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Adipose-Derived Stem Cells and Vascularized Lymph Node Transfers Successfully Treat Mouse Hindlimb Secondary Lymphedema by Early Re-connection of the Lymphatic System and Lymphangiogenesis

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Running head: Successful treatment with vascularized lymph node transfer and adipose stem cells in a mouse hindlimb lymphedema model
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Abstract

Background: Secondary lymphedema is often observed in post-malignancy treatment of the breast and the gynecologic organs, but effective therapies have not been established in chronic cases even with advanced physiological surgeries. Currently, reconstructive surgery with novel approaches has been attempted.

Methods: The hindlimbs of 10-week-old male C57BL/6J mice, after 30 Gy X-ray radiation, surgical lymph node dissection, and 5-mm gap creation, were divided into 4 groups, with vascularized lymph node transfer (VLNT) abdominal flap, and $1.0 \times 10^4$ adipose-derived stem cells (ADSC). Lymphatic flow assessment, a water-displacement plethysmometer paw volumetry test, tissue quantification of lymphatic vessels, and functional analysis of lymphatic vessels and nodes were performed.

Results: Photo Dynamic Eye (PDE) images using, indocyanine green fluorescence, demonstrated immediate staining in subiliac lymph nodes, and linear pattern imaging of the proximal region was observed in the combined treatment of ADSC and VLNT. Both percent improvement and percent deterioration in the combined treatment of ADSC and VLNT were significantly better than in other treatments ($p<0.05$). The numbers of lymphatic vessels with LYVE-1 immunoreactivity significantly increased when treated with ADSCs ($P<0.05$) and B16 melanoma cells were metastasized in groups treated with VLNTs by day 28.

Conclusion: ADSCs increase the number of lymphatic vessels and VLNTs induce the lymphatic flow drainage to the circulatory system. Combined ADSC and VLNT treatment in a secondary lymphedema may effectively decrease edema volume and lymphatic function by lymphangiogenesis and the lymphatic to venous circulation route.
INTRODUCTION

Lymphedema is caused by, and consists of, chronic inflammation and the impairment of the lymphatic systems of collection, drainage, and circulation of interstitial protein-rich fluid.

Among lymphedema, secondary lymphedema is acquired as a result of trauma, surgery, radiotherapy, infection, or a combination of these, and it occurs more frequently than primary lymphedema. Cancer therapy with radical surgical lymph node dissection and radiotherapy may result in severe impairment of the lymphatic systems, and radiation causes tissue fibrosis and further destruction of the lympho-reticuloendothelial system.

Treatment of lymphedema is still challenging even with surgeries that are performed in severe and refractory cases as well as conservative therapies (1).

Lymphovenous anastomoses (LVA), which create a bypass for the lymphatic fluid return to venous systems and require refined surgical skills, may be effective in early-stage lymphedema, but not in later-stage or advanced-stage lymphedemas (2,3), possibly due to the loss of the lymphatic vessels’ ability to transfer lymph fluid in later stages.
Another physiological surgical option is a vascularized lymph node transfer (VLNT), and in this method, vascularized lymph nodes are transferred into areas where lymph nodes have been dissected for cancer treatment or into distal regions of lymphedematous distal tissue, such as limbs, to restore lymphatic drainage function. Becker et al. (4) reported, for the first time, post-mastectomy clinical cases of VLNT with promising results and has been followed by other such cases in upper limbs (5, 6) and lower limbs (7). Despite promising clinical results, widespread application of VLNT is not yet underway.

Aside from such transplanting and reconstructive surgeries, pharmacologic agents like VEGF-C are potently lymphangiogenic (8), which was elucidated in an overexpression transgenic model targeting the skin and lymphatic endothelial cells, and the signal of VEGF-C was transduced by VEGFR-3, and shown to be involved in growth, survival, and migration (9).

Adipose-derived stem cells, ADSCs, are candidates for a novel therapeutic modality because of their multi-differential capacities and marked enhancement of lymphatic endothelial cell (LEC)
proliferation in vitro, as well as the tube formation, migration, and expression of lymphangiogenic factors and the regulation of Prox-1 and VEGFR-3 expression (10). ADSCs successfully induced lymphangiogenesis (11) and VEGF-C in a hydrogel with ADSCs demonstrated decreased dermal edema and enhanced lymphatic vessel regeneration (12).

