Role of Glial Cell Line-Derived Neurotrophic Factor in Adipose Tissue Extract-Induced Angiogenesis in Mice

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Abstract

Our present study is aimed to evaluate the effects of adipose-derived extracts (AT-Ex) and GDNF within the extracts on skin graft. AT-Ex was harvest from fresh human lipoaspirates with centrifugation, emulsification and lysing by cycles of freeze and thawing. Concentrations of GDNF, VEGF and bFGF were detected by ELISA. AT-Ex and anti-GDNF-antibody-coupled AT-Ex were further used to test their ability to promote tube formation using human umbilical vein endothelial cells (HUVECs) and stimulate angiogenesis in nude skin-graft models. The results demonstrated that abundant GDNF, VEGF and bFGF were detected in AT-Ex, with GDNF displaying the highest concentration. AT-Ex significantly promoted the tube formation ability of HUVECs *in vitro*, with a dosage-dependent manner, while this ability was partially impaired when the anti-GDNF antibody was conjugated. *In vivo*, The AT-Ex treatment increased dermal thickness, augmented dermal proliferation and increased vascular density and GDNF contributed greatly to the AT-Ex effect in improvement the grafted skin condition by promoting angiogenesis *in vivo*. Our results suggested that critical effect of GDNF from AT-Ex on improvement skin graft condition.

Introduction

Adipose tissue can provide an abundant source of adipose-derived stem cells (ASCs) or stromal vascular fraction (SVF) for the treatment of various diseases, including photo-aged skin, ischemic conditions, multiple sclerosis and tissue defects [1–4]. We previously [5] also show the ASCs serve as a source of SMCs and ECs in blood vessel engineering. Recently, Wu LW et al [6] demonstrates that ASCs enhance wound healing via platelet-derived growth factor-AA (PDGF-AA). Moreover, Zografou A et al [7] shows that ASCs increase skin-graft survival by secreting abundant growth factors. Adipose tissue is a known source of growth factors [8], including the vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF). These adipose-derived growth factors can induce angiogenesis and contribute to the SVF- and ASCs-based therapies. However, there are still several disadvantages with regards to the current therapies.

ASCs require the enzymatic digestion, the isolation and culture are time consuming. Although the SVF is more easily obtained, the low survival rates and the risk of tumorigenicity limit the cell therapies' clinical application. Recently, an emulsified adipose tissue production from the mechanical process has been reported [9], which may address this problem. It demonstrates that the emulsified adipose tissue effectively enriches adipose-derived stromal cells and the product may be therapeutic. Theoretically, the supernatant extract from this emulsified adipose tissue lysate could provide a cell-free product with abundant growth factors. Sarkanen JR et al [10] produces a cell-free extract from mature human adipose tissue and names it adipose tissue extract (ATE). This ATE is produced by cutting the adipose tissue into small pieces and incubating in PBS for growth factor secretion. In fact, this extract is the adipose tissue-derived secretome because the adipose tissue is left for a minimum of 15 min for growth factor secretion. And there is no clear evidence to show that which detailed composition has the angiogenic potential. Here, we used the "adipose tissue extract (AT-Ex)" to distinguish from the ATE (adipose tissue-derived extract/secretome) produced by Sarkanen JR et al.

In this study, we investigated the level of several growth factors contained by AT-Ex, and demonstrated that the AT-Ex had strong angiogenic potential. We further indicated that the GDNF (glial-derived neurotrophic factor), not the well-known VEGF and bFGF, in the AT-Ex was a key mediator in improvement skin graft condition.

Materials And Methods

Preparation of human adipose tissue extract

Fresh human lipoaspirates were isolated from healthy patients following approval of the Research Ethics Committee of Shanghai Ninth People's Hospital, as our previously described [5]. Informed consent was obtained from all the patients. The lipoaspirates were centrifuged at 2000×g for 5min to harvest the middle fat layer. The fat emulsification was performed manually with syringes following the method described by Mashiko T et al[9]. The same volume of PBS was added into the emulsified fat, the mixture was lysed by three cycles of freeze and thawing. Then the lysate was centrifuged at 12000×g for 5min. The AT-Ex was produced by filtered the supernatant with 0.22µm filters. The AT-Ex was stored at -20°C.

Measurement of growth factor concentration

The levels of GDNF, VEGF and bFGF of the AT-Ex were measured by ELISA kits (R&D systems) as our previously described[11].

