이학석사 학위논문

Enhanced effect of secondary administration of adipose-derived stem cells concurrent with fat grafting

지방줄기세포 기반 지방이식술에서 줄기세포의 2차 주입에 따른 생존율 향상

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의과대학 줄기세포생물학

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이 논문을 이학석사 학위논문으로 제출함

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Abstract

Enhanced effect of secondary administration of adipose-derived stem cells concurrent with fat grafting

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Autologous fat grafting is commonly used in plastic and aesthetic surgery. However, because of the rapid absorption of grafted fat, predicting its long-term effect is difficult. Therefore, cell-assisted lipotransfer, a fat graft combined with adipose-derived stem cells, has been introduced to overcome these disadvantages. Furthermore, the efficacy of cell-assisted lipotransfer has been proven in preclinical and clinical studies.

In our previous study, we employed a modified cell-assisted lipotransfer animal model to demonstrate the effects of intravenously injected adipose-derived stem cells in fat grafting for the first time. However, a protocol for the intravenous administration of adipose-derived stem cells in fat grafting has not been established. In our present work, the effects of a secondary intravenous injection of adipose-derived stem cells on fat grafting were investigated.

In this study, wild-type C57BL/6J (B6) mice were employed as both donors and recipients of grafted fat. Adipose-derived stem cells were harvested from green fluorescent protein and DsRed B6 mice. The recipient mice were divided into three groups: SI group (single injection), RI1 group (repeated injection with a 1-week interval), and RI2 group (repeated injection with a 2-week interval). All groups received intravenous injections of green fluorescent protein-adipose-derived stem cells immediately after fat grafting. The RI1 and RI2 groups received repeated intravenous injections of DsRed adipose-derived stem cells at 1 and 2 weeks, respectively, after fat grafting. After final intravenous injection of adipose-derived stem cells, the stem cells were tracked by an in vivo imaging system. The grafted fat was observed by immunofluorescent staining at postoperative week 3. The grafted fat volume was measured using micro-computed tomography. Gene expression was analyzed by real-time polymerase chain reaction.

The result showed that, in all three groups, green fluorescent protein and DsRed adipose-derived stem cells were homing to the graft site. The *Sdf-1* gene, a stem cell homing gene, was shown to be more expressed in

grafted fat than in other fat tissue. The *Cxcr4* gene, a receptor for Sdf-1, was shown to be more abundant in adipose-derived stem cells than in adipose tissue. We concluded that *Sdf-1* from grafted fat recruited the stem cells expressing the Cxcr4 receptor via chemoattraction toward a gradient of *Sdf-1*. The RI2 group has the highest retention of graft volume and vascular density, followed by the RI1 and the SI groups. Angiogenesis genes, *Vegf* and *Fgf2*, were also expressed at the highest levels in the RI2 group, followed by the RI1 and SI groups.

Secondarily injected DsRed adipose-derived stem cells were recruited to the grafted fat, consequently, higher graft volume and vascular density were retained. The grafted fat volume and vascular density of the RI2 group were higher than those of the SI and RI1 groups. Thus, a 2-week interval secondary intravenous injection of adipose-derived stem cells improved the effect of adipose-derived stem cell enrichment on fat grafting. Our findings improve clinical practices and boost the therapeutic potential of cell-assisted lipotransfer.

Keyword: Adipocytes; Cell-assisted lipotransfer, Adipose-derived Stem Cells, Stem cell transplantation; Intravenous injections

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1. Introduction

Autologous fat grafting has been widely accepted as a primary surgical procedure for soft tissue augmentation and reconstruction. Despite various advantages of the procedure, unpredictable long-term outcomes related to poor graft retention are critical drawbacks and limits. Cell-assisted lipotransfer (Fig.1.) is a fat grafting technique using adipose tissue mixed with stromal vascular fraction cells or adipose-derived stem cells.[1] This technique enables the conversion of stem cell-poor aspirated fat to stem cell-rich fat prior to grafting. In 2013, a clinical trial was conducted, 13 patients were grafted fat or fat with ASCs in the arm, after 121 days, and the MRI data showed when only fat grafting was performed, 16.3% of fat rem ained, whereas grafted with ASCs showed 81% of fat remaining[2]. Thus, cell-assisted lipotransfer has been clinically applied and studied for aesthetic and reconstructive reasons.

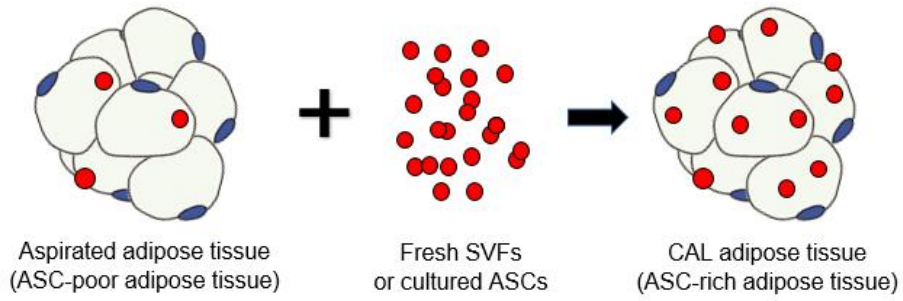

Fig.1. Schematic illustration of cell-assisted lipotransfer (CAL).

To elucidate the underlying mechanism of cell-assisted lipotransfer in terms of improved retention of the grafted fat, we previously generated an animal model of cell-assisted lipotransfer using transgenic mice to trace the grafted fat and enriched adipose-derived stem cells.[3] Tracing analyses indicated that adipose-derived stem cell supplementation promoted angiogenesis and adipogenesis in both donor and recipient, leading to a favorable microenvironment for the uptake of grafted fat. Moreover, a subset of donor adipose-derived stem cells can differentiate into blood vessels and fat, partially contributing to grafted fat retention. In a subsequent study, in addition to the direct delivery of adipose-derived stem cells mixed with the fat to be grafted, adipose-derived stem cells were intra-venously delivered concurrently with fat grafting (Fig.2.).[4] The intravenous delivery of adipose-derived stem cells, like the direct delivery of adipose-derived stem cells, also showed superior

outcomes of fat graft retention, but there were only few blood vessels and fat that differentiated from donor adipose-derived stem cells, which indicates that paracrine action rather than direct differentiation is the main mechanism of fat graft retention.