We investigated both VLNT and ADSCs in a mouse hindlimb chronic secondary lymphedema model, which was created by a single dose of 30 Gy of radiation, surgical removal of complete lymph nodes and lymphatic systems in situ. The mice underwent transfer of an abdominal flap, plus or minus vascularized lymph nodes, plus or minus ADSCs. This may be more consistent with and reflects clinical secondary lymphedema, as radiation therapy and surgery are often used as an adjuvant or mainstay of the treatment (13-17). Our findings may provide information about the pathogenesis of secondary lymphedema and the possible implications of both adipose-
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derived stem cell therapy and VLNT as potential therapeutic modalities.
MATERIALS AND METHODS

Secondary lymphedema mouse model

Lymphedema was established in the left hind limbs of 10-week-old male C57BL/6J mice (Charles River Laboratories Japan, Inc.). All studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Nagasaki University, #1007150867-4.

In order to establish a lymphedema model, the mice were subjected to X-ray radiation in the left inguinal region at 30 Gy in a single dose 7 days prior to the surgery. After radiation, mice were then subjected to circumferential incision in the inguinal region of the muscle layer. Under a microscope, the popliteal lymph nodes were removed, and the superficial collecting lymph vessels were cut and cauterized, and the 5-mm wide gap was left open (Figure 1)(Table 1)(11).

Vascularized lymph node transfer (VLNT) surgery

After simultaneously establishing a lymphedema mouse model, an ipsilateral left abdomino-cutaneous flap based on the left superficial inferior epigastric artery, containing subiliac lymph nodes, was
meticulously elevated en bloc using dissecting scissors under a microscope (Figure 2)(Figure 3). The elevated flap contained skin, subcutaneous fat, and subiliac lymph nodes. The flap was transferred and inset into the 5-mm wide inguinal defects to set the vascularized subiliac lymph nodes onto the site where popliteal lymph nodes had been removed from. Skin at donor and recipient sites was directly closed.

Preparation of adipose-derived stem cells

Adipose-derived stem cells (ADSCs) were isolated as previously described (11). ADSCs were harvested from the adipose tissue of 10 individual animals of the same species of 10-week-old male C57BL/6J mice. An average of 2.89 ± 0.5 g of adipose tissue was harvested from the intra-abdominal and inguinal regions, taking care to identify and remove lymph nodes. Harvested adipose tissue was minced into pieces smaller than 3 mm. ADSCs from the first to three passages
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were used for cell transplantation. The ADSCs were counted with a Beckman Coulter Z1 (Beckman Coulter, Inc., Japan).

**Grouping of the experimental animals**

The mice prepared for lymphedema were divided into 4 groups. Both no VLNT and VLNT groups were subdivided into another two groups according to whether ADSCs were transplanted or not. In no VLNT groups, each mouse had the subiliac lymph nodes carefully removed from the flap under a microscope, and then the flap with no lymph nodes was transferred. In the ADSC groups, each mouse had $1 \times 10^4$ ADSCs injected with 0.3 ml of phosphate-buffered saline (PBS). Each solution was injected subcutaneously into the entire flap, proximal (to the flap) limbs, and distal (to the flap) limbs equally, just after surgery, with a 30G needle. In no ADSC groups, the mock injection of PBS solutions was performed in a similar manner.
Group 1 (control), no VLNT group, n = 5, was injected with only 0.3 ml PBS.

Group 2, VLNT group, n = 5, was injected with only 0.3 ml PBS.

Group 3, no VLNT group, n = 5, was injected with $1.0 \times 10^4$ ADSCs with 0.3 ml PBS.

Group 4, VLNT group, n = 5, was injected with $1.0 \times 10^4$ ADSCs with 0.3 ml PBS.

**Assessment using a near-infrared video camera system (PDE)**

Lymphatic flow assessment using a fluorescence near-infrared video camera system, Photodynamic Eye® (Pde-neo®, Hamamatsu, Japan), was performed with the intradermal injection of Indocyanine Green (ICG), Diagnogreen® (Daiichi Sankyo Company, Ltd., Tokyo, Japan). 0.1 mg of ICG was injected into the left paw. After 5 minutes, the observation was carried out.

