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ORIGINAL ARTICLE

TERM

Effects of Adipose-Derived Stem Cells on Keloid Fibroblasts Based on Paracrine Function

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Adipose-derived stem cells (ASCs) possess multipotent differentiation capabilities, including the ability to repair and regenerate injured tissues. Recent studies have reported that ASCs have effects on fibroblasts that are related to wound healing. On the other hand, keloid is a result of pathologic wound healing, and keloid fibroblasts are known to have higher growth potential and an abnormal balance between proliferation and apoptosis. This study aimed to explore the paracrine effects of ASCs on normal fibroblasts (NF) and keloid fibroblasts (KF). NF and KF were cultured alone and in combination with ASCs using a trans-well cell-contact-independent cell culture system. Cell morphology was observed, cell proliferation was assessed using a CCK-8 kit, and apoptosis was assessed by live-dead-cell staining on days 1, 3, and 7. Western blot analysis at 24 hours after culture was used to investigate the production of type 1 collagen and alpha-smooth muscle actin (α-SMA). Co-culture with ASCs resulted in increased proliferation of both NF and KF. In the co-culture with ASCs, collagen production decreased and expression of α-SMA increased in both the NF and KF. Comparison between the four groups revealed a significant difference in the expression of both type I collagen and α-SMA (p=0.016 and 0.022; Kruskal-Wallis test). Our findings are different from previous studies that used ASCs for normal dermal fibroblast and normal wound healing. However, considering pathologic mechanisms of keloid, ASCs may have the different effect on keloid fibroblasts, which could be another effect of ASCs modulating keloid during healing process.

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Key Words: Adipose-derived stem cells; Fibroblast; Co-culture; Keloid

INTRODUCTION

Adipose-derived stem cells (ASCs) are multipotent cells in adipose tissue with characteristics similar to bone marrow-derived stem cells (BMSCs). However, in contrast to the invasive procedure and low yield of BMSCs harvesting, ASCs have the advantages of accessibility and minimal morbidity involved in their harvest. Since the first report by Zuk et al. [1,2], many studies have confirmed that ASCs have favorable effects, such as wound repair, immunomodulation, and anti-apoptotic activity [1-5].

Fibroblasts regulate the production and degradation of the extracellular matrix (ECM), glycoproteins, and cytokines; they thus repair wounds and maintain the integrity and suppleness of the skin. In the early stage of wound healing, fibroblasts migrate to the affected area and produce collagen-based ECM to replace missing tissue and help to reapproximate the wound edges by their contractile properties [6,7]. On the other hand, Keloids are pathologic structures resulting from the excessive accumulation of connective tissue during the healing process [8]. They result from several processes, such as fibroblast hyperproliferation, increased collagen synthesis, and reduced collagen [9,10]. Keloid fibroblasts have higher growth potential and an abnormal balance between proliferation and apoptosis [11,12].

Recent studies have reported that ASCs enhance the wound-healing potential of fibroblasts by stimulating collagen synthesis and by increasing the proliferation and migration of fibroblasts [13,14]. In this study, we investigated the effects of ASCs on NF and KF based on paracrine function to explore the modulation of a keloid using ASCs.

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MATERIALS AND METHODS

Isolation and culture of normal fibroblasts and keloid fibroblasts

The procedures in this study were approved by the Institutional Review Board (IRB) of the Catholic University of Korea (KCMC06BR067). NF and KF were obtained from tissue excised from a 43-year-old male patient who underwent surgery to excise a keloid scar in his anterior chest wall. Informed consent was obtained from the patient before surgery. KF were obtained from the central dermal layer of the keloid scar, and normal fibroblasts (NF) from the normal dermal layer of the same patient. The keloid scar was identified by a clinician and a pathologist. The tissue was washed with Gibco phosphate buffered saline (PBS, Thermo Fisher Scientific Inc., Waltham, MA, USA), cut into 1 mm^3 pieces and placed in culture dishes. They were incubated 2 hours in Dispase II solution (neutral protease, grade II, Roche, Germany). Then, minced after dermis was separated without epidermis and digested with 0.1% collagenase (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline for 2 hours at 37°C in a 5% CO_2 atmosphere with gentle agitation. The collagenase was inactivated with an equal volume of complete media (DMEM; Dulbecco's modified Eagle's medium, Thermo Fisher Scientific Inc., USA) containing 10% fetal bovine serum (FBS, Wisent Inc., CA, USA) and 1% antibiotic/antimycotic (Life Technologies). Cells were harvested by centrifugation at 200 g for 5 minutes and filtered 100 μm mesh to remove debris. Cells were plated onto conventional culture plates with complete media at 37°C in a 5% CO_2 atmosphere, and the media was further replaced every three days. The resulting cells were passaged when 70−80% confluent and not used until at least passaged three times (P3).

