Substance P Modulates Properties of Normal and Diabetic Dermal Fibroblasts

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Dermal fibroblasts play essential roles in wound healing. However, they lose their normal regenerative functions under certain pathologic conditions such as in chronic diabetic wounds. Here, we show that substance P (SP) rescues the malfunctions of dermal fibroblasts in diabetes. SP increased the proliferation of diabetic dermal fibroblasts dose-dependently, although the effect was lower compared to the SP-stimulated proliferation of normal dermal fibroblasts. In contrast to normal dermal fibroblasts, SP increased the expression level of vascular endothelial growth factor (VEGF) and stromal cell-derived factor-1 (SDF-1) in diabetic dermal fibroblast hence, rescuing their angiogenic potential. The cellular characteristics of diabetic dermal fibroblasts modulated by SP would be able to accelerate the wound healing process through faster wound contraction and improved angiogenesis in diabetic chronic wounds. Moreover, SP pretreatment into dermal fibroblasts isolated from diabetic patients would be a promising strategy to develop autologous cell therapy for treating diabetic chronic wounds.

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INTRODUCTION

Skin wound healing is achieved through individual events of different cell types and their coordinated interactions [1,2]. Dermal fibroblasts play essential roles in wound healing mainly through the synthesis and secretion of extracellular matrix (ECM) components including type I and type III collagen [3-5]. This ECM creates a scaffold structure that provides structural integrity, cellular adhesion surface, and serves as a reservoir of cytokines and growth factors that lead to wound contraction [6]. Therefore, both the proliferation of dermal fibroblasts during wound healing and their migration from the wound edges into the wounds are important in the process of tissue regeneration [7].

Wound healing is mediated by multiple cellular and molecular processes including inflammation, angiogenesis, ECM synthesis, and tissue remodeling. Dysregulation of these processes can induce the formation of chronic wounds such as diabetic wounds and pressure ulcers [1,7]. For example, cell death following poor angiogenesis is a typical cause of the impaired re-

generation in diabetic wounds.

Substance P (SP) is an 11-amino acid peptide that is involved in pain perception, mediates the mobilization of bone marrow-derived mesenchymal stem cells into peripheral blood and modulates inflammation [8,9]. It is also known that SP accelerates the wound healing process both in normal and diabetic wounds [10-12]. Therefore, we decided to examine whether SP, a wound healing accelerator, would modulate the properties of dermal fibroblasts that play essential roles in the wound healing process.

MATERIALS AND METHODS

Mice

C5BL/6 male mice and db/db male mice were purchased from Central Lab. Animal Inc. (Seoul, Korea) and Laboratory Animal Resource Center of Korea Research Institute of Bioscience & Biotechnology (Daejeon, Korea), respectively. Mice were maintained under controlled temperature (21–22°C) and light (12 hours light/12 hours dark cycles) with free access to drinking water and food. Eight to ten-weeks old mice were used for primary culture of dermal fibroblasts. All animal experiments were performed in compliance with protocols approved by the Institutional Animal Care and Use of Kyung Hee University.

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Primary culture of dermal fibroblasts

Fibroblasts were isolated from dermis of normal or db/db mice through the treatment of Type I collagenase (Washington Biochemical Corp., Freehold, NJ, USA). Dermal fibroblasts were cultured with fibroblast growth media (FGM2, Lonza, Switzerland) containing 2% fetal bovine serum, 5 µg/mL of insulin, 1 ng/mL of basic fibroblast growth factor, and 50 µg/mL of gentamicin. After passage number 3, the dermal fibroblasts were used for experiments.

Immunocytochemistry

Dermal fibroblasts were seeded on type I collagen (0.5 µg/mL, Nitta Gelatin NA Inc., Osaka, Japan) coated coverslips in 24 well plates at a density of 3×10^4 cells/well with FGM2 media. The cells were incubated for 2 days and fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10 minutes on ice. Then, immunocytochemistry was performed using a general procedure [8] with the primary antibodies against the following proteins: α -smooth muscle actin (α -SMA, DAKO, Denmark), vimentin (Millipore, Billerica, MA, USA), CD31 (BD, San Jose, CA, USA), and Keratin 5 (K5, Abcam, Cambridge, MA, USA). The following secondary antibodies (Life Technologies, Carlsbad, CA, USA) were used at a 1:1000 dilution: anti-mouse IgG-Alexa 488, anti-rabbit IgG-Alexa 488, anti-rat IgG-Alexa 488, and anti-goat IgG-Alex-488. Stained cells were imaged using a Zeiss LSM 700 confocal microscope or a Leica DMI4000 fluorescence microscope.