**Measurement of hind paw edema volume**
The left hind paw volume was measured with a water-displacement plethysmometer (MK-101 CMP; Muromachi Kikai Co., Ltd., Japan). Quantitative measurements at the same site (the musculotendinous junction of the gastrocnemius muscle) were performed under anesthesia before the surgery, 2 days after surgery, and 14 days after surgery, by blinded evaluators devoid of knowing the attributing groups. Measurements of the left hind paw volume were repeated 3 times at each time point, and the mean values were statistically analyzed. The effectiveness of treatment improvement were quantitatively calculated using the mean percent $\triangle$ value normalized by each volume at 2 days, on which swelling was most severe. Each $\triangle$ value was calculated by subtracting the volume at 2 days from the volume at 14 days, in which the effect is considered most profound. Similarly, the percent deterioration was quantitatively calculated by the mean percent $\triangle$ value normalized by each pre-surgery volume. Each $\triangle$ value was calculated by subtracting the pre-surgery volume from the volume at day 14. The study design is summarized in Table 1.
Histological examination

After the evaluation of the dynamic changes of PDE, the transferred flaps, including their vascularized lymph nodes, were carefully harvested for tissue sampling.

LYVE-1 immunoreactivity

The tissue was fixed immediately with 4% paraformaldehyde and embedded in paraffin. The embedded specimens were sectioned (5 μm) along the longitudinal axis of the flaps, immersed in (pure) xylene for 20 minutes each, and then sequentially immersed in 80%, 90%, 95%, and 100% ethanol for 5 minutes for deparaffinization. After antigen retrieval with microwave treatment in citrate buffer at 120 °C for 10 min, sections were pre-incubated with 10% normal goat serum. After immersion in 0.3% H₂O₂, tissues were incubated overnight at 4 °C with anti-LYVE-1 antibodies, a lymphatic vessel marker (Mouse LYVE-1 Antibody Polyclonal Goat IgG, AF 2125, R&D Systems), at a 1:100 dilution. The slides were subsequently simple
stained with goat MAX-PO, and then visualized with the chromogenic substrate diaminobenzidine (DAB).

Sections stained with LYVE-1 were scanned at low magnification (20×) to select areas containing the most lymphatic vessels (hot spots). Five hot spots within each section were measured at high magnification, and the lymphatic vessel density was calculated as the mean number of lymphatic vessels in hot spots per field.

**VEGF-C and VEGF-R3 immunoreactivity**

After deparaffinization and antigen retrieval, tissues were incubated overnight at 4 °C with anti-VEGF-C polyclonal antibodies (Rabbit polyclonal antibody to VEGF-C, GTX113574, Genetex) at 1:100 dilution or anti-VEGF receptor 3 monoclonal antibodies (Rabbit polyclonal antibody to VEGF Receptor 3, ab27278, Abcam) at 1:100 dilution. The slides were subsequently labeled with Biotin (LSAB2), and then visualized with DAB. Sections stained with VEGF-C, or VEGF-R3, were evaluated at high magnification.
Analysis of lymphatic vessel and lymph node function

To determine whether the lymphatic fluid is passing through the transferred lymph nodes or not in such conditions, 5×10⁵ B16 mouse melanoma cells (JCRB0202, JCRB, Japan) were transplanted subcutaneously into the left paw at the same time of VLNT alone, VLNT plus /ADSC minus group (similar to Group 2: n=6) and VLNT and ADSCs, VLNT plus /ADSC plus group (similar to Group 4: n=6). The lymph nodes and metastatic skin tumors were harvested from the mice, for histology, at 21 days (VLNT plus /ADSC minus group: n=3 and VLNT plus /ADSC minus-plus group: n=3) and 28 days (VLNT plus /ADSC minus group: n=3) after transplantation. The tissues were evaluated using Melan-A immunoreactivity. After immersion in 1.0% H₂O₂, the tissues were incubated overnight at 4 °C with mouse monoclonal antibodies (DT101 + BC199) to Melan-A (ab731, Abcam) at a 1:100 dilution. The slides were subsequently labeled with anti-mouse immunoglobulins/HRP, and then visualized with amino-ethylcarbazole (AEC). Melan-A positive cells were determined for each section.
Statistical analysis

All statistical analyses were performed using Statview version 5 for Windows (SAS Institute Inc., Cary NC).

