Multiple Mechanisms for Anti-Fibrotic Functions of Statins on Radiotherapy Induced Fibrosis

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Abstract: Radiotherapy-induced fibrosis (RTIF) presents a challenge in radiotherapy for cancer patients. Although numerous studies have attempted to elucidate the mechanisms leading to RTIF, the pathogenesis of RTIF at the cellular and molecular level is still incompletely described. One key component involved in the post-radiation injury is the pleuripotent cytokine transforming growth factor (TGF)-β. TGF-β signaling pathway has been under intensive investigation about its critical role in radiation-induced fibroproliferative disease. Connective tissue growth factor (CTGF), also known as insulin-like growth factor binding protein-related protein 2 (IGFBP-rP2) is a potent regulator of fibroblast proliferation, cell adhesion, and stimulation of extracellular matrix production. CTGF is known as a major downstream mediator of the chronic fibrotic effects of TGF-β. Here we have demonstrated that irradiation and TGF-β induced CTGF, subsequently upregulates fibrotic factors such as fibronectin and type IV collagen. Furthermore, as HMG-CoA reductase inhibitors, statins inhibit expressions of CTGF and downstream fibrotic proteins in both normal human fetal fibroblasts (HFL-1) and human dermal fibroblasts (HDF) on TGF-β treatment or irradiation. Our study also demonstrates that simvastatin not only suppressed TGF-β-induced fibrosis through inhibition of CTGF production but also CTGF-induced fibrosis. We further show that simvastatin may act in a TGF-β-independent manner by inhibiting Rho kinase pathway. Taken together, these data suggest that radiotherapy may upregulate CTGF expression in a TGF-β-dependent and -independent manner, thereby enhancing expression of profibrotic factors and inducing lung fibrosis.

Keywords: CTGF, Statins, Fibrosis, TGF-β, Radiation, Rho/ROCK pathway.

INTRODUCTION

Radiation induced lung injury is the main dose-limiting factor in patients with lung cancer who receive radiation treatment. Based on the onset, radiation induced lung injury can be categorized into two phases: acute radiation pneumonitis (early stage) and pulmonary fibrosis late stage toxicity [1, 2]. Radiotherapy-induced fibrosis (RTIF) is confined to the irradiated area of the lung with a complex process of repair following activation of fibroblasts and local release of pro-fibrotic factors such as transforming growth factor (TGF)-β [3], connective tissue growth factor (CTGF) [4] and platelet derived growth factor (PDGF) [1]. CTGF is a mediator, downstream of TGF ß1 and these two cytokines act together as co-factors in fibrogenesis [5]. Targeting the TGF-β signaling pathway represents a rewarding treatment to reduce radiation-induced fibrosis [3]. However, Anscher *et al.* have shown that blocking the effects of TGF-β with an anti-TGF-β antibody does not completely eliminate RTIF in the rat model [6]. CTGF, also known as insulin-like growth factor binding protein-related protein 2 (IGFBP-rP2) and a member of the CCN family, is a secreted matricellular protein that has multiple effects on development, cellular differentiation, homeostasis and fibroproliferative diseases as well as certain types of cancer [4, 5, 7]. CTGF facilitates cell proliferation, extracellular matrix deposition such as fibronectin and collagen synthesis, angiogenesis, wound repair and phenotype change from fibroblast differentiation into myofibroblast [5, 8]. Further studies revealed that CTGF can also be induced in a TGF-β-independent manner and contribute to the development of fibrosis in fibroblasts [5, 7]. This presents CTGF as a potential anti-fibrotic target that leads to the suppression of fibrotic proteins and inhibits fibrotic response in many fibrotic diseases [9, 10]. Simvastatin, one of HMG-CoA reductase inhibitors, originally applied in the treatment of cardiovascular diseases, has been demonstrated to attenuate or inhibit RTIF *in vitro* and *in vivo* systems [11, 12]. This study focuses on determining if pro-fibrotic markers such as fibronectin and collagen IV can be induced further downstream in the TGF-β pathway by inducing CTGF alone as a TGF-β independent effect and if simvastatin is capable of inhibiting fibrosis by targeting more than one site in the pathway in a TGF-β dependent and an independent manner. There

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are reports that suggest that in addition to the TGF-β pathway, another pathway that has been studied with regards to fibrosis is the Rho/ROCK pathway. Rho-proteins belong to a family of small GTPases that are responsible for a wide range of cellular functions and these functions largely depend on the activation of their effectors downstream, Rho kinase (ROCK) [13]. Inhibition of Rho-kinase (ROCK) has been shown to mediate LPS mediated induction of CTGF in renal mesengial cells [14]. This led us to investigate the effect of statins on CTGF-induced pro-fibrotic factors with respect to the Rho/ROCK pathway in RTIF.