Bromo-2'-Deoxyuridine incorporation assay

The primary cultured dermal fibroblasts were starved with mitogen-free, serum-starved media (FBM) for 18 hours. Cells were treated with SP for 48 hours. For the last 9 hours, the cells were treated with 20 µM 5-Bromo-2'-Deoxyuridine (BrdU, Sigma, St. Louis, MO, USA). Then, cells were fixed with 4% PFA in PBS for 10 minutes on ice. Fixed cells were stained with primary antibody against BrdU (Roche, Switzerland) followed by secondary antibody, anti-mouse IgG-Alexa 488 (Life Technologies). Stained cells were imaged using a Zeiss LSM 700 confocal microscope. BrdU-positive cells were counted from more than 5 random images of each experimental group to measure the cellular proliferation.

RNA isolation and reverse transcription-polymerase chain reaction analysis

Total RNA was extracted from fibroblasts using TRIzol™ reagent (Life Technologies) according to the manufacturer's instructions. Five µg of total RNA were used for single strand complementary DNA (cDNA) synthesis using Superscript First-Strand cDNA Synthesis System (Life Technologies) according to

the manufacturer's instructions. After reverse transcript reaction, RNA was degraded by E.coli RNase H (Life Technologies). Real-time polymerase chain reaction (PCR) was performed using Power SYBR Green PCR Master Mix (Life Technologies). Mouse ribosomal protein 36B4 gene was used as an endogenous control. The following primers were used to detect the expression of Type I collagen (Col I), Type III collagen (Col III), SDF-1, VEGF, and 36B4: Col I (sense): 5'-CAGGCCAGTGGC AATGTAAAGA-3', Col I (antisense): 5'-ACTGTTGCCTTCG CCTCTG-3', Col III (sense): 5'-CAGGCCAGTGGCAATG TAAAGA-3', Col III (antisense): 5'-CTCATTGCCTTGCGT GTTTGATA-3', SDF-1 (sense): 5'-GTCCTCTTGCTGTCCA GCTC-3', SDF-1 (antisense): 5'-AGATGCTTGACGTTGGC TCT-3', VEGF (sense): 5'-AGAGCA ACATACCA GCAG-3', VEGF (antisense): 5'-CAGTGAACGCTCCAGGATTT-3', 36B4 (sense): 5'-GAACATCTCCCCCTTCTCCTT-3', and 36B4 (antisense): 5'-GCAGGGCCTGCTCTGTGAT-3'.

Statistical analysis

Quantitative data were presented as mean $\pm$ SD. Unpaired Student t-test was applied to evaluate differences between two groups. Values of $p < 0.05$ were considered statistically significant. All statistical analyses were performed using GraphPad version 5.01 (GraphPad Software, USA; <http://www.graphpad.com>).

RESULTS

Identification of dermal fibroblasts derived from normal mice and type 2 diabetic mice

Primary culture of dermal fibroblasts derived from normal mice and type 2 diabetic mice (db/db mice) was performed in order to observe the effects of SP on the characteristics of dermal fibroblasts that play important roles in the wound healing process. Cells were isolated from dermis of the back skin of mice and cultured for 3 passages under media condition which only permits the growth of fibroblasts. Firstly, we carried out immunocytochemistry against the marker proteins of primary cultured-dermal fibroblasts, vimentin and α -SMA. All of the primary cultured cells established both from normal mice and db/db mice expressed vimentin (Fig. 1A and B). α -SMA was also expressed in more than 87% of the primary cultured cells established from normal mice ($87.35 \pm 2.109\%$, $n=3$ mice) and db/db mice ($87.67 \pm 2.376\%$, $n=6$ mice) (Fig. 1C and D). Importantly, none of the primary cultured cells expressed either CD31, a marker protein of endothelial cells (Fig. 1E) or K5, a marker protein of epithelial cells (Fig. 1F). Therefore, these results suggest that the primary cultured cells derived from dermis of normal mice and db/db mice were dermal fibroblasts without contamination of endothelial or epithelial cells.

