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Smad, but not MAPK, pathway mediates the expression of type I collagen in radiation induced fibrosis

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ABSTRACT

Radiation induced fibrosis occurs following a therapeutic or accidental radiation exposure in normal tissues. Tissue fibrosis is the excessive accumulation of collagen and other extracellular matrix components. This study investigated how ionizing radiation affects the expression level and signal pathway of type I collagen. Real time RT-RCR showed that both α1and α2 chain of type I collagen mRNA were elevated from 48 h after irradiation with 10 Gy in NIH3T3 cells. The relative luciferase activities of both genes and type I collagen marker were elevated at 72 h. TGF-β1 mRNA was elevated earlier than those of type I collagen genes. A Western blot analysis showed the elevation of Smad phosphorylation at 72 h. Conversely, treatment with TGF-β receptor inhibitor inhibited the mRNA and relative luciferase activity of type I collagen. The phosphorylation of Smad was repressed with the inhibitor, and the luciferase activity was cancelled using a mutant construct of Smad binding site of α2(I) collagen gene. However, the MAPK pathways, p38, ERK1/2 and JNK, were not affected with specific inhibitors or siRNA. The data showed that the Smad pathway mediated the expression of type I collagen in radiation induced fibrosis.

1. Introduction

Fibrosis is the excessive accumulation of collagen and other extracellular matrix (ECM) components following breakdown in the normal balance of ECM synthesis and degradation. Ionizing radiation is a highly effective modality for local control of tumor growth. However ionizing radiation can induce normal tissue to undergo fibrogenesis [1]. Radiation induced fibrosis (RIF) is assumed to occur as a result of a coordinated response to ionizing radiation involving several cytokines and growth factors, fibroblast proliferation and differentiation, and also remodeling of the ECM [2–4]. However, the intracellular signaling and mechanism of gene expression that promote the RIF process are not yet fully understood.

Collagens, which are the major constituents of extracellular matrices, are critical for the formation and function of the organs in the body [5]. There are 46 human collagen genes, each encoding procollagen molecules that coalesce to form long triple helices [6]. Type I collagen is the major component of the ECM, which is a heterotrimer composed of coordinately expressed two α1 chains and one α2 chain. Their expression is modulated by various cytokines [7]. Transforming growth factor (TGF)-β is a member of a superfamily of multifunctional cytokines which function during development, wound repair, and other pathologic processes [8]. TGF-β is a potent inducer of ECM protein synthesis and accumulation and has been implicated as the key mediator of various fibrosis [9,10]. Therefore, TGF-β strongly contributes to fibrotic disorders as various fibrogenous diseases, hepatic cirrhosis, diabetic nephropathy, and rheumatoid arthritis.

TGF-β mediates signals through pairs of specific plasma membrane receptors (types I and II serine/threonine kinases). TGF-β binds and activates the type II receptor, which then phosphorylates the type I receptor. The active receptor complex then phosphorylates the so-called R (receptor)-Smad2 or Smad3. The phosphorylation of Smad2/3 propagates the signal by binding with Smad4 (a so-called co-Smad). This complex translocates into the nucleus and binds transcriptional co-activators such as p300 and Creb-binding-protein (CBP), which regulate the transcriptional activity of various TGF-β target genes [11]. Furthermore, recent evidence has indicated that TGF-β transduces through another pathway – mitogen activated protein kinase (MAPK) [12,13] and

Abbreviations: RIF, radiation induced fibrosis; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay.

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cross-talk between the MAPK and Smad pathway in response to TGF-β signaling have been suggested [14].

This study investigated how ionizing radiation affects the expression level and signal pathway of type I collagen, which is associated with the RIF process.

2. Materials and methods

2.1. Cell culture

The cell lines used in this study were mouse fibroblast NIH-3T3 cell and mouse embryonic fibroblast cells.

The mouse embryonic fibroblast cells were isolated from embryonic mice. The ICR mice were purchased from commercial sources (Yoshitomi, Fukuoka, Japan). The animals were treated in accordance with the Oita University Guidelines for the Care and Use of Laboratory Animals based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS; Sanko Junyaku, Tokyo, Japan) at 37 °C in a 5% CO_2/air environment.