RESULTS

TGF-β and Irradiation Induce CTGF and its Downstream Pro-Fibrotic Proteins in Human Lung Fibroblasts

CTGF has been shown as a major downstream fibrotic factor of TGF-β-induced fibrosis. To confirm the effect of TGF-β and irradiation on induction of CTGF and downstream fibrotic targets such as fibronectin and collagen type IV (Col IV), HFL-1 cells were treated with TGF-β or irradiated and both mRNA and protein levels were determined. The levels of CTGF, fibronectin and Col IV increased in a dose- dependent manner, on TGF-β treatment and irradiation, at both mRNA (Figure **1A** and **1C**) and protein levels (Figure **1B** and **1D**). A time-dependent increase in the expression levels of CTGF and fibronectin was observed when the cells were irradiated with 5Gy (Figure **1E**). We also performed experiments in human dermal fibroblasts (HDF) and observed similar results (SI-1A, 1B). These data indicate that CTGF and related pro-fibrotic downstream proteins can be induced by TGF-β and radiation in a dose- and time-dependent manner.

Statins Attenuate Effects of TGF-β and Radiation on Expression of CTGF and Downstream Targets

Simvastatin (SIM), a HMG-CoA reductase inhibitor, has been shown to have anti-fibrotic activity [11]. We observed that SIM inhibited TGF-β-induced CTGF and downstream targets at both mRNA and protein levels in dose-dependent manner (Figure **2A(i)** and **2A(ii)**). Similar effect of SIM was also observed in HFL-1 post-irradiation (Figure **2B(i)** and **2B(ii)**). To further explore effects of other potential HMG-CoA inhibitors on radiation-induced CTGF expression, HFL-1 were incubated with pravastatin (PRA), mevinolin (MVO), mevastatin (MVS) and SR12813, in addition to SIM (Figure **2C**). More potential anti-fibrotic effect was observed in the cells treated with SIM, MVO and MVS other than with PRA and SR 12813 compared with control. We also tested effect of SIM on TGF-β- and irradiation induced CTGF and pro-fibrotic factors in HDFs and found that SIM inhibits CTGF, fibronectin and ColIV in HDFs also (SI-1C,1D). Together these data suggest that statins may be used for mitigation and treatment of RTIF through inhibition of CTGF and downstream pro-fibrotic proteins expression.

TGF-β Independent Signaling Pathway may be Involved in RTIF

To investigate if the induction of CTGF expression in TGF-β- treated or irradiated HFL-1 cells is primarily *via* the TGF-β pathway, these cells were treated with TGF-β neutralizing antibody. The neutralizing antibody completely abolished CTGF expression in TGF-β treated cells (Figure **3A**), but the decrease was not significant in the irradiated cells (Figure **3B**). On treating TGF-β- treated or irradiated HFL-1 cells with SIM, the inhibitory effect on CTGF was more potent in both the cells. Our observation is in line with Anscher *et al.* report that blocking TGF-β1 function cannot completely prevent fibrogenesis caused by high-dose radiation in a rat model using TGF-β1 neutralizing antibody [6]. These data suggest that radiation-induced CTGF upregulation occurs partially through TGF-β-independent signaling pathway and that SIM may inhibit CTGF upregulation occurring *via* the TGF-β-independent pathway as well.

SIM Inhibits CTGF Induced Pro-Fibrotic Markers in HFL-1 Cells

We and others have established that TGF-β-induced CTGF and fibrotic markers can be inhibited by SIM. We next investigated if SIM can act further downstream, i.e. if it can inhibit CTGF-induced fibronectin. In order to investigate this, HFL-1 cells were infected with adenoviral plasmids containing CTGF cDNA sequence, with or without SIM treatment. Figure **4** shows that SIM also inhibits CTGF-induced fibronectin at both mRNA (Figure **4A**) and protein (Figure **4B**) levels while it could not suppress ectopic expression of CTGF.