Preparations of AT-Ex without GDNF

Anti-GDNF antibody(Abcam, USA) was coupled to NHS-activated Sepharose 4 Fast Flow agarose (GE Healthcare) overnight at 4 °C following the manufacturer' instructions. Control (empty) beads were prepared the same way without adding the antibody. Then the antibody-binding beads (AT-Ex+Ab group) or control beads (AT-Ex group) was incubated with the AT-Ex for 2 hours with gentle rotation. Samples were centrifuged and ready to use.

Tube formation assay

Tube formation assay was performed as our previously described [5]. Briefly, HUVECs were cultured on the growth factor-reduced Matrigel (BD Biosciences) coated wells. AT-Ex, AT-Ex+Ab or PBS was added to culture and incubated for 8 hours. The pictures were captured to calculate the tube number and tube length per field.

Cell proliferation assay

The Human umbilical vein endothelial cells (HUVECs) were seeded onto the coverslips in 24-well plates in the presence or absence of AT-Ex for 48hours, and the wells were incubated with 10µM bromodeoxyuridine (BrdU, Sigma) for 2 hours. Then cells were fixed with 4%PFA and processed for immunocytochemistry.

Full-thickness skin grafting

Recipient nude mice were anaesthetized using 50mg/ml pentobarbital solution. The back skin was washed with 70% ethanol. The 1.5 cm² square of full-thickness defect was created with scissors. The experiments were approved by the Institutional Animal Care and Use Committee of Shanghai Jiaotong University School of Medicine.

Prior to skin grafting, 100µl AT-Ex or AT-Ex incubated with antibody against GDNF was topical injected into the facial layer of the recipient bed (20µl per sites, 5 equally distributed sites in total) in the AT-Ex group or AT-Ex +Ab group, respectively (n=9 for each group). The same volume of PBS was applied in the control group(n=9). Then the full-thickness skin grafts from newborn C57/B6 mice were grafted onto the null mice to assess the fate of the grafts. Digital photographs were taken to analyze the grafts condition. The graft take was determined by the physical appearance. The histology examinations were also performed to provide evidence for the graft survival.

Histology

The skin samples of the grafts were harvested on postoperative day 9. The rectangle-shaped grafted samples, including the surrounding normal nude mouse skin on both sides, were harvested. The collected tissues were fixed in 4% paraformaldehyde (PFA). Then the samples were embedded in paraffin for paraffin section. Based on the images from H&E sections, the dermal thickness was measured from basement membrane to the hypodermis. The thickness was calculated from 15 randomly selected fields with Photoshop CS4(Abode, CA).

Immunohistochemistry

For paraffin sections, the sections were performed with heat-induced antigen retrieval and permeabilized with 0.1% TritonX-100. After blocking with the 10% normal donkey serum, the sections were incubated overnight at 4°C with primary antibodies. The primary antibodies used in this analysis were as follows: rabbit anti-CD31(ab28364, abcam, USA), mouse anti-K14 (ab7800, abcam, USA), rabbit anti-Ki67 (ab16667, abcam, USA). After washing with 0.5% PBST three times, the sections were incubated with fluorochrome-conjugated secondary antibody (anti-mouse IgG-Alexa 488 and anti-rabbit IgG-Alexa 594) for 1 hour at room temperature, and nuclei were stained by 4',6'-diam idino-2- phenylidole (DAPI). All the slides were viewed with fluorescence microscopy and images were obtained. The apoptosis was assessed by the in situ cell death detection kit (Roche, Tdt-mediated dUTP-biotin nick-end labelling (TUNEL) kit). The vascular density was estimated with CD31 staining by calculating the number of CD31⁺ blood vessels per field. And the average number of blood vessels was assayed.

Statistical analysis

All data collected and analyzed with Statistical Package of the Social Sciences Windows version 12.0 (SPSS, USA). The t-test was used for analyzing the differences between the two groups. All data were

expressed as mean±standard error of the mean. A value of $P<0.05$ was considered statistically significant.

