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CXCR4 antagonist AMD3100 protects TGFβ1 induced viability and migration in rat cardiac fibroblasts.

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Abstract

Proliferation and migration of Cardiac Fibroblasts (CFBs) are important in early stage of myocardial fibrosis. The purpose of the present study was to investigate the effect of CXCR4 antagonist AMD3100 treatment on transforming growth factor (TGF)β1induced CFBs abnormal proliferation and migration, and to elucidate the underlying mechanisms. The cell proliferation was evaluated by a MTT assay. Cell transwell migration assay and wound scratch assay were used to detect the cells migration. Measurement of ROS levels were determined by the DCF-DA dye. TGFβ1 stimulation in CFBs resulted in increased proliferation, migration and ROS generation. In conclusion, the present study revealed that AMD3100 treatment inhibited CFBs proliferation and migration induced by TGFβ1, at least in part through suppression of ROS signaling.

Keywords: CXCR4, AMD3100, TGFβ1, Cardiac fibroblasts, Proliferation, Migration.

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Introduction

Cardiac fibroblast abnormal proliferation, migration and collagen proteins deposition in Extracellular Matrix (ECM) induces cardiac fibrosis, which is involved in myocardial tissue remodelling and dysfunction [1-3]. Cardiac tissue remodelling ultimately leads to elevated regional myocardial stiffness, left ventricular diastolic dysfunction, reduced the vascular resistance of the coronary arteries and sudden cardiac death [4-6].

Immune responses, ischemia-reperfusion injury and hyperglycemia induces cardiac fibrosis *via* activating the renninangiotensinaldosterone system, the collagen proteins generation and depredating cytokines production [7]. Thus, investigation of the pathogenesis and molecular mechanisms of cardiac fibrosis is necessary for the severe cardiovascular events prevention and treatment.

Transforming growth factor (TGF)β1 has been regarded as a multifunctional peptide [8]. TGFβ1 has a key role in various biological activities, especially in the cardiovascular system [9,10]. TGFβ1 modulates the transformed phenotype of fibroblasts and elevates the generation of collagen proteins and fibrosis [11-13].

Previous studies have also confirmed that TGFβ1 inhibits the activity of matrix metalloproteinase and inhibits ECM degradation in prostate cancer (PrCa) cells [14]. Specifically, TGFβ1 is involved in the modulation of multiple transduction pathways and directly stimulates the proliferation of CFBs [15,16].

More, TGF-β1 increased pancreatic carcinoma cells, human dermal fibroblasts and CFBs migration [17-19]. However, the molecular mechanisms underlying this abnormal alteration are poorly understood.

The previous studies demonstrated that the CXCR4 plays a key role in the pathogenesis of CFBs [20]. CXCR4 antagonist AMD3100 promoted wound healing in diabetic mice by increasing cytokine production and elevating the activity of fibroblasts [21].

Many studies found that TGF-β1 regulates CXCR4 expression in both mRNA and protein level, which then affected stem cell migration and adhesion [22,23]. However, whether TGF-β1 induces proliferation and migration of CFBs by CXCR4 remains unclear.

The aim of the present study was investigated whether treatment with AMD3100 could abolish the alterations in CFBs and ROS generation induced TGF-β1 and the molecular mechanisms that underlie the protective effects of AMD3100 on CFBs function with the suppression of the ROS pathway.

Materials and Methods

Materials

TGF-β1 was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). AMD3100 and Fetal Bovine Serum (FBS) were purchased from *Sigma*-Aldrich (St. Louis, MO, USA). Fibroblast Medium-2 was from Sciencell (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Thermo Scientific, Grand Island, NY, USA).

Cell culture

Rat cardiac fibroblasts were obtained from Sciencell (R6300, Shanghai, China). The cells were cultured in fibroblast medium-2 (2331, Sciencell, Shanghai, China) with 10% fetal bovine serum (FBS, *Sigma*-Aldrich, St. Louis, MO, USA) and 1% penicillin and streptomycin at 37°C incubator with 5% CO_2. All experiments were carried out with cardiac fibroblast from 2-4 generation.