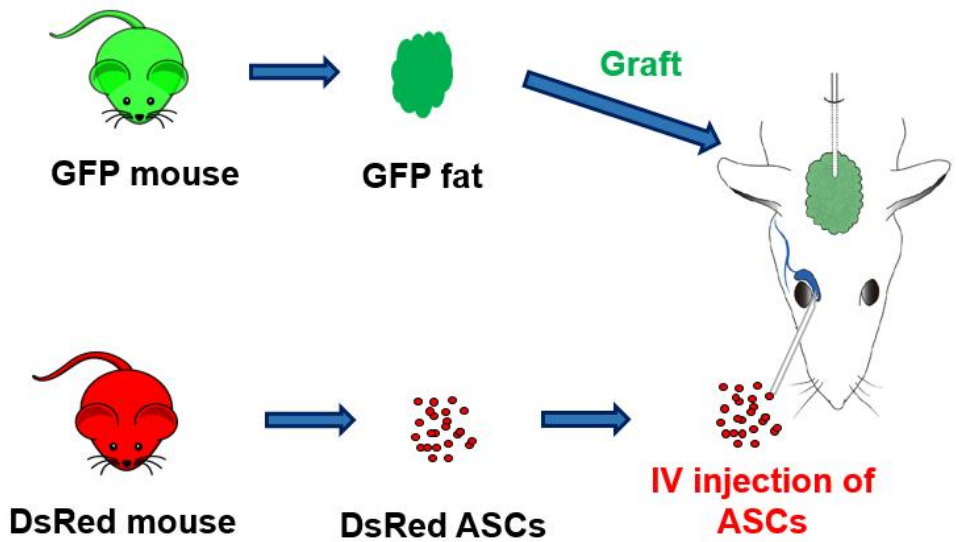

Fig.2. Schematic illustration of our previous study. We modified introduced animal model of CAL using two transgenic reporter mouse. GFP gat tissue was grafted to subperiosteal plane. On the same day, DsRed adipose-derived stem cells were injected intravenously. According to the study, we confirmed that intravenous injection of ASCs could improve the fat graft retention.

We have confirmed that intravenous injection of adipose-derived stem cells has a positive effect on the survival rate of fat grafts. The intravenous delivery of adipose-derived stem cells, including multiple injections with various combinations of cell numbers and intervals, has several advantages. Multiple injections of

stem cells are clinically challenging in terms of efficacy, safety, and cost. Nonetheless, prior research using animal models in various diseases and settings such as diabetes mellitus,[5] spinal cord injury,[6] ischemic cardiomyopathy,[7] and vascularized composite allotransplantation[8] suggested the potential for the clinical application of multiple injections of mesenchymal stem cells. However, the efficacy of multiple intravenous injections of adipose-derived stem cells for fat grafting has not yet been studied. In this study, we investigated the effects of a secondary intravenous injection of adipose-derived stem cells on fat grafting.

2. Materials and Methods

2.1 Animal model

The experimental procedures and animal care in this study were approved by the Institutional Animal Care and Use Committee (No. SNU-180611-1-3) of Seoul National University. All the mice were bred in specific pathogen-free animal facilities at the same institute. Mice were anesthetized with 3% isoflurane in 100% oxygen at a delivery rate of 5 L/min for induction until loss of righting reflex.

The animal model of cell-assisted lipotransfer used in a previous study, with two transgenic reporter mouse strains expressing different fluorescent signals,[4] was modified. Green fluorescent protein and DsRed adipose-derived stem cells were harvested from green fluorescent protein[9] and DsRed C57BL/6J (B6) mice[10], respectively. Wild-type B6 mice were used as donors for the grafted fat and as recipients.

For a primary culture of green fluorescent protein and DsRed adipose-derived stem cells, murine inguinal adipose tissues were digested with 0.2% collagenase type I (Worthington Biochemical Corp., Lakewood, N.J.) at 37°C for 30 min and then centrifuged at 1200 rpm for 3 min. The cell pellet was resuspended in Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F12; Gibco Life

Technologies, Carlsbad, Calif.) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotic agent (Gibco Life Technologies) and then seeded in cell culture dishes until passages 2 to 5.

In the graft experiments, 100 μl of wild-type fat tissue was injected into a recipient mouse at the supraperiosteal plane of the skull using a 16-gauge needle. Next, 1.0×10^5 resuspended and filtered green fluorescent protein adipose-derived stem cells in 50 μl of phosphate-buffered saline were injected by a retrobulbar approach through the retro-orbital sinus. After that, we divided the mice into three groups: a control group that received a single injection of green fluorescent protein adipose-derived stem cells (SI group) (n=10), and other groups with repeated injections of DsRed adipose-derived stem cells (1.0×10^5) at postoperative week 1 (RI1 group) (n=10) and 2 (RI2 group) (n=11) (Fig. 1). Grafted fat volume was measured using micro-computed tomography at postoperative week 8.

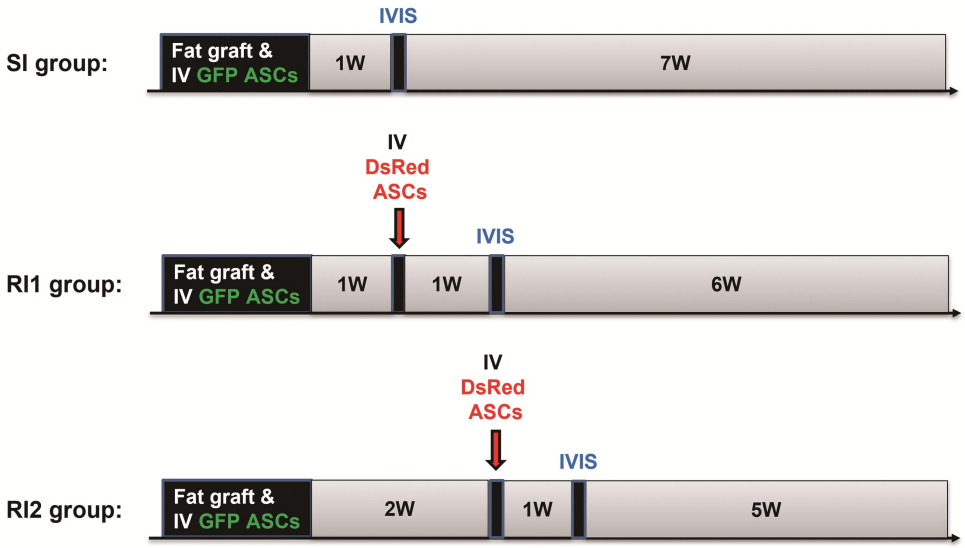

Fig. 3. The timeline of the present study. Donor fat (100 μl) from wild-type C57BL/6J mice was grafted into the supraperiosteal plane of the skull, followed by intravenous injection of 1.0×10^5 green fluorescent protein (GFP) adipose-derived stem cells (ASCs). SI group received single injection of GFP adipose-derived stem cells, groups with repeated intravenous injection of DsRed adipose-derived stem cells (1.0×10^5) at postoperative week 1 (RI1 group) and 2 (RI2 group). Bioluminescent imaging was taken at one week after the last injection of adipose-derived stem cells by in vivo imaging system (IVIS).