2.2. Ionizing irradiation of cells in culture

The cells were irradiated with ^{137}Cs γ-ray using a Gammacell 40 (MDS Nordion, Ottawa, Canada) at a dose rate of approximately 1.0 Gy/min. The cells were irradiated to the total does called for in the experimental design for each experiment. The radiation doses used in this experiment was 10 Gy. Ionizing irradiation was performed at the initial point of 0 h, when ionizing irradiation was completed. Before irradiation, the cells were incubated in DMEM supplemented with 10% FBS for 24 h. The effect of ionizing irradiation on type I collagen expression levels and the signal pathway was confirmed at various times.

2.3. RNA isolation and real-time PCR

Total RNA was isolated from cultured cells using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. RNA was quantified by optical density (A260) and stored at −80 °C. One microgram of total RNA was reverse transcribed by random primer using ReverTra Ace (Toyobo, Tokyo, Japan). Real-time PCR was performed using a LightCycler Taqman Master (Roche, Indianapolis, IN, USA). The thermal cycling conditions included 1cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 1 s. The relative mRNA expression levels were normalized against that of GAPDH gene from the same RNA preparations, using a comparative threshold cycle method. The primer sets are listed in Table S1.

2.4. Measurement of the Cross-linked N-telopeptides of type I collagen (NTx)

The cells were placed in 35 mm dishes. Twenty mL of the medium was used in subsequent assays. The concentration of NTx in the medium was measured with an enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's protocol (Cusabio Biotech, Wuhan, China).

2.5. Transient transfection and luciferase assays

Plasmid constructs used in these experiments were previously described [15]. The cells were plated at a density of 2×10^5 per 35-mm dish 18 h before transfection. For transient transfection, 10 μg of plasmid DNA was transfected into these cells by using calcium phosphate precipitation methods. Plasmid pRL-TK vector (Promega, Madison, WI, USA) was always cotransfected as an internal control for transfection efficiency. After additional cultivation for 24 h, the transfected cells were irradiated with dose of 10 Gy. At 72 h after ionizing irradiation, the cells were harvested, lysed, centrifuged to pellet the debris, and subjected to luciferase assay. The luciferase activities were measured as chemiluminescence in a luminometer (Lumat LB 9507, Perkin–Elmer Life Sciences, Waltham, MA, USA) using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

2.6. Preparation of cell lysates and Western blot analysis

The cells were treated with ionizing irradiation, washed twice with ice-cold phosphate-buffered saline (PBS), and lysed on ice in RIPA buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% Na-deoxycholate) containing protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM NaF, 1 g/mL aprotinin, 1 g/mL leupeptin, 1 g/mL pepstatin). Cell extracts were then centrifuged at 15,000*g* for 10 min at 4 °C, supernatants were collected, and protein content was determined using a bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL, USA). Fresh cell extracts were prepared in sample buffer (0.125 M Tris–HCl [pH 6.8]) 4% SDS, 20% glycerol, 0.002% bromophenol blue, and 10% 2-mercaptoethanol). Samples were separated by SDS–PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane. Equal loading and appropriate transfer of each lane was confirmed by staining the PVDF membrane with the ponceau S solution (Sigma–Aldrich, St. Louis, MO, USA). The membrane were blocked with 5% skim milk or 5% BSA in Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature, incubated with anti-Smad2/3 antibody, or anti-phospho-Smad2/3 antibody overnight at 4 °C, and then incubated in the horseradish peroxidase (HRP)-conjugated antirabbit IgG for 1 h at room temperature. The signals were enhanced using a chemiluminescence system (ECL plus; Amersham Pharmacia Biotech, Buckinghamshire, UK) and exposed to X-ray film.

2.7. Reagents

SB431542, a specific inhibitor of TGF-β type I receptor, was purchased (Sigma). ERK1/2 inhibitor (PD98059), p38 MAPK inhibitor (SB203580) and JNK inhibitor (SP600125) were purchased from Calbiochem (San Diego, CA, USA). The cells were preincubated with those reagents for 1 h before ionizing radiation.

2.8. siRNA transfection

The siRNA cocktails targeting mouse MAPK (ERK 1/2, p38 MAPK) or mouse Smad3 were purchased (Sigma). The cells were transfected using Lipofectamine 2000 (Invitrogen Co., Carlsbad, CA, USA) to achieve a final siRNA concentration of 50 nM. The specificity of MAPK (ERK 1/2, p38 MAPK) and mouse Smad3 knockdown was confirmed by a real-time PCR reaction.