Results

Angiogenic potential of growth factors-rich AT-Ex

The concentrations of growth factors in the AT-Ex were measured with ELISA (Fig. 1A). The GDNF concentration was abundant ($701.0 \pm 12.00\text{pg/ml}$), and the VEGF and bFGF concentration was 147 ± 9 and $119 \pm 2\text{pg/ml}$, respectively. To investigate the angiogenic effect of AT-Ex on HUVECs, the tube formation assay was performed with various concentrations of AT-Ex (negative control, 5%, 15% and 45%) for 6 hours (Fig. 1B). Treatment with AT-Ex significantly promoted the tube formation in a dose-dependent manner (Fig. 1C, 1D, tube number per field NC: 4 ± 0.57 , 5%AT-Ex: 7 ± 0.54 ; 15% AT-Ex 13.3 ± 0.81 and 45% AT-Ex: 17.6 ± 0.78).

Effects of GDNF on AT-Ex-mediated angiogenic capacity *in vitro*

As the effects of VEGF and bFGF on tube formation were well-studied and the levels of GDNF was abundant, we focused on the contributions of GDNF in AT-Ex by pre-incubated the AT-Ex with antibody against the GDNF. Interestingly, compared with the control group, the treatment of HUVECs with 15% AT-Ex (AT-Ex group) or with 15% AT-Ex and GDNF antibody (AT-Ex +Ab group) both significantly promoted the tube formation (Fig. 2A). Compared with the AT-Ex group, treatment of HUVECs with AT-Ex and antibody significantly suppressed the tube formation (Fig. 2B,2C). To further investigated the effects of GDNF in AT-Ex on endothelial cell growth, the MTT and Brdu assay were performed. The cell proliferation was significantly promoted in AT-Ex group and AT-Ex+Ab group, and GDNF antibody partially abolished the stimulation effect of cell proliferation (Fig. 2D-2F).

Effects of AT-Ex on skin grafts *in vivo*

To further confirm the effect of AT-Ex on angiogenic capacity *in vivo* and the potential application of GDNF in plastic and reconstructive surgery, the skin grafts nude mice model was performed. At postoperative day 9, the grafted skin in the control group was still pink, while the grafts in the AT-Ex group or AT-Ex+Ab group had obvious pigment and hairs (Fig. 3A). To evaluate the contribution of GDNF in AT-Ex, the skin grafts were harvested to perform the histological assay. The H&E staining showed that the grafts in all groups showed the typical skin structures, including hair follicle and sebaceous glands formation (Fig. 3B). The skin grafts in the AT-Ex had the thickest dermis and the grafts in the AT-Ex+Ab group showed the thicker dermis than that in the control group (Fig. 3C, 3D).

Effects of GDNF on AT-Ex-mediated angiogenic capacity *in vivo*

As angiogenesis was critical for the skin grafts, we measured the vascular density in the grafts. The vascular density (CD31 positive staining) in the AT-Ex group was significantly higher than that in the control group, and GDNF antibody partially decreased the CD31 positive vascular number (Fig. 4A, 4B).

We also investigated the cell proliferation and apoptosis in the skin grafts, and Ki67 positive cells in the dermis was significantly increased in the AT-Ex group, as expected, addition of the GDNF antibody partially blocked the effect (Fig. 4C, 4D). TUNEL staining showed the AT-Ex didn't influence the apoptosis (Fig. 4E).

Discussion

The adipose tissue has currently gained translational significance as its derived stromal vascular fraction (SVF) and adipose-derived stem cells (ASCs) have been widely known for their therapeutic potential in the field of ischemia diseases and regenerative medicine. Recent cytotherapy strategies using stem cells demonstrated the stem cells promoted wound healing due to their paracrine effects. Previous studies showed the SVF and ASCs can promote the angiogenesis via secreting various growth factors, such as VEGF and bFGF [12]. However, the SVF contains abundant of adipose-derived stem cells, endothelial precursor cells, macrophages and others, the tumorigenic potential of both SVF and ASCs is major concern for clinical applications [13]. Therefore, a safer treatment strategy needs to be developed.