2.2 Histomorphometric analysis

At postoperative weeks 3 and 8, grafted fat, subcutaneous inguinal and visceral epididymal fat were harvested. All the tissues were fixed for 1 h with 1% paraformaldehyde in phosphate-buffered saline at room temperature. For immunofluorescent staining of adipose tissues, the whole-mount staining method was used.[11]

Adipose tissues were blocked with 5% goat serum (Jackson

ImmunoResearch, West Grove, Pa.) in phosphate-buffered saline with 0.03% Triton X-100 for 1 h, incubated overnight at 4°C with either a guinea pig anti-mouse perilipin antibody (20R-PP004; Fitzgerald, Acton, Mass.) or an armenian hamster anti-mouse CD31 antibody (clone 2H8; Merck Millipore, Darmstadt, Germany), and then washed with phosphate-buffered saline with 0.03% Triton X-100. Next, the tissues were incubated with either a goat Cy5-conjugated anti-guinea pig or an anti-armenian hamster antibody (Jackson ImmunoResearch) for 2 h at room temperature. Immunofluorescent staining images were visualized using a confocal microscope (LSM 800; Zeiss, Oberkochen, Germany). Morphometric analyses were conducted using the ImageJ (Fiji) software (National Institutes of Health, Bethesda, Md.).

2.3 In vivo imaging study

Mice were examined 1 week after the last adipose-derived stem cell injection in all the groups. An in vivo imaging system (IVIS Lumina II Imaging System; PerkinElmer, Waltham, Mass.) was used according to the manufacturer's instructions to detect fluorescent signals from green fluorescent protein and DsRed adipose-derived stem cells. Images of mice were taken with a 20-cm field of view and an exposure time of 1 s. The excitation and emission filters were set for the green fluorescent protein signals from 480 to 520 nm and for the

DsRed signal from 560 to 620 nm.

2.4 Gene expression analysis

Total RNA from inguinal and grafted fat at postoperative week 3 was extracted using a RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was performed using TOPscript™ RT DryMIX (Enzynomics, Inc., Daejeon, Korea) and quantitative real-time polymerase chain reaction was performed using the SYBR Green system (Enzynomics, Inc.). Vascular endothelial growth factor (*Vegf*)[12] and fibroblast growth factor 2 (*Fgf2*)[13] were used as potent angiogenic markers. For stem cell homing, the stromal-derived factor-1 (*Sdf-1*) and C-X-C chemokine receptor type 4 (*Cxcr4*) axis was analysed.[14] Housekeeping gene (*Gapdh*) was used as an internal reference. Primer sequences used in this study are listed in Table 1.

2.5 Statistical analysis

All results are shown as mean ± standard deviation. Statistically significant differences in the mean values ($p < 0.05$) were analyzed using the Mann-Whitney *U* test or one-way analysis of variance with the Bonferroni post hoc test using IBM SPSS Version 25 (IBM Corp., Armonk, N.Y.).

Table 1. Primer sequences for quantitative real-time polymerase chain reaction

Gene		Sequence
Cxcr4	Forward	5'-TCA TCA AGC AAG GGT GTG AG-3'
	Reverse	5'-GGC TCC AAG GAAAGC ATA GA-3'
Fgf2	Forward	5'-GACCCACACGTCAAACTACA-3'
	Reverse	5'-GCCGTCCATCTTCCTTCATAG-3'
Gapdh	Forward	5'-GTCGTGGAGTCTACTGGTGTCTTCAC-3'
	Reverse	5'-GTTGTCATATTTCTCGTGGTTCACACCC-3'
Sdf-1	Forward	5´-CAGAGCCAACGTCAAGCA-3´
	Reverse	5´-AGGTACTCTTGGATCCAC-3´
Vegf	Forward	5'-TATTGGTGACTGAATGCGGCGGTG-3'
	Reverse	5'-ATGTACACGATGCCATGCTGGTCAC-3'

3. Results

3.1 Distribution of intravenously injected green fluorescent protein and DsRed adipose-derived stem cells

During the study period, all the mice survived without any critical complications. We investigated the presence and distribution of intravenously injected green fluorescent protein and DsRed adipose-derived stem cells (Fig. 2) by using an in vivo imaging system.

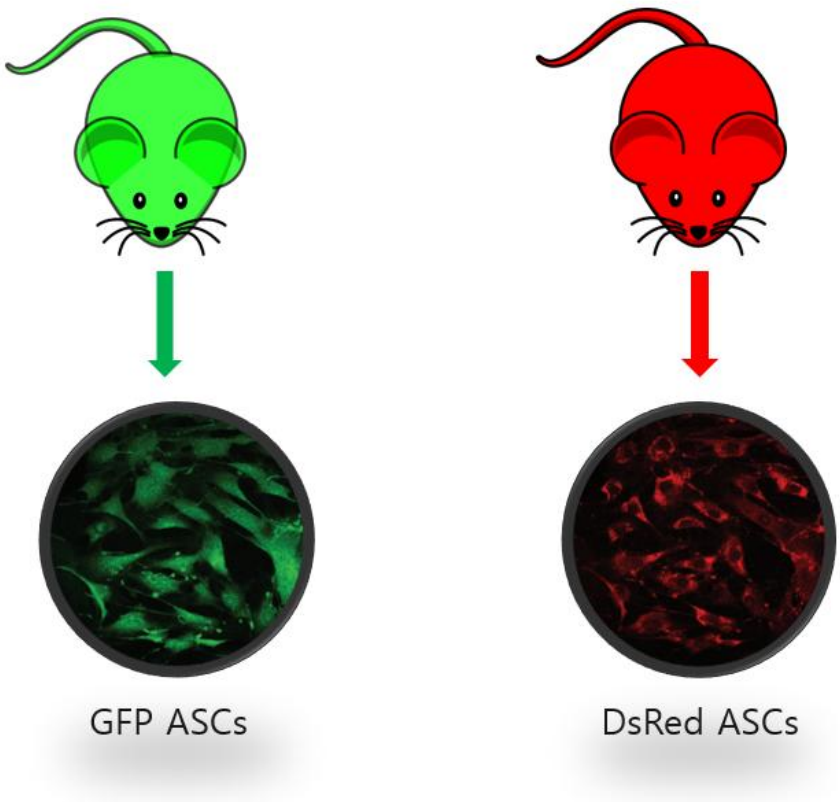

Figure. 4. Fluorescence microscopic image of green fluorescent protein and DsRed adipose-derived stem cells at passage 3.

In all groups, fluorescent signals from green fluorescent protein and DsRed adipose-derived stem cells were detected mainly in the grafted fat (Fig. 3).