An overall difference between the groups was determined by one-way ANOVA. Post hoc multiple comparisons were made by using a Tukey-Kramer all-pairwise-comparison test for parametric analysis. The values are expressed as means ± standard deviation (SD) and p-values less than 0.05 were considered significant.

Animals are inbred, handling animals, surgical and management procedures are uniformly established. According to this method, the experiment should be of an appropriate size if the error degrees of freedom in analysis of variance (ANOVA) area somewhere 10 and 20 (18, 19).

The equation is calculated as below:
X = N - T - B + 1

N = total number of animals
T = the number of treatments
B = the number of groups

In this manuscript, N = 20, T = 4 and B = 4

Therefore, X = 20 - 4 - 4 + 1 = 13

This number “13” is sought to be considered appropriate for the experimental design.

RESULTS

Apparent swelling peaked macroscopically at 2 days after surgery.
The appearances of hind limbs varied at 14 days, at which treatment was stabilized (Figure 4-7).
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PDE images

PDE images of normal mouse hindlimbs

Immediately after the injection of ICG to the paw, a bright spot was seen on the foot. Within 5 minutes, lymphatic flow was visualized as bright ICG fluorescence reaching the popliteal and subiliac lymph nodes in prone and supine positions.

Group 1

The features in the fluorescent imaging seemed to be spotted or uniform. Fluorescent imaging toward the proximal trunk was not observed in the irradiated region and thus the lymphedema was lasting, which indicates no lymph flow as deep as can be observed with the PDE. No fluorescent staining was present in the flaps (Figure 8).

Group 2

Fluorescent imaging toward the proximal trunks was not observed in the irradiated region. However, in the transferred flap, the
superficial inferior epigastric vein was detected by ICG staining through the transferred lymph nodes. The flap was gradually stained with mild lymph flows and the ICG passed through the efferent lymphatic vessels into the venous system (Figure 9).

**Group 3**

Small linear fluorescent imaging was observed in the proximal region of the flap, which was supposed to restore superficial collecting lymph vessels running along the ischiatic vein. Staining was not observed within the flap (Figure 10).

**Group 4**

Through the transferred subiliac lymph nodes, the flap was stained immediately. The lymph node flap shunted lymphatic fluid from the recipient bed, via lymph nodes, into the flap’s pedicle vein. Linear fluorescent imaging was also observed in the proximal region of the flap as in Group 3 (Figure 11).
Volumetric analysis

The ranges of values of each group at 14 days after surgery were 0.294 to 0.480, 0.284 to 0.453, 0.312 to 0.438, and 0.241 to 0.265 ml in Group 1, Group 2, Group 3 and Group 4, respectively.

The ranges of values of each group at 2 days after surgery were 0.317 to 0.513, 0.307 to 0.502, 0.343 to 0.548, and 0.370 to 0.539 ml in Group 1, Group 2, Group 3 and Group 4, respectively.

The ranges of values of each group at pre-surgery were 0.109 to 0.149, 0.114 to 0.142, 0.119 to 0.153, and 0.102 and 0.145 ml in Group 1, Group 2, Group 3 and Group 4, respectively.

The hind paw volume of Group 4 was significantly lower than in the other groups at 14 days after surgery (p<0.05) (Figure 12). The percentage of improved difference was 10.0 ± 3.7 (range: 6.4 to 15.0), 7.9 ± 3.8 (range: 4.3 to 14.3), 13.0 ± 5.0 (range: 7.6 to 20.1), and 46.0 ± 7.0 (range: 34.0 to 50.9) in Group 1, Group 2, Group 3, and Group 4, respectively, in between 2 days and 14 days after surgery. The ratio
was significantly improved in Group 4 compared with other groups (p<0.05) (Figure 13).