Culture of adipose-derived stem cells

Human adipose tissue, collected as waste product from liposaspiration or lipectomy procedures, were obtained from otherwise healthy patients with informed consent, and with the approval of the IRB of the Catholic University of Korea. The specimens were washed in PBS, minced, and digested with 0.5% type I collagenase (Sigma-Aldrich, St. Louis, MO, USA) under gentle agitation for 60 min at 37°C. The resulting suspensions were centrifuged at 300 g for 5 minutes, and the supernatants removed. The retrieved cell fractions were cultured in complete media, at 37°C and with 5% CO_2 atmosphere. The medium was changed every 3 days at 80−90% confluence. These human ASCs were expanded in compelete media and used after passage 3 (P3).

Culture of fibroblasts and ASCs

We used four culture conditions: 1) NF monoculture, 2) KF monoculture, 3) NF/ASCs co-culture, and 4) KF/ASCs co-culture. The cells were grown in a trans-well co-culture system (6 wells, 3 μm pore diameter; Millicell®, Millipore, Billerica, MA, USA). For the monoculture groups, 10000 NF or KF were seeded in the bottom chambers. For the co-culture groups, 10000 NF or KF were seeded into the bottom chambers, and 10000 ASCs into the top chambers. All the plates were filled with complete media, and this medium was changed every 3 days. The cultures were repeated three times to validate the findings.

Cell morphology

Cell morphology was observed on days 1, 3, and 7. The insert well was removed for direct visualization of the fibroblasts in the bottom chamber, and images were captured with a CKX41 low magnification camera adapter (Olympus, Tokyo, Japan).

Cell counts

In order to evaluate the growth rate of the cells, cells numbers were counted on days 1, 3, and 7 using a Cell Count Kit-8 (CCK-8 kit; Dojindo, Santa Clara, CA, USA). The CCK-8 solution was mixed with complete media at a ratio of 1:9 and, after the removal of the inset wells, 400 μL of the mixture was added to each well. The wells were incubated for 2 hours at 37°C under 5% CO_2, and 100 μL aliquots of supernatant were transferred to 96-well plates, which were read at 450 nm using a SoftMax Pro5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Optical density values were converted to cell numbers using a standard curve.

Apoptosis

Apoptosis/necrosis was quantified with a Live-Dead Cell Staining kit (Bio Vision, Milpitas, CA, USA) on days 1, 3, and 7. The fibroblasts in the bottom chamber were washed twice with PBS and mixed with 500 μL kit solution (a mixture of 1 μL solution A, 1 μL solution B, and 1 mL staining buffer). The plates were then incubated for 15 min at 37°C, and immediately observed under a Axiovert 200 fluorescence microscope (Zeiss, Oberkochen, Germany). In this system, live cells stain only with the cell-permeable live-dye and fluoresce green, while dead cells stain with both the cell-permeable live-dye and the cell impermeable propidium iodide (PI), and fluoresce yellow-red.