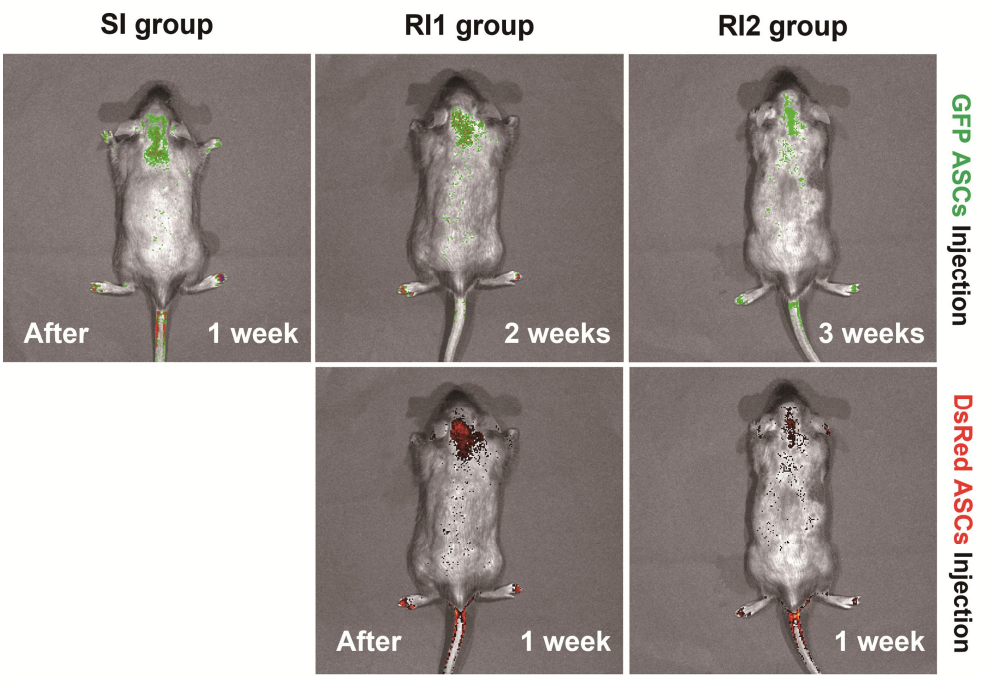

Fig. 5. Bioluminescent imaging of green fluorescent protein (GFP) and DsRed adipose-derived stem cells with in vivo imaging system. In the SI group, the image was taken 1 week after injection of GFP adipose-derived stem cells. In the RI1 group, the image was taken 2 weeks and 1 week after injections of GFP and DsRed adipose-derived stem cells, respectively. In the RI2 group, the image was taken 3 weeks and 1 week after injections of GFP and DsRed adipose-derived stem cells, respectively. In all groups, GFP and DsRed adipose-derived stem cells were mainly detected on the grafted area.

Similar to the bioluminescent imaging results, green fluorescent protein and DsRed adipose-derived stem cells were observed in the grafted fat using immunofluorescent staining. (Fig. 4).

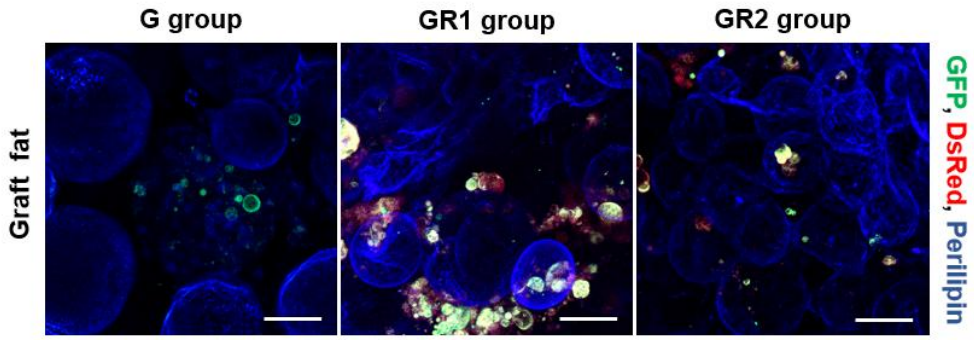

Fig. 6. Immunofluorescent staining of grafted fat at postoperative week 3. Green fluorescent protein (GFP) and/or DsRed adipose-derived stem cells were observed in the grafted fat. Scale bars = 20 μm.

In inguinal fat and epididymal fat, neither green fluorescent protein nor DsRed adipose-derived stem cells were detected by immunofluorescent staining. (Fig. 5)

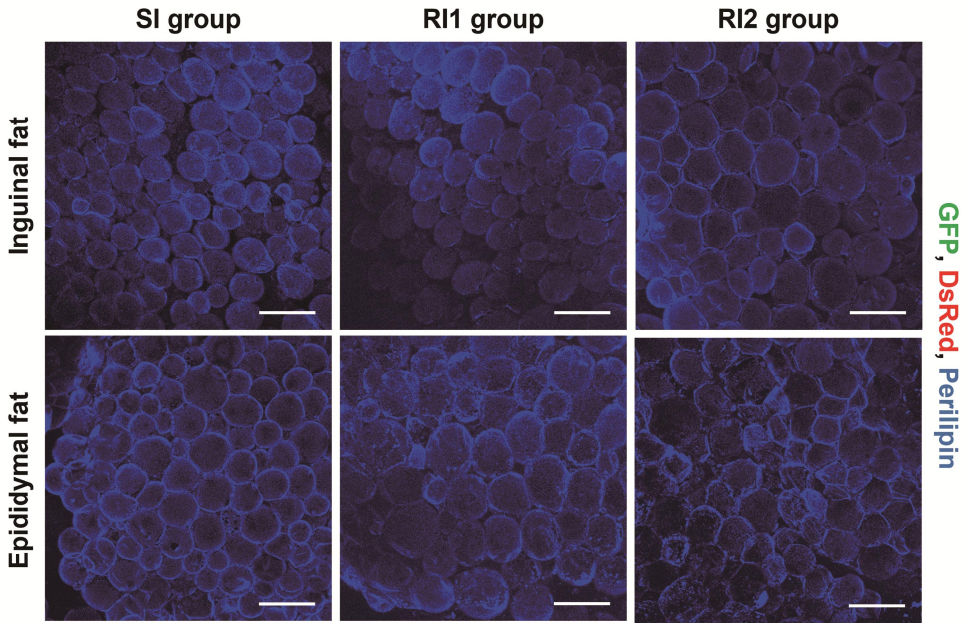

Fig. 7. Inguinal fat and Epididymal fat immunofluorescent staining at week 3. No fluorescent signals were observed in epididymal fat and inguinal fat at postoperative week 3. Scale bars=100 μm.

These findings indicated that not only green fluorescent protein adipose-derived stem cells but also DsRed adipose-derived stem cells injected at intervals of 1 or 2 weeks after fat grafting were recruited to the graft site.

