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Protective effect of Tranilast on radiation-induced heart fibrosis in C57BL/6 mouse

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ABSTRACT

The heart is a major dose-limiting organ for radiotherapy of cancer in the thoracic region. The purpose of this study was to examine the protective effect of tranilast on the radiation-induced heart fibrosis model using the C57BL/6 murine strain. A significant reduction in the expression of TGF-β1, collagen type Ⅰ and collagen type Ⅲ was observed in the radiation plus tranilast group. The authors also suggest the use of tranilast in a clinical trial for the prevention of radiation-induced heart fibrosis.

***Keywords**: Heart Fibrosis, Radiation, Tranilast*

1. INTRODUCTION

1.1 General Appearance

The cardiac structures (heart and pericardium) are recognized as being sufficiently radiosensitive to be dose-limiting structures during radiotherapy for cancers in the thoracic region. The term "radiation-induced heart disease" (RIHD) was coined in the 1960s [1],[2] and describes the conglomerate of structural alterations produced by radiation of the cardiac tissues and the clinical manifestations of these alterations RIHD may vary depending on the total dose of radiation administered, the fractionation schedule, the volume of the heart radiated, the existence of prior heart disease, and the use of chemotherapeutic medications in the management of the disease.

Clinically evident RIHD has a characteristic morphology. The most common morphologic alterations occur in the pericardium [3]. The parietal pericardium develops variable degrees of fibrosis that replace the outer adipose tissue. The visceral pericardium also develops fibrosis, although not to the same degree as the parietal pericardium. The myocardium is involved less frequently than the pericardium, but it develops a more serious lesion [3],[4]. The valvular lesions induced by radiation are nonspecific and could be the result of conditions other than RIHD. The morphology of coronary artery disease after radiation is not different from that of spontaneous atherosclerosis. Therefore, these lesions cannot be diagnosed as specifically associated with radiation therapy.

The underlying mechanisms of radiation-induced fibrosis remain unresolved. Based on our present knowledge of radiation-induced changes in cells and tissues, however, it can be concluded that radiation-induced fibrosis is due to an interplay of cellular and molecular events among several cell systems that are engaged in fibrotic reactions. Radiation of the accompanying parenchymal cell induces an immediate synthesis of specific cytokines, such as transforming growth factor-beta (TGF-β), which then alter the interaction of the parenchymal cells with the fibroblast cell system. Growth factors, especially TGF-β, are believed to have an essential role in the development of radiation-induced heart fibrosis. Although all three isoforms of TGF-β (TGF-β1, TGF-β2, TGF-β3) are present in the heart, the amount of TGF-β1 in particular, appears to be related to the development of radiation-induced heart fibrosis [5]-[7].

Tranilast [N-(3,4dimethoxycinnamoyl) anthranilic acid] has long been used clinically to treat allergic disease such as bronchial asthma, atopic dermatitis and allergic rhinitis. The efficacy of tranilast in the treatment of these allergic diseases is based on the inhibition of antigen-induced chemical mediator release from mast cells and basophils. Recently, tranilast has also been approved for the treatment of hypertrophic scar and keloid. In animal studies, tranilast reduced pathological matrix accumulation in hypertensive heart disease [8] and in diabetic nephropathy [9], although its effects in the radiation-induced heart fibrosis mouse model have not been examined previously.

The purpose of this study was to examine the protective effect of tranilast on the radiation-induced heart fibrosis model using the C57BL/6 murine strain.

2. MATERIALS AND METHODS

2.1 Animals

For this study we selected C57BL/6 mice, which are

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well-known for their radiosensitivity. Adult female mice, 8 weeks old and approximately 20 g in weight, were housed 4 per cage and allowed to acclimate from transportation for 1 week prior to treatment.

2.2 Control and treatment groups

The C57BL/6 mice were divided into control, tranilast, radiation, radiation plus tranilast groups with 4 mice in each group).