Western blotting

For Western blotting, extracts of the fibroblasts in the bottom chambers were prepared after 24 hours. Aliquots were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% resolving gels. The proteins were

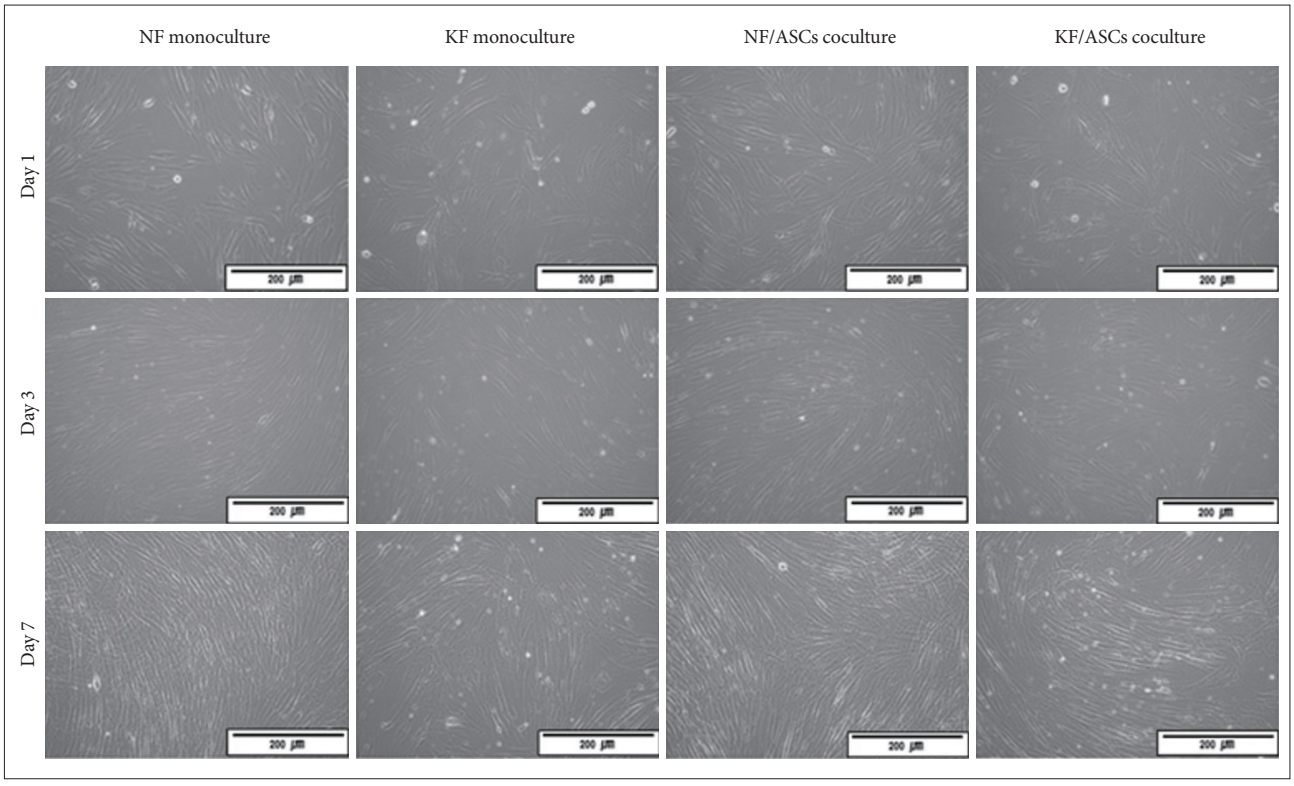

Figure 1. The morphology of the fibroblasts was determined on days 1, 3, and 7 by direct observation with a light microscope. NF: normal fibroblasts, KF: keloid fibroblasts, ASCs: adipose-derived stem cells.

