Inhibition of Transforming Growth Factor-β, Hypoxia-inducible Factor-1α and Vascular Endothelial Growth Factor Reduced Late Rectal Injury Induced by Irradiation

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Tumor hypoxia and angiogenesis associated with malignant progression have been studied widely. The efficacy of angiogenesis inhibition combined with radiotherapy has been demonstrated in cancer treatment. Here, we studied the effect of hypoxia and angiogenesis inhibition on radiation-induced late rectal injury. The rectum of C57BL/6N mice was irradiated locally with a single dose of 25 Gy. Radiation-induced histological changes were examined at 90 days after irradiation by hematoxylin-eosin (H.E.) staining and azan staining. Pimonidazole was administered and its distribution was assayed by immunohistochemistry staining. Expression of transforming growth factor β1 (TGF-β1), hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) was assessed on the fibrotic region using real-time PCR and immunohistochemistry. In addition, the effects of TGF-β, VEGF and HIF-1α on radiation-induced injury were investigated by the administration of neutralizing antibody of TGF-β, antibody of VEGF or YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole) which was developed as an agent for inhibiting HIF-1 expression after irradiation respectively. Fibrosis and uptake of pimonidazole were found 90 days after irradiation. The expression of TGF-β1, HIF-1α and VEGF significantly increased with the formation of fibrosis induced by irradiation compared with unirradiated controls. In addition, treatment of neutralizing antibody of TGF-β, antibody of VEGF or YC-1 reduced the development of radiation-induced injury. Our results suggested that radiation-induced hypoxia may play an important role in late rectal injury. Although the inhibition of HIF-1α and VEGF reduced the radiation induced late injury, the precise mechanism is still unclear.

INTRODUCTION

Radiation therapy comes into existence in the balance between tumor control and normal tissue injury inducing irreversible sequelae. If a dose that kills tumor cells is less than the dose inducing late normal tissue injury, radiotherapy can be safely conducted. The pathology of late injury is mostly related to the sequences of fibrosis and tissue remodeling in irradiated tissues.1) Late injury impairs tissue function and is sometimes potentially life-threatening. Moreover, once it happens, no curative, only palliative treatment is left.

Previous studies have demonstrated that the late response results from a continuous production of various cytokines and growth factors including transforming growth factor β1 (TGF-β1), which is considered to be a main switch of fibrotic process.2) Early and persistent endothelial and vascular injury in irradiated tissues is a characteristic feature of response to irradiation. The reducing of oxygen tension in skin has been demonstrated after irradiation.3) A recent study indicates that post-radiation hypoxia may contribute to the late lung injury.5) Our previous study also reported that post-radiation hypoxia may play a role in radiation-induced rectal fibrosis by involving TGF-β1, hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF).5) In addition, angiogenesis inhibition, mainly by VEGF inhibition, was demonstrated to be effective for improving the tumor response to radiotherapy for experimental tumors.6,7) However, the effect of angiogenesis inhibition on radiation-induced normal tissue injury is unclear.

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Radiation proctitis featured by fibrosis often occurs after pelvic radiotherapy, particularly after treatment of prostatic and cervical cancer. It is a common side effect that worsens quality-of-life in patients receiving radiotherapy because of bleeding, atrophy and strictures of the rectum. In this study, we used mouse rectum to investigate: (1) the expression of hypoxia in radiation-induced late injury and (2) the effect of neutralizing antibody of TGF-β, antibody of VEGF and HIF-1α inhibitor on prevention of fibrosis in irradiated rectal tissues.

MATERIALS AND METHODS

Animals
Female C57BL/6N mice, 10 weeks old, were purchased from Japan Charles River Laboratories (Yokohama, Japan). The animals were housed in a pathogen-free room under controlled temperature, humidity and 12-hour dark/light cycles, and were allowed to acclimate from shipping for 2 weeks before irradiation treatment. All animal experiments were carried out according to the Guidelines for Animal Experimentation of Hirosaki University.