2.3 Radiation schedule

A plastic jig was used to restrain the mice and lead strips were placed to shield the head and abdomen. The radiation characteristics were as follows: beam energy, 6-MV linear accelerator (Digital Mevatron MX2, Simens, Concord, MA, U.S.A); dose-rate, 3 Gy/min; source surface distance (SSD), 1m; size of the radiation field (FS), 5 cm × 20 cm. Film dosimetry was used to determine the relative dose distribution. Dosimetry was performed with a cylindrical ionization chamber. The depth of the maximum dose of the 6-MV photon beam could be reduced significantly by the tissue-equivalent plastic material (thickness 10 mm) of the restraining jig. Therefore, the buildup region of the 6-MV photon beam was located in the plastic material and achieved an acceptable dose uniformity throughout the thoraces of the mice. The total 20 Gy of radiation was delivered in a single fraction through a posterior port. After the radiation, the mice were maintained, 4 per cage, in laminar flow hoods in pathogen-free rooms to minimize pulmonary infections, and they were supplied with autoclaved diet and water. Age-matched controls were maintained under identical conditions during the course of the study. Treated and control mice were sacrificed by an overdose of an anesthetic drug at the time that corresponded to the fibrotic phase (18 weeks after radiation).

2.4 Administration of tranilast

The C57BL/6 mice, in both the tranilast and radiation plus tranilast groups, were treated with tranilast (100 mg/kg per day) six times a week through intraperitoneal injections, starting 3 days before radiation, for 18 weeks.

2.5 Histopathology and immunohistochemistry

Immediately before death, the entire hearts were removed. The hearts were placed in a fixative for histologic and immunohistochemical analyses. The heart tissues from the four different groups were analyzed by a combination of light microscopy and immunohistochemistry.

2.5.1 Hematoxylin-eosin (HE) stain and Masson's trichrome (MT) stain: For histologic analysis, the hearts were fixed in 10% neutral buffered formalin for 24 hours, paraffin-embedded, and sectioned at an average thickness of 1-2 mm. The mounted sections were subjected to hematoxylin-eosin stain as well as to Masson's trichrome stain for fibrosis.

2.5.2 Immunohistochemistry for TGF-β1, collagen type Ⅰ and collagen type Ⅲ: The expression of TGF-β1, and the amounts of collagen type Ⅰ and collagen type Ⅲ were assessed by immunohistochemical assays of the ABC method (avidin-biotin enzyme complex method). Briefly, 4 μm sections from the 4 mice in each group were cut transversely, treated with 3% H_2O_2 in phosphate-buffered saline (PBS), and incubated with 1% goat serum for 10 minutes to block nonspecific antibodies before the addition of a primary antibody. For immunohistochemical detection of TGF-β1, collagen type Ⅰ and collagen type Ⅲ, the heart sections were incubated overnight at 4℃ with either a primary monoclonal Ab, or a negative control mouse serum, instead of a primary antibody. The primary antibodies used were anti-TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-collagen type Ⅰ (Santa Cruz Biotechnology, Santa Cruz, CA), anti-collagen type Ⅲ (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactivity was detected by sequential incubations of heart sections with a biotinylated secondary antibody for 30 minutes. Finally, the heart sections were counterstained with hematoxylin and mounted in aqueous mounting media, and then observed under a microscope.