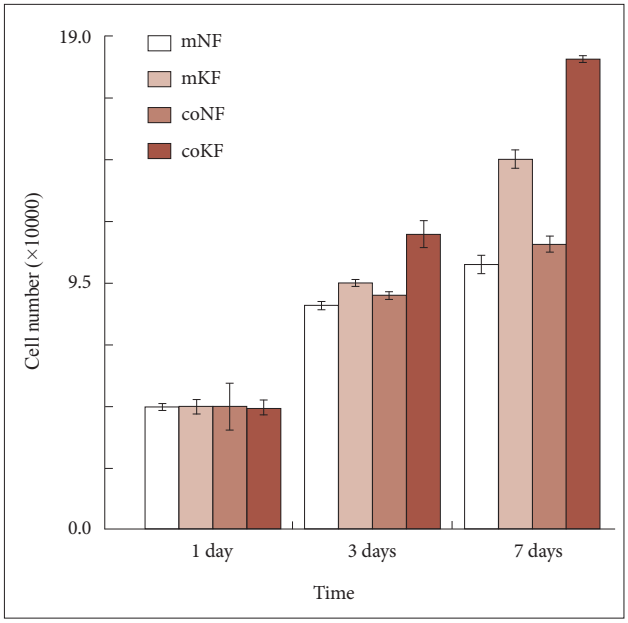

Figure 2. The cell counts were analyzed using CCK-8 kits (Dojindo) on days 1, 3, and 7. KF: keloid fibroblasts, ASCs: adipose-derived stem cells.

then transferred to nitrocellulose membranes (Millipore). The membranes were incubated for 24 hours with primary antibodies against type I collagen (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA) or alpha-smooth muscle actin (Sigma Aldrich) and incubated with secondary antibody (anti-rabbit IgG-HRP conjugate; 1:5000; Santa Cruz Biotechnology) for 2 hours at room temperature. The blots were developed with an ECL kit (GE Healthcare, Buckinghamshire, UK) and quantified with a LAS-4000 luminescent image analyzer (Science Lab, FUJI, Tokyo, Japan).

Statistical evaluation of results

A Kruskal-Wallis test was used to analyze differences between the four groups, and a Mann-Whitney test was used to examine differences between pairs of groups. Nonparametric tests were used because the variables of interest were not normally distributed. The Shapiro-Wilk test was used to test for normality. $p<0.05$ was adopted as the level of significance.

RESULTS

Morphology of fibroblasts

The morphology of the NF and KF cultured with and without ASCs, was determined on days 1, 3, and 7 by direct observation with a light microscope. The NF formed a closely packed, nearly compact arrangement, and the successive configurations of the spindle-like fibroblasts pointed in regular directions. The KF

