Hypoxia Expression in Radiation-induced Late Rectal Injury

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Tumor hypoxia and angiogenesis have been studied extensively. However, the relation between normal tissue injury and hypoxia is still unclear. In this study, we investigated the effect of hypoxia on radiation-induced late rectal injury in mice. The rectum of C57BL/6N mice was irradiated locally with a single dose of 25 Gy and the following experiments were performed including hematoxylin-eosin (H. E.) staining, azan staining, real-time PCR, immunohistochemistry and immunofluorescence. Radiation-induced fibrotic changes were observed from 14 days and reached the peak 30 days after irradiation. The expression of transforming growth factor β1 (TGF-β1), hypoxia-inducible factor-1α (HIF-1α), vascular endothelial growth factor (VEGF) and endothelial cell marker CD31 increased significantly with the formation of fibrosis induced by irradiation compared with unirradiated control. In addition, the maximum expression of TGF-β1, HIF-1α and VEGF was found at 14, 30 and 90 days after irradiation, respectively. The temporal changes of cytokines were consistent with the dynamic change of fibrosis. Our data suggests that late normal tissue injury involved various cytokines including hypoxia-induced angiogenic cytokines. These results may have important implications in the understanding of radiation-induced late normal tissue injury.

INTRODUCTION

The dose-limiting factor of radiotherapy is the extent and severity of late complications found in normal tissues surrounding tumors. Lesions characterized by fibrotic changes in stroma were found in various tissues where sufficient doses were given, which can impair tissue function, worsen the quality of life and even potentially threaten life. However, the molecular mechanism remains unclear.

Previous studies have postulated that the initial response of late injury results from a continuous production of cytokines and growth factors including interleukin 1α (IL-1α), platelet derived growth factor-AA (PDGF-AA) and transforming growth factor β1 (TGF-β1) which is considered as a master switch of the fibrotic process. Recently, attention to the role of the hypoxia has contributed to the understanding of radiation-induced late normal tissue response in many tissues including lung and central nervous system (CNS).

Hypoxia is a common characteristic of tumors which contributes to malignant progression including increasing probability of regional spread and distant metastasis, and then inducing the resistant response to radio- and chemotherapy. Under hypoxic conditions, the diverse hypoxia-driven genes are regulated by a transcriptional factor, hypoxia-inducible factor-1 (HIF-1). HIF-1 is a heterodimer consisting of HIF-1α and HIF-1β subunits. HIF-1β is expressed constitutively, while HIF-1α is rapidly degraded by E3 ubiquitin ligase complex under normoxic conditions. Under hypoxic conditions, HIF-1α stabilizes and translocates to the nucleus, where it heterodimerizes with HIF-1β. The resultant product is an active HIF-1 protein that binds to specific hypoxic response elements of target genes and then activates transcription of these genes including vascular endothelial growth factor (VEGF). VEGF is essential for many angiogenic processes in tissues by stimulating vascular endothelial cell proliferation, such as tumor vascularization, mainly by interacting with two tyrosine kinase receptors, VEGFR-1 and VEGFR-2.

The rectum is a highly critical organ which is almost invariably exposed during radiotherapy for abdominal and pelvic cancer including uterine, prostate and rectum cancers. In our previous study, the animal model of radiation-induced late rectal injury was established successfully. Here, we investigated the effect of radiation-induced...
hypoaxia in late normal tissue injury using the model.

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 they were allowed to acclimate from shipping for 2 weeks before irradiation treatment. All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation of Hiroaki 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) as described previously. Briefly, the unanesthetized mice were confined to plastic jigs and irradiation was applied locally to the rectums (10 mm in diameter). The rest of the body was shielded by lead collimators. The irradiation was performed with a single dose of 25 Gy, which was chosen because fibrosis was induced in more than 80% of all irradiated mice and they survived for a long enough time after rectal irradiation in our previous study. After irradiation, the mice were housed in plastic cages (four to six mice per cage) in pathogen-free room and supplied with standard laboratory diet and water ad libitum.