Irradiation
X-ray irradiation (RT) was performed using 150 kV, 5 mA, with 0.5 mm Al filter, at a dose rate of 1.26 Gy/min (Hitachi MBR-1505R2, Hitachi, Japan). Briefly, the unanesthetized mice were confined to plastic jigs and irradiation was performed locally to the rectums and anuses (10 mm in diameter). The irradiation dose of 25 Gy was chosen because fibrosis was induced in more than 80% of irradiated mice and they survived for a sufficiently long time according to our previous study. After irradiation, the mice were maintained four to six per cage in a pathogen-free room supplied with standard laboratory diet and water ad libitum.

Administration of antibodies and inhibitor
After irradiation, four to six irradiated mice received intraperitoneal (i.p.) injection of neutralizing antibodies of TGF-β (β1, β2, β3) (anti-TGF-β) (R&D Systems, Minneapolis, MN), VEGF (R&D Systems) (anti-VEGF) or YC-1 (3-(5’-hydroxymethyl-2’-furyl)-1-benzylindazole) (Cayman Chemical, Ann Arbor, MI) which is a small molecule inhibitor of HIF-1α expression, respectively, as illustrated in Fig. 1. Group 1 received a single injection of anti-TGF-β (1 mg/kg) immediately after irradiation; Group 2 received injection of anti-TGF-β (1 mg/kg) weekly for 28 days; Group 3 received injection of anti-TGF-β (1 mg/kg) weekly for 84 days; Group 4 received injection of anti-VEGF (500 μg/kg) weekly from the 37th day to the 86th day after irradiation; Group 5 received injection of YC-1 in dimethyl sulfoxide (DMSO) (30 mg/kg) daily from the 31st day to 89th day after irradiation as a control of the Groups of antibody injection, and Group 7 received the vehicle (DMSO) daily from the 31st day to 89th day after irradiation as a control group of YC-1 treatment.

Histological analysis
The mice were sacrificed for collecting of rectal tissues by cervical dislocation at 90 days after irradiation. The samples were partly fixed by 10% neutral-buffered formalin, and then embedded in paraffin. Sections were cut at 4 μm-thickness...
for hematoxylin-eosin (H.E.), azan staining or immunohistochemical examination. Other samples were immediately frozen in liquid N₂ and then stored at –80°C until use for RNA isolation.

For a quantitative analysis of severity of fibrosis, the level of fibrosis was measured as a blue pixel number by Azan staining using Adobe Photoshop (Adobe Systems, San Jose, CA) according to the analytical digital photomicroscopy (ADP) technique. Briefly, adjusted the brightness and contrast of each image, chose blue range which best represents collagen and fibrosis in azan staining, and then the pixel number of it was recorded.

RNA isolation and real-time polymerase chain reaction (Real-time PCR)

Total RNA was obtained from rectum tissue by RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer’s instruction. Complementary DNA (cDNA) was synthesized from 1 μg total RNA and amplification reactions were performed with iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) by ICycler IQ Real-Time PCR Detection System (Bio-Rad Laboratories) in a 20 μl final volume containing 10 μl SYBR Green reagent, 0.5 μl each primer and 9 μl cDNA according to the real-time PCR temperature profiles described previously. Glyceraldehyde-3-A phosphate-dehydrogenase (GAPDH) was used as an internal control. The real-time PCR primer sequences used were follows: TGF-β1 forward, 5'-GACTCTCCACCTGCAAGACCATT-3'; TGF-β1 reverse, 5'-GGGACTGGCGACTGACTG-3'; HIF-1α forward, 5'-AGCCCTAGATGGCTTTGTA-3'; HIF-1α reverse, 5'-TATCGAGGCTGTGTCTAGAG-3'; VEGF-A forward, 5'-GCAGATGTGAACTCAGACAAAA-3'; VEGF-A reverse, 5'-GCAGATGTGAA-

