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Original Article

Cytokines released from human adipose tissue-derived stem cells by bFGF stimulation: Effects of IL-8 and CXCL-1 on wound healing

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ABSTRACT

Objectives: Adipose-derived stem cells (ADSCs) are widely used in wound care because they release a variety of cytokines. However, the molecular mechanism of paracrine action remains unclear. It has been reported that basic fibroblast growth factor (bFGF) enhances the therapeutic potential of ADSCs. In this study, we searched for cytokines whose release from ADSCs is enhanced by bFGF stimulation.
Results: Quantitative RT-PCR and ELISA analyses revealed that bFGF upregulates CXCL-1 and IL-8 mRNA synthesis and secretion from ADSCs. Both cytokines showed the ability to promote important processes for wound healing, including tube formation of vascular and lymphatic endothelial cells and cell migration of fibroblasts *in vitro*.
Conclusions: These results suggest that bFGF stimulation increases the secretion of CXCL-1 and IL-8 from ADSCs and that these cytokines may promote angiogenesis, lymphangiogenesis, and cell migration, leading to enhanced efficiency of wound healing.

Significance statement

ADSCs release cytokines for wound healing, and bFGF enhances their therapeutic potential. However, its molecular mechanism remains unclear. In this study, the authors searched cytokines whose release from ADSCs were promoted by bFGF stimulation, and identified two cytokines, CXCL-1 and IL-8. Both cytokines promoted several important processes in wound healing, including regeneration of blood and lymph vessels, and fibroblast migration. Collectively, this study demonstrated that bFGF stimulation increases CXCL-1 and IL-8 release from ADSCs, leading to wound healing (93 words).

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1. Introduction

The field of the plastic surgery treats intractable wounds, such as limb ischemia, lymphedema, and ulcers from venous insufficiency, diabetes or burns. However, these diseases have limited treatment options [1,2], and delayed healing can lead to infection and even limb amputation. Therapy using stem cells has reported to improve vascular and lymph vessel networks and heal compromised wounds [3,4]. Adipose-derived stem cells (ADSCs) are recognized as one of the most promising stem cells in cell therapy due to their ability to differentiate in several cellular lineages and release a variety of cytokines [5–10]. When compared with other stem cells, ADSCs can be harvested easily from fat tissue with less invasion. Moreover, there are fewer ethical concerns compared to embryonic stem (ES) cells or induced pluripotent stem (iPS) cells. In fact, ADSCs have been already used in several clinical trials for wound treatment, limb ischemia, muscle regeneration, and lymphedema [6,11,12].

Interestingly, co-administration of bFGF is well-known to enhance the therapeutic effect of ADSC transplantation [3,13,14]. However, its molecular mechanism is largely unknown, and this has long hindered better therapeutic development.

We hypothesize that bFGF enhances the therapeutic effect of ADSCs by promoting the secretion of cytokines from ADSCs that are effective in wound healing. In this study, therefore, we searched for cytokines whose secretion from ADSCs was enhanced by bFGF stimulation, and identified CXCL-1 and IL-8 as such cytokines. We also examined the effects of these cytokines on important processes in wound healing *in vitro*, including angiogenesis, lymphangiogenesis and fibroblast migration.

2. Materials and methods

2.1. Cell culture and reagents

Human ADSCs were obtained from Lonza (Basel, Switzerland) and cultured in KBM ADSC-1 medium (Kohjin Bio, Saitama, Japan). HUVECs (PromoCell, Heidelberg, Germany), TIME-GFP cells (ATCC, Manassas, VA), and HDLECs (Takara Bio, Shiga, Japan) were cultured in endothelial cell growth medium 2 (PromoCell), vascular cell growth medium (ATCC), and endothelial cell growth medium MV2 (PromoCell), respectively. TIG114 fibroblasts cells (JCRB Cell Bank, Tokyo, Japan) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All cells were cultured at 37 °C with 5% CO_2. Recombinant human bFGF was purchased from PeproTech (Rocky Hill, NJ), while CXCL-1 and IL-8 were obtained from R&D Systems (Minneapolis, MN).