SIM Inhibits TGF-β- and CTGF- Induced Pro-Fibrotic Markers through the Rho-ROCK Pathway

To determine the pathway in which CTGF-induced pro-fibrotic markers are inhibited, HFL-1 cells were treated with either SIM or ROCK inhibitor (Y27632), followed by treatment with CTGF. Figure **4C** and **4D** show that Y-27632 also inhibits CTGF-induced

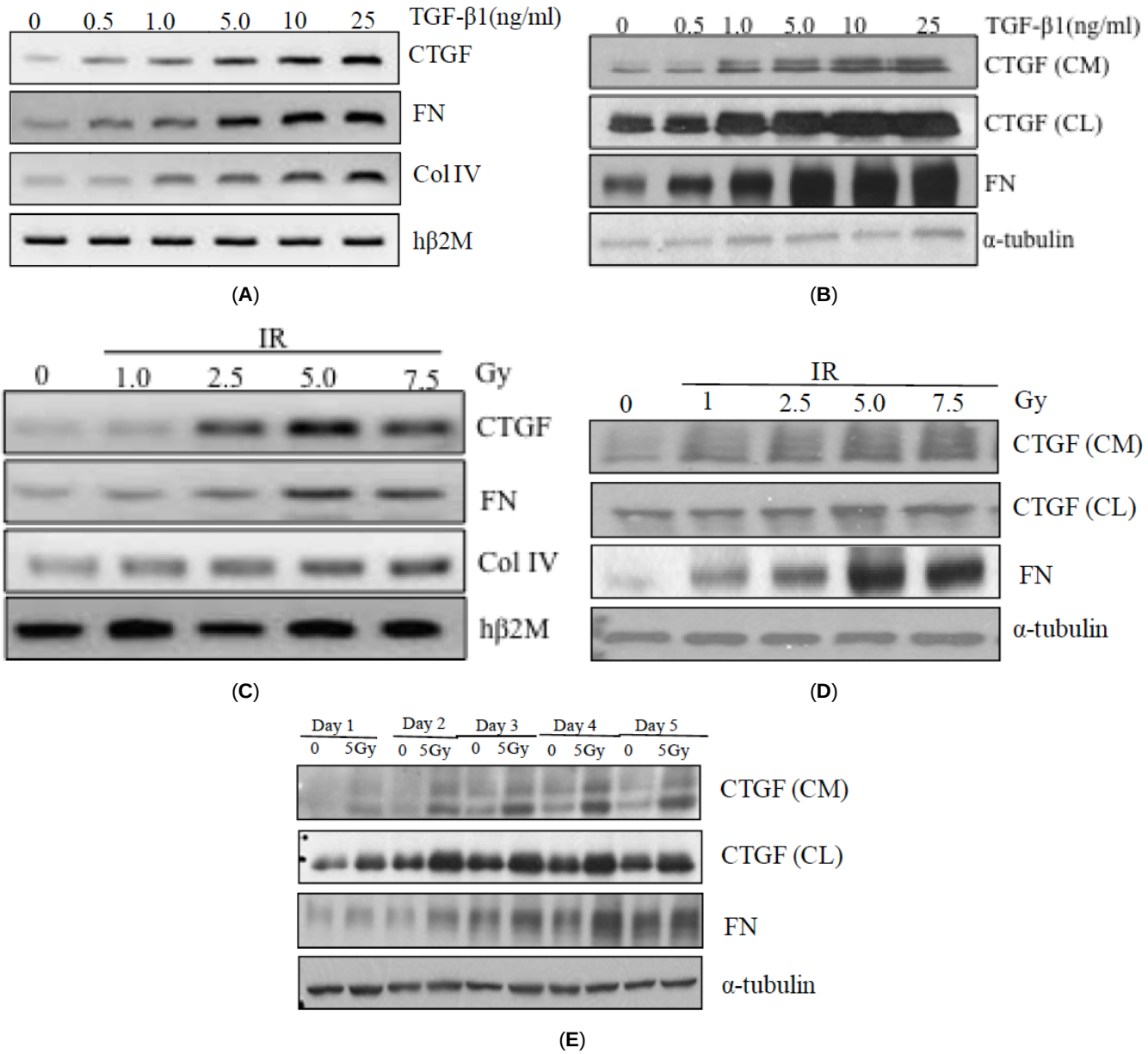

Figure 1: TGF-β_1 and irradiation-induced CTGF expression in HFL-1 cells. (**A**) TGF-β1 induced CTGF expression in normal lung fibroblasts (HFL-1) was determined by RT-PCR. CTGF, Fibronectin (FN), Collagen type IV (COL IV) expression at mRNA level in TGF-β1-treated HFL-1 after 6-hour-treatment. hβ_2M was set as the internal control. (**B**) Western immunoblot analysis of expressions of CTGF in conditioned media (CM) and cell lysate (CL), and FN in CL after 3 days' treatment. (**C**) CTGF, FN, Col IV expression at mRNA level in Radiation-treated HFL-1. Cells were collected 20 hours later postirradiation and subjected to RT-PCR. (**D**) Western immunoblot analysis of expressions of CTGF in CM and CL, and FN in CL 3 days postirradiation. (**E**) CM and CL were collected for western blot analysis. CTGF and fibronectin expression at different time points post irradiation with 5 Gy dose in HFL-1 cells.

fibronectin similar to SIM suggesting that induction of fibronectin by CTGF could also be through the Rho-ROCK pathway and also that SIM may be inhibiting fibronectin *via* the Rho-ROCK pathway in addition to the TGF-β pathway.

