Astragalus saponin attenuates the expression of fibrosis-related molecules in irradiated cardiac fibroblasts

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Introduction

Radiotherapy treatment for thoracic and abdominal tumors can affect the heart which locates in the mediastinum and cause damage to the heart, known as radiation-induced heart disease (RIHD). The incidence of RIHD has been increasing and is becoming a popular research topic. However, the pathogenesis of RIHD remains unclear and there are no effective treatments. Radiation-induced cardiac damage is described as an outside-in progressive process of fibrosis and is mainly characterized by the increase of cardiac fibroblasts (CFs) and an increased expression of myocardial collagen [1]. CFs are the main affected cells in cardiac fibrosis [2,3] and therefore are rationally chosen for the study of radioactive fibrosis damage. TGF-β1 and collagen, which are synthesized by CFs, are indicators of fibrosis damage, as they are directly involved in the fibrotic process. Fibrosis is a multifactorial disease in which imbalanced expression of cytokines occurs.

Free radicals and reactive oxygen species (ROS) are the products of ionizing radiation in biological systems. On the one hand, oxidative stress caused by ROS is an important mechanism of radiation therapy for tumor cells; on the other hand, ROS also damages the normal cells. It is widely believed that oxidative stress is the main pathogenesis of radiation-induced inflammation and fibrosis [4–6]. Therefore, antioxidant treatment for radiation-induced fibrosis has attracted increasing attention in recent years.

Astragalus saponin (AST) is a pharmacologically active component isolated from the Chinese herb Astragalus membranaceus, a well-known traditional Chinese medicine, used for the treatment of cardiovascular diseases [7]. Recently, AST was shown to act as an antioxidant [8]. Increasing evidence has indicated that Astragalus membranaceus relies on...
its antioxidant properties to effectively attenuate pulmonary fibrosis [9,10]. In the present study, we explored the effects and mechanism of AST on the expressions of fibrosis-related molecules in irradiated CFs in vitro, and then proposed the possibility of using AST to treat radiation-induced cardiac fibrosis in clinic.

Materials and Methods

Reagents
The SYBR Premix Ex Taq and Prime Script RT reagents were purchased from Promega (Madison, USA). Rat polyclonal anti-β-actin (BioVision, Milpitas, USA), rabbit polyclonal anti-TGF-β1, anti-p-Smad2/3, and anti-Col-1 were purchased from Santa Cruz (Santa Cruz, USA). Dulbecco’s modified Eagle’s medium (DMEM)/F-12 (1 : 1) and fetal calf serum (FCS) were purchased from HyClone (Jiangyin, China) and cultured in DMEM/F-12 (1 : 1) supplemented with 10% FCS, penicillin (50 U/ml), and streptomycin (50 μg/ml) in a humidified incubator at 37°C with 5% CO2 for up to 72 h. Culture medium was replaced every 48 h.

Preparation of AST
AST was extracted according to the method of Ma et al. [11] with some minor modifications. In brief, 500 g of crude herb was refluxed with 2% potassium hydroxide in methanol for 1 h. Butan-1-ol was added to the reconstituted residue from above for phase separation to obtain total saponin. The dried and lyophilized AST powder (0.6%; w/w) was reconstituted in ultrapure water to make a 10 mg/ml stock and stored at −20°C.

Cell culture
Rat CFs were obtained from CHI Scientific (MCI003; Jiangyin, China) and cultured in DMEM/F-12 (1 : 1), supplemented with 10% FCS, penicillin (50 U/ml), and streptomycin (50 μg/ml) in a humidified incubator at 37°C with 5% CO2. The CFs were detached using 0.25% Trypsin-EDTA and randomly divided into various experimental and control groups prior to X-ray irradiation.

Irradiation procedure
Cell cultures received fresh culture medium 12 h prior to irradiation. A single dose of 0.5, 1, 2, 3, or 4-Gy X-ray was administered to cells, using a Siemens PRIMUS high-energy linear accelerator (Erlangen, Germany) at a dose-pulse rate of 6 MV/min with a source-to-skin distance of 100 cm. After irradiation, the cell cultures were placed in the incubator and maintained at 37°C with 5% CO2 for up to 72 h. Culture medium was replaced every 48 h.