2.2. Cytokine screening

ADSCs were cultured until they became confluent. After one day of starvation, ADSCs were stimulated with and without 20 ng/mL human bFGF for 4 h. Afterward, quantitative RT-PCR was performed using three kinds of Taqman™ Fast 96 wells (Thermo Fisher Scientific, Waltham, MA): Human cytokine network, Human angiogenesis, and Human growth factors, which carry 181 different genes in total (Fig. 1A). Out of 181 genes, we selected the genes of cytokines that consistently exhibited a 1.5-fold increase or more and an average of 2-fold increase or more in the expression level after stimulation with bFGF compared to unstimulated controls.

2.3. Quantitative RT-PCR (qRT-PCR) and ELISA analysis

Total RNA underwent reverse transcription reaction with ReverTra Ace (Toyobo, Osaka, Japan). Real-time PCR was performed with TaqMan probes for human IL-8, CXCL-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Thermo Fisher Scientific) using 7500 Fast or QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific). ELISA analysis of conditioned medium was performed using Quantikine ELISA kits (R&D Systems).

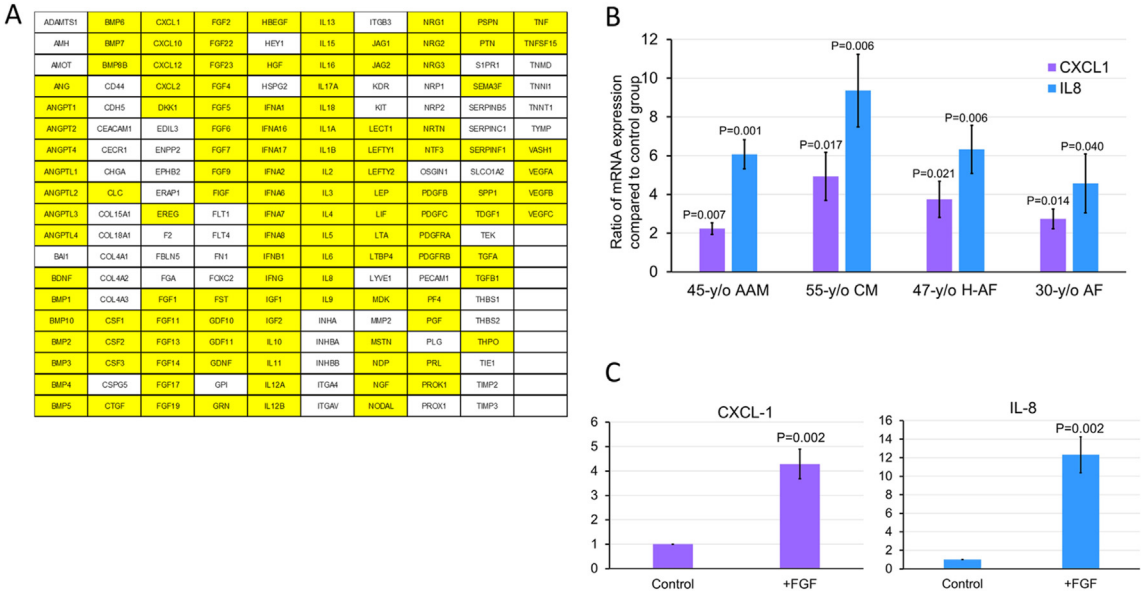

Fig. 1. Basic FGF stimulation promotes secretion of CXCL-1 and IL-8 from ADSCs. A, The list of 181 genes analyzed by qRT-PCR using three kinds of Taqman™ Fast 96 wells (Thermo Fisher Scientific): Human cytokine network, Human angiogenesis, and Human growth factors. Cytokine genes are indicated in yellow. B, Stimulation with bFGF increases the mRNA levels of CXCL-1 and IL-8 in ADSCs of four individuals with different backgrounds. After 4-h stimulation with bFGF, qRT-PCR was performed to examine the expression levels of CXCL-1 (*violet bars*) and IL-8 (*blue bars*) mRNAs in ADSCs from 45-year-old (-y/o) African male (AAM), 55-year-old Caucasian male (CM), 47-year-old mixed Hispanic-African female (H-AF), and 30-year-old Asian female (AF). The ratios to unstimulated controls are shown. GAPDH was used as an internal control. Bars and error bars represent the means and standard errors, respectively. C, Stimulation with bFGF increases the secretion of CXCL-1 and IL-8 from ADSCs. After 1-day stimulation with bFGF, conditioned medium was subjected to ELISA analyses. The amounts of CXCL-1 and IL-8 in unstimulated control cells were set to 1.0. Bars and error bars represent the means and standard errors, respectively (n = 3).