DISCUSSION

Radiotherapy is the most important non-surgical alternative for treatment of lung cancer patients. However, radiation pneumonitis and subsequent radiotherapy-induced lung fibrosis (RTIF) are the two main dose-limiting factors when irradiation is administered to lung cancer [15]. Although numerous studies have attempted to elucidate the mechanisms of RTIF, the pathogenesis of RTIF at the cellular and molecular level still is not well understood.

In this study, we confirmed previous reports that TGF-β and irradiation can induce CTGF and fibrotic

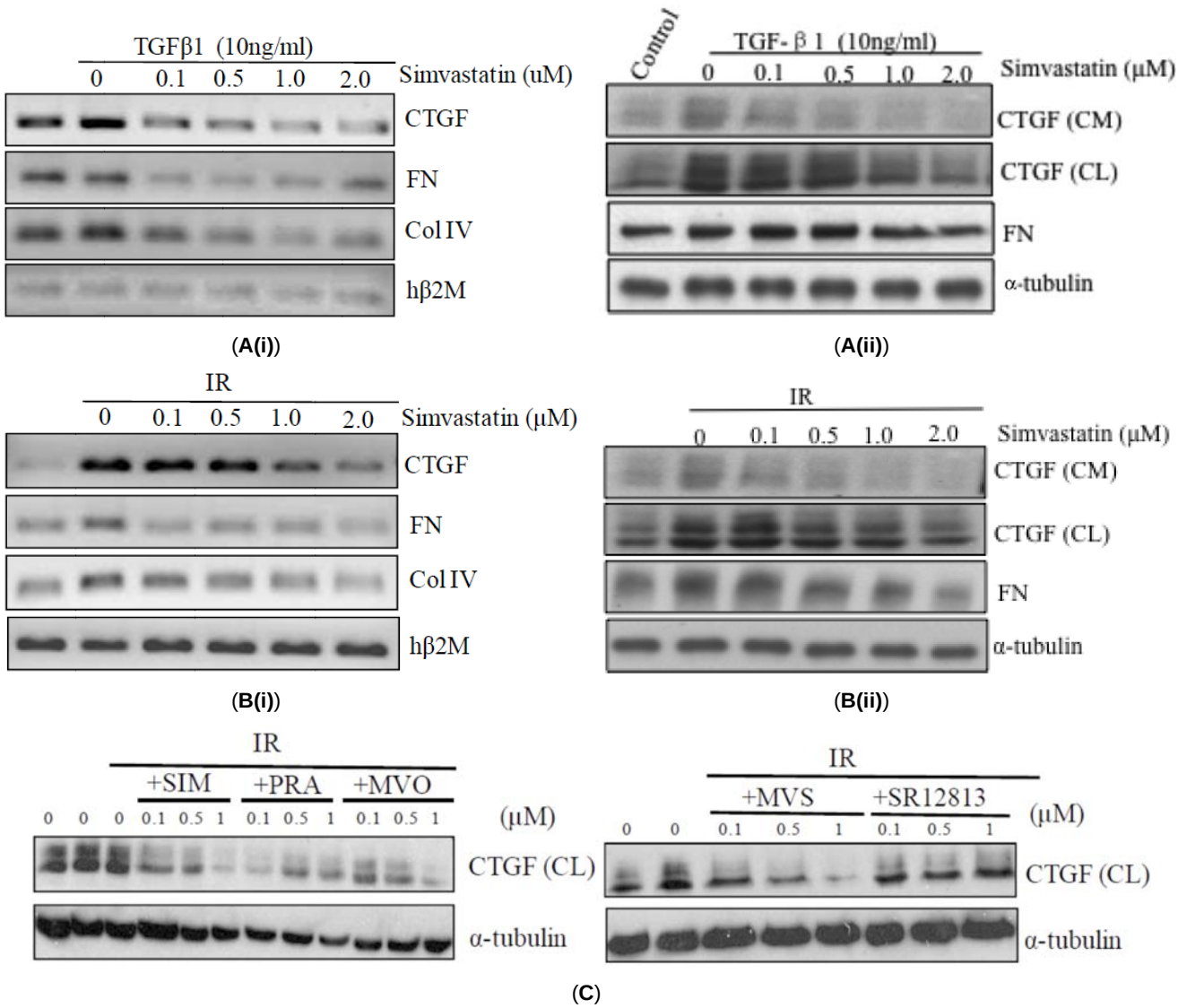

Figure 2: SIM suppressed TGF-β_1 and irradiation-induced expression of CTGF and downstream factors. (**A**) Inhibitory effect of SIM on TGF-β_1 -induced expression of CTGF and pro-fibrotic factors in HFL-1 at the mRNA (**i**) and protein (**ii**) levels. Cells were treated with the indicated amount of SIM in presence of 10 ng/ml TGF-β1 for 20 hours (RT-PCR) and 3 days (Western blot) in the medium. (**B**) Inhibitory effect of SIM on irradiation induced expression of CTGF and pro-fibrotic factors in HFL-1 cells at the mRNA (**i**) and protein (**ii**) levels. Cells were treated with the indicated amount of SIM for 6 hours before subjecting to 10 Gy radiation and incubated for 20 hours (RT-PCR) in the medium whereas 5 Gy radiation and incubated for 3 days in the serum free medium (Western blot). (**C**) Inhibitory effect of Statins