Laser Doppler imaging evaluation of adipogenesis after adipose tissue-derived stem cell implantation

Ran Ito, M.D.*[1], Naoki Morimoto, M.D., Ph.D.[2], Shigehiko Suzuki, M.D., Ph.D.[3]

[1] *Department of Plastic Surgery, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA*

[2.] *Department of Plastic and Reconstructive Surgery, Kansai Medical University, Osaka, Japan.*

[3.] *Department of Plastic and Reconstructive Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan.*

**Corresponding author: Ran Ito, MD, 1. Department of Plastic Surgery, Unit 1488, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA. E-mail: ranito.1978@gmail.com, Phone: +1-832-314-6514, Fax: +1-713-563-8041*

2. Department of Plastic and Reconstructive Surgery, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Syogoin, Sakyo-ku, Kyoto, 6068507, Japan. E-mail: ran_ito@kuhp.kyoto-u.ac.jp, Phone: +81-75-751-3613, Fax: +81-75-751-4340

Second corresponding author: Naoki Morimoto, MD, PhD, Department of Plastic and Reconstructive Surgery, Kansai Medical University, 10-15 Fumizono, Moriguchi, Osaka, 570-8507, Japan. E-mail: mnaoki22@kuhp.kyoto-u.ac.jp, Phone: +81-6-6992-1001, Fax: +81-75-751-4340

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The Field of my research: tissue engineering, biomaterials, artificial skin

Abstract

We need a better method of assessing adipose tissue formation non-invasively than the current one, which requires resecting tissue samples in vivo. The aim of this study was to establish a system to evaluate adipogenesis using laser Doppler imaging (LDI) to measure subcutaneous microcirculation.

CGSs containing adipose stem cells with or without bFGF were implanted in the backs of 30 mice. Once per week after implantation, LDI was used to evaluate blood flow at the implantation site. The implantation sites were resected at 6 weeks, and the tissue was weighed.

Six weeks after implantation, LDI showed that mice who received CGS with 1 $\mu g/cm^2$ bFGF had the greatest mean blood flow, and these mice had the heaviest resected specimens, which contained the most newly formed adipose tissue. The findings with LDI and the weight findings were compatible.

This study indicates that LDI could be used to assess subcutaneous tissue regeneration in vivo in a real-time, non-invasive manner.

Introduction

Combining cells with an artificial matrix consisting of a scaffold and growth factors enables the generation of various tissues [1, 2], including adipose tissue [3, 4]. An area of refinement in tissue engineering has been the incorporation of sustained-release growth factors. Tsuji *et al*. (2009), in a study of adipose tissue regeneration using collagen scaffolds and gelatin microspheres that gradually release basic fibroblast growth factor (bFGF) [5], found that the sustained-release bFGF more effectively promoted adipose tissue regeneration than aqueous bFGF [6].

My group successfully developed a novel scaffold, a collagen/gelatin sponge (CGS) that provides a sustained release of bFGF. We confirmed that bFGF is released gradually as the CGS biodegrades and CGS impregnated with bFGF promote greater adipose tissue formation in vivo. 6 weeks after implantation than CGS without bFGF [7]. However, to facilitate animal studies and enable translation into the clinic, we needed a better method of assessing adipose tissue formation than the current one, which requires resecting tissue samples. Recently we have succeeded in non-invasive detection of

neovascularization in a grafted bilayered artificial dermis using laser Doppler imaging (LDI). The laser Doppler imaging also enabled to evaluate the maturation of formed dermis-like tissue [8].

The aim of this study was to establish a system to evaluate adipogenesis using LDI to measure subcutaneous microcirculation. If successful, LDI could be used to estimate subcutaneous tissue regeneration non-invasively. To my knowledge, there are no other reports of a method to measure adipogenesis and angiogenesis in a real-time, non-invasive manner.