**Detection of tissue hypoxia**

The tissue hypoxia was detected using Hypoxyprobe™-1 Kit (CHEMICON International, Inc., CA) according to the manufacturer’s instructions. Briefly, a dose of 60 mg/kg hypoxyprobe-1 (pimonidazole) was injected (i.p.). Sixty minutes after injection, rectum was obtained, fixed in formalin, and paraffin-embedded sections were processed. Sections were deparaffinized and then incubated with Hypoxyprobe-1 Mab1 (1:50, CHEMICON International, Inc., CA) 60 minutes at room temperature. The slides were washed three times for 5 min in PBS and incubated with 1:500 dilutions of the secondary antibody, biotin-SP-conjugated F(ab’)2 fragment of rabbit anti-mouse IgG (Accurate Chemical Scientific Corp., Westbury, NY).

**Immunohistochemistry**

The sections were deparaffinized for immunohistochemical staining. The sections on slides were treated with rabbit anti-TGF-β1 polyclonal antibody (1:150, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-HIF-1α polyclonal antibody (1:150, Santa Cruz Biotechnology) or rabbit anti-VEGF polyclonal antibody (1:150, Santa Cruz Biotechnology) as a primary antibody for overnight at 4°C. The sections were washed and then treated with a secondary antibody using Histofine SAB-PO(R) Kit (Nichirei Co., Japan) and examined on a microscope (BX50, Olympus, Japan).

**Statistical analysis**

All experiments were performed at least three times. Data

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**Fig. 2.** Representative histological changes in rectal tissue after irradiation. Blue stain indicates fibrosis in azan section. Cont: unirradiated control mice; RT: 90 days after irradiation. Magnification: ×40 (A); ×100 (B).
are presented as mean ± standard deviation. Statistic analyses were performed using Student t-test with KaleidaGraph (Version 4.0, Synergy Software). P value < 0.05 was considered statistically significant.

RESULTS

Radiation-induced histological change in rectal tissues
Histological changes in rectal tissue after irradiation were analyzed by H.E and azan staining (Fig. 2). The results showed that the histological changes characterized by loss of surface epithelium, deep erosion of mucosa, thickening of lamina propria and submucosa, collagen deposition and fibrosis were found on day 90 after irradiation (Fig. 2). In unirradiated control rectum, no lesions were observed.

Transcription factor and cytokine mRNA expression in rectal tissues after irradiation
The mRNA levels of TGF-β1, HIF-1α and VEGF following irradiation with a single dose of 25 Gy were determined on day 90 after irradiation (Fig. 3). Results showed that TGF-β1 (Fig. 3A), HIF-1α (Fig. 3B) and VEGF (Fig. 3C) mRNAs expression increased significantly on day 90 after irradiation compared with the unirradiated mice.

Pimonidazole deposition in irradiated rectum
The deposition of the tissue hypoxia marker pimonidazole was analyzed by immunohistochemistry. Pimonidazole was accumulated significantly in irradiated rectum corresponding to the fibrotic foci compared with unirradiated control (Fig. 4).

Expression of TGF-β1, HIF-1α and VEGF proteins in irradiated rectal tissues
The expression of cytokine proteins in irradiated rectal tissues was analyzed by immunohistochemistry. In addition,
TGF-β1, HIF-1α and VEGF proteins also increased significantly on day 90 after irradiation (Fig. 4). These results were consistent with those mRNAs expression (Fig. 3) and suggested that radiation-induced TGF-β1 and angiogenic cytokines may contribute to late rectal injury after irradiation.