on radiation-induced CTGF expression in HFL-1. Cells were treated with the indicated amount of Statins for 6 hours before subjected to 5 Gy radiation and incubated for 3 days in serum free medium. Cell lysate were collected for Western blot analysis. SR12813, a HMG-CoA inhibitor, which is not a statin, but a cholesterol lowering agent was also used in this experiment.

proteins further downstream, such as fibronectin and collagen type IV in a dose- and time- dependent manner (Figure **1**) [16]. We further showed that statins, a clinically approved class of HMG-CoA reductase inhibitor, inhibits the expression of TGF-β and irradiation induced-CTGF and subsequent targets in HFL-1 (Figure **2**), however irradiation induced CTGF upregulation cannot be completely abrogated by TGF-β neutralizing antibody. This suggests the involvement of a TGF-β-independent signaling pathway in RTIF (Figure **3**). An interesting finding is that downstream in the TGF-β pathway, overexpression of CTGF alone, without any treatment with TGF-β can also induce the levels of pro-fibrotic factors and this induction can be inhibited by administration of SIM in HFL-1 cells (Figure **4**). This suggests that statins may inhibit RTIF at multiple levels including inhibition of TGF-β-induced CTGF production as well as CTGF-enhanced fibrotic proteins expression. However it is still possible that statins may also utilize other pathways to suppress RTIF.