MTT assay
Cells (1 × 10^4) were seeded into 96-well plates and cultured for 12 h, and then treated with 0, 0.5, 1, 2, 3, or 4-Gy X-ray irradiation. After being incubated for 12–72 h, 20 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well then with a 4-h incubation. The medium was then discarded and replaced with 150 μl of dimethyl sulfoxide in each well. The mixtures were then incubated for 10 min. The OD_{570 nm} was measured and the proliferation rate was calculated. MTT was also used in an AST cytotoxicity assay.

Morphologic observation
The endoplasmic reticulum (ER) is an organelle that is responsible for the synthesis, modification, and correct folding of a vast array of proteins [12]. Cells were treated with 0 or 1-Gy X-ray irradiation and cultured for 48 h, then immobilized in 3% glutaraldehyde. The ultrastructure of ER was observed using a JEM 1230 transmission electron microscope (JEOL, Tokyo, Japan).

Western blot analysis
CFs were treated as indicated, and then lysed. The protein concentrations were measured using a Bradford assay kit (Roche, Basel, Switzerland). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, USA). Membranes were blocked with non-fat milk and probed with primary antibodies (anti-TGF-β1, anti-p-Smad2/3, anti-Col-1, or anti-β-actin). Membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz). Antibody-bound protein bands on the immunoblot were visualized using a ChemiDOC XRS + Gel imaging analysis system (Bio-Rad, Hercules, USA).

Real-time quantitative PCR
The cells were irradiated with 0, 0.5, 1, 2, or 4-Gy X-rays. Total RNA was extracted from cells at 12, 24, 48, or 72 h post-irradiation using a Trizol reagent (Invitrogen, Carlsbad, USA), and was used to obtain complementary DNA (cDNA). All primers used for PCR amplification of TGF-β1, Col-1, and β-actin genes were designed and synthesized by Invitrogen. RT-PCR detection was performed in a cycler (Bio-Rad).

Real-time PCR-based array analysis
Total RNA extraction and all samples were stored at −80°C. First-strand cDNA synthesis was performed with 1 μg of total RNA using an RT² first strand kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. The diluted first-strand cDNA was mixed with RT² SYBR® Green qPCR Mastermix (Qiagen) and pipetted into 96-well PCR-array plates (RT² Profiler™ PCR Array, Fibrosis PAMM-120Z; Qiagen) to evaluate the expression of 84 key genes involved in fibrosis. Real-time PCR detection was performed in a cycler by heating the plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The CT data were uploaded using a PCR-array data analysis software.
spreadsheet template on the PCR-Array Data Analysis website (www.SABiosciences.com/pcarraydataanalysis.php). At this website, the web-based software automatically quantified gene expression using the ΔΔCT method. Quality controls were performed to confirm that there was no DNA contamination and no factors affecting the reverse transcription and PCR in the array plates. Housekeeping genes *Gusb*, *B2m*, *HSP90ab1*, and *Actb* were used to normalize the data. After normalization, the relative expression level of each gene was averaged for the three samples in each group. The assay was repeated twice.

**Flow cytometric analysis**
Flow cytometry was used to assess the level of ROS induced by radiation. Briefly, CFs were labeled with 0.1 mM DCFH-DA (Sigma, St Louis, USA). In the presence of ROS, DCFH-DA is oxidized into fluorescent DCF, allowing ROS levels to be determined indirectly by flow cytometry.

**Statistical analysis**
Data were expressed as the mean ± standard deviation (SD). Statistical and graphical analysis was performed using the SPSS version 18.0 software package. Statistical significance was determined using a one-way ANOVA. *P* < 0.05 was considered statistically significant.

**Results**

**Establishing a fibrosis damage model in irradiated CFs**
An MTT assay was used to evaluate the effects of irradiation on the proliferation of CFs by calculating the proliferation rate after CFs were irradiated with different doses of X-ray. Inhibition of cell proliferation was observed at all time points when CFs were irradiated with 4 and 8-Gy X-rays (*P* < 0.05). However, significant inhibition was only observed in CFs that had been irradiated with 2 and 1-Gy X-rays for 48 and 72 h, respectively (*P* < 0.05) (**Fig. 1A**). There was almost no effect on cell proliferation at all time points when CFs were irradiated with 0.5-Gy X-ray compared with 0 Gy. Based on these results, 48 h of irradiation with 1 and 2 Gy was selected to develop the CFs fibrosis damage model.