The percentage of deterioration difference in each group was 198.2 ± 51.9 (range: 150.4 to 272.9), 186.2 ± 50.6 (range: 109.7 to 234.1), 199.7 ± 45.2 (range: 144.7 to 258.8), and 92.9 ± 36.8 (range: 68.8 to 154.5) in Group 1, Group 2, Group 3, and Group 4, respectively. The volumetric deterioration significantly recovered in Group 4 compared with other groups (p <0.05) (Figure 14).

**Histological analysis**

There were no signs of transferred lymph node ischemia or necrosis in both Group 2 and Group 4. Lymphatic vessels detected with LYVE-1 immunoreactivity were seen around the transferred lymph nodes.

**The number of lymphatic vessels with LYVE-1 immunoreactivity**

The numbers of LYVE-1-positive lymphatic vessels were 7.4 ± 0.9 (range: 6.4 to 8.6), 8.0 ± 0.6 (range: 7.4 to 8.6), 11.7 ± 0.4 (range: 11.2 to 12.2).
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to 12.4), and 11.5 ± 1.4 (range: 9.2 to 12.6) per field in Group 1, Group 2, Group 3, and Group 4, respectively. The numbers of lymphatic vessels in Group 3 and Group 4 were significantly increased compared with those in Group 1 and Group 2 (Figure 15) (p<0.05).

VEGF-C and VEGF-R3 immunoreactivity

There were no VEGF-C or VEGF-R3 expressing cells in the lymphatic vessels of any of the groups.

Analysis of lymphatic transport capacity and function

At 21 days after melanoma cell transplantation, only one of three mice in VLNT plus /ADSC minus group developed lymph node metastasis. In contrast, all mice in VLNT plus /ADSC plus group (three of three mice) developed lymph node metastases. In addition, there were metastatic skin tumors on the trunks of the mice in VLNT plus /ADSC plus group only (Figure 16, 17).

All Group 4 mice died of tumor progression by day 25.
By day 28, all mice in Group 2 (three of three mice) developed gross lymph node metastases and in-transit metastases in their flaps. The transferred lymph nodes were able to trap metastatic tumor cells by forming new lymphatic vessels. Mice in Group 4 developed lymph node metastases more quickly than those in Group 2. These findings suggested that recanalization and reanastomoses of the lymphatic vessels between the recipient and the transferred lymph node occurred, as well as lymphangiogenesis, and efferent lymphatic fluid was routed through the transferred lymph nodes and superficial collecting lymph vessels (Figure 18, 19).
Discussion

In this experiment, PDE fluorescent imaging clearly depicted the vascularized lymph node groups. In VLNT plus / ADSCs minus, the superficial inferior epigastric vein was detected through the transferred lymph nodes, and this may contribute to decreasing the lymphedema, whereas in mice with VLNT plus and ADSCs plus, the subiliac lymph nodes in the flap were stained immediately, then the lymph node flap re-connected lymphatic fluid from the recipient bed, via lymph nodes, into the flap’s pedicle vessels and linear fluorescent imaging was also observed in the proximal region of the flap. In this experiment, the gap difference was 5 mm and the abdominal flap, in fact a bit bigger than 5 mm in width as it shrinks after elevation, are tested in this experiment. Regardless of VLNT minus and ADSCs minus, group 1, either VLNT plus and ADSCS minus, group, 2 or VLNT minus and ADSCs plus, group 3, failed the effective recovery of lymph edema.

A relatively smaller dose of ADSCs, at $1.0 \times 10^4$ cells, successfully demonstrated linear fluorescent imaging in the proximal region of
the flap, which was supposed to restore superficial collecting lymph vessels running along the ischiatic vein in mice with ADSCs plus and VLNT minus. This finding is consistent with our previous data (11), even though this current model is different in the manner in which the vascularized flap was inserted.