2.9. Statistical analysis

The values are presented as the mean ± SD of multiple independent tests and Student's *t*-test was used to evaluate the statistical differences between groups, and a $p < 0.05$ was considered to be significant. Each experiment was performed at least three times and individual samples were run in triplicate.

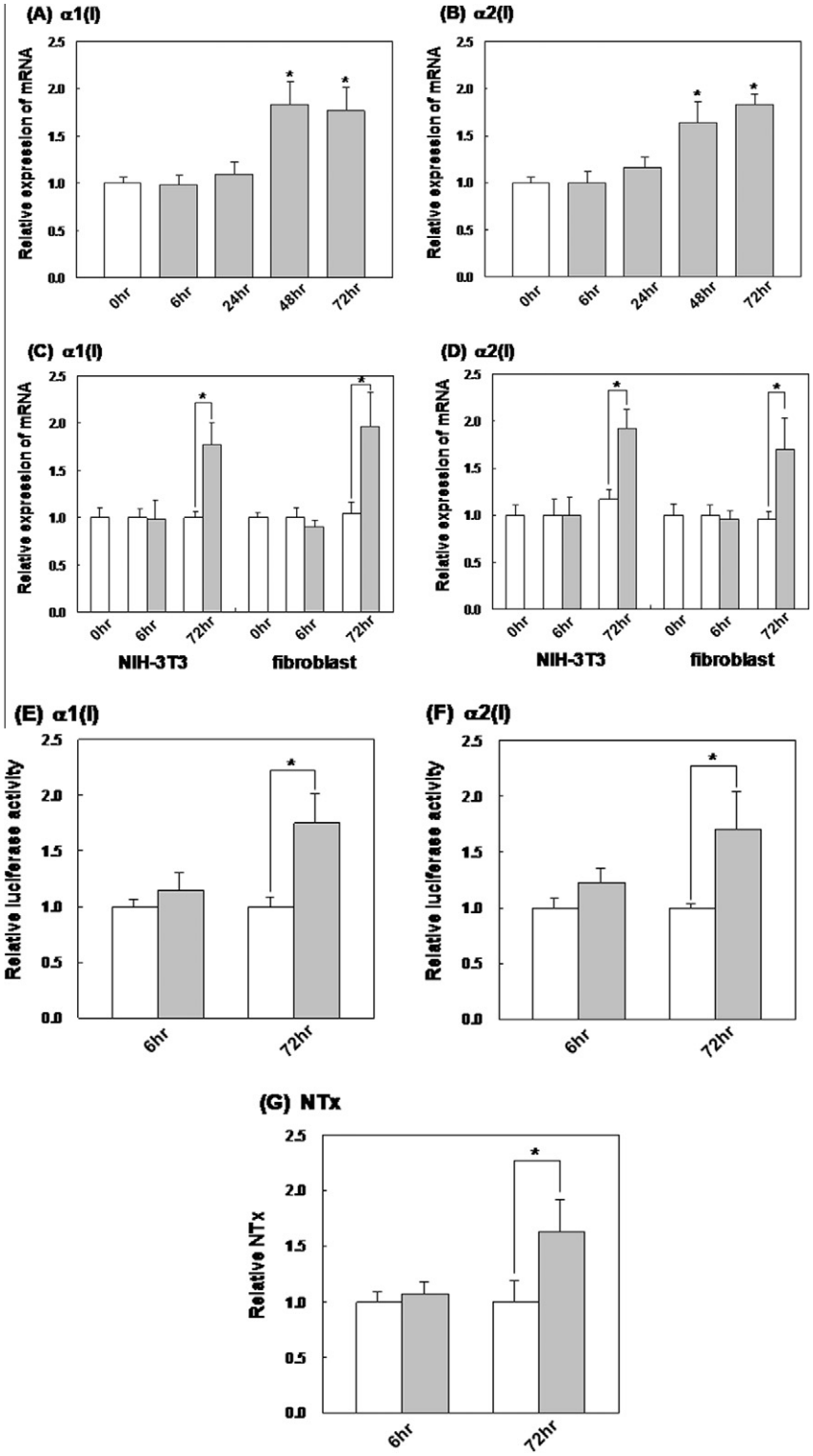

Fig. 1. Effect of irradiation with 10 Gy on the expression of type I collagen. Real time RT-PCR for collagen α1(I) (A) and α2(I) (B) chain genes before (0 h) and after radiation at 6, 24, 48 and 72 h in NIH3T3. Each value is normalized to that before radiation (white column). Asterisks indicate statically significant in comparison to the value before radiation (A and B). Real time RT-PCR for collagen α1(I) (C) and α2(I) (D) using mouse embryonic fibroblast. The gray and white columns in each time point show treatment with and without radiation. Each value is normalized to that before radiation. Luciferase assay for collagen α1(I) (E) and α2(I) (F) chain genes. Each value is normalized to the value without radiation at 6 h after radiation. ELISA for measurement of concentration of NTx in culture medium of NIH3T3 (G). Asterisks indicate statically significant in comparison to the values with and without radiation at each time point (C–G). All date represent the mean ± SD of results in three independent experiments. $^{*}p < 0.05$.