We speculated that the cell-free adipose tissue extract (AT-Ex) is a promising therapy method via its contained growth factors. To address this hypothesis, we first detected several growth factors' levels in AT-Ex using ELISA kit. The results showed that AT-Ex was rich in growth factors, especially GDNF, VEGF and bFGF, in which the level of GDNF was highest ($701.0 \pm 12.00\text{pg/ml}$). Besides, AT-Ex promoted tube formation in a dose-dependent manner. Then we investigated the effect of detailed composition in AT-Ex. VEGF and bFGF are well-known factors which have angiogenic potential [14, 15], while the role of GDNF in adipose tissue-induced angiogenesis are not fully understood. GDNF belongs to the transforming growth factor- β superfamily and has the function of maintaining tem cells survival and promoting their differentiation and proliferation [16]. Moreover, Nakasatomi M et al found that GDNF enhances hepatocyte growth factor-induced tube formation in HUVECs [17]. Consistent with this research, both 15% AT-Ex-treated HUVECs (AT-Ex group) and 15% AT-Ex with GDNF antibody pre-treated HUVECs (AT-Ex+Ab group) had significant angiogenesis *in vitro*. Furthermore, the tube number and cumulative tube length were relatively inhibited in the AT-Ex+Ab group compared with the AT-Ex group. In addition, the further experiments indicated that AT-Ex promoted the proliferation of HUVECs, while the number of HUVECs decreased with the pre-incubation of anti-GDNF antibodies. Therefore, the endothelial cell proliferation and tube formation assay confirmed that AT-Ex possessed the angiogenic potential *in vitro*, and we showed that GDNF played a crucial role in AT-Ex-induced angiogenesis.

Full-thickness skin grafts are common methods for the treatments of wounds. Recently, ASCs had been used clinically to rescue the patient with ischemic fasciocutaneous flap via vascular reconstruction [18], but long-term side effects of stem cell (such as the tumorigenicity) and specific factors are not clear. In present study, we found that AT-Ex significantly increased the dermal thickness of the grafted skin and promoted the regeneration of the hair follicle and its sebaceous glands *in vivo*. More importantly, the effect of AT-Ex was significantly weakened with adding the anti-GDNF antibody. These evidences suggested that GDNF in AT-Ex has the ability to promote dermal condition. To elucidate the underlying

mechanism, we further explored the vascular density of grafted skin in different treatment groups. As expected, the CD31 positive vascular number in the dermis in the AT-Ex group was significantly more than in the control group and the AT-Ex+Ab group and GDNF contributed greatly to the AT-Ex effect in improvement the grafted skin condition by promoting angiogenesis *in vivo*. In this study, we clarify the effect of GDNF derived from AT-Ex on skin graft.

Conclusion

The present study showed the growth factor-rich AT-Ex possessed the angiogenic potential *in vitro*, which is partially blocked by pre-incubation with anti-GDNF antibody. The AT-Ex provides a cell-free therapeutic method to promote angiogenesis and PDGF contributes mostly to the AT-Ex effects in improvement the skin grafts condition *in vivo*.

Declarations

Conflict of interest: None of Renpeng Zhou, Chuang Yin, Weiwei Bian, Chen Wang have any financial interest in any products, devices or drugs used in the manuscript. There is no conflict of interest related to any commercial associations or financial relationships.

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Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Figures

Figure 1

The effect of AT-Ex on angiogenesis in vitro. (A) The concentrations of growth factors in the AT-Ex samples. (B) Phase-contrast image demonstrating tube formation of HUVECs and (C, D) Quantitative analysis. *, statistical significance. Scale bar=500μm.

Figure 2

The effect of GDNF in AT-Ex on angiogenesis in vitro. (A) Phase-contrast image demonstrating that depletion of GDNF partly inhibited AT-Ex-induced tube formation, Scale bar=50μm, and (B, C) Quantitative analysis. (D) The MTT assay and (E,F) The BrdU staining and quantitative analysis showing that AT-Ex stimulated HUVECs cell proliferation whereas addition of GDNF antibody attenuated the effect . Scale bar=50μm. *, statistical significance.

Figure 3

Effect of GDNF in AT-Ex on full-thickness skin grafts (A) Representative photos of skin grafts in the control group, AT-Ex treated group and AT-Ex+ Ab group. (B) The representative panoramic view (40 $\times$) of skin graft section in each group. The black dashed line indicated the approximate border between the grafts and normal nude skin(recipient). (C) The representative micrographs showing thicker dermis in the AT-Ex

treated group. e, epidermis; d, dermis; p, panniculus carnosus. Scale bar=100μm. (D) Quantitative analysis of dermal thickness. *, statistical significance.

Figure 4

Immunohistochemistry comparison between the grafted skins. (A) The CD31 staining and (B) Quantitative analysis showing that compared to control, AT-Ex-treated group had significantly more CD31

staining per field, whereas AT-Ex+ Ab group partly abolished the effect. (C) The dermal proliferation and (D) Quantitative analysis. (E) The apoptosis in dermis *, statistical significance. Scale bar=50µm.