Measurements of paw volume, represented in Figure 12, quantitative measurements at the same site (the musculotendinous junction of the gastrocnemius muscle), which seem much easier, more repeatable, and more precise than circumferential measurements (11), were determined by a water-displacement plethysmometer, which can be compared with the angiotensin II type 1 receptor adjuvant-induced arthritis rat model (20). It is dependent on the time course of “edema” of this model. After establishing the animal model by radiation, removal, flap or cell injection, the degree of “edema” is peaking at day 2 and gradually the degree decreases by day 14, where the “edema” reaches within the “plateau”. Thus, both day 2 and day 14 are chosen for analysis in this experiment. This animal model is similarly confirmed in our previous study (11). In prevention
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of lymphedematous mouse hindlimb model with non-vascularized lymph node transplantation (21), demonstrated the effects of VLNT, ADSC, and their combination, and only the combination of VLNT and ADSCs significantly improved the edema (close to 50%). Furthermore, the percent deterioration in VLNT plus / ADSCs plus group is less than 2-fold, while the other groups demonstrated around 10% improvement and deteriorations approximately in the order of 3-fold.

Both VEGF-C and VEGFR3 immunoreactivity were not observed. In the previous model, both VEGF-C and VEGFR3 were dose-dependently increased from $1.0 \times 10^4$, $1.0 \times 10^5$ to $1.0 \times 10^6$ cells in the similar, but no vascularized flap inserted limb model (11). This model is very different in terms of the local expression of VEGF-C and VEGFR3, because VEGF-C/VEGFR3 are negatively regulated by the action of fibroblast growth factors (FGFs) in an autocrine manner as the inhibition of FGFR signaling in mouse mammary carcinoma and rat glioma cancer cells suppresses VEGF-C expression in a COX-2 (cyclooxygenase-2) or HIF1A (hypoxia-inducible factor 1-a) independent manner (22). Also, a fibronectin fiber guided assay
provides far stronger sprouting and guidance cues to endothelial cells. VEGF-A, but not VEGF-C, stimulates the collective outgrowth of lymphatic endothelial cells (LEC) (23) and Neuropilin-2 can mediate lymphangiogenesis via an integrin α9β1/FAK/Erk pathway but is independent of VEGFC/VEGFR3 signaling in colorectal carcinoma (24). These findings suggest that VEGF-C and VEGF-C/VEGFR3 are not the only primary induction factors in lymphangiogenesis. Lymphatic vessels are specifically immunoreactive to LYVE-1 and the degree of augmentation with ADSCs is almost equal to that in the presence or no presence of VLNTs. In VLNT groups, B16 melanoma transplantation into the paw led to all three animals when with ADSCs to die of tumor progression by day 25. Gross lymph node metastasis and in-transit metastasis was present in the flaps of all experimented animals with VLNT by day 28. As lymphatic drainage from murine B16 melanomas in syngeneic, immune-competent C57Bl/6 mice is associated with LN enlargement (25). In this experiment, function of lymphatic transport capacity was tested in groups of VLNT plus / ADSCs minus or VLNT plus /
ADSCs plus. In time course, all animals are dead by day 28 in VLNT plus / ADSCs plus group. This would explain the VLNTs are able to transport the lymphatic flow and more enhanced with existence of ADSCs, because the rate of the lymph node metastases at day 21 and more aggressive systemic effects by 28. There is no statistical analysis in this specific experiment but all animals are dead in VLNT plus / ADSCs plus group may lead to the enormous effects by both VLNT and ADSCs, thus in clinical situation, it is highly cautious when the “malignancy” exists.

Treatment model of secondary lymphedema by both ADSCs and VLNTs was proposed through lymphangiogenesis and the decrease of edema volumes through accelerated lymphatic drainage to the venous systems.
Figure Legends

Figure 1
Experimental design. X-ray radiation in the left inguinal region.

Figure 2
Anatomical features of vascularized lymph nodes. Arrow, the superficial inferior epigastric artery; circle, subiliac lymph nodes.

Figure 3
Elevated vascularized flap with visible subiliac lymph nodes (top left). VLNT to the ipsilateral 5-mm gap in the inguinal region.

Figure 4
The appearances of hind limbs at 14 days after surgery in Group 1 (control)

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The appearances of hind limbs at 14 days after surgery in Group 2 (VLNT alone)

Figure 6

The appearances of hind limbs at 14 days after surgery in Group 3 (ADSCs alone)

Figure 7

The appearances of hind limbs at 14 days after surgery in Group 4 (VLNT and ADSCs)

Figure 8

PDE images at 14 days after surgery in Group 1 (control).

The arrow points the transferred flap. The fluorescent imaging representing lymphatic flow is not observed.