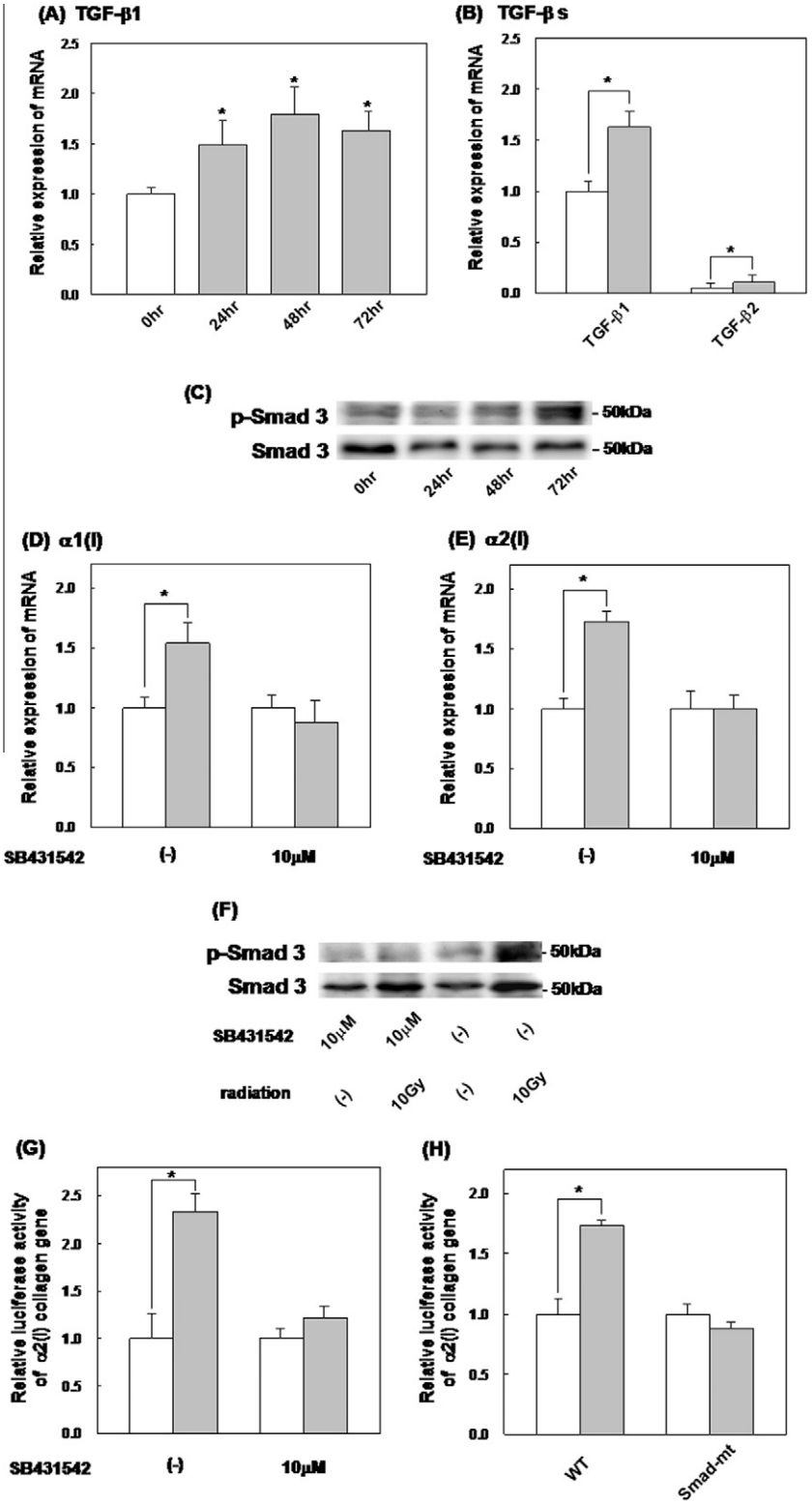

Fig. 2. Effect of irradiation with 10 Gy on the expression of TGF-β and Smad. (A) Real time RT-PCR for TGF-β1before (0 h) and after radiation at 24, 48 and 72 h in NIH3T3. Each value is normalized to that before radiation (white column). Asterisks indicate statically significant in comparison to the value before radiation. (B) Real time RT-PCR for TGF-β1 and TGF-β2 at 72 h in NIH3T3. Each value is normalized to the value without radiation of TGF-β1. (C) Western blotting for Smad3 and phospho-Smad3. Relative expression α1(I) chain (D) and α2(I) chain (E) treated with and without TGF-β receptor inhibitor SB431542 (10 μM) at 72 h after radiation in NIH3T3. Effect of SB431542 on phosphorylation of Smad3 using Western blotting (F) and luciferase activity of collagen α2(I) gene (G). Luciferase assay using Smad mutant construct of α2(I) chain collagen promoter (H) .Each value is normalized to that without radiation and with SB431542 (D, E and G) or without radiation and with wild construct (WT) (H). Asterisks indicate statically significant compared to the value without radiation (B, D, E, G and H). Date represent the mean ± SD of results in three independent experiments. $^{*}p < 0.05$. (F).

3. Results

3.1. Up-regulation of type I collagen expression in NIH-3T3 cells and mouse embryonic fibroblast on ionizing radiation

The expression of α1 and α2 chain of type I collagen genes were initially examined by real-time PCR in NIH-3T3 cells (Fig. 1A and B). They were almost no change at 24 h and increased at 48 and 72 h after irradiation. Similarly, they were up-regulated at 72 h in mouse embryonic fibroblast (Fig. 1C and D).

A luciferase assay was performed using the constructs of the basal promoter of the α1and α2 chain of type I collagen gene [15]. The both activity did not changed at 6 h and increased at 72 h in NIH3T3 cells (Fig. 1E and F).