Cell treatment

CFBs were pre-treated with CXCR4 inhibitor AMD3100 (0-1 μg/ml), LY294002 (10 μM) for 30 min at 37°C and then added to TGF-β1 with different concentrations (0-20 ng/ml) for 24 h. The control group were added with PBS alone. After treatment, the cells were collected for further investigation.

Cell proliferation assay

Cells (5×10^3 cells/well) were treated with TGF-β1 and AMD3100 for 24 h. The cells were washed twice with PBS, and were incubated with 5 mg/ml MTT reagent (20 μl, Beyotime Technology) at 37°C for 4 h, the cells were dissolved in DMSO (150 μl, Sigma-Aldrich, Inc., USA). The absorbance was measured by a microplate reader (Bio-Rad, CA, USA) at 490 nm.

Cell migration assay

CFBs migration was determined by using transwell culture plates with 8.0 μm pore polycarbonate membrane (Corning Costar, Cambridge, MA, USA). CFBs (2×10^4 cells/well) was seeded to the upper insert, the lower chamber were added 500 μL of DMEM medium containing TGF-β1 with different concentrations (0-20 ng/ml) at 37°C in a humidified, 5% CO_2 atmosphere for 12 h. Then, the DMEM medium was removed from the upper insert and non-migrated cells were removed by scraping. The migrated cells were stained with Calcein-AM at 37°C in the dark for 30 min. Migrated cells were quantified with a light microscope (200x magnification ,Carl Zeiss Microimaging, Thornwood, NY, USA).

Wound scratch assay

CFBs (2×10^4 cells/well) were suspended in 6-well plates (Thermo Fisher Scientific, Waltham, USA) at 70-80% confluence. Then 24 h later, the cell monolayer was scratched with a yellow pipette tip. TGF-β1 (20 ng/ml) and AMD3100 (1 μg/ml) was added to each well. Images were analyzed at 0 and 24 h with a light microscope (Carl Zeiss Microimaging, Thornwood, NY, USA) and the scratch area was measured by the Image-Pro Plus software.

Measurement of ROS levels

CFBs were incubated with 10 μM 2', 7'-dichlorofluorescein diacetate (DCF-DA, Sigma-Aldrich, USA) at 37°C for 30 min. The fluorescence intensities of DCF were monitored with a microscope (Carl Zeiss Microimaging, Thornwood, NY, USA) at excitation (488 nm) and emission (525 nm).

Statistical analysis

All data are presented as the mean ± SD of at least three experiments. Comparisons between two groups were analyzed with student's t-test (SPSS 23.0; SPSS Inc., Chicago, IL, USA). $P<0.05$ were considered to be statistically significant.

Results

The effect of AMD3100 on TGF-β1 induced the cell proliferation of CFBs

We first examined whether TGFβ1 and AMD3100 has cytotoxic effects in rat CFBs, the proliferation of CFBs were investigated by MTT assay. TGFβ1 treatment stimulated a substantially increase in CFBs proliferation compared with control group (Figure 1A). AMD3100 treatment did not affect CFBs proliferation (Figure 1B). However, TGFβ1induced increase in CFBs proliferation was suppressed by pre-treatment with AMD3100 (Figure 1C). So, AMD3100 (1 μg/ml) and TGF-β1 (20 ng/ml) were used as the optimal concentration for the next experiments.