Materials and Methods

We prepared CGSs and isolated human adipose tissue–derived stem cells (ASCs) as described previously [9]. CGSs (each 8 mm in diameter and 3 mm thick) were prepared and placed in 10-cm tissue culture dishes (BD Falcon; BD Biosciences, Bedford, MA, USA). CGSs were then impregnated with normal saline solution (NSS; Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) and with different concentrations of bFGF (1 and 14 μg/cm^2). Next, CGSs were incubated for 1 h in a CO_2 incubator at 37°C. Human ASCs (5.0×10^4) were suspended in 50 μl of Medium 199 (Invitrogen, Tokyo, Japan) and seeded on CGSs in the tissue culture dishes, and the seeded CGSs were incubated in a CO_2 incubator overnight as described previously [7].

Animal experiments were reviewed by the Committee on the Ethics of Animal Experiments (Faculty of Medicine, Kyoto University, Kyoto, Japan). Institutional guidelines for the care and use of laboratory animals have been observed. Thirty 7-week-old male BALB/c nude mice (three groups of 10; Shimizu Laboratory Supplies Co., Ltd., Kyoto, Japan) were used in this study. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg; Dainippon Sumitomo Pharmaceutical Co., Ltd., Osaka, Japan). The ASC-seeded CGSs were implanted in the subcutis in the backs of the mice after each group received CGSs impregnated with NSS, 1 μg/cm^2 bFGF, or 14 μg/cm^2 bFGF (2 sites per mouse).

The CGSs and newly formed adipose tissue were resected at 3 in half mice and 6 weeks after

implantation in others. Each specimen was weighed using an electronic scale. The specimens were fixed with 20% formalin fluid, paraffin-embedded, and sectioned, and then stained with hematoxylin and eosin. Using 4 μm-thick paraffin-embedded sections, immunohistological staining with von Willebrand factor was performed to detect newly formed capillaries in the CGS. After sections had been dewaxed and rehydrated, they were incubated in PBS with 0.1% trypsin (Vector Laboratories Inc., Burlingame, CA, USA) for 15 minutes at 37°C. Anti-Von Willebrand factor rabbit polyclonal antibody (Dako Japan, Tokyo, Japan) was used as the primary antibody (1: 250 dilution), and En Vision +Rabbit/HRP (Dako Japan) was used as the secondary antibody. These sections were exposed to DAB (3-3'-diaminobenzidine-4HCl) (Dako Japan) for 2 minutes at room temperature, and counterstaining was performed with hematoxylin.

Once per week for 6 weeks after the implantations, blood flow at the implantation sites was evaluated using LDI (moorLDI2-IR; Moor Instruments Ltd., Axminster, Devon, UK). Mean blood flow of 2 implanted sites per mouse was calculated using the software provided with the device (Moor LDI Image Review version 2.3; Moor Instruments Ltd.). LDI is a technique to determine the blood flow as flux (perfusion unit) over a skin area without direct contact with the skin surface using laser beam (785nm [8]). Flux is a relative measure of blood flow represents the average speed and number of blood cells about 1-1.5mm in depth [10].

All data are expressed as means ± standard errors. An analysis of variance and Fisher's protected least significant difference test were used to assess differences between the three groups. $P < 0.05$ was considered statistically significant. Microsoft Excel software (Microsoft Corp., Redmond, WA, USA) with the Statcel software add-in (OMS Publishing Inc., Tokyo, Japan) was used for all statistical analyses.

Results

The gross appearance of newly formed adipose tissue 3 and 6 weeks after the implantation of the CGSs is shown in Figure 1A and 1B. Newly formed adipose tissue containing CGS with 1 μg/cm^2 bFGF weighed significantly more than tissue containing CGS with 14 μg/cm^2 bFGF or NSS at 3 and 6 weeks

(Figure 1C). Light microphotographs of histological sections of implanted sites 6 weeks after implantation of CGS incorporating ASCs containing different concentrations of bFGF and NSS are shown in Figure 2A. The adipose tissue was regenerated in the bFGF-administered group. Light microphotographs of newly formed adipose tissue at 6 weeks that had been immunohistologically stained with von Willebrand factor are shown in Figure 2B. In the newly formed adipose tissue area in the bFGF-administered group, the vWf-positive capillaries were stained. Conversely, in the bFGF non-administered group, no capillaries were detected in the adipose tissue.