Tissue sampling and histological analysis

The mice were sacrificed by cervical dislocation for collecting rectal tissue at day 1, 7, 14, 30 and 90 after irradiation. Rectal tissue was obtained and sectioned lengthwise.

Half of the sections were fixed by 10% neutral-buffered formalin, and then embedded in paraffin. Sections were cut with 4 µm-thickness for hematoxylin-eosin (H. E.), azan staining or immunohistochemical examination. The rest of the samples were flash-frozen in liquid N2 and then stored at −80°C until to be used for RNA isolation.

For analysis of severity of fibrosis, the level of fibrosis was measured by observing the extent of mucosal depletion, submucosal thickening and collagen deposition by microscopy and graded as shown in Fig. 1.

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

In brief, total RNA was obtained from rectum tissue by RNaseasy Mini Kit (QIAGEN, Germany) according to the manufacture’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, USA) 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 as follows: TGF-β1 forward, 5'-GAC-TCTCCAAGGTCAAGCCAT-3'; TGF-β1 reverse, 5'-GG-GACTGCGAGCCTTAGTT-3'; HIF-1α forward, 5'-AGGCCTAGATGGCTTTGTGA-3'; HIF-1α reverse, 5'-TATCGAGCGCTGTGTCGACTG-3'; VEGF-A forward, 5'-GAGCAGATGTGTAAGGACCA-3'; VEGF-A reverse, 5'-GCAGATGTGTAAGGACCA-3'; GAPDH forward, 5'-TGAAGGTGCGGTTGAAGGATTT-3'; GAPDH reverse, 5'-ACGACACTCTGACCGGCTTCAC-3'.

Fig. 1. Representative histological changes in irradiated rectal tissue. Blue stain indicates fibrosis in Azan section. Cont: unirradiated control mice; RT: irradiated mice. Magnification: × 100.
**Immunohistochemistry**

The sections were deparaffinized and processed for immunohistochemical staining. Briefly, the slides were treated with rabbit anti-TGF-β1 polyclonal antibody (1:150, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-HIF-1α polyclonal antibody (1:150, Santa Cruz Biotechnology), rabbit anti-VEGF polyclonal antibody (1:150, Santa Cruz Biotechnology) or rabbit anti-PECAM-1 (CD31) polyclonal antibody (1:150, Santa Cruz Biotechnology) as a primary antibody for overnight at 4°C. The sections were then treated with secondary antibody using Histofine SAB-PO(R) Kit (Nichirei Co., Japan) and examined on a microscope (BX50, Olympus, Japan). The negative staining was performed using normal sera instead of the primary antibodies.

**Double immunofluorescence**

To investigate the relationship between HIF-1α and VEGF, or HIF-1α and CD31, double-fluorescence immunostaining was performed. Briefly, sections were deparaffinized with xylene, graded ethanols and PBS. They were then incubated with a mixture of two primary antibodies overnight at 4°C. Goat anti-HIF-1α polyclonal antibody (Santa Cruz Biotechnology), rabbit anti-VEGF polyclonal antibody (Santa Cruz Biotechnology) and rabbit anti-PECAM-1 (CD31) polyclonal antibody (Santa Cruz Biotechnology) were diluted at 1:150. The slides were washed three times for 5 min in PBS and incubated with 1:5000 dilutions of the secondary antibodies, tetramethylrhodamine-5(and-6)-isothiocyanate (TRITC)-conjugated rabbit F(ab')2 fragment to goat IgG (MP Biomedicals, Solon, OH, USA) and FITC conjugated goat affinity purified IgG against rabbit IgG (MP Biomedicals). The immunofluorescent staining was observed on a fluorescence microscope (IX71, Olympus, Japan) and a CCD digital camera. The negative staining was performed using normal sera instead of the primary antibodies.

**Statistical analysis**

All experiments were performed at least three times. Five mice per time point were analyzed. Data are presented as mean ± standard deviation (SD). Statistical analyses were performed using Student t-test with KaleidaGraph (Version 4.0, Synergy Software, USA). P value < 0.05 was considered to be statistically significant.