We did not observe any obvious alteration of CFs under a phase-contrast microscope after irradiation (**Fig. 1B**). While dilatation, obvious proliferation and a disordered arrangement of ER were observed in irradiated CFs under the electron microscope (**Fig. 1C**). Morphologic changes of ER and structural changes of the plasma membrane would inevitably affect its function of protein processing.

To assess whether the fibrotic change was caused by the above conditions, the expression of molecules associated with fibrosis was studied in CFs after 48 h of irradiation with 0.5, 1, and 2-Gy X-rays. The mRNA expression of *TGF-β1* was increased by 19.54%, 138.64%, and 88.03%, respectively, compared with the control group (0 Gy) (*P* < 0.01) and the mRNA expression of *Col-1* was increased by 109.65% (1 Gy) and 80.62% (2 Gy), respectively, compared with the control group (*P* < 0.01), but with no obvious change at 0.5 Gy. When the radiation dose was increased to 4 Gy, the above mRNA expression was lower than the control group (**Fig. 1D**). A similar trend was found at protein expression level as the irradiation dose was gradually increased. The relative level of the TGF-β1 protein was increased by 160.05% after the 1-Gy X-ray irradiation and by 98.95% after 2-Gy irradiation, compared with the control group (*P* < 0.01). Col-1 was increased by 201.37% with 1 Gy irradiation and by 129.54% with 2-Gy irradiation (*P* < 0.01). While the expression was down-regulated in CFs irradiated with 4 Gy (**Fig. 1E**).

**Effects of AST on the proliferation of irradiated CFs**
Then we investigated whether the fibrotic effect induced by irradiation could be attenuated by AST. AST was used to pretreat the irradiated CFs for 1 h. First, MTT assay was used to evaluate the effects of AST on the proliferation of irradiated CFs and to determine the optimal AST concentration. Inhibition of proliferation was observed in groups pretreated with 40 and 80 μg/ml AST in which the proliferation rates were decreased by ~80% and 60%, compared with the control group (0 μg/ml AST) (*P* < 0.05), respectively. The proliferation rates were not significantly affected in irradiated CFs pretreated with 10 and 20 μg/ml AST at which the proliferation activity of irradiated CFs was not affected, to ensure that the attenuate effect of AST on the expression of fibrosis molecules was not caused by its cytotoxic damage due to large doses.

**Effects of AST on ROS induced by irradiation**
Previous studies have shown that radiation could induce the production of ROS [4–6], which played a key role in cardiac fibrosis. As AST was shown to possess some antioxidative activity, we examined the effect of AST (0, 10, 20 μg/ml) on radiation-induced intracellular ROS generation. AST suppressed ROS formation in CFs as measured after irradiation with 1-Gy X-ray, while AST alone had no effect on ROS generation (**Fig. 3**). These data clearly demonstrated that AST could decrease radiation-induced ROS production.

**AST attenuated the fibrotic damage induced by irradiation**
CFs were pretreated with AST (0, 10, 20 μg/ml) for 1 h before irradiation and the expression of molecules associated with fibrosis was measured. AST significantly reduced the radiation-induced up-regulation of expression. The mRNA of *Col-1* was decreased by 24.51% (10 μg/ml AST) and
Figure 1. Establishing radiation-induced fibrosis damage model in CFs with low-dose X-ray irradiation

(A) Effects of irradiation on the proliferation of CFs were determined using an MTT assay through determining the relative proliferation rate of CFs treated with 0, 0.5, 1, 2, 3, or 4-Gy X-rays for 12–72 h. *P < 0.05, **P < 0.01 vs. control group (0 Gy). (B) Almost no obvious alteration of CFs under a phase-contrast microscope after irradiation. (C) The morphologic changes of ER were clearly observed in irradiated CFs under the electron microscope. (D) The mRNA expression of TGF-β1 and Col-1 in CFs was assessed by RT-PCR. (E) The protein expression of TGF-β1 and Col-1 in CFs was analyzed by western blot analysis. The average fluorescence intensity of each band is shown. All data were representative of three independent experiments. **P < 0.01 vs. control group (0 Gy).
Astragalus saponin attenuates radiation-induced fibrosis damage in CFs

Effects of AST on the proliferation of irradiated CFs

Effects of AST on the proliferation of irradiated CFs were determined using an MTT assay through determining the relative proliferation rate of CFs pretreated with AST (0, 10, 20, 40, 80 μg/ml). AST was used to pretreat cells for 1 h prior to irradiation with 1-Gy X-ray. All data were representative of three independent experiments. *P < 0.05 vs. 0 μg/ml AST group.