2.5.3 Quantitation of matrix deposition, TGF-β1, collagen type Ⅰ and collagen type Ⅲ expression: Because all heart tissue sections were treated in the same manner and stained simultaneously under the same conditions, the intensity of immunostain could be compared. All heart specimens were subjected to a blinded evaluation. To ensure objectivity, a standardized procedure was established and used consistently throughout the study. After an examination of the entire heart section and an estimate of the extent of altered heart parenchyma, representative, noncontiguous, and non-overlapping fields were selected systematically and analyzed. The accumulation of matrix, and the extent of TGF-β1, collagen type Ⅰ and collagen type Ⅲ were quantified by computer-assisted image analysis, as reported previously [10],[11]. Twenty random non-overlapping fields per section from the 4 mice per group were assessed by a color image analyzer (TDI Scope Eye ™ Version 3.0 for Windows). After an image was captured, the accumulation of matrix and the extent of TGF-β1, collagen type Ⅰ and collagen type Ⅲ were quantified by the Optimas 6.51 program, which counted the percentages of the areas stained. Each field measured 42,942 μm^2 and the total cardiac surface explored per section was 858,840 μm^2. The measurements from the twenty fields were totaled, and the arithmetic mean was calculated. This method is highly reproducible and reflects consistently the visually appreciated immunoreactivity, even in specimens where stain and radiation injury are not distributed uniformly.

2.6 Statistical analysis

The data were presented as mean ± standard error of the mean. SPSS software was used for all statistical calculations. Independent sample T-test with SPSS software was used to test the statistical significance of any differences among the groups. The results were considered significantly different at a value of $P < 0.05$.

3. RESULTS

3.1 Results of Hematoxylin-eosin and Masson's trichrome stain

Hematoxylin-eosin stain demonstrated mononuclear cell infiltration, and marked swelling of endothelial cells with narrowing of the capillary lumen in the radiation group (Fig. 1C).

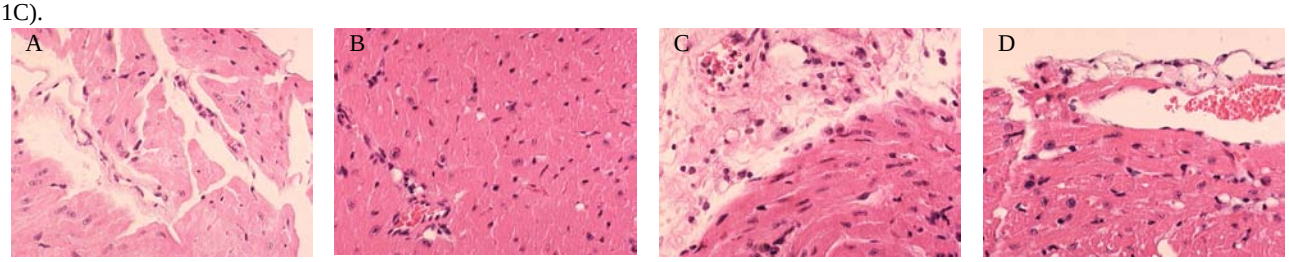

Fig. 1. Histologic comparison by hematoxylin-eosin stain among the groups of control (A), tranilast (B), radiation (C), radiation plus tranilast (D) at 18 weeks after 20 Gy of single radiation. C57BL/6 mice treated with single radiation plus tranilast (100 mg/kg) showed mild degree of mononuclear cell infiltration, minimal endothelial changes, mild to moderate degree of myocardial degeneration compared to C57BL/6 mice received single radiation alone. These photomicrographs are representative of results obtained from four animals in each group. Magnification: × 400.