3.2 Homing of adipose-derived stem cells via the stromal-derived factor-1/C-X-C chemokine receptor type 4 axis

To explore the mechanism underlying adipose-derived stem cell homing, we investigated the involvement of the Sdf-1/Cxcr4 axis in our model. In the third week after green fluorescent protein adipose-derived stem cell injection, we measured the expression of the *Sdf-1* gene in the two types of fat. The expression of *Sdf-1* gene was significantly higher in the grafted fat than in the inguinal fat (inguinal fat: median = 1.69, interquartile range = 1.31 to 2.08; grafted fat: median = 18.75, interquartile range = 14.66 to 22.56; $p < 0.05$) (Fig. 6). This result indicated that grafted fat, rather than inguinal fat, could be a potential source of Sdf-1. To confirm the expression of *Cxcr4* gene in adipose-derived stem cells, we measured and compared the gene expression of *Cxcr4* in fat (inguinal fat) and adipose-derived stem cells. The expression of *Cxcr4* gene was significantly higher in adipose-derived stem cells than in normal fat (fat: median = 1.70, interquartile range = 1.00 to 2.25; adipose-derived stem cells: median = 13.10, interquartile range = 10.81 to 17.15; $p < 0.05$) (Fig. 6). Taken together, these data indicate that both green fluorescent protein and DsRed adipose-derived stem cells can be recruited to grafted fat and not to other adipose tissues via Sdf-1/Cxcr4 axis.

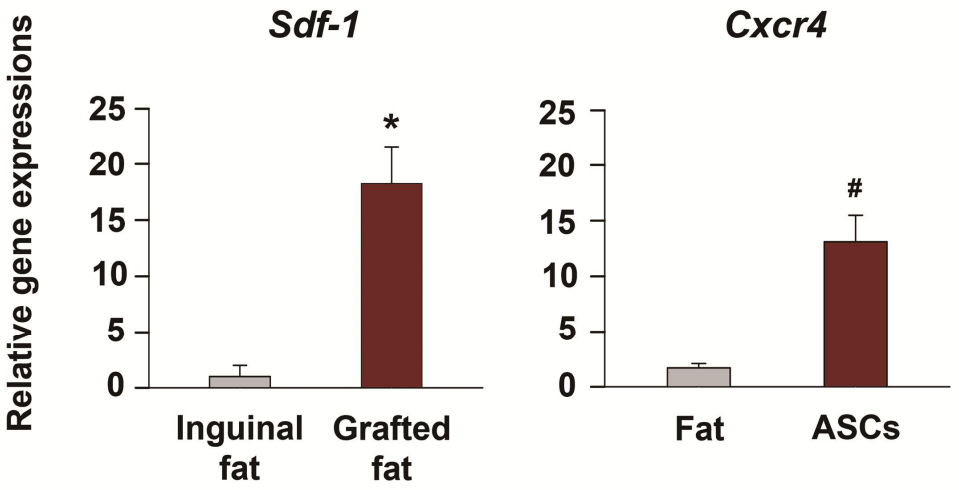

Fig. 8. Gene expression analyses of stromal-derived factor-1 (*Sdf1*) and C-X-C chemokine receptor type 4 (*Cxcr4*) by quantitative real-time polymerase chain reaction at postoperative week 3, normalized to *Gapdh* expression. The expressions of *Sdf1* gene were compared between inguinal and grafted fat, and those of *Cxcr4* gene compared between fat (inguinal fat) and adipose-derived stem cells (ASCs). *p < 0.05 versus the inguinal fat. #p < 0.05 versus the fat.

3.3 Quantitative analyses of fat graft retention

Next, we investigated the effect of secondary administration of adipose-derived stem cells on fat graft retention in all groups at postoperative week 8. Interestingly, the volume of grafted fat was higher in the RI1 group than in the SI group (Fig. 7). Moreover, the volume of grafted fat in the RI2 group was higher than that in both the SI and RI1 groups (SI group: median = 28.8 mm^3, interquartile range = 25.3 to 34.6 mm^3; RI1 group: median = 60.2 mm^3, interquartile range = 53.1 to 63.1 mm^3; RI2 group: median = 78.9 mm^3, interquartile range = 76.0 to 93.2 mm^3; $p < 0.05$) (Fig. 7). These data demonstrate that the groups with secondary administration of adipose-derived stem cells had a higher volume of grafted fat than the group with a single injection of adipose-derived stem cells. In addition, the secondary administration of adipose-derived stem cells at the 2-week-interval was more effective than that at the 1-week-interval in terms of fat graft retention.

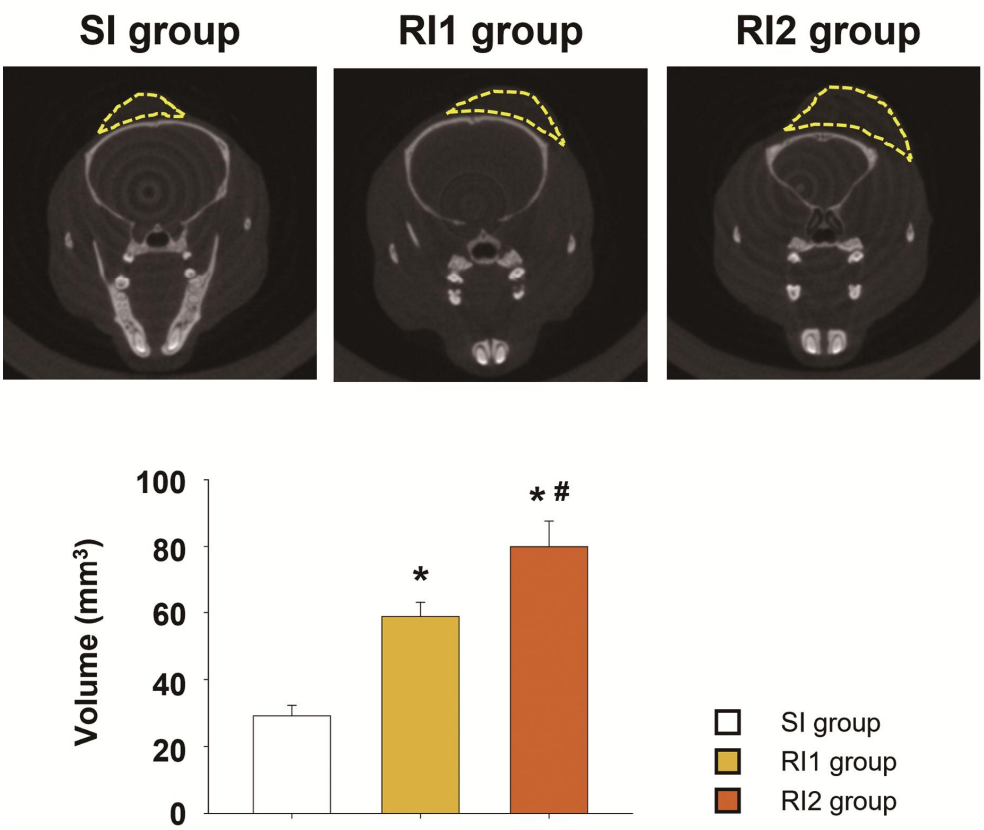

Fig. 9. The volume of grafted fat measured by micro-computed tomography at postoperative week 8. Grafted fat is outlined by a yellow circle. The volume of grafted fat was greater in the RI1 group than in the SI group. Moreover, the volume of grafted fat in the RI2 group was greater than that in both the SI and RI1 groups. *p < 0.05 versus the SI group. #p < 0.05 versus the RI1 group.