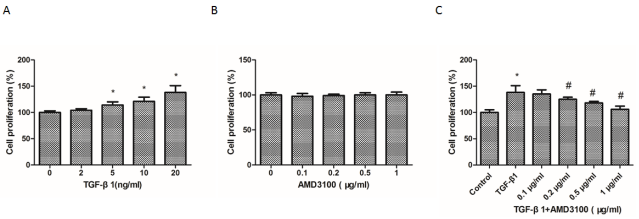

Figure 1. *The effect of AMD3100 on TGF-β1 induced proliferation of rat CFBs. Cell proliferation assay was performed by a colorimetric method using MTT assay. (A) CFBs were incubated with TGF-β1 (0-20 ng/ml) for 24 h; (B) CFBs were incubated with AMD3100 (0-1 μg/ml) for 24 h; (C) CFBs were pre-incubated with 0-1 μg/ml AMD3100 for 30 min, and then treated with TGF-β1 (20 ng/ml) for 24 h. Results are expressed as mean ± SD (n=3); $^*p<0.05$ versus control; $^\#p<0.05$ versus TGF-β1.*

The effect of AMD3100 on TGF-β1 induced the cell migration of CFBs

We next detected the effects of TGFβ1 and AMD3100 on migration of rat CFBs by a Boyden chamber transwell assay. TGFβ1 significantly induced CFBs migration (Figure 2A).

AMD3100 treatment did not affect CFBs migration (Figure 2B). However, TGFβ1induced increase in CFBs migration was inhibited by pre-treatment with AMD3100 (Figures 2C and 2D).

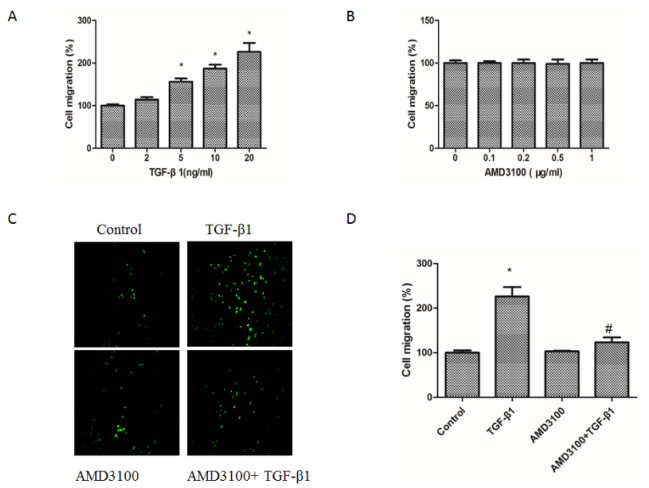

Figure 2. *The effect of AMD3100 on TGF-β1 induced migration of rat CFBs. Cell migration assay was performed by a Boyden chamber transwell assay. (A) CFBs were incubated with TGF-β1 (0-20 ng/ml) for 12 h; (B) CFBs were incubated with AMD3100 (0-1 μg/ml) for 12 h; (C) CFBs were pre-incubated with 0-1 μg/ml AMD3100 for 30 min, and then treated with TGF-β1 (20 ng/ml) for 12 h; (D) Quantification of migration of rat CFBs as in (C). Results are expressed as mean ± SD (n=3); *p<0.05 versus control; #p<0.05 versus TGF-β1.*

Next, we assessed CFBs migration by scratch assay. In the control group and AMD3100, there were no differences in migration of the CFBs (Figure 3). In the TGF-β1 group, CFBs showed significantly enhanced migration ability (Figure 3). However, TGFβ1 mediated increase in CFB migration was abolished by pre-treatment with AMD3100 (Figure 3), as presented by a wider scratch area.

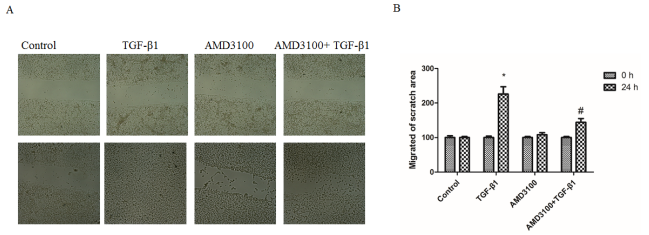

Figure 3. *The effect of AMD3100 on TGF-β1 induced migration of rat CFBs. Cell migration assay was performed by a scratch assay. (A) CFBs were pre-incubated with 0-1 μg/ml AMD3100 for 30 min, and then treated with TGF-β1 (20 ng/ml) for 24 h; (B) Quantification of scratch area of rat CFBs as in (A). Results are expressed as mean ± SD (n=3); *p<0.05 versus control; #p<0.05 versus TGF-β1.*