The concentration of N-telopeptide (NTx) from type I collagen molecule in the medium of NIH3T3 cells was measured using ELISA to examine whether the increase of collagen mRNA expression was followed by an increase in collagen protein synthesis, (Fig. 1G). The data were consistent with the mRNA and promoter activity (Fig. 1G).

3.2. Effects of ionizing radiation on the expression of TGF-β that regulates collagen expression

Many studies have suggested that several growth factor and cytokines released from fibroblast play an important role in the expression of type I collagen [11]. TGF-β is a potent regulator of type I collagen synthesis. Real time RT-PCR was performed to assess the affect of TGF-β on type I collagen expression after ionizing radiation. TGF-β1 mRNA was already elevated at 24 h (Fig. 2A), while those of α1 and α2 chain of type I collagen were not (Fig. 1A and B). TGF-β1 expression was still elevated at 72 h after ionizing radiation and approximately 20 times higher than TGF-β2, which was also increased after ionizing radiation, in NIH3T3 (Fig. 2B).

TGF-β signals from the cell surface are transduced to the nucleus by Smad2/3 and 4 complexes. The phosphorylation of Smad2/3 is crucial for this downstream signaling cascade. The levels of Smad3 and phospho-Smad3 were measured using Western blotting. The level of phospho-Smad3 was significantly increased at 72 h after irradiation (Fig. 2C).

The expression of type I collagen was measured after treating cells with SB431542, a specific inhibitor of the TGF-β receptor. This suppressed the increased expression of type I collagen chains after ionizing radiation (Fig. 2D and E). The level of phospho-Smad3 was also suppressed with treatment of SB431542 at 72 h after irradiation using Western blotting (Fig. 2F). Furthermore, the promoter activity of α2(I) collagen gene after ionizing radiation was suppressed with SB431542 (Fig. 2G). Similarly, the luciferase activity was examined using a mutant Smad binding site construct (Smad-mut/Luc) [15]. The effect of ionizing radiation was not inhibited using the mutant construct (Fig. 2H).

