined groups after radiation 3 months (Figure 3A) and so did 5 months postirradiation (Figure 3C). However, contrast to

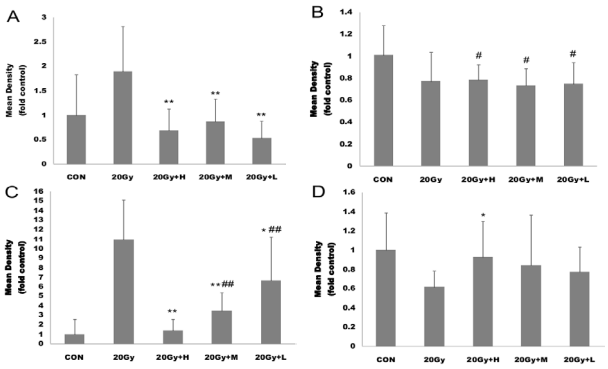

Figure 3. The Effect of CBLB502 on the Designated Indicator LN and SP-B Postirradiation. (A) LN levels of each group 3 months after irradiation treatment were analyzed with Immunohistochemistry. Pictures (at least 10) were assayed by software Image Pro Plus 6.0. The mean density was analyzed. **Significant difference between irradiated-alone group and CBLB502-combined radiation groups ($p<0.01$). (B) SP-B levels of each group 3 months after irradiation treatment were analyzed with Immunohistochemistry. #Significant difference between untreated control group and CBLB502-combined radiation groups ($p<0.05$). (C) LN levels of each group 5 months after irradiation treatment were analyzed with Immunohistochemistry. *Significant difference between irradiated-alone group and CBLB502-combined radiation groups ($p<0.05$). ##Significant difference between untreated control group and CBLB502-combined radiation groups ($p<0.01$). (D) SP-B levels of each group 5 months after irradiation treatment were analyzed with Immunohistochemistry

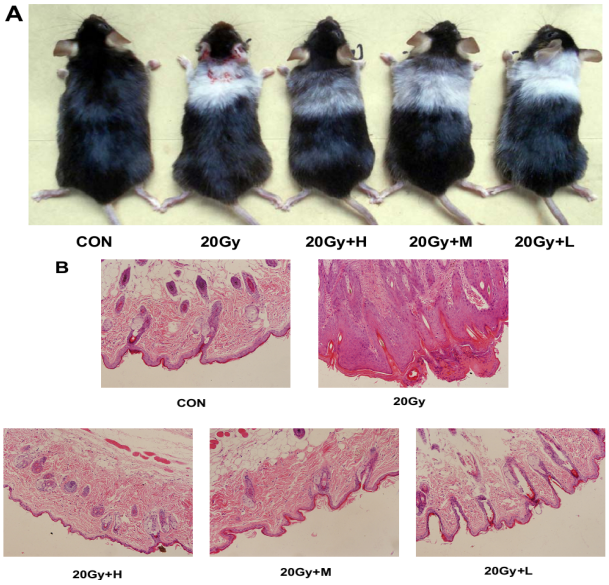

Figure 4. CBLB502 Affects the Alteration of Irradiated Region Color (A) and Skin Histopathology (B, 400X) 5 Months Postirradiation

LN, SP-B was increasingly lower after radiation and the CBLB502 treatment hardly retrieved the expression of SP-B at the 3rd month postirradiation (Figure 3B). Despite CBLB502 did not show effect of promotion at 3 months, it had somewhat elevation of CBLB502-combined groups than the irradiated-alone mice at the 5th month, especially 20Gy+H (Figure 3D).

CBLB502 alleviates the radiation-induced hair and skin injury

We discovered 5 months after radiation that the hair

and skin of irradiated region showed phenotypic changes. The C57BL/6J mice, which was known as black, turned into dramatically white at the thoracic irradiated area. This alteration of irradiated-alone group displayed extremely apparent, and less in the CBLB502-combined groups, particularly 20Gy+H. The radiation-induced dermal ulcer was significantly observed in irradiated -alone mice, but not presented in other groups (Figure 4A). The irradiated neck skin at the same area of each group was subjected to hematoxylin and eosin (H&E) staining. Under the microscope, compared with other groups, IR-alone mice showed atrophy of the hair follicles, hyperkeratosis, hyperemia and hemorrhage, hyperplasia of the epidermis with papillary thickening of its layers. CBLB502 administrated groups had no noteworthy difference between each other and with untreated control group.20Gy+L appeared diffusion of dermis cells with a mass of inflammatory cells infiltration (Figure 4B).

Discussion

In our research, we found that administrating CBLB502 30 minutes before radiation could alleviate local radiation-induced radiation pneumonitis and pulmonary fibrosis, which was thought closely correlated with the apoptosis of pulmonary cells. Other evidences showed the protective effect of CBLB502 on the local radiation-induced skin injury.