Figure 1. Identification of primary cultured dermal fibroblasts derived from normal or db/db mice. (A and B) Immunocytochemistry for vimentin in primary cultured cells derived from normal mice (CON) or db/db mice (db/db). Vimentin was immunostained in green and nuclei were stained with DAPI in blue. The percentages represent the number of vimentin positive cells from total cells in (B). Mean values are shown in red. (C and D) Immunocytochemistry for α -smooth muscle actin (α -SMA) in primary cultured cells derived from normal mice (CON) or db/db mice (db/db). α -SMA was immunostained in green and nuclei were stained with DAPI in blue. The percentages represent the number of α -SMA positive cells from total cells in (D). Mean values are shown in red. (E and F) Immunocytochemistry for CD31 or cytokeratin 5 (K5) using the primary cultured cells derived from normal mice (CON) or db/db mice (db/db). CD31 (E) or K5 (F) were immunostained in green and nuclei were stained with DAPI in blue. Scale bar, 100 μ m.

Substance P-induced proliferation of diabetic dermal fibroblasts is lower than that of normal dermal fibroblasts

It has been shown that SP induces the proliferation of dermal fibroblasts. Therefore, we tested the effects of SP on the proliferation of diabetic dermal fibroblast and normal dermal fibroblasts to determine if SP would affect differently each cell group.

Figure 2. Substance P (SP) is less effective to induce the proliferation of diabetic dermal fibroblast compared to normal dermal fibroblasts. The percentages represent the number of BrdU incorporated cells in normal dermal fibroblasts (CON) or diabetic dermal fibroblasts (db/db) after SP treatment (0, 1, 10, 100, 300 nM). Mean values are shown in black. BrdU: Bromo-2'-Deoxyuridine.

SP enhanced the proliferation of both normal and diabetic dermal fibroblasts in a dose-dependent manner (Fig. 2). The proliferation of normal fibroblasts treated with 300 nM SP increased 17.7-fold, compared to non-treated cells. However, the treatment of 300 nM SP into diabetic dermal fibroblasts only resulted in a 4.1-fold increase in the proliferation, compared to non-treated cells (Fig. 2). Importantly, there was no difference in the expression level of NK-1, SP receptor, between normal and diabetic fibroblasts (data not shown). Therefore, it is very likely that SP induces the proliferation of diabetic dermal fibroblasts. However, the effect of SP on inducing cellular proliferation is greater on normal compared to diabetic dermal fibroblasts.

Substance P does not affect the synthesis of type I and type III collagens in dermal fibroblasts

Previous studies show that SP accelerates the wound healing process [10-12]. Dermal fibroblasts synthesize ECM proteins that play essential roles in the regeneration of injured tissues [13,14]. Type I and type III collagens are major components of the ECM proteins of the dermis [15]. Therefore, we examined whether SP would affect the synthesis of type I and type III collagens from normal and diabetic dermal fibroblasts. We found no difference in the messenger RNA (mRNA) expression level of type I collagen between normal and diabetic dermal fibroblasts in response to SP treatment (Fig. 3). Both the diabetic dermal fibroblasts and the normal dermal fibroblasts expressed type III collagen, although the expression level was significantly lower than the expression level of type I collagen. However, SP

Figure 3. The expression of type I and type III collagens in dermal fibroblasts is not changed by substance P (SP). (A) The level of type I collagen (Col I) mRNA was checked in the normal dermal fibroblasts (CON) and the diabetic dermal fibroblasts (db/db) after SP treatment (0 or 100 nM). (B) The level of type III collagen (Col III) mRNA was checked in the normal dermal fibroblasts (CON) and diabetic dermal fibroblasts (db/db) after SP treatment (0 or 100 nM). Results are shown as mean $\pm$ SD for each group after normalization with 36B4 (n=2 mice per each experimental group). mRNA: messenger RNA.

Figure 4. The expressions of VEGF and SDF-1 in diabetic dermal fibroblasts are increased by substance P (SP). (A) The level of VEGF mRNA was checked in normal dermal fibroblasts (CON) and the diabetic dermal fibroblasts (db/db) after SP treatment (0 or 100 nM). (B) The level of SDF-1 mRNA was checked in the normal dermal fibroblasts (CON) and the diabetic dermal fibroblasts (db/db) after SP treatment (0 or 100 nM). Results are shown as mean $\pm$ SD for each group after normalization with 36B4 (n=2 mice per each experimental group).

treatment into fibroblasts was unable to increase the synthesis of type III collagen in both groups of fibroblasts (Fig. 3B). Therefore, these results suggest that SP does not affect the expression of type I and type III collagens in both normal and diabetic dermal fibroblasts.