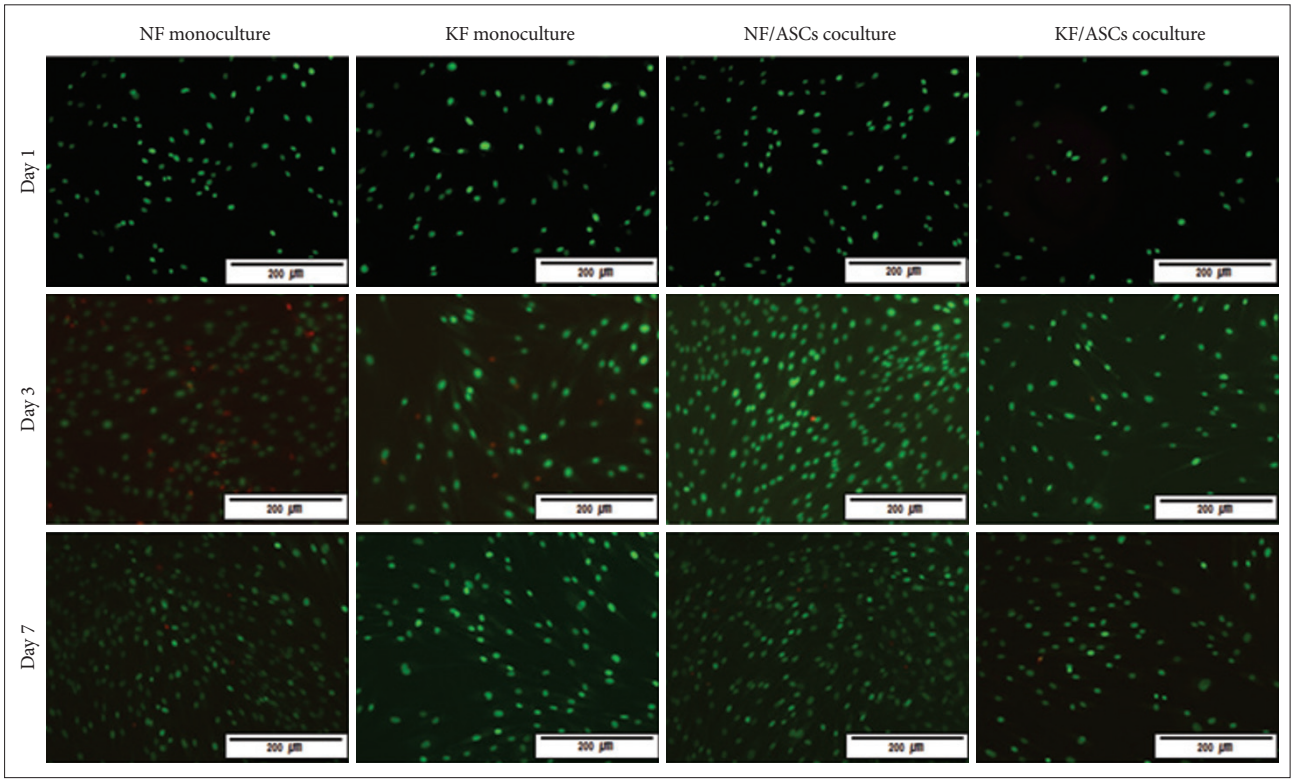

Figure 3. Quantification of cell apoptosis/necrosis using a Live-Dead Cell Staining Kit (BioVision) on days 1, 3, and 7. Live cells stain with the cell-permeable live-dye (green), while dead cells stain with the cell-permeable live-dye and the cell impermeable propidium iodide (yellow-red). NF: normal fibroblasts, KF: keloid fibroblasts, ASCs: adipose-derived stem cells, α-SMA: alpha-smooth muscle actin.

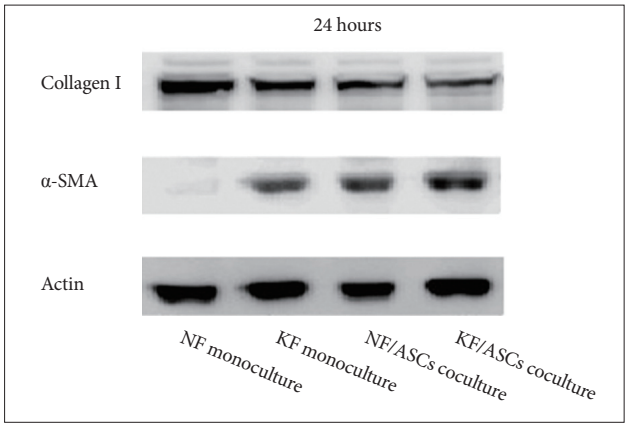

Figure 4. Production of type I collagen and alpha-smooth muscle actin by NF and KF was measured by the Western blot analysis at 24 hours. NF: normal fibroblasts, KF: keloid fibroblasts, ASCs: adipose-derived stem cells.

were more flattened and larger, and formed more processes than the NF. Thus, the KF occupied more space and formed abundant ECM, creating larger intercellular spaces. After co-culture with ASCs, the NF were elongated forming more complex shapes, whereas the KF, though also elongated and enlarged, formed a more regular arrangement with cells pointing in regular directions (Fig. 1).

Effects of ASCs on NF and KF proliferation and apoptosis

The cell counts of each group were analyzed using CCK-8 kits and counted with a microplate reader on days 1, 3, and 7 (Fig. 2). The KF grew faster than the NF in monoculture, and the trans-well co-culture with ASCs stimulated the growth of both NF and KF. When the four groups were compared, there was no significant difference between them on days 1 and 7, but on day 3 they were significantly different (p=0.033).

We observed fewer apoptotic cells in the KF monoculture than in the NF monocultures, and in both cases the number of apoptotic cells was lower in the co-cultures with ASCs, which correlates with the finding of increased proliferation after co-culture with ASCs (Fig. 3).