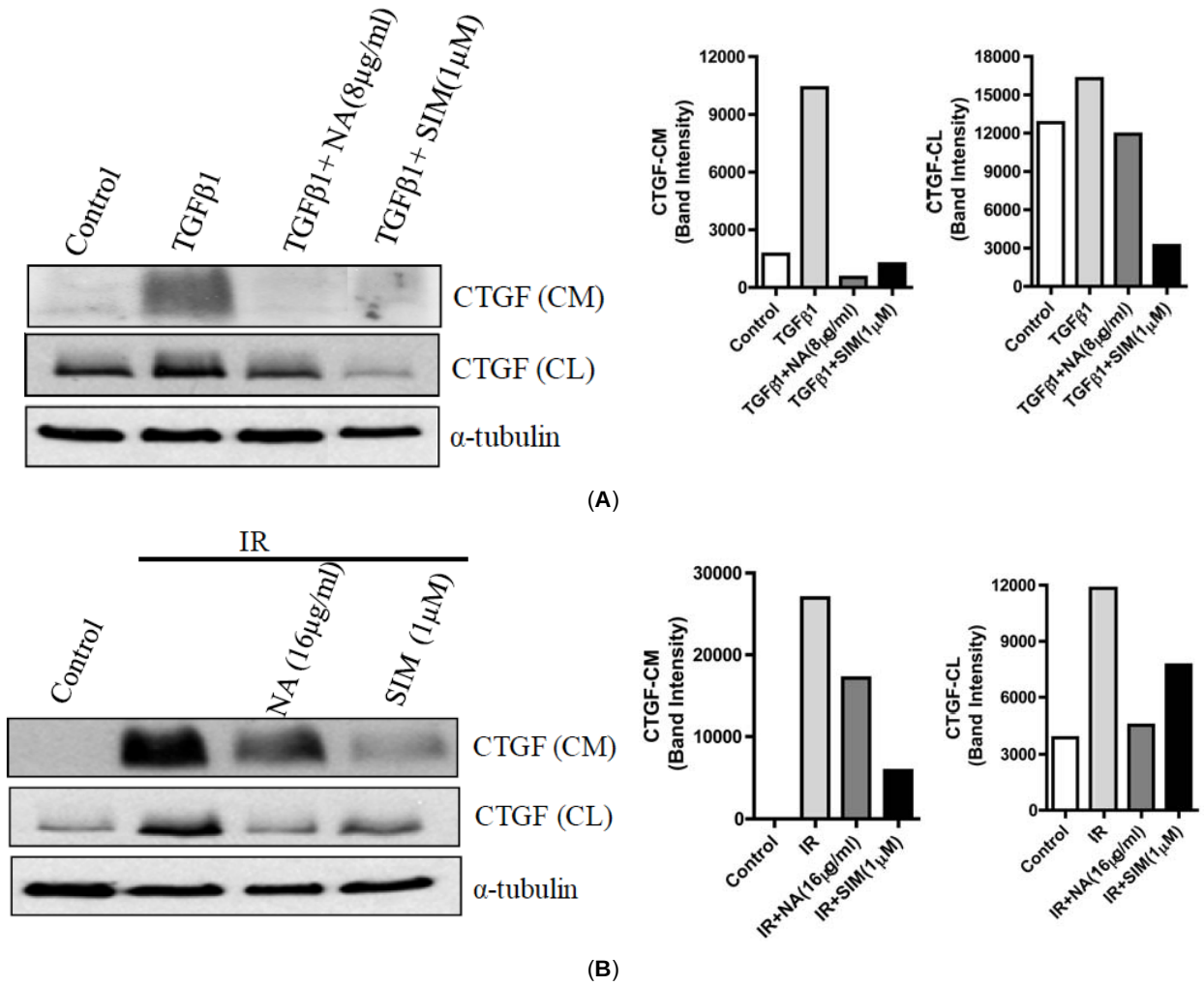

Figure 3: TGF-β_1-independent signaling pathway may be involved in radiation-induced CTGF upregulation. (**A**) Blocking of TGF-β1-induced CTGF expression in HFL-1 cells by TGF-β1 neutralizing antibody (8 μg/ml). Proteins levels of CTGF expression in CM and CL were confirmed by Western lot. (**B**) Radiation-induced CTGF upregulation cannot be completely inhibited by TGF-β_1 neutralizing antibody. HFL-1 cells were pre-treated with TGF-β_1 neutralizing antibody 16 μg/ml for 6 hours prior to irradiation. Densitometric analysis of CTGF bands after normalization to α-tubulin is also shown graphically.

Previous studies reported that increasing serum CTGF expression was observed in patients with systemic sclerosis and associated with the extent of skin sclerosis and the severity of pulmonary fibrosis [17]. Lopes *et al.* in their study show that analogues of small heat shock proteins decrease the expression of CTGF and collagen type I induced by TGF-β in human dermal keloid fibroblasts and that has a potential of preventing excessive tissue scarring [18]. Statins have also shown to be effective for the treatment of systemic sclerosis and digital ulcers [19]. We in our study have shown that TGF-β and irradiation induced CTGF, fibronectin and ColIV can be inhibited by SIM in a dose dependent manner in dermal fibroblasts as well (Figure **SI-1**).

Statins have also shown anti-fibrotic functions in a variety of mammalian cell lines or tissues through interference with the Rho/ROCK/CCN2/-ECM cascade [11]. The correlation between Rho/ROCK pathway and fibrogenic signaling pathway has gained more attentions in recent years. Reports show that inhibition of Rho activation by statins (inhibition of Rho isoprenylation) or ROCK kinase inhibitor decreased CTGF expression and subsequent extracellular matrix deposition *in vitro* [12]. Our *in vitro* data indicated that anti-fibrotic action of SIM not only blocked TGF-β- or irradiation- induced CTGF expression but repressed CTGF induced upregulation of pro-fibrotic factors as well. Data presented in Figure **3B** imply that there is a pathway in addition to the TGF-β pathway that is involved in the induction of CTGF. Indeed, our results in Figure **4** demonstrate that suggesting specific inhibition of Rho/ROCK and TGF-β signaling pathway may provide a synergistic anti-fibrotic therapy for irradiation-induced fibrosis.