Substance P increases the expression of VEGF and SDF-1 in diabetic dermal fibroblasts, but not in the normal dermal fibroblasts

VEGF [6] and SDF-1 [16,17] are essential factors of the wound healing process and tissue repair through different mechanisms. Fibroblasts express VEGF [18] although it was found that its expression level is low in fibroblasts and vascular endothelial cells of dermis [19]. Fibroblasts can also synthesize SDF-1 [20]. Therefore, we compared the expression levels of VEGF and SDF-1 between normal and diabetic dermal fibroblasts. We also determined if SP would affect the expression levels of VEGF and SDF-1 in both groups of fibroblasts. Importantly, the expressions of VEGF and SDF-1 were down-regulated in diabetic dermal fibroblasts compared to normal dermal fibroblasts (Fig. 4). SP treatment did not increase the expression level of VEGF in normal dermal fibroblasts. However, SP caused a slight increase (1.24-fold) in the expression level of VEGF in diabetic dermal fibroblasts (Fig. 4A). SP did not change SDF-1 expression level in normal dermal fibroblasts. On the other hand, diabetic dermal fibroblasts treated with SP increased the expression level of SDF-1 by approximately 1.56-fold compared to untreated diabetic dermal fibroblasts (Fig. 4B). Therefore, these results suggest that SP rescues the impaired expressions of both VEGF and SDF-1 in diabetic dermal fibroblasts.

DISCUSSION

This study shows that SP treatment induced the proliferation of dermal fibroblasts derived from normal and diabetic mice. In addition, the expression levels of both VEGF and SDF-1 increased in diabetic dermal fibroblasts in response to SP treatment. However, SP did not affect mRNA levels of VEGF and SDF-1 in normal dermal fibroblasts.

In the wound repair process of injured skin, dermal fibroblasts proliferate at the wound edge and subsequently migrate into the matrix deposit localized at the center of the wound [6]. The cellular proliferation is downregulated in diabetic dermal fibroblasts, compared to normal dermal fibroblasts [21,22]. Therefore, the regulation of the proliferation of fibroblasts would be important in the treatment of diabetic wounds. Importantly, our current study demonstrated that SP increased the proliferation of the diabetic dermal fibroblasts. However, SP-mediated increase in the proliferation of diabetic dermal fibroblasts was less prominent than that in normal dermal fibroblasts. This may be due to different intrinsic properties of normal and diabetic dermal fibroblasts. Further studies are required to identify the different intrinsic cellular factors. Interestingly, we found that SP receptor, NK-1R, was expressed at similar levels on both fibroblasts. It would be also interesting to further compare the expression level or the activity of SP degrading neutral endopeptidase on fibroblasts.

The regulation of angiogenesis is essential for wound healing [6]. It has been shown that VEGF is one of major regulators of angiogenesis [23] and its expression is massively up-regulated after injury [24]. Reduced expression or enhanced degradation

of VEGF is found in chronic wounds [25]. In contrast, ectopic expression of VEGF accelerates wound healing [26]. SDF-1 also modulates angiogenesis [27]. In order to lead to the acceleration of wound healing in diabetic wounds, SDF-1 mobilizes endothelial progenitor cells into wounds [28] as well as bone marrow-derived mesenchymal stem cells [29]. To our surprise, SP increased the expression of VEGF and SDF-1 in diabetic dermal fibroblasts, although it did not change their expression in normal dermal fibroblasts in response to SP treatment.

ECM is also important in wound healing [6]. Contrary to our expectation, SP did not increase the expression of ECM of dermal fibroblasts. However, SP treatment would indirectly enhance ECM deposition in wounds because it increased the proliferation of dermal fibroblasts consequently increasing the number of these cells in wounds.

In conclusion, SP treatment would enhance the wound healing process under normal and diabetic conditions through the modulation of the cellular and molecular characteristics of dermal fibroblasts. Dermal fibroblasts treated with SP would enhance angiogenesis in diabetic wounds via SDF-1 and VEGF. Also, dermal fibroblasts treated with SP would enhance wound contraction via providing more ECM and more myofibroblasts in wounds through the increase in cellular proliferation. Importantly, transplantation of autologous or allogeneic skin fibroblasts has been developed for the treatment of diabetic ulcers [30,31]. SP treatment into cultured dermal fibroblasts would be more effective in fibroblast cell therapy that accelerates wound healing of diabetic wounds.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

This study was approved by the Kyung Hee University Animal Institutional Review Board.

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