2.4. Tube formation assay

Five thousand HUVECs, TIME-GFP, and HDLECs were cultured in a 96-well plate on 50 μl of growth factor-reduced Matrigel basement membrane matrix (Corning Life Sciences, Corning, NY) with or without 20 ng/ml human CXCL-1 or IL-8. For experiments using neutralizing antibodies (NAbs), cells were cultured in a 50-fold diluted conditioned medium from ADSCs, together with 10 ng/ml anti-CXCL-1 NAb, anti-IL-8 NAb, or control mouse IgG, instead of cytokines.

After a 6-h incubation, images of each well were taken using a BZ-X800 fluorescence microscope (Keyence, Osaka, Japan) at 4x magnification and analyzed with Image J software in a 350× 350-pixel square area at the center. Tube formation rate (%) was calculated by dividing the assembled tube's area (pixels) by the total selected viewing area (pixels).

2.5. Scratch assay

TIG114 cells were cultured in 48-well dishes with 10% FBS in DMEM until confluent. After scratching, cells were incubated for 6 h in DMEM with 1% FBS, with or without 10 ng/ml CXCL-1 or IL-8. Images were captured before and after incubation, and the scratched area was measured using ImageJ software within a 350 × 350-pixel square area at the well center. Cell migration rate (%) was calculated by dividing the difference in scratch area (in pixels) before and after the 6-h incubation by 350 × 350 pixels.

2.6. Statistical analysis

All statistical analyses were performed using EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [17]. Continuous data were presented as average ± standard error (SE). For descriptive statistics, the student t-test was used to analyze continuous data, and values of $P < 0.05$ were defined as statistically significant.

3. Results

3.1. Identification of cytokines released from ADSCs upon bFGF stimulation

First, using a combination of TaqMan array and qRT-PCR analysis, we searched for cytokines whose secretion from ADSCs is enhanced by bFGF stimulation using 45-year-old healthy African American male ADSCs. Out of 122 cytokines (Fig. 1A), CXCL-1 (2.24 ± 0.3, $P = 0.007$), HB-EGF (4.155 ± 0.538, $P = 0.002$), IL-1β (2.733 ± 0.283, $P = 0.001$), and IL-8 (6.072 ± 0.744, $P = 0.001$) showed significant and reproducible upregulation by bFGF stimulation (Fig. 1B, Supplemental digital contents 1)

When we measured the amount of these cytokines secreted into the medium by ELISA, secretions of CXCL-1 (4.283 ± 0.6-fold, $P = 0.002$) and IL-8 (12.326 ± 1.939-fold, $P = 0.002$) protein from ADSCs increased after 1-day bFGF stimulation (Fig. 1C), while those of HB-EGF and IL-1β were undetectable. Consequently, we further analyzed CXCL-1 and IL-8.

To investigate if CXCL-1 and IL-8 mRNA upregulation by bFGF is specific to the African American male studied, we next analyzed three additional individuals with different characteristics (Fig. 1B). Compared to their control groups, bFGF stimulation of ADSCs from a 55-year-old Caucasian male increased the expression of CXCL-1 (4.931 ± 1.238, $P = 0.017$) and IL-8 (9.362 ± 1.873, $P = 0.006$). Similar upregulation of CXCL-1 and IL-8 was observed in ADSCs