Histopathology analyzing the pulmonary tissue of 1 month, 3 months and 5 months postirradiation, we found that the pathological changes of CBLB502 pretreated groups showed comparatively moderated than IR-alone group. Previous report (Brickey et al., 2012) in their research utilizing wild type and Myd88-deficient C57BL/6J mice for exploring survival ratio, pulmonary injury pathologic difference and inflammatory factor expression after radiation, found that Myd88-deficient mice displayed more sensitive to thoracic single flushing dose of radiation resulting in higher mortality and increasingly expression of fibrotic marker gene. However, it has been confirmed firstly in 2008 (Burdelya et al., 2008) that the agonist of TLR5, flagellin and the designated CBLB502, had radioprotective effects to mice and primate animals that received whole body irradiation. Subsequent experiments found that flagellin and CBLB502 protected against hematopoietic system and gastrointestinal tract acute radiation syndromes. CBLB502, as the agonist of TLR5, could activate downstream NF-κB which transcribes a variety of factors that could promote cell regeneration through TLR5 signal pathway and thereby suppress intestinal tract epithelial and vascular endothelial apoptosis (Tallant et al., 2004). Consistent with previous reports, we administrated CBLB502 subcutaneously prior to radiation and it could efficiently decrease the apoptosis of pulmonary cells. This suggested that CBLB502 protects pulmonary cells from radiation-induced apoptosis as well as intestinal tract, thus influents the process of radiation pneumonitis and pulmonary fibrosis. It was assumed that CBLB502 playing a protective role against apoptosis of pneumonocyte, known as one major inducement of radiation pneumonitis and pulmonary fibrosis, made the main reason why it functioned as a radioprotectant. It was also reported that mice were protected from radiation by flagellin through in a TLR5/MyD88-dependent manner (Vijay-Kumar et al., 2008). According to the reports and our research, we could conclude that CBLB502 depends on TLR5/MyD88 pathway to activate transcription factor NF-κB, inhibits the apoptosis of pulmonary cells and ultimately ameliorates radiation pneumonitis and radiation pulmonary fibrosis.

Another potential mechanism that might contribute to moderate the process of radiation pneumonitis and radiation pulmonary fibrosis is that diverse cytokines and chemotactic factors suppress the fibrotic process, such as IFN-γ, IFN-α/β, IL-10, IL-12, CXCL10, CXCL11 (Wynn, 2007) and SOD (Delanian et al., 1994). CBLB502 interacting with TLR5 not only activates transcription factor NF-κB, but stimulates many kinds of cytokines and enzyme for regulation, including radioprotective compounds such as SOD2 (Epperly et al., 2002), G-CSF (Dale, 2002) and IL-6 (Atkinson et al.1995). It has reported that eliminating the activity of G-CSF/ IL-6 could impressively reduce the radioprotective capacity of CBLB502 with no matter mice, primates or dogs (Krivokrysenko et al., 2012). Besides, SOD displays dramatically anti-fibrotic process induced by radiation as well as radioprotective effectiveness. This indicates that those molecules take an essential part in the radioprotective process of CBLB502.

Radiated skin injury is another main complication in the radiotherapy of thoracic neoplasms. It is obvious in our research that the hair and skin subjected to irradiation turned into white 5 months postirradiation and dermal ulcer was observed in IR-alone group. CBLB502-combined groups showed concentration-dependence which suggests the radioprotection of skin injury. Previous research (Burdelya et al., 2012) has confirmed CBLB502 protects mice from dermatitis and mucositis caused by local radiation, which is consistent with our results. Whereas radiological dose reaches as highly as 20Gy to 40Gy, skin stem cells in basal lamina could not form new matured cells and meanwhile skin cells in upper layer continuously eliminate, blood capillary expands, becomes circuity, microthrombosis, ischemia and necrosis which result in desquamation of epithelium and ultimately dermal ulcer. High energy ray directly insulting the DNA of epidermal cells and stem cells suppress their ability of proliferation and fission.CBLB502 activates NF-κB to transcribe lots of anti-apoptotic cytokines and chemokines that efficiently decrease radiation-induced DNA damage and thereby protects skin from radiation injury.

To summarize, in our studies, it has been proved that preventively CBLB502 administration could alleviate the occurrence of radiation pneumonitis and pulmonary fibrosis as well as radiation-induced skin injury. Rich of reports have confirmed that CBLB502 was able to inhibit various normocellular apoptosis without influencing the radiosensitivity of tumor cells. That indicates the implemented prospect of CBLB502 in the radiotherapy of thoracic neoplasms.

Acknowledgements

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