Production of fibroblast proteins

Expression of type I collagen and α-SMA was compared by Western blot analysis (Fig. 4). In the co-cultures with ASCs, collagen production decreased in both NF and KF (Fig. 5). Conversely, expression of α-SMA was detected in both monoculture groups, and was higher in the co-culture groups (Fig. 6). Comparison between the four groups revealed a significant difference in the expression of both type I collagen and α-SMA

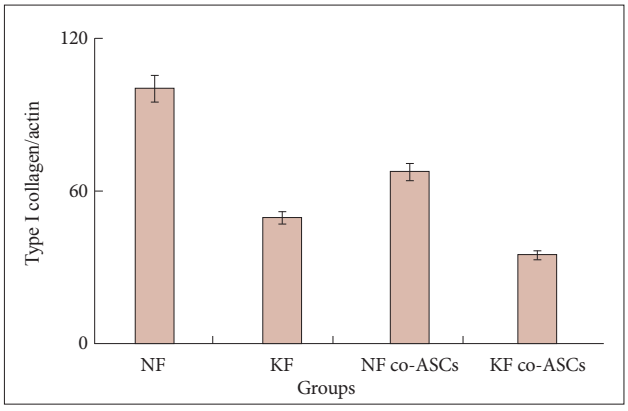

Figure 5. Expression of type I collagen production in the NF, KF, and those cultured with ASCs were compared by the Western blot analysis at 24 hours. After co-cultures with ASCs, collagen production decreased in both NF and KF. Comparison between the four groups revealed a significant difference in the expression of type I collagen (p=0.016; Kruskal-Wallis test). NF: normal fibroblasts, ASCs: adipose-derived stem cells, KF: keloid fibroblasts.

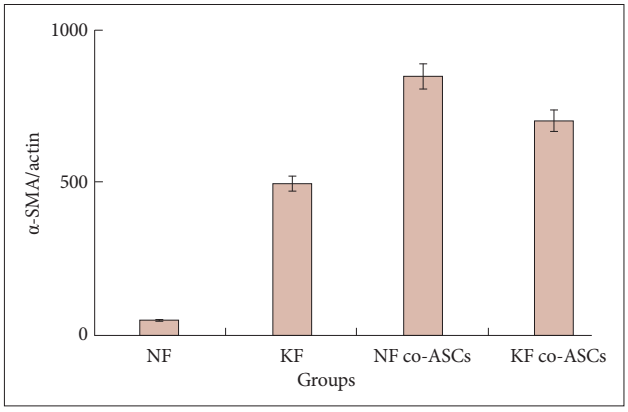

Figure 6. Levels of α-SMA in the NF, KF, and those cultured with ASCs were compared by Western blot analysis at 24 hours. The expression of α-SMA was detected in both monoculture groups, and was higher in the co-culture groups. Comparison between the four groups revealed a significant difference in the expression of α-SMA (p=0.022; Kruskal-Wallis test). α-SMA: alpha-smooth muscle actin, KF: keloid fibroblasts, ASCs: adipose-derived stem cells.

(p=0.016 and 0.022; Kruskal-Wallis test).

DISCUSSION

Many studies have confirmed the beneficial paracrine activity of ASCs, such as wound repair, immunomodulation, anti-apoptotic effects, and induction of differentiation. A few articles have demonstrated that ASCs have effects on fibroblasts that are related to wound healing and photo-aging. Kim et al. [19-21] reported that conditioned medium by ASCs promoted the proliferation of fibroblasts and their collagen production, and suggested that factors secreted by ASCs promote wound healing by the fibroblasts. Another study found that ASCs induced the proliferation of photo-aged human dermal fibroblasts, increased type I collagen production and decreased their MMP-1 production, suggesting an anti-aging effect of the ASCs [22,23].

Keloids are pathologic structures resulting from the excessive accumulation of connective tissue during the healing process [8]. They result from several processes, such as fibroblast hyperproliferation, increased collagen synthesis stimulated by keratinocytes, release of transforming growth factor beta1 (TGF-β1) and vascular endothelial growth factor (VEGF) from fibroblasts, and reduced collagen degradation resulting from the presence of a collagen inhibitor such as plasminogen activator inhibitor that inhibits procollagenase activation [9,10]. KF are similar to NF in size and shape, but show a greater capacity to proliferate, and produce high levels of ECM components such as collagen, fibronectin, elastin, and proteoglycans [24-26]. KF have higher growth potential and an abnormal balance between proliferation and apoptosis [11,12].