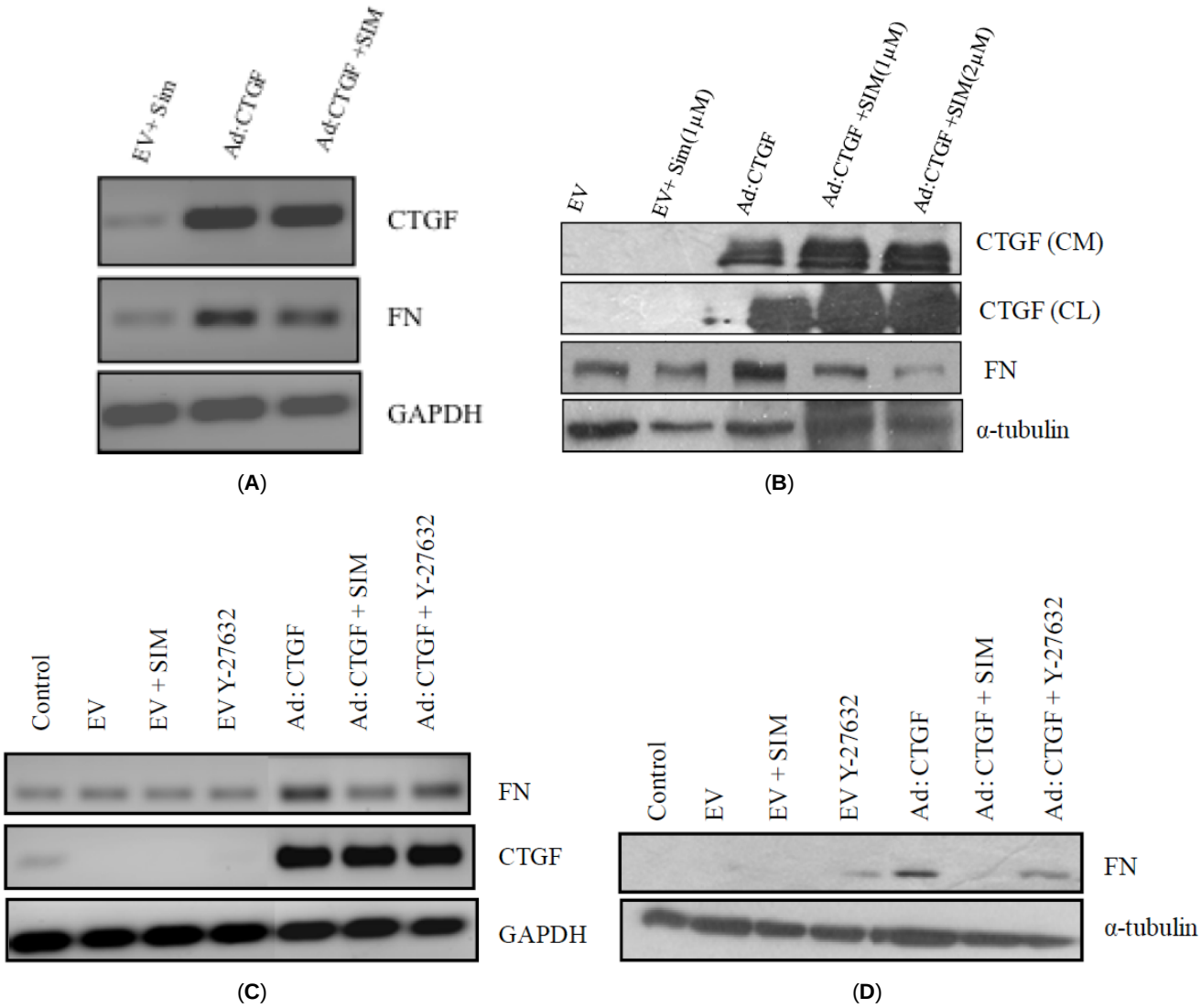

Figure 4: SIM inhibits CTGF- induced pro-fibrotic markers through the Rho-ROCK pathway. HFL-1 cells were pretreated with SIM 6 hours prior to adenoviral infection with CTGF vector (MOI 500). Cells were incubated for 24 hours and 48 hours for mRNA (**A**) and protein expression (**B**) respectively post-infection. HFL-1 cells were treated with SIM for 6 hours and 50μM ROCK inhibitor (Y-27632) for 3 hours before treatment with TGF-β or CTGF adenovirus and were incubated for 20 hrs and 48 hrs respectively for mRNA (**C**) and protein expression levels (**D**).