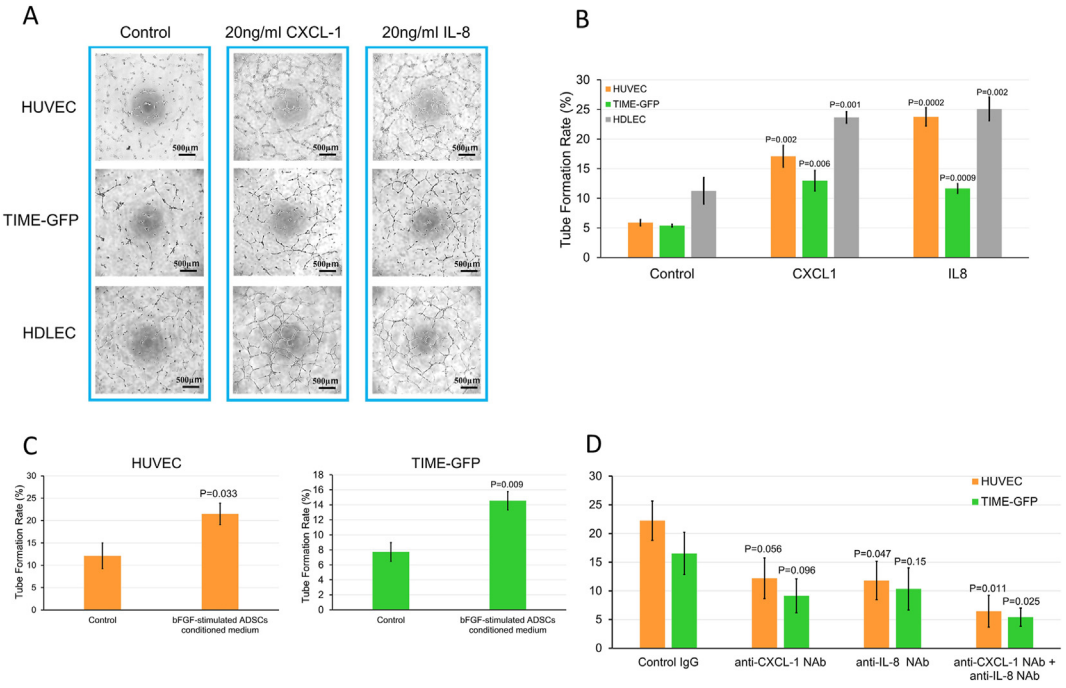

Fig. 2. CXCL-1 and IL-8 promote blood and lymph vessels formation. Recombinant CXCL-1 and IL-8 enhance blood and lymph vessels formation (A, B). A, Representative images of tube formation assay using umbilical vein endothelial cells (HUVEC), microvascular endothelium cells (TIME-GFP), and dermal lymphatic endothelial cells (HDLEC). B, After 6-h incubation with CXCL-1 or IL-8, images of each sample were taken at 4x magnification and were analyzed with Image J software. C, Condition medium from bFGF-stimulated ADSCs promote tube formation of HUVEC or TIME-GFP. HUVEC and TIME-GFP were cultured for 6 h in the presence or absence of the conditioned medium from bFGF-stimulated ADSCs. The tube formation rate (%) was calculated by dividing the area of formed tube by the total area. Bars and error bars represent the means and standard errors, respectively (n = 3). D, Neutralization of CXCL-1 and IL-8 suppresses the promoted tube formation rate of HUVEC and TIME-GFP by conditioned medium from bFGF-stimulated ADSCs. HUVEC and TIME-GFP were cultured in the presence of conditioned medium from ADSCs, together with 10 ng/ml anti-CXCL-1 neutralizing antibody (NAb), anti-IL-8 NAb or control mouse IgG. After 6-h incubation, entire images of each sample were taken with a microscope at 4x magnification and were analyzed with Image J software. Bars and error bars represent the means and standard errors, respectively (n = 3).

from a 47-year-old mixed Hispanic-African female (CXCL-1, 3.74 ± 0.93, $P = 0.021$; IL-8, 6.326 ± 1.24, $P = 0.006$), and a 30-year-old Asian female (CXCL-1, 2.738 ± 0.515, $P = 0.014$; IL-8, 4.57 ± 1.523, $P = 0.04$). These results suggest that CXCL-1 and IL-8 mRNA upregulation by bFGF is not specific to the African American male.