We examined whether ASCs have any effect on KF and can reverse their abnormalities. KF grew faster than NF, and co-culture with ASCs increased the proliferation of both KF and NF. This agreed with the results of live-dead cell staining, which revealed fewer apoptotic cells among the KF than among the NF. Moreover, both the NF and KF co-cultures contained reduced numbers of apoptotic cells, which agree with their increased rate of proliferation. We found that the KF occupied more space than the NF, with abundant ECM, so increasing intercellular space. However, in co-culture with ASCs, the KF were more condensed and compact, resembling NF. We also studied proteins associated with wound healing. Collagen is produced mainly by fibroblasts, and by endothelial cells. KF have a greater capacity to produce collagen autonomously. Some, but not all, cultures of KF contain higher levels of type I collagen than do NF [11,12,27,28]. After 24 hours of co-culture with ASCs, collagen production decreased compared with monoculture in both the NF and KF. This result is inconsistent with previous studies that used ASCs for dermal wound healing, which found that ASCs were well-suited for wound healing since they increased collagen synthesis by fibroblasts and stimulated their proliferation [19,20,22]. However, considering mechanisms of keloid, reduction of collagen synthesis could be another effect of ASCs, the first step for regulating keloid abnormalities.

Moreover, in view of the anti-fibrotic effects, BMSCs are effective in reducing fibrosis in liver cirrhosis and myocardial disease [29]. Co-culture of BMSCs and activated hepatic cells led to decreased collagen deposition and cell proliferation, and induced apoptosis of the activated hepatic cells [29]. Mesenchymal stem cells delivered into the infarcted porcine heart tissue improved cardiac function by reducing infarct size, increasing

cell survival, and reducing collagen deposition [30]. ASCs may also have anti-fibrotic effects in pathologic conditions like keloids, and act differently from dermal wound healing.

α-SMA is a form of actin that is well-known for its role in myofibroblast contractility. Immunohistochemical studies have shown that keloids only contain α-SMA around microvessels, but KF can have detectable levels of α-SMA in culture [27,31]. In our study, expression of α-SMA was detectable in KF and NF. Furthermore, after 24 hours of co-culture with ASCs, α-SMA expression was increased in both KF and NF. Progression through the different stages of normal wound healing leads to the formation of granulation tissue and differentiation of fibroblast into myofibroblasts. In normal wound healing, the myofibroblast mark, α-SMA, peaks between 7−15 days and gradually disappears, and by day 30, the fibroblast population returns to normal. Since the transient appearance of myofibroblasts in a wound is a prerequisite for proper wound healing, the increase of α-SMA after co-culture of ASCs might be additional evidence that ASCs has effect on KF.

Blazić and Brajac [32] hypothesized that keloids are caused by a failure of fibroblast senescence. According to them, progression through the stages of normal wound healing was not accomplished and this led to uncontrolled fibroblast proliferation, and the excessive collagen production resulted in keloid formation. Therefore, controlling fibroblasts and fibroblast activities might help to control keloids. In our study, ASCs modulated the activity of KF. Although their proliferation increased, KF became more condensed and compact, reducing abundant ECM and narrowing the intercellular space. As the result, their production of collagen reduced and that of α-SMA increased. This was only a short term report, but there might be a possibility that ASCs may have effect on keloids and modulate them, which is far different from normal dermal fibroblast and normal wound healing.

Shimode et al. [33] demonstrated that BMSCs had a significant effect on tendon fibroblasts, enhancing their proliferation and migration, but had little effect on ECM production. Kumai et al. [34] reported decreased scar fibroblast cell numbers and collagen production in co-culture with ASCs, and in scar fibroblasts isolated from the vocal folds. On the other hand, Kim et al. [19,20] found increased collagen production in dermal fibroblasts, which is contrary to our findings. These discrepancies between studies might be due to different phenotypes among fibroblasts and different effects of ASCs depending on environmental conditions [17,35,36]. Our findings suggest that ASCs may modulate KF by changing morphology and arrangements and by controlling production of fibroblast proteins. Further *in vivo* and *in vitro* studies on signals, such as cytokines, other growth factors, and their receptors involved in communication between ASC and KF are required for more understanding of the interactions between ASCs and KF.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

There are no animal experiments carried out for this article.

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