Representative two-dimensional images of blood flow and mean blood flow at the implanted sites over time are shown in Figure 3A and 3B. Three and 6 weeks after implantation, LDI showed that mice who received CGS with 1 $\mu g/cm^2$ bFGF had the greatest mean blood flow (Figure 3C). Thus, the LDI findings were compatible with the weight findings.

Discussion

LDI has been investigated and used since 1993 to assess burn wound healing by evaluating skin microcirculation. LDI detects changes in blood flow by measuring the Doppler shift in a laser beam that occurs when the beam is reflected by moving red blood cells in the cutaneous microcirculation at a depth of about 1 to 1.5 mm [11-13]. As clinical interest in soft tissue regeneration techniques increases, adipose tissue engineering will continue to improve. Although other methods such as ultrasonography, computed tomography, and magnetic resonance imaging can measure soft tissue regeneration, the most reliable methods to evaluate subcutaneous neovascularization seem to be histologic and immunohistologic staining of capillaries [7]; however, real-time, non-invasive, quantified measurement of blood flow would be ideal.

LDI non-invasively detected the blood flow in skin, CGSs and subcutaneous tissue around implanted CGSs in mice who received implants of CGS containing ASCs with or without bFGF. The greatest blood flow was seen in the group who had the heaviest resected samples—those with the most newly formed adipose tissue. This group had received CGS with 1 $\mu g/cm^2$ bFGF. Mice who received

CGS without bFGF had the least blood flow on LDI and the lightest resected specimens. There was no obvious difference skin and subcutaneous tissue around CGS implants in gross appearance and H&E staining sections, we confirmed the increase of capillaries in CGS using the immunohistological staining. It indicated that the gross area of capillaries in CGS 6 weeks after implantation was largest in the group impregnated with 1 $\mu g/cm^2$ of bFGF. This suggests that LDI have the potential to evaluate subcutaneous tissue regeneration non-invasively.

LDI detected differences in blood flow between groups by 3 weeks after implantation, and these differences persisted through 6 weeks. However, LDI findings did not change substantially between 3 and 6 weeks, even though the mean weight of the resected specimens containing newly formed tissue was greater at 6 weeks than at 3 weeks. These findings suggest that LDI could predict whether adipose tissue would be formed or not in the earlier phase after implantation.

Further work is needed, but despite the thicker skin and subcutis in humans, LDI holds promise as a real-time, non-invasive technique for detecting perfusion as a marker of subcutaneous tissue regeneration in humans.

Conclusion

LDI is a desirable measurement of blood flow real-time, non-invasive and quantified for subcutaneous tissue regeneration in vivo.

