Inhibitory Effect of Mitoxantrone on Collagen Synthesis in Dermal Fibroblasts

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Background: Fibroblasts produce collagen molecules that support the structure of the skin. The decrease and hypersynthesis of collagen causes skin problems such as skin atrophy, wrinkles and scars.
Objective: The purpose of this study is to investigate the mechanism of mitoxantrone on collagen synthesis in fibroblasts.
Methods: Cultured fibroblasts were treated with mitoxantrone, and then collagen synthesis was confirmed by reverse transcription-polymerase chain reaction and Western blot.
Results: Mitoxantrone inhibited the expression of type I collagen in fibroblasts at both the mRNA and protein levels. In the collagen gel contraction assay, mitoxantrone significantly inhibited gel contraction compared to the control group. Mitoxantrone inhibited transforming growth factor (TGF)-β-induced phosphorylation of SMAD3. Finally, mitoxantrone inhibited the expression of LARP6, an RNA-binding protein that regulates collagen mRNA stability.
Conclusion: These results suggest that mitoxantrone reduces collagen synthesis by inhibiting TGF-β/SMAD signaling and LARP6 expression in fibroblasts, which can be developed as a therapeutic agent for diseases caused by collagen hypersynthesis.

Keywords: Collagen, Fibroblasts, LARP6, Mitoxantrone, SMAD

INTRODUCTION

Fibroblasts are the main cells that make up the dermis and are responsible for producing and secreting extracellular matrix molecules such as collagen, elastin and proteoglycans[1]. Among them, type 1 collagen is the most abundant protein that directly affects skin texture. As aging progresses, fibroblasts are less proliferative and collagen synthesis decreases, resulting in skin atrophy and wrinkles[2]. Conversely, in pathological situations such as keloids, transforming growth factor-β (TGF-β) signal is activated and collagen synthesis increases, so that the scar grows without spontaneous regression[3]. Because collagen synthesis of fibroblasts is associated with healthy skin texture, cosmetic problems, and pathological conditions, many researchers have attempted to develop substances that can regulate fibroblast activity and collagen synthesis.

Recently, drug repositioning technique has been spotlighted as an alternative method for developing new drugs[4,5]. This is because it can significantly save time and money spent on drug development. We purchased Screen-Well® FDA approved drug library V2 (Cat #BML-2843-0100; Enzo Life Sciences Inc., Farmingdale, NY, USA). This drug library contains more than 800 compounds that are FDA-approved and are in clinical use, all of which are well-known and well-characterized for their biological activity, safety and bioavailability. We conducted screening test and found that mitoxantrone has the

effect of inhibiting collagen synthesis in fibroblasts.

Mitoxantrone is an anthraquinone used to treat several cancers including breast and prostate cancer, lymphoma and leukemia[6]. Mitoxantrone has been shown to inhibit TGF-β-induced transcription of collagen type I α1 chain (COL1A1) by suppressing the binding of the SP1 transcription factor to the *COL1A1* gene promoter in cultured human dermal fibroblasts[7]. However, the exact action mechanism of mitoxantrone on collagen synthesis is not well understood. In this study, we show that mitoxantrone inhibits SMAD signaling and also inhibits the expression of RNA-binding protein LARP6 (La ribonucleoprotein 6, translational regulator), which is involved in the post-transcriptional regulation of collagen mRNA.

MATERIALS AND METHODS

The study was approved by the Institutional Review Board of the Chungnam National University Hospital (IRB no. 2016-07-009). Written informed consent was obtained from all donors.

Cell culture

Normal human skin fibroblasts were cultured as previously described[8]. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Welgene, Gyeongsan, Korea) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). Mitoxantrone was purchased from Enzo Life Sciences and dissolved as 1,000× concentrate in dimetylsulfoxide (DMSO) and diluted with culture medium. The control group was treated with the same amount of DMSO. For mitoxantrone treatment, cells were seeded in 60-mm culture dish and incubated overnight. The next day, an appropriate amount of mitoxantrone was added directly to the culture medium and the cells were incubated for an additional 24 hours. For treatment with TGF-β, cells were seeded in 60-mm culture dish and incubated overnight. Then, the cells were washed twice with phosphate-buffered saline, replaced with serum-free DMEM, and incubated overnight. The next day, TGF-β (R&D systems, Minneapolis, MN, USA) was added directly to the culture medium. Cells were cultured for an additional 1 hour (for detection of phosphor-SMAD) or 24 hours (for detection of collagens, LARP6, and HNRNPK [heterogeneous nuclear ribonucleoprotein K]).