3.2. Tube formation assay

In wound healing, ADSCs are thought to play several roles, including promoting angiogenesis and lymphangiogenesis. To assess the impact of CXCL-1 and IL-8 upregulation by bFGF on blood and lymphatic vessel formation, we conducted a tube formation assay with two kinds of human vascular endothelial cells, umbilical vein endothelial cell (HUVEC) and microvascular endothelium cell (TIME-GFP), and one kind of lymphatic endothelial cells, human dermal lymphatic endothelial cell (HDLEC). CXCL-1 stimulation led to a 2.909-, 2.401-, and 2.098-fold increase in tube formation for HUVECs (17.076% vs. 5.871%, $P = 0.002$), TIME-GFP (12.957% vs. 5.397%, $P = 0.006$), and HDLECs (23.654% vs. 11.272%, $P = 0.001$), respectively, compared to the control. Similarly, IL-8 stimulation resulted in a 4.047-, 2.160-, and 2.226-fold increase in tube formation for HUVECs (23.759% vs. 5.871%, $P = 0.0002$), TIME-GFP (11.656% vs. 5.397%, $P = 0.0009$), and HDLECs (25.093% vs. 11.272%, $P = 0.002$), respectively (Fig. 2A and B).

Next, to examine the requirement of CXCL-1 and IL-8 for the promotion of angiogenesis by ADSCs, we investigated the effect of neutralizing CXCL-1 and IL-8 on tube formation. Incubation with conditioned medium from bFGF-stimulated ADSCs increased tube formation rates by 1.774-fold for HUVECs ($P = 0.033$) and 1.880-fold