3.4 Quantitative analyses of angiogenesis in grafted fat

Since angiogenesis is considered one of the microenvironmental factors that lead to a change in fat graft retention, we measured the vascular density of the grafted fat by immunofluorescent staining of CD31 at postoperative week 8. The vascular density of the RI1 group

was higher than that of the SI group, and the vascular density of the RI2 group was higher than that of the other two groups (SI group: median = 11.0 percent, interquartile range = 6.0 to 17.5 percent; RI1 group: median = 20.8 percent, interquartile range = 18.3 to 24.8 percent; RI2 group: median = 26.9 percent, interquartile range = 19.3 to 29.7 percent; $p < 0.05$) (Fig. 8).

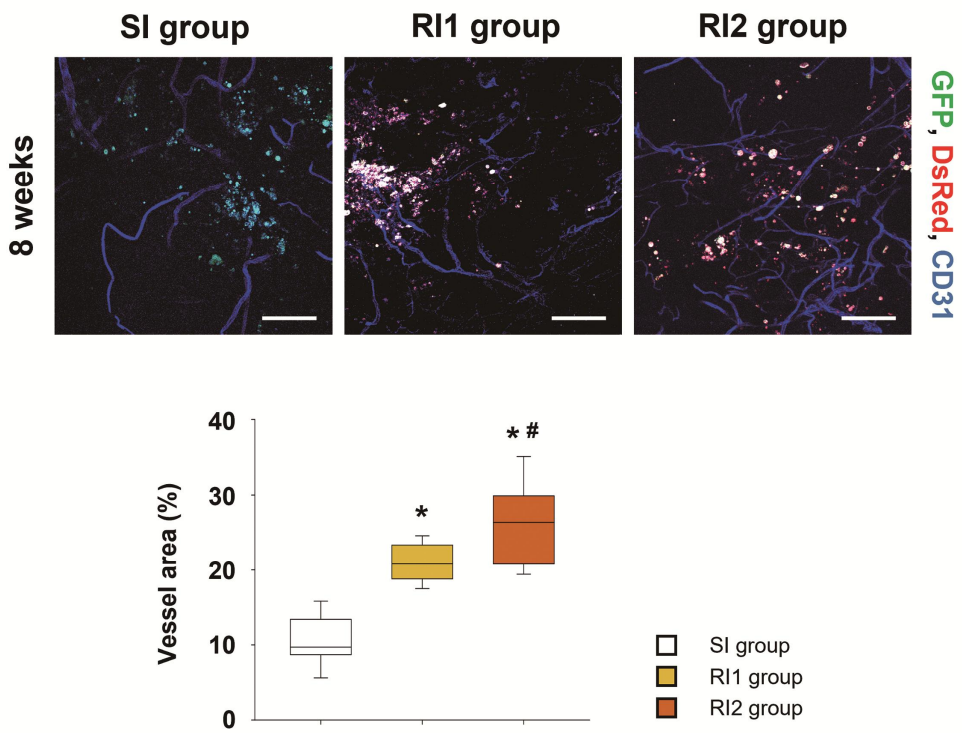

Fig. 10. Vascular density of the grafted fat measured by quantification of CD31 immunofluorescent staining at postoperative week 8. The blood vessel density of the RI1 group is higher than that of the SI group, and the blood vessel density of the RI2 group is higher than that of the other two groups. *$p < 0.05$ versus the SI group. #$p < 0.05$ versus the RI1 group. Scale bars = 100 μm. GFP, green fluorescent protein.

In accordance with the results of immunofluorescent staining, the gene expression of *Vegf* (SI group: median = 1.13, interquartile range = 0.89 to 1.31; RI1 group: median = 2.21, interquartile range = 2.10 to 2.41; RI2 group: median = 4.82, interquartile range = 3.93 to 5.22; $p < 0.05$) (Fig. 9) and *Fgf2* (SI group: median = 1.01, interquartile range = 0.64 to 1.46; RI1 group: median = 2.00, interquartile range = 1.48 to 2.82; RI2 group: median = 2.33, interquartile range = 1.56 to 3.21; $p < 0.05$) (Fig. 9) at postoperative week 3 were highest in the RI2 group. Taken together, the groups with secondary administration of adipose-derived stem cells showed a higher density of blood vessels and higher expression of angiogenic genes in the grafted fat than the group with a single injection of adipose-derived stem cells. Thus, these data suggest that secondary administration of adipose-derived stem cells promotes angiogenesis in grafted fat, and a 2-week-interval is more effective.

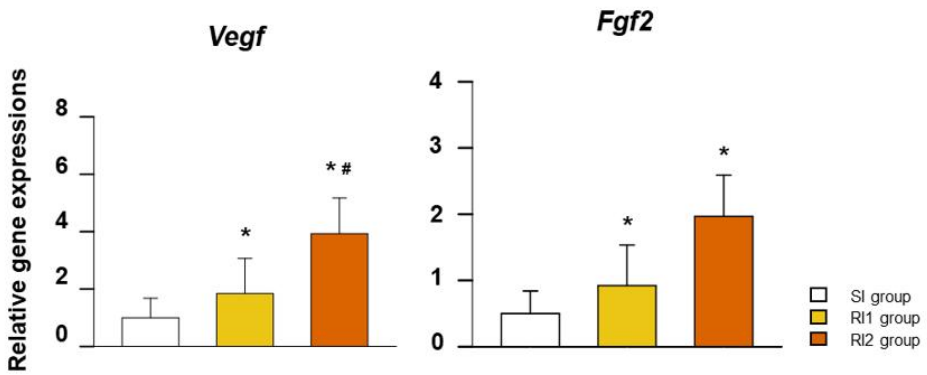

Fig. 11. Gene expression analyses of *Vegf* and Fgf2 in the grafted fat by quantitative real-time polymerase chain reaction at postoperative week 3. **p* < 0.05 versus the SI group. #*p* < 0.05 versus the RI1 group.

4. Discussion

In the present study, we investigated the effects of a secondary intravenous injection of adipose-derived stem cells and the interval between the two injections on the survival of fat grafts. Mice receiving one more injection of adipose-derived stem cells at a 2-week interval showed greater volume retention and vascular density of grafted fat. To the best of our knowledge, this is the first study to investigate the effects of repeated intravenous injections of adipose-derived stem cells on fat grafting.

We demonstrated that both green fluorescent protein and DsRed adipose-derived stem cells injected at different time points were recruited into the grafted fat. The process of cell migration and engraftment into the target tissue is called homing.[15] In this study, we noted that DsRed adipose-derived stem cells injected 1 or 2 weeks after fat grafting also showed homing to grafted fat. In our previous study, injury due to the fat grafting procedure at the recipient site was considered as one of the main causes of stem cell homing.[4] However, since DsRed adipose-derived stem cells injected at postoperative week 1 or 2, which is regarded as the time point when the injury at the recipient site is nearly resolved, were also recruited to grafted fat, specific mechanisms resulting from injury had to be considered. Among these, we investigated chemo-attractants related to stem cell

homing.