Cell viability test

To examine the effect on cell viability, MTT assay was performed. Normal human skin fibroblasts were seeded in 12-well culture plate at a density of 1×10^5/well and treated with mitoxantrone for 24 hours. Then medium was replaced with fresh medium containing 0.5 mg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). Cells were incubated for an additional 4 hours, and then formazan crystal was dissolved in DMSO. The optical density was measured at 570 nm using a microplate reader.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from fibroblasts using Easy-blue RNA extraction kit (Intron Biotechnology, Seongnam, Korea). Two μg of total RNAs were reversed transcribed with moloney-murine leukaemia virus (M-MLV) reverse transcriptase (RTase) (Elpis Biotech, Daejeon, Korea). A portion of cDNA was taken and used for the PCR reaction. The sequences for primers were as follows: COL1A1, 5'-ccaaatctgtctccccagaa and 5'-tcaaaaacgaaggggagatg; COL1A2, 5'-ctgcaagaacagcattgcat and 5'-ggcgtgatggcttatttgtt; LARP6, 5'-ttacacgggactggagaacc and 5'-cagctctctcccaggtttga; HNRNPK, 5'-atgtccctggcatctgttca and 5'-actgctccccaccttagttc; GAPDH, 5'-GTCAGTGGTG-GACCTGACCT and 5'-AGGGGTCTACATGGCAACTG.

Collagen gel contraction assay

Gel contractions assay was performed using a Cell contraction assay kit (Cell Biolabs Inc., San Diego, CA, USA), according to the manufacturer's protocol. Briefly, 5×10^5 cells were mixed with collagen gel and polymerized in Transwell permeable supports (Merck KGaA, Darmstadt, Germany). After collagen polymerizaiton, 5 ml of medium was added atop each collagen gel lattice.

Western blot

Cells were harvested by centrifugation and then lysed in Pro-Prep protein extraction solution (Intron Biotechnology). After vigorous pipetting, the extract was centrifuged for 15 minutes at 15,000 rpm. Total protein was measured using a BCA protein assay kit (Thermo Fisher Scientific). Samples (20~30 μg protein per lane) were run on SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and incubated with appropriate antibody at 4℃ overnight with gentle agitation. The blot was then incubated with peroxidase-conjugated secondary

antibody for 30 minutes at room temperature and visualized by enhanced chemiluminescence (Intron Biotechnology). The following primary antibodies were used in this study: COL1A1 (sc-8783), COL1A2 (sc-8786), HNRNPK (sc-28380), actin beta (ACTB) (sc-47778) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA); LARP6 (H00055323-B01P; Abnova, Taipei, Taiwan); phosphorylated-SMAD family member 2 (p-SMAD2) (3108S), phosphorylated-SMAD family member 3 (p-SMAD2) (9520S) (Cell Signaling Technology, Danvers, MA, USA).

Statistical analysis

Data were evaluated statistically by one-way ANOVA or Student's t-test using IBM SPSS software v 22.0 (IBM Corp., Armonk, NY, USA). Statistical significance was set at $p<0.05$.

RESULTS

Our pilot screening study showed that mitoxantrone inhibits collagen synthesis. To investigate the effect of mitoxantrone on cell viability, normal human skin fibroblasts were treated with various concentrations of mitoxantrone and MTT assay was performed. As a result, mitoxantrone induced cell death at doses of 1 μM or higher (Fig. 1A). To examine the effect of mitoxantrone, on collagen synthesis, we performed RT-PCR and Western blot analysis. As a result of mitoxantrone treatment,

the mRNA expression of COL1A1 and COL1A2 decreased in a dose-dependent manner (Fig. 1A). Consistent with these results, the protein levels of COL1A1 and COL1A2 were also decreased by mitoxantrone (Fig. 1B).

To further verify the effect of mitoxantrone, we performed the collagen gel contraction assay that well reflects collagen production by fibroblasts[9]. Gel contraction occurred well over time in the control groups (non-treated and DMSO-treated groups). However, gel contraction was remarkably suppressed in the mitoxantrone-treated group (Fig. 2). Together, these data clearly showed that mitoxantrone has the inhibitory effect on collagen production of fibroblasts.