Impact of AST on the expression profile of 84 fibrosis-related genes in irradiated CFs

Given that 1-Gy X-ray resulted in the increased expression of TGF-β1 and Col-1 in CFs and AST pretreatment could hamper such fibrotic effect, we further investigated the impact of AST on the expression profile of 84 fibrosis-related genes in CFs with an RT² profiler PCR array.

Table 2 showed the changes of gene expression in CFs exposed to 1 Gy with or without 20 μg/ml AST. The number of significantly altered genes was ~40. The individual genes are listed in Table 2 and Supplementary Table S1. It was obvious that more genes were up-regulated when treated with 1-Gy X-ray. However, pretreatment with 20 μg/ml AST reversed the expression changes of majority of genes caused by irradiation. Interestingly, Col-1A2 and Col-3A1 of extracellular matrix, MMP14, MMP3, and MMP8 of remodeling enzymes, CXCR4, IL-10, IL-13, IL-13ra2, IL-1α, IL-1β, and TNF of inflammatory cytokines/chemokines, TGF-β1, Smad2, Smad3, Smad4, and Cav1 of TGF-β superfamily, and CTGF, NF-κB1, Fasl were up-regulated when treated with 1-Gy X-ray, whereas these genes were down-regulated when pretreatment with 20 μg/ml AST. In addition, TIMP1 and Smad7 that were down-regulated treated with 1 Gy X-ray were up-regulated when pretreated with 20 μg/ml AST.

Discussion

Until recently, most RIHD studies have focused on histopathological observation in experimental animals. The current study observed the fibrosis damage by irradiating CFs at a cellular level. This study showed that X-ray irradiation significantly decreased the proliferation rate of CFs in a dose- and time-dependent manner compared with the control group. Low-dose X-ray did not cause serious damage to cells without obvious morphologic changes. While obvious morphologic changes of ER were caused by irradiation, which might suggest that the protein processing function of ER was imbalanced or enhanced, in order to adapt to the large number of synthetic matrix proteins (such as Col-1) caused by radiation. Morphologic changes of ER could be used as the indirect indicator of fibrosis damage caused by irradiation.

Radiation has a direct pro-fibrosis effect. Herskind et al. [13] reported that radiation could induce differentiation of fibroblasts into fibrocytes, which were responsible for fibrosis due to their increased production of TGF-β1 and Col-1 after irradiation. In the present study, fibrosis-related molecules in CFs were detected to further clarify the molding conditions of radioactive fibrosis damage. Results showed that 1 or 2-Gy X-ray had the most obvious pro-fibrotic effect. Based on the comprehensive consideration for the effects of radiation on CFs proliferation and fibrosis molecular expression, 1 or 2-Gy X-ray was confirmed as the proper conditions for CFs fibrosis damage modeling and 48 h was the proper irradiation time. Under these modeling conditions, CF proliferation activity was not apparently affected, and fibrosis performance was most significant. The modeling conditions are supported by Boerma et al. [14]. In their study, the average expression of fibroblast growth factor-2...
and Col-1 mRNAs was significantly elevated, between 4 and 48 h, in rat CFs after a dose of 2.0 Gy. However, this pro-fibrosis effect was weakened when the irradiation dose was increased to 4 Gy. Maybe 4-Gy X-ray produced severe damage to CFs, which was demonstrated not only by the significantly decreased expression of fibrosis molecules, but also by the cell proliferation rate. High doses of radiation caused severe damage and resulted in disorders in cell function, which was difficult for the body to repair or rescue these cells and finally often led to apoptosis and necrosis, while low dose (1 or 2 Gy) of irradiation did not severely damage cells. But the resultant fibrosis pathological changes are the root cause for organ function damage caused by radiation. Research on the ‘early damage effects of low-dose irradiation’ is seemingly more in line with the actual clinical situation regarding the development of side effects caused by radiotherapy.