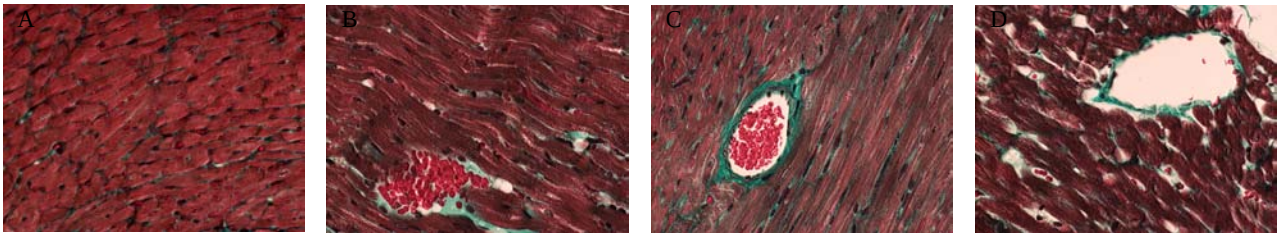

Fig. 2. Histologic comparison by Masson's trichrome stain among the groups of control (A), tranilast (B), radiation (C), radiation plus tranilast (D) at 18 weeks after 20 Gy of single radiation. C57BL/6 mice treated with single radiation plus tranilast (100 mg/kg) showed less collagenous matrix deposition than C57BL/6 mice received single radiation alone. These photomicrographs are representative of results obtained from four animals in each group. Magnification: × 400.

The radiation plus tranilast group demonstrated only a mild degree of mononuclear cell infiltration, minimal endothelial changes, and mild to moderate degree of myocardial degeneration (Fig. 1D). Masson's trichrome stain demonstrated an increased matrix accumulation in both the perivascular and interstitial regions in the radiation group (Fig. 2C). The extent and severity of matrix deposition was significantly reduced, and a mild degree of interstitial fibrosis was observed in the radiation plus tranilast group (Fig. 2D). There was a statistically significant difference in the extent and severity of the matrix deposition between the radiation and radiation plus tranilast groups (Fig. 3, $P < 0.01$). The hearts of C57BL/6 mice in the control and tranilast groups demonstrated no noticeable changes (Figs. 1A, 1B, 2A, 2B).

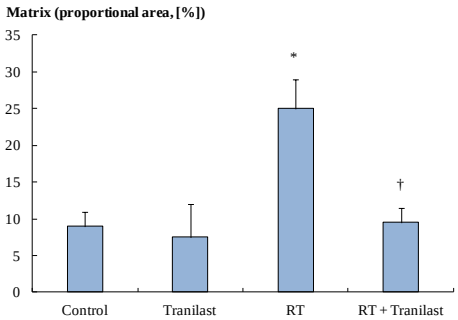

Fig. 3. Cardiac extracellular matrix deposition as assessed by proportional area on sections stained with Masson's trichrome in control, tranilast, radiation, radiation plus tranilast groups.

$^{*}P < 0.01$ versus control; $^{\dagger}P < 0.01$ versus radiation group

3.2 Results of immunohistochemical stain

Strongly stained signals of TGF-β1, collagen type Ⅰ and collagen type Ⅲ were observed mainly around the fibrotic foci, such as the perivascular and interstitial fibrosis in the radiation group (Figs. 4C, 5C, 6C). A significant reduction in the expression of TGF-β1, collagen type Ⅰ and collagen type Ⅲ was observed in the radiation plus tranilast group (Figs. 4D, 5D, 6D). There was a statistically significant difference between the radiation and radiation plus tranilast groups in the extent of immunoreactivity for TGF-β1, collagen type Ⅰ and collagen type Ⅲ (Figs. 7, 8, 9, $P < 0.01$). The hearts of C57BL/6 mice in the control and tranilast groups demonstrated no noticeable immunoreactivity (Figs. 4A, 4B, 5A, 5B, 6A, 6B).

4. DISCUSSION

This study is the first to demonstrate that tranilast with radiation could modulate late radiation-induced heart fibrosis by suppressing the expression of TGF-β1, collagen type I and collagen type Ⅲ. In this study, radiation-induced heart fibrosis was associated with increased deposition of collagenous matrix and evidence of TGF-β1 activation in the hearts of C57BL/6 mice. The antifibrotic agent tranilast, not only attenuated collagen production, but also reduced the pathological fibrosis that develops in radiation-induced heart injury *in vivo*.