It is well known that TGF-β/SMAD signaling plays a central role in collagen synthesis[10,11]. To investigate the mechanism of action, we first identified the effect of mitoxantrone on TGF-β/SMAD signaling. When fibroblasts were treated with TGF-β, phosphorylation of SMAD2 and SMAD3 was induced. Pre-treatment of mitoxantrone did not affect TGF-β-induced phosphorylation of SMAD2, whereas phosphorylation of SMAD3 was slightly but significantly inhibited by mitoxantrone (Fig. 3A). As expected, mitoxantrone weakly but significantly inhibited TGF-β-induced collagen synthesis (Fig. 3B).

Although mitoxantrone inhibited TGF-β-induced collagen synthesis, we found that mitoxantrone could still inhibit collagen synthesis even in the absence of TGF-β. Therefore, we
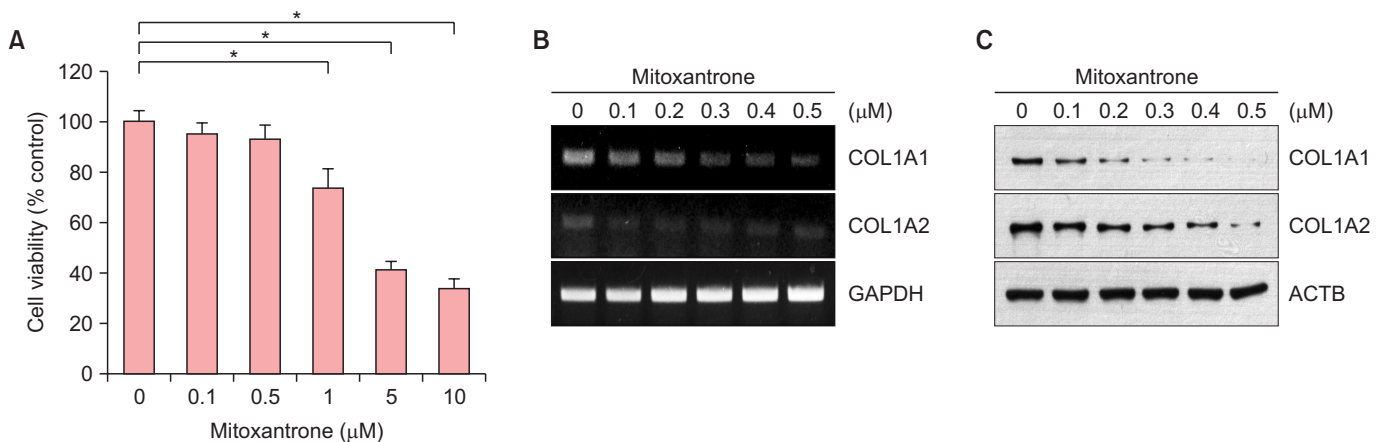