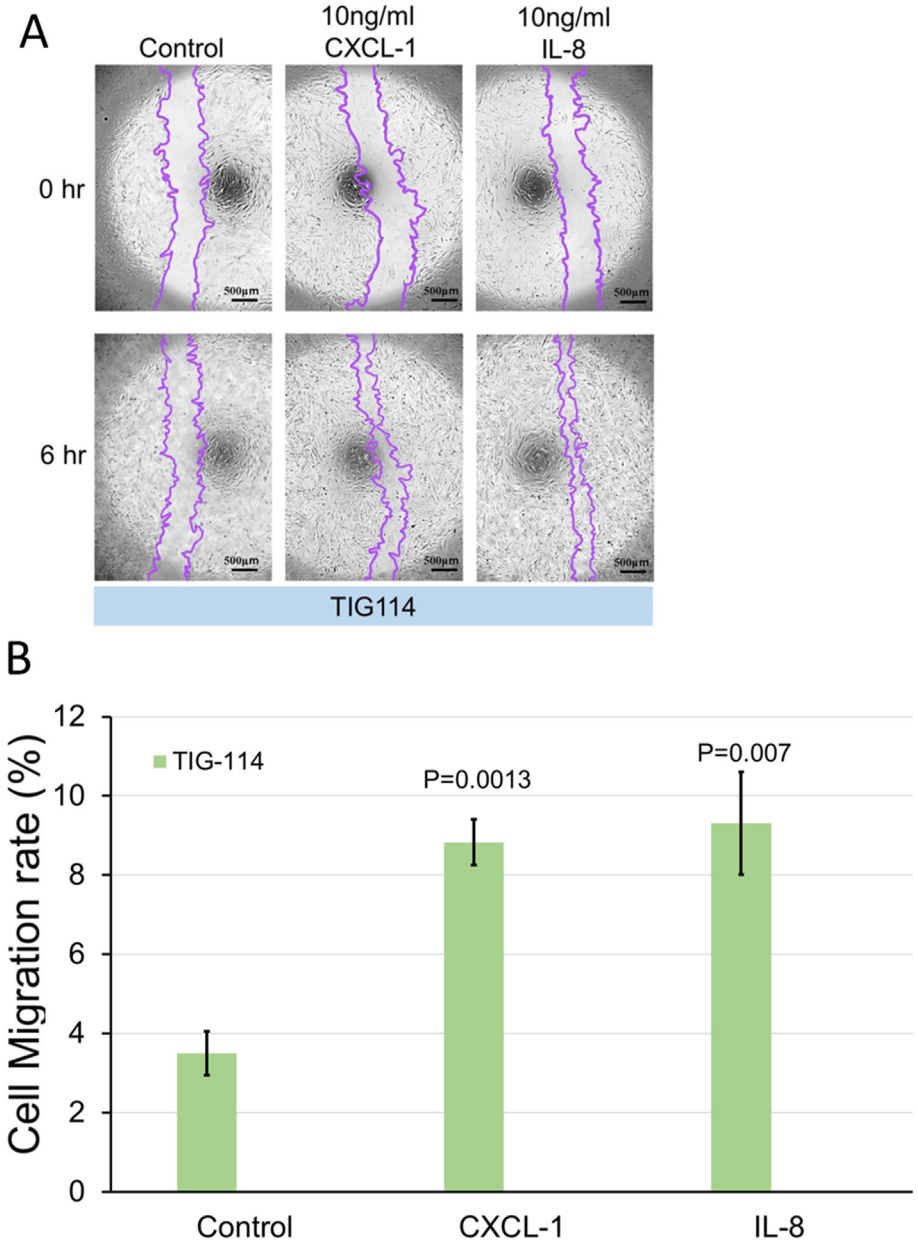

Fig. 3. CXCL-1 and IL-8 promote migration of TIG114 fibroblasts. A, Representative images of scratch assay after scratch. TIG114 were incubated in the presence or absence of CXCL-1 or IL-8 for 6 h. B, Cell migration rate was calculated by dividing the difference between scratch area before and after incubation. Bars and error bars represent the means and standard errors, respectively (n = 3).

for TIME-GFP (P = 0.009) (Fig. 2C), suggesting the presence of activity that promotes angiogenesis in the conditioned medium from ADSCs. When neutralizing antibodies (NAbs) were added to inhibit CXCL-1 and/or IL-8 function in the conditioned medium, the tube formation rate of HUVECs decreased by 1.820-fold with anti-CXCL-1 NAb (12.222%, $P = 0.056$), 1.884-fold with anti-IL-8 NAb (11.810%, $P = 0.047$), and 3.457-fold with both NAbs (6.436%, $P = 0.011$) (Fig. 2D). Similarly, the tube formation rate of TIME-GFP incubated with the conditioned medium was decreased by 1.812-fold by anti-CXCL-1 NAb (9.132%, P = 0.096), 1.598-fold by anti-IL-8 NAb (10.352%, P = 0.150), and 3.056-fold by both NAbs (5.413%, P = 0.025). These results suggest that CXCL-1 and IL-8 are active factors promoting angiogenesis in the conditioned medium.

3.3. Scratch assay

In wound healing, fibroblasts migrate and gather at the wound site. We therefore examined the effect of CXCL-1 and IL-8 on the migration of TIG114 fibroblast cells using scratch assay (Fig. 3A and B). TIG114 cells showed 3.499% migration rate after 6 h in the absence of cytokines. The migration rate was significantly increased by the presence of CXCL-1 (8.830%, $P = 0.001$) and IL-8 (9.304%, $P = 0.007$), suggesting that both CXCL-1 and IL-8 promote fibroblast migration.

4. Discussion

In this study, to understand the molecular mechanism of the cooperative effect between ADSCs and bFGF on wound healing, we searched for cytokines released from ADSCs upon bFGF stimulation using human ADSCs. We found that bFGF stimulation upregulated IL-8 and CXCL-1 mRNA synthesis, and increased the secretion of these cytokines from ADSCs. We also found that these cytokines promote important processes for wound healing, such as angiogenesis, lymphangiogenesis, and cell migration. The present results may provide very useful information for the use of ADSCs in wound healing therapy.

Although we found that CXCL-1, IL-8, IL-1β, and HB-EGF mRNA expression was increased by bFGF stimulation, only CXCL-1 and IL-8 proteins were accumulated in the culture medium, while IL-1β and HB-EGF were undetectable. IL-1β exists intracellularly as a precursor, and is cleaved by caspase-1 for extracellular secretion [15]. Similarly, HB-EGF is released from a membrane-anchored protein by cleavage at the membrane junction by ADAM family. Lack of protein secretion for IL-1β and HB-EGF may be due to absence of these cleavage enzymes in ADSCs [16].