Figure 9

PDE images at 14 days after surgery in Group 2 (VLNT alone).
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The arrow points the transferred flap, the superficial inferior epigastric vein was detected by ICG staining through the transferred lymph nodes. Fluorescent imaging toward the proximal trunks was not observed.

Figure 10
PDE images at 14 days after surgery in Group 3 (ADSCs alone).
The circle points small linear fluorescent imaging was observed in the proximal region of the flap, which was supposed to restore superficial collecting lymph vessels running along the ischiatic vein. Staining was not observed within the flap as indicated by the arrow.

Figure 11
PDE images at 14 days after surgery in Group 4 (VLNT and ADSCs).
The arrow indicated the flap the transferred subiliac lymph nodes was stained immediately. The lymph node flap shunted lymphatic fluid from the recipient bed, via lymph nodes, into the flap’s pedicle.
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vein. The circle represents linear fluorescent imaging was also observed in the proximal region of the flap as in Group 3.

Figure 12
Hind paw volume measurement. In all groups, hind limb lymphedema was observed at 2 days after surgery. A significantly lower paw volume was detected in Group 4 compared with other groups at 14 days after surgery (p<0.05).

Figure 13
The ratio of hind paw volume improvement. The percentage of improved difference was significantly greater in Group 4 compared with other groups (p<0.05).

Figure 14
The percentage of deterioration difference was significantly lower in Group 4 compared with other groups (p<0.05).
Figure 15
The number of lymphatic vessels with LYVE-1 immunoreactivity.
The numbers of lymphatic vessels in Group 3 and Group 4 were significantly increased compared with those in Group 1 and Group 2 (p<0.05).

Figure 16
Representative photographs of the hind limbs in VLNT plus / ADSCs minus group. 28 days after tumor cell transplantation, gross metastasis to the lymph nodes and in-transit metastasis in their flaps were seen.

Figure 17
Representative photographs of the hind limbs in VLNT plus / ADSCs plus group. 21 days after tumor cell transplantation, there were metastatic skin tumors on the trunks. The arrow indicates transferred lymph nodes. The circle indicates metastatic skin tumor.
Figure 18
B16 Melanoma cells caused transferred lymph node metastases in both VLNT plus / ADSCs minus and VLNT plus / ADSCs plus groups and multiple skin metastases in VLNT plus / ADSCs plus group. Melanoma cells were immunoreactive to Melan-A.
Transferred subiliac lymph node at high magnification in VLNT plus / ADSCs minus group.

Figure 19
B16 Melanoma cells caused transferred lymph node metastases in both VLNT plus / ADSCs minus and VLNT plus / ADSCs plus groups and multiple skin metastases in VLNT plus / ADSCs plus group. Melanoma cells were immunoreactive to Melan-A.
The metastatic skin tumor at high magnification in VLNT plus / ADSCs plus group.

Table 1
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Study design
References


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Figure 2
Figure 6
Figure 12

![Graph showing paw volume (ml) pre-surgery, 2 days after surgery, and 14 days after surgery for Group 1 (n = 5), Group 2 (n = 5), Group 3 (n = 5), and Group 4 (n = 5).](image)
\[ \Delta \text{value} = \frac{(2 \text{ days after operation}) - (14 \text{ days after surgery})}{(2 \text{ days after surgery})} \times 100 \]
Figure 14

\[ \Delta \text{value} = \frac{(14 \text{ days after surgery}) - (before \text{ surgery})}{(before \text{ surgery})} \times 100 \]

The percentage of deterioration difference \( \Delta \text{value} \)

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<tr>
<td>VLNT(-)</td>
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<td>VLNT(-)</td>
<td>VLNT(+)</td>
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<tr>
<td>ADSC(-)</td>
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<tr>
<td>(n = 5)</td>
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</tr>
</tbody>
</table>
Figure 16
Table 1

- 7 days
  - 30 Gy radiation

0  2 days
  - Paw volume measurement
  - VLNT
  - ADSC transplantation
  - Lymphatic vessel removal

14 days
  - Paw volume measurement
  - PDE lymphatic flow
  - Histology and immunohistochemistry

Gy: Grays