Fig. 1. Effect of mitoxantrone on collagen synthesis. (A) Normal human skin fibroblasts were treated with mitoxantrone at the indicated concentrations for 24 hours. MTT assay was performed to determine cell viability. Mitoxantrone induced cell death at doses of 1 μM or higher. (B) Skin fibroblasts were treated with mitoxantrone at the indicated concentration for 24 hours. The mRNA levels of collagen type I α1 chain (COL1A1) and collagen type I α2 chain (COL1A2) were determined by reverse transcription-polymerase chain reaction (RT-PCR). Mitoxantrone decreased the mRNA expression of COL1A1 and COL1A2 in a dose-dependent manner. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (C) The protein levels of COL1A1 and COL1A2 were determined by Western blot. Mitoxantrone decreased the protein level of COL1A1 and COL1A2 in a dose-dependent manner. Actin beta (ACTB) was used as a loading control. *$p<0.05$.
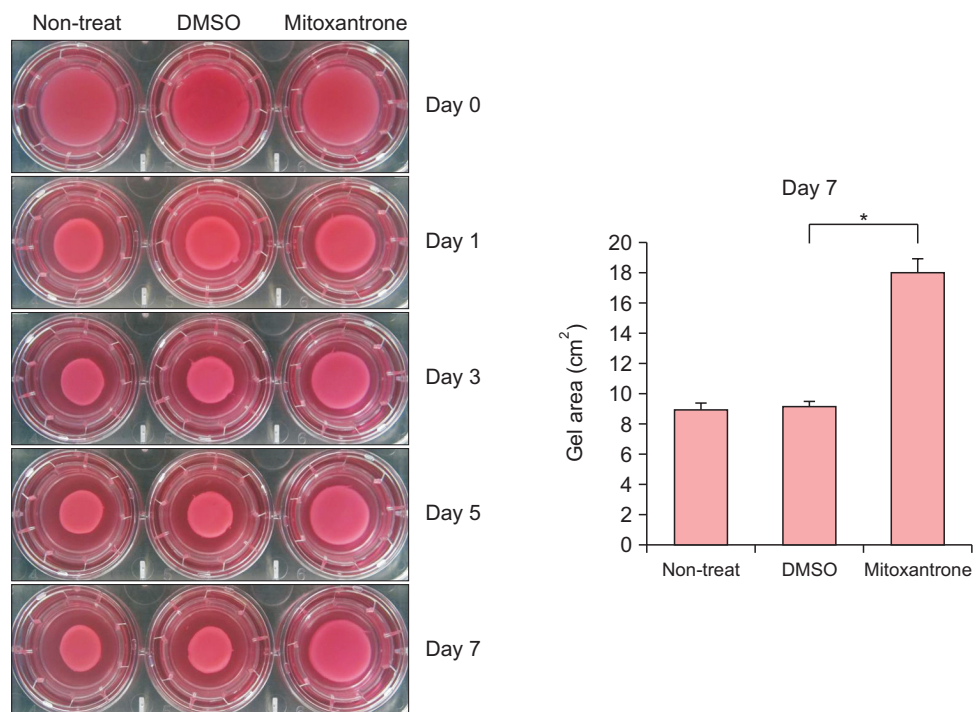
Fig. 2. Collagen gel contraction assay. Collagen gels containing fibroblasts were prepared then treated with 0.5 µM of mitoxantrone for 7 days. Gel contraction was significantly inhibited in mitoxantrone treated group compared to control group. Dimethyl sulfoxide (DMSO) was used as a vehicle control. Right graph shows the results measured from three independent experiments. *$p < 0.05$.
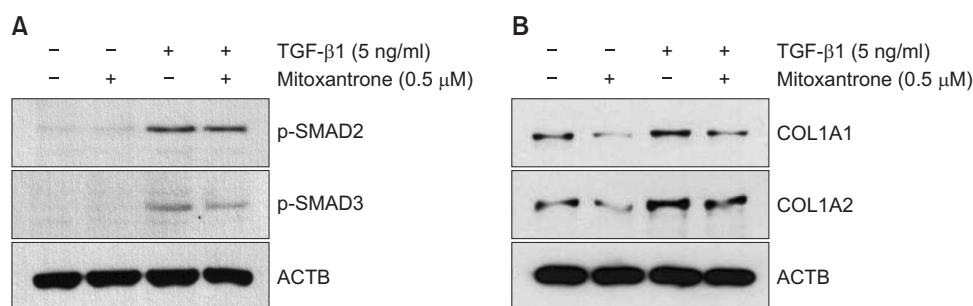

Fig. 3. Effect of mitoxantrone on transforming growth factor-β (TGF-β)/SMAD signaling. (A) Fibroblasts were treated with mitoxantrone for 1 hour then stimulated with TGF-β for 1 hour. Phosphorylations of SMAD2 and SMAD3 were determined by Western blot. Mitoxantrone slightly inhibited TGF-β-induced phosphorylation of SMAD3. (B) The protein levels of collagen type I α1 chain (COL1A1) and collagen type I α2 chain (COL1A2) were determined by Western blot. Mitoxantrone weakly but significantly inhibited TGF-β-induced collagen synthesis. Actin beta (ACTB) was used as a loading control.

tried to find other targets that mitoxantrone could have an effect on. Among the factors that regulate collagen synthesis, RNA-binding proteins including LARP6 and HNRNPK are known to play important roles by stabilizing collagen mRNA[12,13]. Therefore, we examined how mitoxantrone affects the expression of LARP6 and HNRNPK. As a result, mitoxantrone inhibited the expression of LARP6 at both the mRNA and protein levels. However, the expression of HNRNPK was not significantly affected by mitoxantrone (Fig. 4). These results suggest that mitoxantrone influences collagen synthesis by regulating the expression of the RNA-binding protein LARP6 in addition to inhibiting TGF-β/SMAD signaling.