Previous studies have shown that ADSCs have potential for angiogenic cell-based therapy in ischemic disease treatment. Transplantation of ADSCs into ischemic mouse hindlimb models has successfully induced neovascularization and vasculogenesis [3,17–19]. In our study, we demonstrated that CXCL-1 and IL-8 promoted capillary tube formation in human vascular endothelial cells using *in vitro* models of angiogenesis. Similar results have been reported by other groups [20]. Additionally, neutralizing antibodies against CXCL-1 and IL-8 hindered the tube formation promoted by the conditioned medium of ADSCs, indicating that these cytokines play a significant role in ADSC-mediated angiogenesis.

The lymphatic system's role in wound healing is significant, and lymph vessel tube formation enhances wound edema reduction, regulates tissue pressure, and boosts immune response to prevent infection [21]. ADSCs have a strong paracrine effect that promotes lymphangiogenesis [4,8]. Our study shows that CXCL-1 and IL-8 promote lymph vessel tube formation using HDLECs as a model for lymphangiogenesis, suggesting that these cytokines may play an important role in ADSC-mediated promotion of lymphatic tube formation.

In wound healing, ADSCs play several roles, besides promoting angiogenesis and lymphangiogenesis. For example, due to its great migration ability, ADSC is rapidly recruited into wounded sites, where they possibly undergo differentiation towards dermal fibroblasts, endothelial cells, and keratinocytes. Additionally, ADSCs are the major sources of extracellular matrix proteins involved in maintaining skin structure and function [22]. In this study, a scratch assay using normal skin fibroblasts showed that CXCL-1 and IL-8 promote fibroblast migration, which is necessary for wound healing. Since this study demonstrated that bFGF stimulation induces secretion of these cytokines from ADSCs, these results suggest that promoting fibroblast migration may be another role of ADSCs in wound healing.

5. Conclusions

This study showed that bFGF stimulation promotes the release of CXCL-1 and IL-8 from ADSCs, and suggested that these cytokines may promote angiogenesis, lymphangiogenesis and fibroblast cell migration. Taken together, these results suggest that CXCL-1 and IL-8, whose secretion is enhanced when ADSCs are stimulated with bFGF, may promote ADSC-mediated wound healing, transplant fat engraftment, and lymphangiogenesis of lymphedema. The results could provide clues for the effective use of ADSCs in clinical treatment.

Disclosures

The authors have no financial interest to declare in relation to the content of this article. All authors have completed the ICMJE uniform disclosure form.

Ethics

All cells are purchased from company, thus informed consent from patients was not required.

Author contributions

1. Chihiro Matsui: Conception and design, financial support, Collection and/or assembly of data manuscript writing, Data analysis and interpretation, Final approval of manuscript.
2. Hiroshi Koide: Conception and design, provision of study material or patients, Supervision, Manuscript writing, Writing – review & editing, Final approval of manuscript.
3. Tomomi Ikeda: Collection and/or assembly of data, Data analysis and interpretation, Final approval of manuscript.
4. Takako Ikegami: Collection and/or assembly of data, Data analysis and interpretation, Final approval of manuscript.
5. Takumi Yamamoto: Conception and design, Supervision, Writing – review & editing, Final approval of manuscript.
6. Joseph M. Escandón: Administrative support, Data analysis and interpretation, Writing – review & editing.
7. Arbab Mohammad: Administrative support, Data analysis and interpretation, Writing – review & editing.
8. Tomoyuki Ito: Administrative support, Data analysis and interpretation, Final approval of manuscript.
9. Hiroshi Mizuno: Conception and design, Financial support, Supervision, Writing – review & editing.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Presentation

Presented at the American Society for Reconstructive Microsurgery (ASRM) 2023 Annual Meeting in Miami, FL, USA. January 20th–24th, 2023.

Presented at the Plastic Surgery Research Council (PSRC) 68th Annual Meeting in Cleveland, OH, USA. April 13–16th, 2023.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.06.008.

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