AST could inhibit the increase of methane dicarboxylic aldehyde (MDA) [15], improve the activity of superoxide dismutase (SOD) [16], and reduce the myocardial oxidative stress injury. Our results showed that the ROS generation in

Table 1. Number of genes in CFs significantly altered after 1 Gy irradiation with/without AST

<table>
<thead>
<tr>
<th>Treatment/control</th>
<th>Number of altered genes</th>
<th>Up-regulated genes a</th>
<th>Down-regulated genes a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Gy/0 Gy</td>
<td>44</td>
<td>30</td>
<td>14</td>
</tr>
<tr>
<td>(AST + 1 Gy)</td>
<td>42</td>
<td>14</td>
<td>28</td>
</tr>
</tbody>
</table>

aP < 0.05 and ≥2-fold changes compared with the control group.

Figure 4. AST attenuated the fibrotic effect induced by irradiation

AST (0, 10, 20 μg/ml) was used to pretreat cells for 1 h prior to irradiation with 0 or 1-Gy X-ray. (A,C) The mRNA expression of Col-1 and TGF-β1 was assessed by RT-PCR. (B,D) The protein expression of Col-1, TGF-β1, and p-Smad2/3 in CFs was determined by western blot analysis. The average fluorescence intensity of each band is shown. All data were representative of three independent experiments. **P < 0.01 vs. control group (0 Gy); ##P < 0.01 vs. (1 Gy + 0 μg/ml AST) group.
irradiated CFs was inhibited by AST in a dose-dependent manner, which was indicated by the observation that the inhibitory effect of 20 μg/ml AST was stronger than that of 10 μg/ml AST. Meanwhile, enhanced TGF-β1 and Col-1 expression in irradiated CFs was attenuated by AST in a similar manner, as the attenuation of 20 μg/ml AST was more obvious than that of 10 μg/ml AST. These findings implied that AST may attenuate radiation-induced fibrotic responses through its inhibition of ROS in irradiated CFs. This speculation could be supported by Liu et al. [17]. In their study, it was reported that Astragalus membranaceus, through its antioxidative action, reduced the release of inflammatory cytokines and therefore relieved cell damage and fibrosis caused by inflammation.

Considering the complex molecular networks involved in fibrosis diseases, we conducted a PCR array for fibrosis gene expression analysis. This array was used to preliminarily screen fibrosis molecules at mRNA level in irradiated CFs pretreated with/without AST. In experiments involving treatment with 1 Gy, it was obvious that more genes were up-regulated. However, pretreatment with 20 μg/ml AST reversed the majority of genes that had been changed by irradiation. These genes were functionally associated with extracellular matrix, remodeling enzymes, inflammatory cytokines/chemokines, and TGF-β superfamily. These preliminary results demonstrated AST’s potential anti-fibrosis activity.

Organ fibrosis is a multifactorial chronic inflammatory disease with excessive deposition of extracellular matrix, mainly composed of Col-1 and Col-3. ECM-secreting myofibroblasts are central to the pathogenesis of all fibrotic diseases [3,18]. Results in some organ fibrosis models have suggested that there may be therapeutic benefit in targeting myofibroblasts [19]. Interestingly, the results of PCR array showed that Col-1A2 and Col-3A1 of extracellular matrix were up-regulated under irradiation with 1-Gy X-ray, whereas these genes were down-regulated when pretreated with AST. Meanwhile, the protein expression of Col-1 was significantly decreased by 20 μg/ml AST. These results indicated that AST prevented the secretion of ECM in irradiated CFs, thus might prevent the radiation-induced fibrosis effect. Our results were consistent with a previous study, which reported the attenuation effect of Astragalus membranaceus on collagen secretion in renal tubular epithelial cells [20].

The net amount of collagen deposited by fibroblasts is regulated continuously by collagen synthesis and collagen