3.3. MAPK pathway on the type I collagen expression after ionizing radiation

Finally, study investigated whether the MAPK pathway is involved in the expression of type I collagen after ionizing radiation. The MAPK pathway was inhibited with PD98059, SB203580 and SP600125, which are specific inhibitors for ERK1/2, p38 MAPK, and JNK, respectively. These inhibitor treatments had very little effect on the type I collagen mRNA expression induced by ionizing radiation (Fig. 3A). In addition, these inhibitors had very little effect on the transcriptional activity of type I collagen following ionizing radiation (Fig. 3B).

Similarly, ERK1/2, p38 MAPK and Smad3 were selectively inhibited by specific siRNAs. These siRNAs specifically inhibited

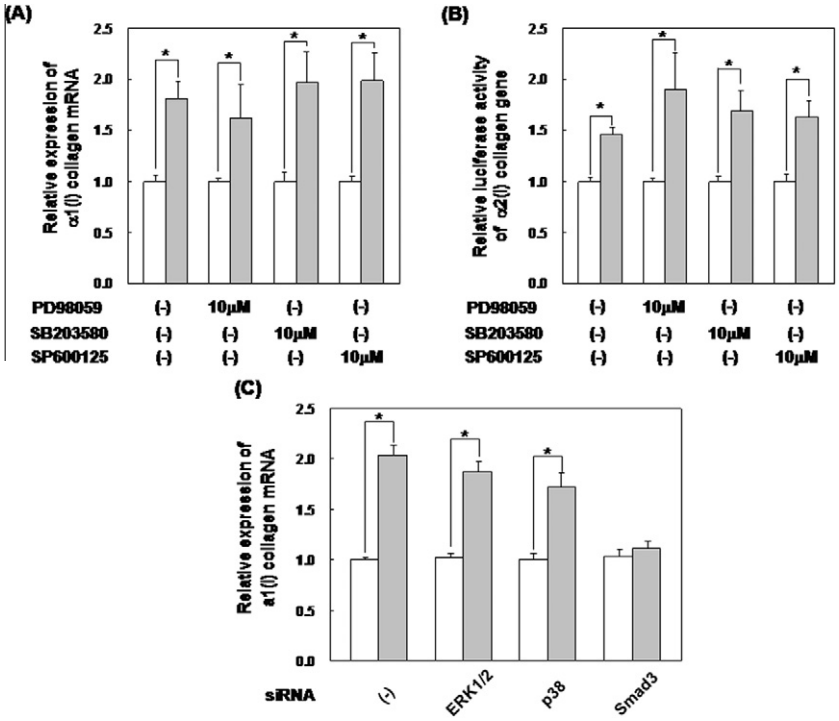

Fig. 3. Effect of MAPK pathway on type I collagen mRNA expression induced by ionizing radiation. Relative mRNA expression of α1(I) collagen (A) and luciferase activity of α2(I) collagen gene (B) treated with or without ERK inhibitor PD98059 (10 μM), p38 MAPK inhibitor SB203580 (10 μM) and JNK inhibitor SP600125 (10 μM). Relative mRNA expression of α1(I) collagen treated with and without siRNA targeted against ERK1/2, p38 and Smad3(C). The data are after at 72 h in NIH3T3 cells with and without radiation. Each value is normalized to the value without radiation at 72 h. Date represents the mean ± SD of results in three independent experiments. $^{*}p < 0.05$.