Effect of administration of neutralizing anti-TGF-β, anti-VEGF or YC-1 on fibrosis induction induced by irradiation

The effect of anti-TGF-β, anti-VEGF or YC-1 treatment on the rectal injury induced by irradiation was analyzed by histological analysis. These results showed that anti-TGF-β, anti-VEGF or YC-1 administration reduced the injury of mucosal epithelium, submucosa, muscularis propria and the collagen deposition induced by irradiation compared with that of irradiation only mice (Fig. 5A). For a quantitative analysis of fibrosis (Fig. 5B), the rectal injury induced by irradiation was all reduced after anti-TGF-β treatment for 1 day, 28 days and prolonged treatment for 84 days after irradiation. These suggested that the inhibitory effect of anti-TGF-β treatment is independent from the administration schedule of the antibody administration. In addition, we also investigated the effect of anti-VEGF or HIF-1α inhibitor (YC-1) treatment in the late phase after irradiation. Treatment by the anti-VEGF or YC-1 reduced the fibrosis obviously.

DISCUSSION

Radiation-induced late normal tissue injury including fibrosis surrounding tumor is a significant problem in patients treated with radiotherapy. However, the mechanism remains unclear. Almost all studies revealed that profibrotic cytokines, especially TGF-β are involved in late radiation injury by Smad proteins.

In a previous study, we have shown that TGF-β1 mRNA expression related to the change of fibrosis from 1 day to 90 days after irradiation. Both mRNA and protein of TGF-β1 increased significantly with the fibrotic response 90 days after irradiation, suggesting a potential role for TGF-β in the induction of radiation-induced fibrosis. In present study,
we studied the effect of TGF-β inhibition by anti-TGF-β. After the administration of anti-TGF-β for 1 day, 28 days or 84 days after irradiation, radiation-induced fibrosis reduced in grade compared with rectal tissues that received irradiation alone, suggesting that TGF-β expression, probably its early expression, contribute to the late injury in irradiated rectal tissues. Our results are consistent with those of the previous studies on lung, liver and intestine.

Recently, the new concept of radiation-induced lung fibrosis focused on the role of post-radiation hypoxia. Radiation-induced injury in the vascular endothelium and microvasculature could in turn decrease the tissue oxygenation and lead to hypoxia. Further study demonstrated that hypoxia-induced reactive oxygen species (ROS) and macropage accumulation contributed to the development of lung fibrosis after irradiation. Here, the deposit of the specific hypoxia marker pimonidazole was found significantly in irradiated rectal tissue suggesting hypoxia induced by irradiation in rectal tissue.

Under hypoxia condition, the transcription factor HIF-1α stabilizes, translocates to the nucleus and then activates the transcription of downstream genes including VEGF. The effect of VEGF on endothelial cells and as a positive mediator of angiogenesis on revascularization is well-known, and which contributes to the tumor progression and wound healing.

Herein, we hypothesize that the injury induced by irradiation may be severe because of insufficient wound healing by suppression of VEGF or HIF-1α, which is the major upstream factor of VEGF. According to mRNA expression of HIF-1α and VEGF in our previous study, we made the treatment of HIF-1α and VEGF inhibition in the late phase after irradiation. Interestingly, fibrosis was unexpectedly reduced instead of being aggravated. Our results suggested that HIF-1α and VEGF induced by radiation-induced hypoxia contribute to the development of radiation injury. Similar results were also reported by others on radiation-induced central nervous system injury and belomycin-induced lung fibrosis after suppression of VEGF by the transgenic method. Other studies demonstrated that VEGF can play a proinflammatory role in injury by increasing vascular permeability and inducing edema, and then aggravate the injury.

VEGF pathway inhibition treatment combined with radiotherapy is currently available for cancer therapy. It is true that the effect of VEGF inhibition on normal tissue injury in radiotherapy is still unknown, but VEGF inhibition may also have the potential to suppress the normal tissue injury. The current study suggested the complex function of VEGF should be investigated in understanding of radiation-induced normal tissue injury.

In summary, our studies demonstrated that post-radiation tissue hypoxia is critical for fibrosis, which involves profibrotic and angiogenic cytokines in rectal tissues. Further investigation of the relationship between profibrotic cytokines and angiogenic cytokines including complexity of vascular system may be necessary to understand the radiation-induced normal tissue injury.

REFERENCES