DISCUSSION

Collagen is an important factor that plays a decisive role in maintaining the shape of the skin. Too little production can cause wrinkles, but too much production can lead to wounds or keloids. From this point of view, there has been a continuous demand for the development of substances capable of regulating collagen synthesis. In this study, we showed that mitoxantrone has the potential to inhibit collagen synthesis in skin fibroblasts. We also demonstrated that mitoxantrone not only inhibits TGF-β/SMAD signaling but also affects the expression of LARP6, which regulates collagen mRNA stability.
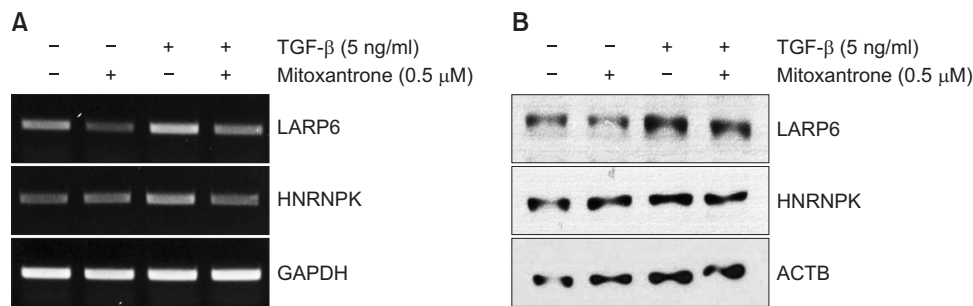
Fig. 4. Effect of mitoxantrone on the expression of RNA-binding proteins. (A) Fibroblasts were treated with mitoxantrone for 1 hour, then stimulated with transforming growth factor-β (TGF-β) for 24 hours. The mRNA levels of LARP6 and HNRNPK were determined by reverse transcription-polymerase chain reaction (RT-PCR). Mitoxantrone inhibited the expression of LARP6 mRNA in both the absence and/or presence of TGF-β. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (B) The protein levels of LARP6 and HNRNPK were determined by Western blot. Mitoxantrone weakly but significantly decreased LARP6 level in both the absence and/or presence of TGF-β. Actin beta (ACTB) was used as a loading control.

The most central intracellular signaling in collagen synthesis is the TGF-β/SMAD pathway, but it has been found that several other important regulators are involved. For example, it has been reported that Wnt/β-catenin signaling contributes to the development of keloids by inducing fibroblast hyperproliferation[14,15]. Other factors involved in collagen synthesis include RNA-binding proteins such as LARP6 and HNRNPK. It is known that the mRNA of type I collagen has an evolutionarily conserved stem-loop structure in the 5' untranslated region. LARP6 binds to this 5' stem-loop in a sequence-specific manner to regulate the stability of collagen mRNA and its translatability. When LARP6 binds to the 5' stem-loop of type I collagen mRNA, LARP6 acts as an adapter recruiting accessory translational factors, increasing the translational competency of type I collagen mRNA[16]. In contrast, HNRNPK increases mRNA stability by binding to the CU-rich element of the 3'untranslated region of type I collagen mRNA[13]. In this study, mitoxantrone inhibits the expression of LARP6, suggesting that mitoxantrone treatment might result in decrease of type I collagen mRNA stability thereby reducing the production of collagen. It is not known how mitoxantrone inhibits LARP6 expression. Interestingly, it has been reported that insulin-like growth factor-1 (IGF-1) promotes the expression of LARP6 via the PI3K/AKT/p70S6K pathway in human aortic smooth muscle cells[17]. Based on these results, it is possible that mitoxantrone suppresses the expression of LARP6 by inhibiting not only the TGF-β/SMAD signal but also the PI3K/AKT signal. The precise mechanism of action underlying the mitoxantrone-suppressed collagen expression should be investigated further.

In summary, we demonstrated that mitoxantrone inhibits the collagen synthesis in dermal fibroblasts. Our data suggest that mitoxantrone can be developed as therapeutics for diseases caused by hypersynthesis of collagen.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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