**RESULTS**

**Radiation-induced histological changes in rectal tissue**

The histological changes after irradiation were characterized by loss of surface epithelium, deep erosion of mucosa, thickening of lamina propria and submucosa, collagen deposition and fibrosis (Fig. 1). In unirradiated control rectal tissue, no such lesions were observed.

The temporal changes of radiation-induced fibrosis are summarized in Table 1. Fibrosis was obvious at the 14th day, and reached the peak at the 30th day after irradiation. However, 90 days after irradiation, the fibrotic reaction in the irradiated rectal tissue was reduced.

<table>
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<tr>
<th>Staining</th>
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<th>1 day</th>
<th>7 days</th>
<th>14 days</th>
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<td>Azan staining</td>
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Intensity of collagen graded as: − none, + mild, ++ strong, +++ intense. Cont: unirradiated control mice.

**Time course of cytokines mRNA expression in rectal tissue after irradiation**

The mRNA levels of TGF-β1, HIF-1α and VEGF following irradiation with a single dose of 25 Gy were investigated from day 1 to day 90 after irradiation (Fig. 2). All three mRNAs increased significantly 90 days after irradiation with the formation of fibrosis compared with unirradiated mice. However, the expression TGF-β1 and HIF-1α mRNA showed different features from the VEGF mRNA expression which increased significantly only 90 days after irradiation (Fig. 2C). As shown in Fig. 2A, TGF-β1 mRNA expression increased significantly not only at day 90 but also at day 1, 14 and 30 after irradiation, which reached a peak at day 14. In contrast, HIF-1α mRNA levels showed a significant increase from day 14 and reached a maximal value at day 30 after irradiation (Fig. 2B).

**Expression of TGF-β1, HIF-1α, VEGF and CD31 proteins in irradiated rectal tissue**

The expression of cytokine proteins in irradiated rectal tissue was analyzed by immunohistochemistry with antibodies against TGF-β1, HIF-1α, VEGF and CD31. As shown in Fig. 3, the four proteins increased significantly 90 days after irradiation compared with the unirradiated rectal tissue. Brown stain indicates the positive staining which increased in proportion to the fibrotic changes in the region 90 days after irradiation.

We further confirmed the relationship between HIF-1α and VEGF, or HIF-1α and CD31 by double immunofluorescence staining. As shown in Fig. 4, HIF-1α, VEGF and CD31 proteins increased specifically and were colocalized in the fibrotic region compared with unirradiated control rectal tissue.

**The possible relationship between radiation-induced fibrosis and cytokines expression**

The temporal histological changes and cytokines mRNA expression are summarized in Fig. 5. Radiation-induced fibrosis was found from 14 days after irradiation. The
Fig. 2. TGF-β1 (A), HIF-1α (B) and VEGF (C) mRNA expression in rectal tissue after irradiation by Real-time PCR. Five mice per point were analyzed. Data is expressed as the relative value of the target gene compared with GAPDH and presented as the mean ± standard deviation. * P < 0.05, ** P < 0.01 when comparing irradiation with unirradiated control. Cont: unirradiated control mice. GAPDH was used as housekeeping gene.

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Fig. 3. Immunohistochemistry staining of irradiated rectal tissue. Brown stain indicates positive staining. Cont: unirradiated control mice. Bars represent 50 μm.

Fig. 4. Double immunofluorescence staining in fibrotic region of irradiated rectal tissue. (A), HIF-1α and VEGF; (B), HIF-1α and CD31. HIF-1α was labeled with red color; VEGF and CD31 were labeled with green color and Merge was shown by yellow color. Cont: unirradiated control mice. Magnification: ×100.

Fig. 5. The temporal changes of cytokines expression and fibrosis after irradiation. TGF-β1 Max. expression: mRNA expression 14 days after irradiation; HIF-1α Max. expression: mRNA expression 30 days after irradiation; VEGF Max. expression: mRNA expression 90 days after irradiation. Cont: unirradiated control mice.
severity of this fibrosis reached a peak at 30 days and then subsided 90 days after irradiation. The change of fibrosis was consistent with HIF-1α expression. Compared with HIF-1α expression, TGF-β1 was induced early and reached a peak at 14 days after irradiation. However, a rising level of VEGF was only found 90 days after irradiation.