The Sdf-1/Cxcr4 axis is one of the major chemokine/receptor pairs governing cell homing and migration. It is known to be associated with homing of hematopoietic cells into the bone marrow during the developmental period, and migration of lymphocytes toward inflammation and injury sites.[16] Sdf-1 is expressed from the injured organ, leading to an elevation of Sdf-1 level and recruitment of stem cells via chemoattraction toward a gradient of Sdf-1.[17] Cxcr4, a receptor of Sdf-1, is expressed by mesenchymal stem cells (MSCs), embryonic stem cells, and hematopoietic stem cells.[18,19] In our model, *Sdf-1* gene was highly expressed in the grafted fat presumably due to grafting procedure itself being performed at the recipient site. Moreover, *Cxcr4* gene was highly expressed in adipose-derived stem cells. Intriguingly, *Sdf-1* gene was still highly expressed in the grafted fat at postoperative week 3. This means that the elevation of Sdf-1 levels after injury can last longer than expected. Thus, both DsRed adipose-derived stem cells injected 1 and 2 weeks after fat grafting could be recruited to the grafted fat. Further studies are needed to define the duration of Sdf-1 elevation after injury.

Repeated injections of adipose-derived stem cells resulted in higher graft volume and vascular density than a single injection of adipose-derived stem cells. Consistent with the results of vascular density at postoperative week 8, *Vegf* and *Fgf2* genes related to

angiogenesis were expressed at higher levels in mice with repeated injections than in those with single injections at postoperative week 3. In a previous study, we suggested that intravenous injection of adipose-derived stem cells could induced angiogenesis in grafted fat via paracrine action.[4] Thus, we can postulate that secondary intravenous administration of adipose-derived stem cells also enhances angiogenesis in the grafted fat, leading to better outcomes in grafted fat retention.

This study still has several issues and limitations that require further investigation. First, the optimal interval between adipose-derived stem cell administrations was unclear. In the groups of repeated injections of adipose-derived stem cells, the interval between adipose-derived stem cell injections was better at 2 weeks than at 1 week. Although there was a difference in angiogenesis between the two groups, the detailed cellular and molecular mechanisms related to the timing of stem cell injection and the outcome of fat grafting have not been elucidated. In a rabbit model of spinal cord injury, repeated intravenous injections of human umbilical cord blood-derived MSCs were proven to be clinically effective. However, recovery was more pronounced in the 3-day interval group than in the 7-day interval group.[6] Therefore, it is assumed that there is an appropriate timing of stem cell injection depending on the purpose and type of cell therapy, and further studies to explain the

underlying mechanism are needed.

Second, optimization of the injection number and cellular dose is needed. In the present study, we performed one or two injections; however, it is not yet clear whether more than three injections are better. In the rat model of chronic ischemic cardiomyopathy treated with c-kit^{+} cardiac progenitor cells, three repeated injections at 35-day intervals were superior to a single injection despite the fact that the total cell number was the same.[20] Considering with first issue, cell therapy should be conducted based on various variables and combinations including injection number, interval, and dose of stem cells.

Third, stem cell therapy cannot rule out oncologic risks in the long term. This study was conducted for eight weeks by modifying the experimental design of a previous study.[4] Although there is a difference in the lifespan of humans and mice, a study with a long-term follow-up is needed to provide experimental evidence of oncologic safety related to cell-assisted lipotransfer.

5. Conclusion

In a modified animal model of cell-assisted lipotransfer, we demonstrated that secondary intravenous administration of adipose-derived stem cells improved retention of grafted fat. Regarding the time point of the boosting effect of secondary administration, the interval between adipose-derived stem cell administrations was better at 2 weeks than at 1 week. Our findings refine clinical protocols and enhance the therapeutic value of cell-assisted lipotransfer.

References

1. Yoshimura, K., et al., *Cell-Assisted Lipotransfer for Cosmetic Breast Augmentation: Supportive Use of Adipose-Derived Stem/Stromal Cells.* Aesthetic Plastic Surgery, 2020. **44**(4): p. 1258-1265.
2. Kolle, S.F.T., et al., *Enrichment of autologous fat grafts with ex-vivo expanded adipose tissue-derived stem cells for graft survival: a randomised placebo-controlled trial.* Lancet, 2013. **382**(9898): p. 1113-1120.
3. Hong, K.Y., et al., *The Fate of the Adipose-derived stem cells during Angiogenesis and Adipogenesis after Cell-Assisted Lipotransfer.* Plastic and Reconstructive Surgery, 2018. **141**(2): p. 365-375.
4. Hong, K.Y., et al., *Systemic Administration of Adipose-derived stem cells Concurrent with Fat Grafting.* Plast Reconstr Surg, 2019. **143**(5): p. 973e-982e.
5. Hao, H.J., et al., *Multiple intravenous infusions of bone marrow mesenchymal stem cells reverse hyperglycemia in experimental type 2 diabetes rats.* Biochemical and Biophysical Research Communications, 2013. **436**(3): p. 418-423.
6. Yang, C.H., et al., *Repeated injections of human umbilical cord blood-derived mesenchymal stem cells significantly promotes functional recovery in rabbits with spinal cord injury of two noncontinuous segments.* Stem Cell Research & Therapy, 2018. **9**:136

7. Guo, S., et al., *Multiple Intravenous Injections of Valproic Acid-Induced Mesenchymal Stem Cell from Human-Induced Pluripotent Stem Cells Improved Cardiac Function in an Acute Myocardial Infarction Rat Model.* Biomed Res Int, 2020. **2020**: p. 2863501.

8. Plock, J.A., et al., *The Influence of Timing and Frequency of Adipose-Derived Mesenchymal Stem Cell Therapy on Immunomodulation Outcomes After Vascularized Composite Allotransplantation.* Transplantation, 2017. **101**(1): p. e1-e11.

9. Zaidi, M.R., T.J. Hornyak, and G. Merlino, *A genetically engineered mouse model with inducible GFP expression in melanocytes.* Pigment Cell & Melanoma Research, 2011. **24**(2): p. 393-394.

10. Zhong, W., et al., *Prox1-GFP/Flt1-DsRed transgenic mice: an animal model for simultaneous live imaging of angiogenesis and lymphangiogenesis.* Angiogenesis, 2017. **20**(4): p. 581-598.

11. Jiang, Y., et al., *Visualization of 3D White Adipose Tissue Structure Using Whole-mount Staining.* Jove-Journal of Visualized Experiments, 2018(141).e58683.