Based on our current knowledge of cell and tissue changes caused by radiation, radiation-induced fibrosis is a complex process that causes inflammation and is mediated by cytokines produced and secreted from a variety of cells, such as

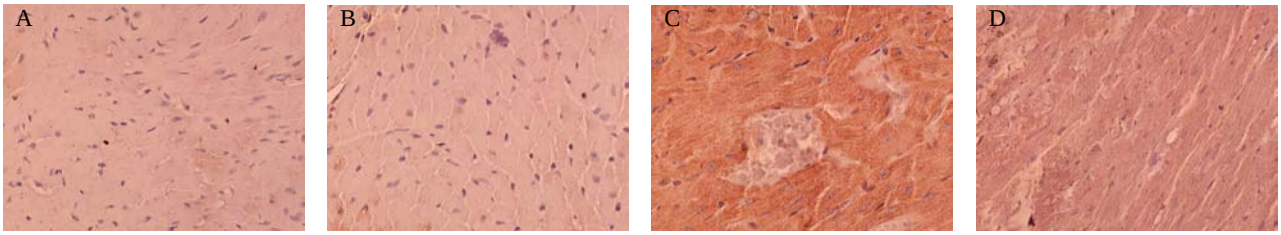

Fig. 4. Immunostaining for TGF-β1 in hearts of control (A), tranilast (B), radiation (C), radiation plus tranilast (D). In control, there was minimal TGF-β1 immunostaining, while radiation group was associated with increased immunostaining interstitial areas. Radiation plus tranilast group was associated with a reduction in TGF-β1 immunostaining. Magnification: × 400. Nuclei were stained blue with hematoxylin.

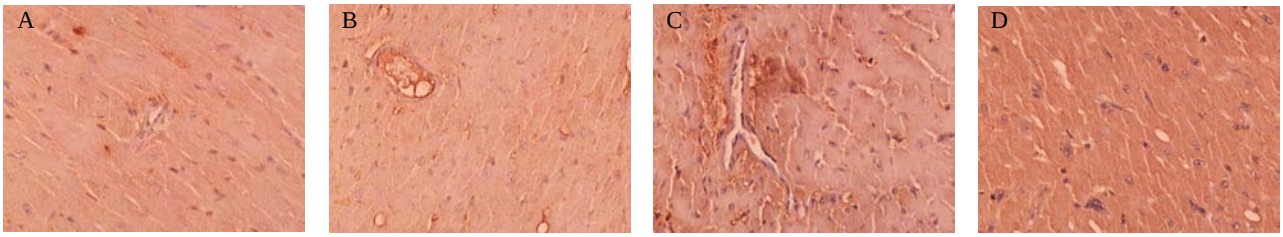

Fig. 5. Immunostaining for collagen type Ⅰ in hearts of control (A), tranilast (B), radiation (C), radiation plus tranilast (D). In control, there was minimal collagen type Ⅰ immunostaining, while radiation group was associated with increased immunostaining interstitial areas. Radiation plus tranilast group was associated with a reduction in collagen type Ⅰ immunostaining. Magnification: ×400. Nuclei were stained blue with hematoxylin.

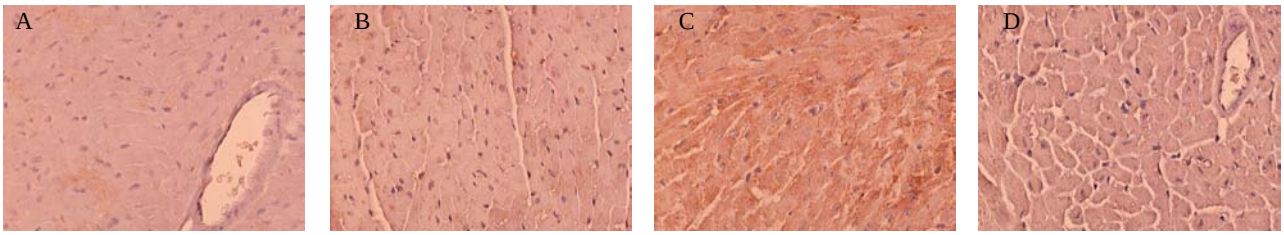

Fig. 6. Immunostaining for collagen type Ⅲ in hearts of control (A), tranilast (B), radiation (C), radiation plus tranilast (D). In control, there was minimal collagen type Ⅲ immunostaining, while radiation group was associated with increased immunostaining interstitial areas. Radiation plus tranilast group was associated with a reduction in collagen type Ⅲ immunostaining. Magnification: ×400. Nuclei were stained blue with hematoxylin.