Acknowledgements

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure 1

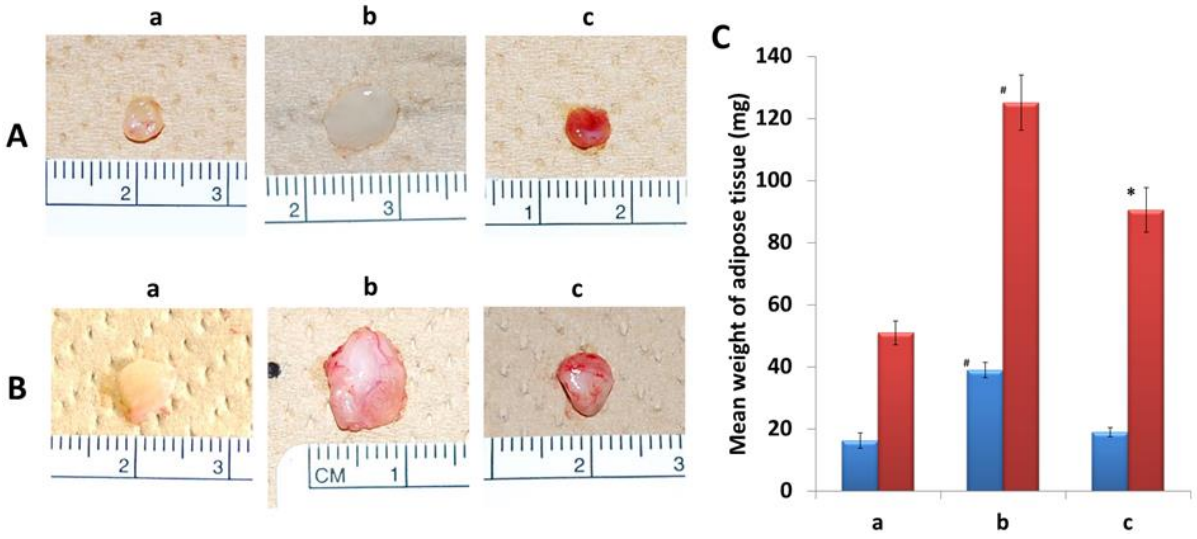

Figure 1. (A and B) Appearance of newly formed adipose tissue 3 (A) and 6 (B) weeks after implantation of CGSs incorporating ASCs and impregnated with NSS (a), 1 μg/cm^2 bFGF (b), or 14 μg/cm^2 bFGF (c). Ruler is in centimeters. (C) Mean weight of resected CGS and newly formed adipose tissue 3 (blue bars) and 6 (red bars) weeks after implantation of CGSs incorporating ASCs impregnated with NSS (a), 1 μg/cm^2 bFGF (b), or 14 μg/cm^2 bFGF (c). $^{\#}p < 0.01$ compared with NSS and 14 μg/cm^2 of bFGF; $*p < 0.01$ compared with NSS.

Figure 2

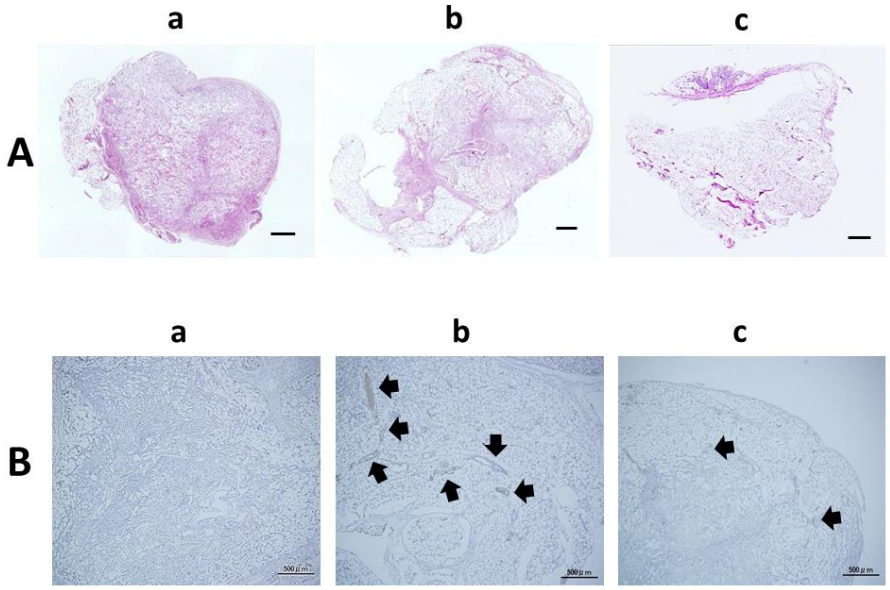

Figure 2. (A) Light microphotographs of histological sections of the implanted site six weeks after implantation of CGS incorporating ASCs impregnated with NSS (a), 1 μg/cm^2 of bFGF (b), 14 μg/cm^2 of bFGF (c). Scale bar: 1 mm. (B) Neovascularization immunostained with von Willebrand Factor in CGS (six weeks after implantation). CGS treated with NSS (a), 1 μg/cm^2 of bFGF (b), 14 μg/cm^2 of bFGF (c). Black arrow indicates newly formed capillaries.