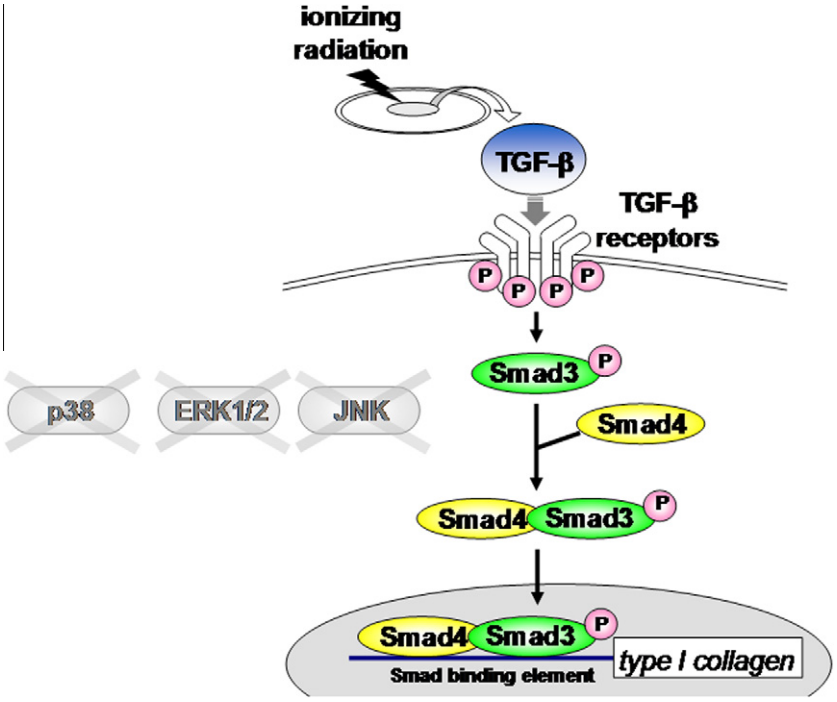

Fig. 4. Schematic illustration of signaling after radiation in NIH3T3 cells.

expression by 50–70% both before and after radiation (Fig. S1). The inhibition by siRNA-mediated knockdown of ERK1/2 and p38 MAPK had no affect on the expression of type I collagen, while that by siRNA Smad3 suppressed the expression of type I collagen induced by ionizing radiation (Fig. 3C).

4. Discussion

The present study examined the effect of ionizing radiation on the expression of type I collagen associated with fibrosis in the fibroblast. Ionizing radiation increased the levels of type I collagen mRNA expression and protein synthesis as well as the promoter activity. The increased expression was seen at 48–72 h, but not 24 h after irradiation. These changes indicate that the effect of ionizing radiation on the expression of type I collagen is a rather delayed response.

TGF-β is a member of a superfamily of multifunctional cytokines which is potent inducer of collagen gene expression during fibrosis. Therefore, TGF-β is considered as a master switch for the formation of RIF. The effect of ionizing radiation on TGF-β expression was seen at 24 h, and it seemed to be earlier than that of collagen expression. The TGF-β signal pathway was inhibited using TGF-β receptor inhibitor and specific siRNA associated with inducing expression of collagen gene after irradiation. Smads operate downstream of the TGF-β superfamily. The luciferase activity was inhibited when the Smad binding site was mutated. This result indicates that ionizing radiation activates Smad mediated TGF-β-dependent transcriptional activity and that this signal pathway contributes to type I collagen gene expression in irradiated cells (Fig. 4). On the other hand, TGF-β also transduces signals through mitogen activated protein kinase (MAPK). The ERK1/2, p38 MAPK and JNK inhibitors did not affect the expression of type I collagen gene induced by ionizing radiation. In addition, the inhibition by specific siRNA-mediated knockdown of ERK1/2 and p38 MAPK had no effect on type I collagen mRNA expression. These results indicates that ionizing radiation up-regulated type I collagen expression through the TGF-Smad signal pathway, and that ERK1/2 and p38 MAPK pathway are not involved in ionizing radiation induced of type I collagen expression in NIH3T3.

However, another mechanism might also work on RIF. Connective tissue growth factor (CTGF) is a matricellular protein that promotes fibroblast proliferation and ECM production, and is a downstream target of TGF-β signaling [16]. Previously studies reported the effect of CTGF on the expression of type I collagen. The selective expression of CTGF promotes systemic tissue fibrosis *in vivo* [17]. A preliminary experiment showed the up-regulation of CTGF mRNA after radiation and it was suppressed when the TGF-β signal pathway was inhibited. Therefore, CTGF might be also involved in RIF via the direct or indirect pathway. Furthermore, the involvement of micro RNAs, which are small, noncoding and approximately 20-nucleotide-length RNAs, were demonstrated in the regulation of the gene expression [18]. Roderburg et al. showed that miR-29 mediates the regulation of liver fibrosis and is part of a signaling molecule involving TGF-β in activated hepatic stellate cells [19]. This pathway might be also involved in RIF as well. Further studies will clarify these mechanisms, which could be useful for the development of novel therapeutic strategies against RIF.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2012.01.039.

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