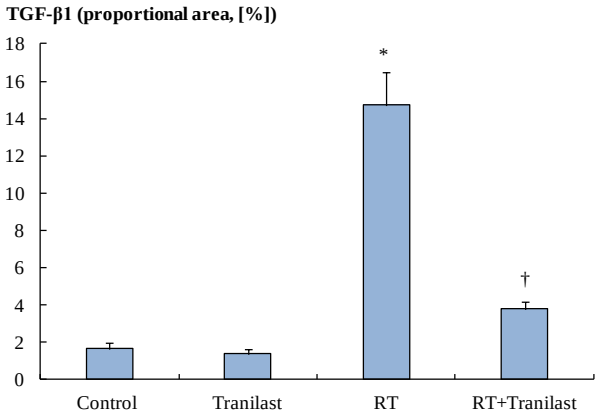

Fig. 7. TGF-β1 in cardiac tissue as assessed by the proportional area of tissue showing positive immunolabelling. $^{*}P < 0.01$ versus control; $^{\dagger}P < 0.01$ versus radiation group

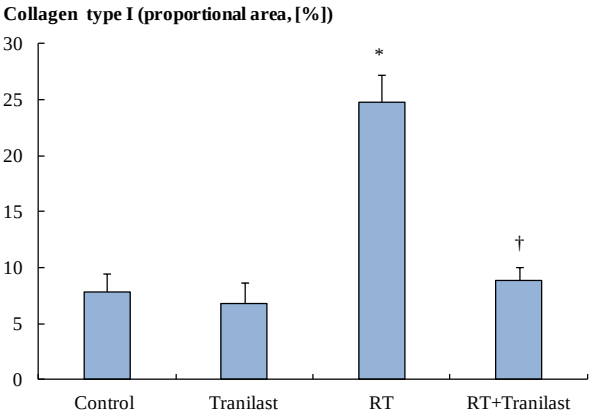

Fig. 8. Collagen type Ⅰ in cardiac tissue as assessed by the proportional area of tissue showing positive immunolabelling. $^{*}P < 0.01$ versus control; $^{\dagger}P < 0.01$ versus radiation group

macrophages, epithelial cells, and fibroblast cells. In this process, the most crucial function is carried out by fibroblast

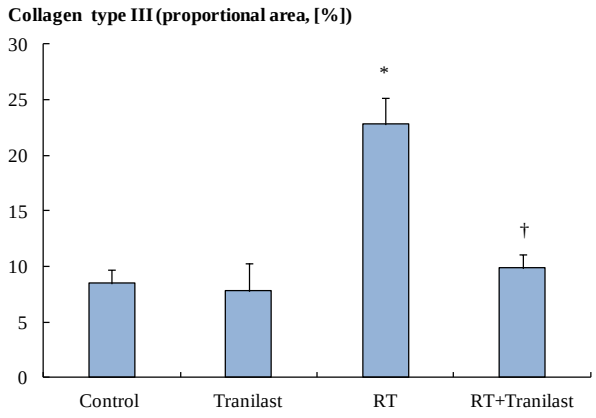

Fig. 9. Collagen type III in cardiac tissue as assessed by the proportional area of tissue showing positive immunolabelling. $^{*}P < 0.01$ versus control; $^{\dagger}P < 0.01$ versus radiation group

cells. Radiation alone can induce a premature terminal differentiation process of the fibroblast/fibrocyte cell system that results in the enhanced accumulation of post-mitotic fibrocytes, which are characterized by a several-fold increase in the synthesis of interstitial collagens. TGF-β1, as the major cytokine responsible for the fibrotic reaction, induces fibroblast proliferation by means of an expansion of the progenitor fibroblast cell types, as well as a premature terminal differentiation of progenitor fibroblasts into post-mitotic fibrocytes. This leads to an accumulation of post-mitotic fibrocytes due to a disturbance of the well-balanced cell type ratio of progenitor fibroblasts and post-mitotic fibrocytes [12]-[14].