### Table 2. Changed expression of genes in CFs after 1 Gy irradiation with/without AST

<table>
<thead>
<tr>
<th>Treatment/control</th>
<th>Functional group</th>
<th>Symbol of up-regulated genes a</th>
<th>Symbol of down-regulated genes a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Gy/0 Gy</td>
<td>Pro-fibrotic</td>
<td>Agt, CCL12</td>
<td>CCL11</td>
</tr>
<tr>
<td></td>
<td>Extracellular matrix</td>
<td>Col-1A2, Col-3A1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Remodeling enzymes</td>
<td>MMP14, MMP3, MMP8, Plau (uPA), Serpin1a</td>
<td>Lox, Plat, Serpine1, TIMP1</td>
</tr>
<tr>
<td></td>
<td>Cellular adhesion</td>
<td>ITGFB3</td>
<td>ITGFox1</td>
</tr>
<tr>
<td></td>
<td>Inflammatory cytokines and chemokines</td>
<td>CXCR4, IL-10, IL-13, IL-13α2, IL-1α, IL-1β, TNF</td>
<td>Cer2</td>
</tr>
<tr>
<td></td>
<td>Growth factors</td>
<td>CTGF, PDGFβ</td>
<td>Edn1</td>
</tr>
<tr>
<td></td>
<td>TGFβ superfamily</td>
<td>BMP7, Cav1, Dcn, TGFβ1, Smad2, Smad3, Smad4, TGIF1</td>
<td>Eng, Ihhbe, Smad7, TGFβr1, Thbs1</td>
</tr>
<tr>
<td></td>
<td>Transcription factors</td>
<td>NF-κB1, Stat1</td>
<td>Sp1</td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td>Fasl</td>
<td></td>
</tr>
<tr>
<td>(AST + 1 Gy)/1 Gy</td>
<td>Pro-fibrotic</td>
<td>CCL12</td>
<td>CTGF</td>
</tr>
<tr>
<td>1 Gy</td>
<td>Extracellular matrix</td>
<td>Col-1A2, Col-3A1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Remodeling enzymes</td>
<td>Plau (uPA), Serpin1a, TIMP1, TIMP 2</td>
<td>MMP14, MMP3, MMP8, Serpine1</td>
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<tr>
<td></td>
<td>Cellular adhesion</td>
<td>ITGFB3</td>
<td>IITGFox1</td>
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<tr>
<td></td>
<td>Inflammatory cytokines and chemokines</td>
<td>IL-1α, CCL-3</td>
<td>IL-10, IL-13, IL-13α2, TNF,</td>
</tr>
<tr>
<td></td>
<td>Growth factors</td>
<td>PDGFβ</td>
<td>CXCR4, IFNG</td>
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<td></td>
<td>TGFβ superfamily</td>
<td>BMP7, Dcn, Smad7, TGIF1</td>
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</tr>
<tr>
<td></td>
<td>Transcription factors</td>
<td>Stat1</td>
<td>TGFβ1, Smad2, Smad3, Smad4,</td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td></td>
<td>TGFβr1, Cav1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NF-κB1, Sp1, Jun</td>
</tr>
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<td></td>
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<td></td>
<td>Fasl</td>
</tr>
</tbody>
</table>

*P < 0.05 and ≥2-fold change compared with the control group.*
catabolism. MMPs and their inhibitors, tissue inhibitors of MMPs (TIMPs), play an important role in the regulation of ECM turnover in fibrotic tissues. While the degradation of pathological fibrillar collagen by MMPs is a key event in the resolution of fibrosis, the degradation of normal ECM components in the early stages of fibrosis promotes deposition of newly synthesized collagen [19]. Furthermore, integrin-mediated TGF-β activation seems to be possible in a protease-dependent or protease-independent manner; protease-dependent TGF-β activation has only been demonstrated for αvβ8 and depends on the binding of the integrin to the rgD site in lap and simultaneous recruitment of MMP14, which then releases TGF-β by proteolytic cleavage [21]. PCR-array results showed that MMP14, MMP3, and MMP8 of remodeling enzymes, were up-regulated by treatment with 1-Gy X-ray, whereas these genes were down-regulated by pretreatment with 20 μg/ml AST. In addition, TIMP1 that was down-regulated by treatment with 1-Gy X-ray was up-regulated when pretreated with 20 μg/ml AST. These findings suggested that the ECM degradation process was activated and tissue reconstruction had begun in the early stage (48 h) of radiation-induced fibrosis. Meanwhile, TGF-β activation mediated by MMP14 was strengthened. Results also showed that AST reversed the radiation effects on the above gene expression, which indicated that AST could regulate the imbalance between TIMPs and MMPs to attenuate fibrosis induced by radiation in cultured CFs. This mediating effect of Astragalus membranaceus on TIMPs/MMPs was also confirmed in rat pulmonary fibrosis [8].