Figure 3

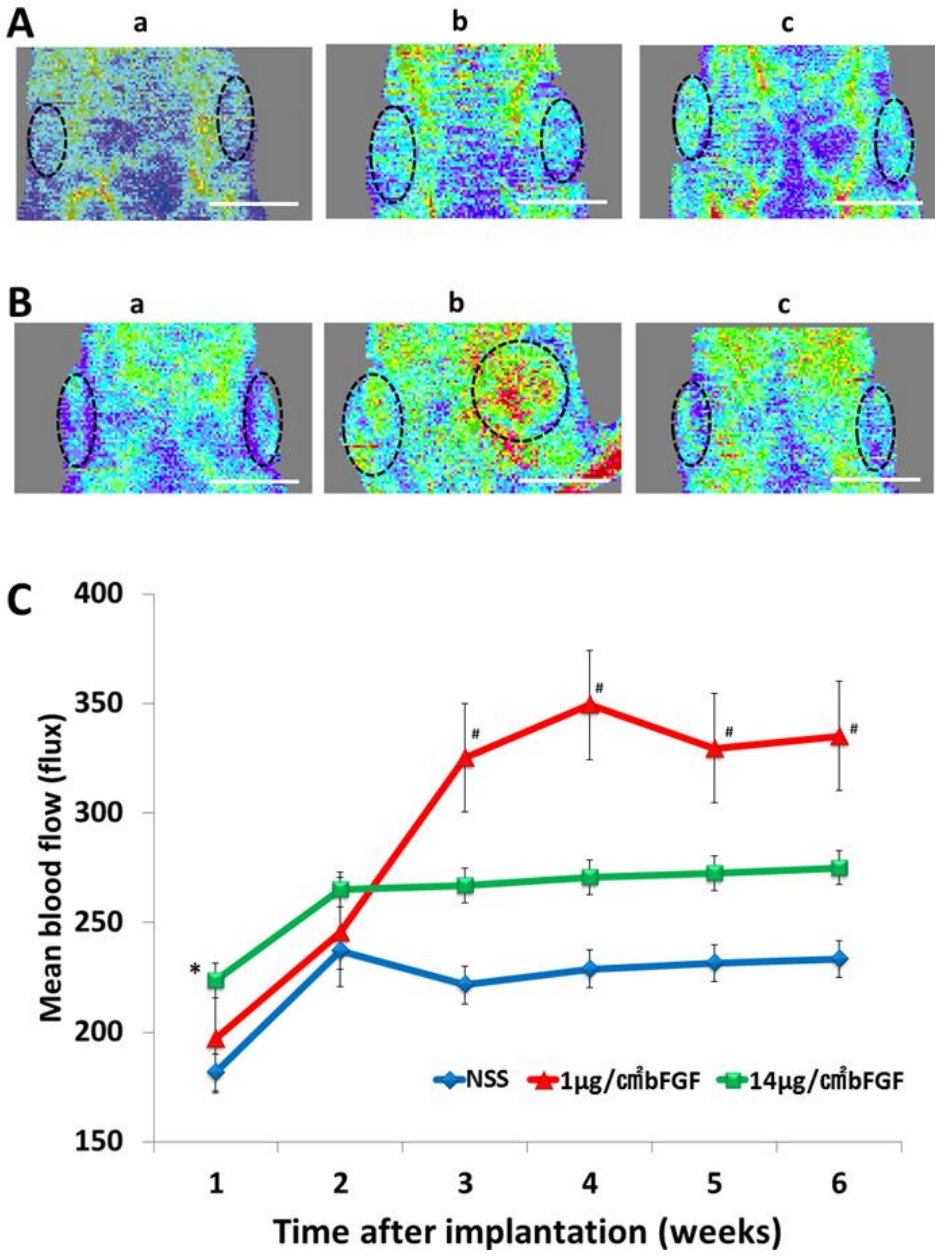

Figure 3. (A and B) LDI appearance of implantation sites 3 (A) and 6 (B) weeks after implantation of CGSs incorporating ASCs impregnated with NSS (a), 1 μg/cm^2 bFGF (b), or 14 μg/cm^2 bFGF (c). Scale bar: 1 cm. The implanted CGSs are encircled by a broken line. (C) Mean blood flow at the implantation site. $^{\#}p < 0.01$ compared with NSS and 14 μg/cm^2 bFGF; $*p < 0.01$ compared with NSS.