12. Holmes, D.I.R. and I. Zachary, *The vascular endothelial growth factor (VEGF) family: angiogenic factors in health and disease.* Genome Biology, 2005. **6**(2):209.

13. Chen, X.S., et al., *Adipose-derived mesenchymal stem cells promote the survival of fat grafts via crosstalk between the Nrf2 and TLR4 pathways.* Cell Death & Disease, 2016. **7**.e2369.

14. Stuermer, E.K., et al., *The role of SDF-1 in homing of human adipose-derived stem cells.* Wound Repair Regen, 2015. **23**(1): p. 82-9.

15. Yagi, H., et al., *Mesenchymal Stem Cells: Mechanisms of Immunomodulation and Homing.* Cell Transplantation, 2010. **19**(6-7): p. 667-679.

16. Horuk, R. and S.C. Peiper, *Chemokines: Molecular double agents.* Current Biology, 1996. **6**(12): p. 1581-1582.

17. Lau, T.T. and D.A. Wang, *Stromal cell-derived factor-1 (SDF-1): homing factor for engineered regenerative medicine.* Expert Opinion on Biological Therapy, 2011. **11**(2): p. 189-197.

18. Cho, H.H., et al., *Overexpression of CXCR4 increases migration and proliferation of human adipose tissue stromal cells.* Stem Cells Dev, 2006. **15**(6): p. 853-64.

19. Baek, S.J., S.K. Kang, and J.C. Ra, *In vitro migration capacity of human adipose tissue-derived mesenchymal stem cells reflects their expression of receptors for chemokines and growth factors.* Exp Mol Med, 2011. **43**(10): p. 596-603.

20. Tang, X.L., et al., *Repeated Administrations of Cardiac Progenitor Cells Are Superior to a Single Administration of an Equivalent Cumulative Dose.* J Am Heart Assoc, 2018. **7**(4).e007400.

국문 초록

지방줄기세포 기반 지방이식술에서 지방줄기세포의 2차 주입에 따른 생존율 향상

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자가지방이식술은 미용 및 재건의 측면에서 수술법이 비교적 간단하여 대중적으로 널리 이용되고 있다. 하지만 이식된 지방조직의 높은 흡수율로 인해 장기적인 이식생착률을 예측하기 어렵다는 단점이 있다. 이러한 단점을 극복하기 위해 고농도의 지방줄기세포를 자가지방에 혼합하여 이식하는 cell-assisted lipotransfer (CAL), 즉 줄기세포 기반

지방이식술이 소개되었고, 여러 임상시험을 통해 CAL이 지방조직의 이식생착률을 증가시킬 수 있는 방법임이 입증되었다.

본 연구진은 선행 연구를 통해 CAL의 마우스 모델을 확립하여 유효성에 대한 기전을 밝힌 바 있으며, 이 마우스 모델을 응용하여 지방줄기세포를 정맥으로 별도로 주입하는 지방이식술을 처음으로 시도하여 지방이식의 생존을 증가시키는 가능성을 확인한 바 있었다. 하지만 지방줄기세포를 정맥으로 투여하는 지방이식술은 아직 프로토콜이나 적합한 조건이 확립되어 있지 않다. 이번 연구에서는 지방줄기세포를 정맥으로 주입하는 지방이식술에서 지방줄기세포의 제공 회수와 간격이 지방이식에 어떠한 영향을 미치는지 관찰하고 이에 대한 기전을 규명하였다.

선행 연구의 CAL 마우스 모델을 응용하여, 공여 지방조직은 8주령의 일반 C57BL/6J (B6) 마우스의 서혜부 지방을 사용하였다. 공여 지방줄기세포는 녹색 형광이 발현되는 green fluorescent protein (GFP) B6 마우스와 빨간색 형광이 발현되는 DsRed B6 마우스의 서혜부 지방에서 각각 기질-혈관 분획 (stromal-vascular fracture, SVF) 세포를 추출하고 이를 배양하여 얻었다 (이하 GFP 지방줄기세포 및 DsRed 지방줄기세포). 수혜 마우스는 일반 B6 마우스를 사용하였으며, 수혜부는 두개골의 골막 윗 공간 (subperiosteal plane)으로 하였다.

수혜 마우스는 SI군 (single injection), RI1군 (repeated injection with 1-week interval), RI2군 (repeated injection with 2-week interval)로 나누어 세 군 모두 지방이식과 같은 날에

GFP 지방줄기세포를 정맥 투여하였다. RI1군과 RI2군은 지방이식 후 각각 1주와 2주째에 DsRed 지방줄기세포를 2차로 정맥 투여하였다. 마지막 지방줄기세포 투여일로부터 1주일 뒤에 in vivo imaging system으로 생체내 지방줄기세포의 형광 발현을 측정하였고, 지방이식 후 3주째에 이식지방을 적출하여 면역형광염색을 시행하였다. 마이크로 컴퓨터 단층촬영 (micro-computed tomography, micro-CT)으로 이식된 지방의 부피를 측정하였고, 실시간 중합효소연쇄반응 (real-time polymerase chain reaction, real-time RCR)으로 줄기세포의 homing과 혈관신생과 관련된 유전자의 발현량을 측정하였다.

세 군 모두 정맥 투여한 GFP 및 DsRed 지방줄기세포가 투여 시기와 상관없이 두부의 이식지방 부위로 homing된 것을 관찰하였다. 줄기세포의 homing과 관련된 *Sdf-1* 유전자가 체내의 다른 지방조직보다 이식지방에서 높게 발현되었고, Sdf-1의 수용체인 *Cxcr4* 유전자가 지방조직보다는 지방줄기세포에 높게 발현된 것을 확인하였다. 이는 Cxcr4를 갖는 지방줄기세포가 Sdf-1의 농도가 상대적으로 높은 이식지방으로 몰려드는 이른 바, Sdf-1/Cxcr4의 화학주성 (chemotaxis)이 지방줄기세포의 homing을 설명하는 하나의 기전임을 증명한 것이다. RI2군에서 이식지방의 부피가 가장 많았고, 혈관의 밀도가 가장 높았으며, RI1군과 SI군 순으로 그 뒤를 이었다. 혈관신생과 관련된 *Vegf*와 *Fgf2* 유전자 발현량도 RI2군에서 가장 높았고, RI1군과 SI군 순으로 그 뒤를 이었다.

지방줄기세포를 정맥으로 주입하는 지방이식술에서 지방줄기세포를

1번 투여하는 것보다는 2번 투여하는 것이, 그리고 2번 투여할 경우 그 간격은 1주보다 2주인 것이 지방이식의 생존률을 더 높일 수 있는 조건임을 확인하였다. 이번 연구가 CAL에 대한 최적의 조건을 찾아 표준화된 프로토콜을 확립하는데 이바지하고, 궁극적으로 세포치료술의 발전에 바탕자료가 될 수 있을 것이라 기대한다.

주요어: 지방 이식, 지방 유래 줄기 세포, 줄기 세포의 이동, 정맥 주사

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