**DISCUSSION**

Radiation-induced late normal tissue injury surrounding tumors is a significant cause of morbidity and mortality in patients treated for tumors. Despite the numerous studies aimed at understanding its etiology of tissue fibrosis after irradiation, the mechanism remains unclear. The previous research on irradiation injury focused on the investigation of profibrotic cytokines, especially TGF-β. Prolonged overexpression of TGF-β induced by chemical or biological injury including irradiation leads to the accumulation of pathological amounts of extracellular matrix in lung tissues by Smad proteins.

A new concept of irradiation injury recently emerged concerning the effect of radiation-induced hypoxia which was studied well as a tumor feature. Radiation-induced change in the microvasculature could in turn have a significant effect on tissue oxygenation and could lead to hypoxia. Under hypoxia conditions, a lot of cytokines including HIF-1α are induced which regulate the irradiation-induced central nervous system injury. However, the underlying mechanism of radiation-induced hypoxia on radiation injury was not known well.

In the present study, we demonstrated not only TGF-β1 but also HIF-1α and VEGF increased significantly with the fibrotic response after irradiation, suggesting a potential role for these cytokines in the induction of radiation-induced late injury. In addition, the colocation of HIF-1α, VEGF and CD31 in the fibrotic region suggested that radiation-induced hypoxia could induce the high expression of angiogenic cytokines. Interestingly, we found that radiation-induced fibrosis, which has commonly been described as irreversible dead scar tissues, was in fact a dynamic process that becomes aggravated and prolonged with time after irradiation. It was found further that the temporal changes of fibrosis were in agreement with radiation-induced profibrotic and angiogenic cytokines expression. By summarizing the temporal changes of histology and cytokines expression, a new paradigm has emerged to understand radiation-induced late rectal injury. This paradigm indicates that profibrotic cytokines including TGF-β1 were induced first after irradiation, which triggered histological lesions including vascular damage, and the resulting hypoxia then led to tissue remodeling when fibrosis reached a peak. Under hypoxia condition, revascularization was induced by VEGF regulated by its upstream cytokine, HIF-1α. This process contributed to wound healing and lead to the alleviation of radiation-induced fibrosis. The consistency between the temporal changes of cytokines and fibrosis indicated that fibrosis is a complex process involving numerous cytokines including angiogenic cytokines.

Recent research has indicated that hypoxia-induced reactive oxygen species (ROS) and macrophage accumulation contributes to the development of radiation-induced late lung injury. In these studies, the increase of VEGF after irradiation was considered to play an important role in radiation-induced injury. Further studies demonstrated that VEGF up-regulation can contribute to central nervous system (CNS) radiation injury by increasing vascular permeability and blood-brain disruption (BBB). These findings were different from our results in the rectum study which showed that the increasing VEGF levels corresponded with the alleviation of fibrosis after irradiation. Their results were also not explained according to the classic understanding of VEGF which considered that VEGF can promote revascularization and wound healing. To explain these conflicting results, the process by which VEGF plays a role as angiogenic cytokine or inflammatory cytokine in radiation-induced injury should be investigated. In addition, these findings indicate that radiation-induced late injury involves a complex process which may have a variable effect within different target organs.

Our current study was designed to explore the effect of cytokines on radiation-induced rectal fibrosis. In order to understand this mechanism in greater depth, further studies including long-time observation and using inhibitors of cytokines will be necessary.

In summary, our studies demonstrated that not only profibrotic cytokines but also angiogenic cytokines resulting from radiation-induced hypoxia may be critical factors in the development of late rectal injury after irradiation. Further investigation of the relationship between profibrotic cytokines and angiogenic cytokines may be necessary to more comprehensively understand the radiation-induced normal tissue injury during radiotherapy.

**REFERENCES**


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