Fibroblasts have the potential to secrete large proinflammatory factors such as TNF-α, IL-1β, IL-3, IL-6, IL-8, IL-10, IL-13, MIP1α/β [22] and chemokines such as CXCL2 and CCL2. These cytokines could directly activate fibroblasts [23,24]. IL-13 and its receptors are regarded as dominant mediators in fibrosis [25,26]. IL-13 has been implicated in radiation-induced fibrosis [27]. On the one hand, IL-13 has been speculated to promote fibrosis by stimulating the production and activation of TGF-β [28]. On the other hand, IL-13 can promote fibrosis by directly enhancing the fibroblast function of synthesis and proliferation [29]. The profibrotic activity of IL-13 is closely related to the abundance of its receptors (IL-13Rα1 and IL-13Rα2) expressed on target cells such as fibroblasts [30,31]. Additionally, fibroblasts could express chemokine receptor such as CXCR4, and be recruited to inflammatory site in a CXCR4-dependent manner [32,33], where they promote the ECM producing fibroblast pool [33]. The results of PCR array showed that CXCR4, IL-13, IL-13Rα2, IL-10, IL-1α, IL-1β, and TNF of inflammatory cytokines/chemokines, were up-regulated when treated with 1-Gy X-ray, whereas these genes were down-regulated when pretreated with 20 μg/ml AST. These results demonstrated that irradiation stimulated the production of inflammatory cytokines and chemokines, and therefore contributed to the fibrosis effect. AST prevented the expression of these cytokines and receptors in irradiated CFs, thus might prevent the development of radiation-induced fibrosis. This speculation was supported by a previous study [9], which revealed that Astragalus membranaceus reduced the release of inflammatory cytokines, and inhibited pulmonary fibrosis of rats [9].

Many studies have shown that the TGF-β signaling pathway was involved in radiation-induced fibrosis [34–36]. The activation of this signaling pathway begins with TGF-β binding to serine/threonine kinase receptor complex, which consists of TGF-βR1 and TGF-βR2, subsequently leading to the recruitment and phosphorylation of the intracellular effector proteins Smad2/Smad3, and then phosphorylated Smad2/Smad3 bind to Smad4 and translocate to the nucleus to initiate gene expression. The TGF-β signaling pathway could be negatively regulated by inhibitory Smads, including Smad6 and Smad7 [37]. PCR-array results showed that TGFβ1, Smad2, Smad3, and Smad4 were up-regulated when treated with 1-Gy X-ray, whereas these genes were down-regulated when pretreated with 20 μg/ml AST. In addition, Smad7 that was down-regulated when treated with 1 Gy X-ray was up-regulated when pretreated with 20 μg/ml AST. Our results suggested that irradiation seemed to promote the activation of TGF-β signaling pathway, meanwhile suppress its negative regulation, and therefore promote the fibrosis development. However, AST completely reversed the above radiation effects. Furthermore, this study showed that the protein levels of TGF-β1 and p-Smad2/3 were also significantly decreased by 20 μg/ml AST. These results indicated that AST could attenuate radiation-induced fibrosis by regulating the TGF-β1-Smad signaling pathway. This viewpoint was supported by several studies [38,39], which reported that Astragalus membranaceus could reduce the expression of TGF-β in fibroblasts.

In the present study, we used PCR array just to observe the differential expression of 84 fibrosis-related genes in CFs. First, we want to preliminarily display the possible anti-fibrosis effect of AST. Second, we also want to preliminarily screen the ‘the possible molecular targets’ for the follow-up study. Our further research will verify the results of PCR array by a classical PCR method and western blot analysis, and to determine whether there is any possible links among these molecules. So this study is a preliminary research, its effectiveness and the exact molecular mechanism still need to be further confirmed by a verification experiment with positive control, in order to complete the basic support for its clinical application.

In conclusion, radiation-induced fibrosis damage was observed at a cellular level. AST attenuated the radiation-induced fibrosis damage effect in CFs and this anti-fibrosis damage effect may be closely related to its antioxidative action. The involvement of fibrosis-related molecules in
irradiated CFs was demonstrated by PCR array for the first time. AST reversed the majority of genes that had been changed by irradiation, confirmed its anti-fibrosis damage effect which was associated with extracellular matrix, remodeling enzymes, inflammatory cytokines/chemokines, and TGF-β. The present *in vitro* findings supported the interpretation that AST is a substance that may be beneficial in protecting the myocardium against radiation-induced fibrosis damage.

**Supplementary Data**

Supplementary data is available at *ABBS* online.

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