Taken together, our study indicated that CTGF plays a critical role for RTIF and serves as a potential fibrotic marker for evaluation of the extent of fibrosis post-irradiation and the response to the drug treatment. Statins inhibit RTIF in normal fibroblasts at multiple levels including inhibition of CTGF through TGF-β and the Rho/ROCK signaling pathway. Our data provide that CTGF can be a potential anti-fibrotic target to develop successful modalities for optimal radiotherapy in the clinic.

MATERIALS AND METHODS

Cell Culture, TGF-β and Radiation Treatment

Normal human fetal lung fibroblasts (HFL-1) (CCL-153) and human dermal fibroblasts (HDF) (PCS-201-012) were purchased from the American Type Culture Collection and cultured in Ham's F12K medium with 10%FBS (GIBCO, 11765) and fibroblast basal media supplemented with fibroblast growth kit respectively. After reaching 75-80% confluence, the medium was changed to serum free medium (SFM) for irradiation with different dose 1, 2.5, 5, 7.5 and 10 Gy by using ^{137}Cesium. Cells were treated with 5-10 ng/ml TGF-ß1 (Sigma, T7039) to stimulate CTGF production. Cells were incubated for 3 days and subjected to Western blot.

Treatment with Statins

Cells were plated into 35mm plates and the following day, the cells were washed with PBS and the

media was changed to SFM. Cells were treated with the indicated amount of Statins for 6 hours before subjecting them to 5Gy irradiation and then incubated for 3 days in SFM.

RT-PCR

Total RNA was extracted from cells, 20 hours post treatment and 1 µg of purified total RNA was used for RT-PCR using the ThermoScript RT-PCR System (Invitrogen). The sequences of the forward and reverse primers were used as follows: CTGF, fwd5'-CTGGTCC AGACCACAGAGTG-3', rev5'-CGGTATGTCTTCATGC TGGT-3';COL-IV,fwd5'-AGCAAGGCAACAGAGGA-CTT-3', rev 5'-GATCTGGGTGGAAGGTGACT-3';FN, fwd,5'-GACTGGAGCTGGAGACATGA-3',rev5'-GTGAT GATGGTGGACTGCTC-3'; β2M fwd, 5-GTGCTCGCG CTCTCTCT-3'; rev,5-CGGCAGGCATACTCATCTTT-3'.The CTGF PCR product is 242 bp in length, COL-138bp, FN-203bp, and hβ2M-278bp. PCR products were run on a 1% agarose gel and visualized by ethidium bromide staining.

Western Blot Analysis

Cell lysates were harvested in HBSST lysis buffer. The primary antibodies selectively recognized CTGF (sc-14939), Collagen type IV (sc-11360), Fibronectin (sc-9068), were purchased from Santa Cruz Biotechnology, Inc and α-tubulin (T9026) from Sigma-Aldrich Inc. Primary antibodies except α-tubulin (1:4,000) were diluted at 1:600 and incubated at 4 °C overnight; and corresponding secondary antibodies at 1:6,000 and incubated at room temperature 1 hour. Antibodies were detected by the enhanced chemiluminescence (PerkinElmer Life Sciences Inc).

Adenoviral Constructs

We used the AdEasy system (Quantum Biotechnologies) to generate Ad:CTGF as described previously [20].

TGF-β1 Neutralizing Antibody Treatment

TGF-β1 was purchased from Sigma-Aldrich, and TGF-β1-neutralizing antibody was purchased from R&D systems (Minneapolis). After achieving 75-80% confluence in 6-well plate, the medium was changed to SFM for treatment. Cells were exposed for 72 hours in each individual well to TGF-β1 (10ng/ml), TGF-β1 (10ng/ml) plus TGF-β1-neutralizing antibody (16µg/ml) (preincubated and shaken together at room temperature for 30 min in 1.5 ml eppendorf tube containing 400 µl SFM before the addition to the cells). Cells were subjected to 5 Gy irradiation after addition of the preincubated complex. Conditioned media and cell lysate were then collected for Western Blot.

Treatment with ROCK-Inhibitor

Cells were plated into 12 well plates and the following day, the cells were washed with PBS and the media was changed to SFM. Cells were treated with the indicated amount of ROCK-inhibitor (Y-27632) (Sigma-Aldrich) for 3 hours before subjecting them to TGF-β treatment or Ad: CTGF infection. The cells were further incubated for 24 hours (RT-PCR) and for 48 hours (western blot) in SFM.

SUPPLEMENTARY FIGURE

The supplementary figures can be downloaded from the journal website along with the article.

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