The effect of AMD3100 on TGF-β1 induced the ROS generation of CFBs

To detect the effects of TGFβ1 and AMD3100 on ROS generation of rat CFBs by a DCFH-DA stained assay. TGFβ1 significantly induced CFBs ROS generation (Figures 4A and 4B). AMD3100 treatment did not affect CFBs ROS generation (Figures 4A and 4B). However, TGFβ1induced increase in CFBs ROS generation was inhibited by pre-treatment with AMD3100 (Figures 4A and 4B).

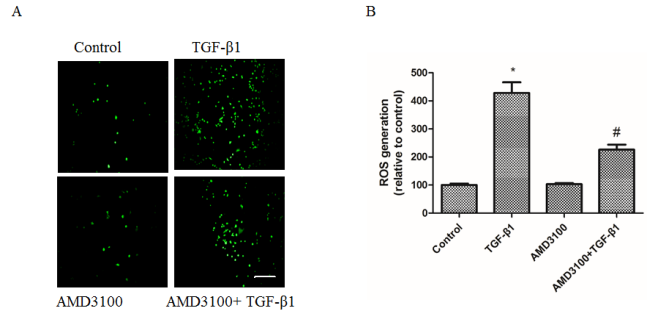

Figure 4. *The effect of AMD3100 on TGF-β1 induced ROS of rat CFBs. ROS production assay was performed by a DCFH-DA stained assay. (A) CFBs were pre-incubated with 0-1 μg/ml AMD3100 for 30 min, and then treated with TGF-β1 (20 ng/ml) for 24 h; (B) Quantification of s ROS generation as in (A). Results are expressed as mean ± SD (n=3); *p<0.05 versus control; #p<0.05 versus TGF-β1, scale bar, 25 μm.*

Discussion

This study demonstrated that AMD3100, CXCR4 antagonist, inhibits TGFβ1 stimulates proliferation and migration of rat CFBs. We also confirmed the molecular mechanisms as the activation of Akt pathway and reduction of ROS generation.

Activation of fibroblasts through proliferation and migration is important in wound healing and maturation of scar tissue after myocardial infarction [24,25]. Thus, the decreased CFBs proliferation and migration in the infarcted area might cause the deactivation of cardiac fibroblasts, which inhibits excessive fibrosis or delays the wound healing and maturation of scar tissue after myocardial infarction. TGFβ1 is an important signaling molecule that induces cardiac fibrosis by activating the proliferation and collagen production of CFBs [26,27]. The excessive TGFβ1 involved in the pathogenesis of maladaptive remodelling and fibrosis [28]. TGFβ1 binds to receptors and induces a number of signal pathways, including Akt signaling pathway. TGFβ1 also induce ROS generation, which activate the Akt signaling pathways [29]. So Akt signaling pathway has an important role in pathogenesis of CFBs.

In previous studies, the results demonstrated selective antagonism of the SDF-1/CXCR4 pathway reversed the development of cardiac fibrosis and left ventricular hypertrophy in the mice diabetes model [30]. In this study, AMD3100 had no effects on proliferation and migration in rat CFBs. It is thus suggested that AMD3100 has no cytotoxicity on rat CFBs. However, AMD3100 significantly inhibited TGFβ1 stimulates proliferation and migration in rat CFBs.

In conclusion, we revealed for the first time that AMD3100 attenuates TGFβ1 stimulates proliferation and migration of rat CFBs, at least partly *via* the activation of ROS pathway. These findings implied that CXCR4 might trigger cardiac fibroblast abnormal alteration and induce the process of cardiac fibrosis.

Acknowledgment

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Conflict of Interest

The authors did not report any conflict of interest.

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