There are many reports about the effects of TGF-β on collagenase and tissue inhibitors of metalloproteinase (TIMP) expression. Overall et al [15] demonstrated that TGF-β suppressed collagenase activity, directly by decreasing procollagenase synthesis, and indirectly by increasing TIMP and plasminogen activator inhibitor (PAI-1) synthesis. Varga et al [16] reported that TGF-β caused a marked enhancement of the production of collagen type Ⅰ and collagen type Ⅲ. We tried to determine whether tranilast would regulate collagen type Ⅰ and collagen type Ⅲ synthesis by inhibiting TGF-β1.

The test animals that mimic the human lesions of RIHD are the New Zealand white rabbits, usually 6 to 10 weeks old [2]. With radiation of a 3×3 cm field that encompasses most of the heart, they have produced consistently the same alterations that are observed in the myocardium and pericardium of humans [17],[5],[6]. Seventy or more days after a single dose of 20 Gy, 94% of the rabbits developed either pericardial fibrosis or diffuse myocardial fibrosis, or both [17]. There was an immediate acute pancarditis that began approximately 6 hours after radiation and reached a peak at approximately 12 hours. Starting at 50 days there was a delayed pericardial fibrosis, pericardial effusion, and diffuse myocardial fibrosis, which reached full development by 90 days [17]. Our choice of radiation dose for this study was based on previous extensive histopathologic dose-response data in order to yield a consistent and significant prevalence of heart fibrosis after thoracic radiation with 20 Gy. We sacrificed control and treated mice at the time that corresponded to the fibrotic phase (18 weeks after radiation).

There are several mechanisms causing the beneficial effects of tranilast in disorders that involve matrix deposition and fibrosis : (1) inhibition of matrix protein synthesis (e.g., collagen); (2) reduction of cell proliferation (e.g., fibroblasts and smooth muscle cells); (3) inhibition of inflammatory responses that result in fibrosis. An in vivo study demonstrated that tranilast suppressed heart fibrosis in hypertensive animals through suppression of TGF-β1 [8,18]. An in vitro study demonstrated that tranilast inhibited monocyte chemoattractant protein (MCP)-1 secretion induced by interleukin (IL)-1β and mRNA expression of MCP-1 in rat mesangial cells [19]. Our in vivo study showed that tranilast with radiation suppressed radiation-induced heart fibrosis by suppressing the expression of TGF-β1, collagen type Ⅰ and collagen type Ⅲ.

When long-term tracking and observation was possible after complete recovery from radiotherapy, adverse effects on the heart were reported in 6.6% of Hodgkin's lymphoma patients and 4.5% of breast cancer patients. Pericardial diseases occur generally between 6 months and 1 year after radiation therapy, and vessel diseases occur between 10 and 15 years after radiation therapy. The heart is a radiation-sensitive organ and is a major factor in limiting the radiation dose for radiotherapy of thoracic malignancies. If less than 25% of the total volume of the heart has been irradiated, the total radiation dose must be limited to less than 40 Gy; and if more than 25% of the total volume of the heart has been irradiated, the total radiation dose must be limited to less than 30 Gy [20], [21]. Consequently,there could be restrictions in radiotherapy of thoracic malignancies, and any treatment that overlooks this runs the risk of increased morbidity from RIHD in addition to that of the tumors. However, no specific prevention and treatment methods have been established yet. Therefore, our results suggest the use of tranilast in a clinical trial designed to prevent radiation-induced heart fibrosis in patients with thoracic malignancies who have an absolute need for radiotherapy.

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