Biomaterials xxx (2011) 1–9

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Therapeutic lymphangiogenesis using stem cell and VEGF-C hydrogel

Ji Hye Hwang [a], In Gul Kim [b], Ji Young Lee [b], Shuyu Piao [b], David S. Lee [c], Tae Seung Lee [d], Jeong Chan Ra [e], Ji Youl Lee [b,*]

[a] Department of Physical and Rehabilitation Medicine, Sungkyunkwan University School of Medicine, Samsung Medical Center, Seoul, Republic of Korea
[b] Department of Urology, The Catholic University of Korea, Seoul St. Mary's Hospital, Republic of Korea
[c] Yongsan International School, Seoul 140-210, Republic of Korea
[d] Department of Surgery, Seoul National University, Bundang Hospital, Republic of Korea
[e] Stem Cell Research Center, RNL Bio Co., Ltd., Republic of Korea

ARTICLE INFO

Article history:
Received 11 February 2011
Accepted 25 February 2011
Available online xxx

Keywords:
Lymphangiogenesis
Stem cell
Hydrogel
VEGF-C

ABSTRACT

Lymphedema is a manifestation of lymphatic system insufficiency. It arises from primary lymphatic dysplasia or secondary obliteration after lymph node dissection or irradiation. Although improvement of swelling can be achieved by comprehensive non-operative therapy, treatment of this condition requires lifelong care and good compliance. Recently molecular-based treatments using VEGF-C have been investigated by several researchers. We designed the present study to determine whether the therapeutic efficacy of implanted human adipose-derived stem cells (hADSCs) could be improved by applying a gelatin hydrogel containing VEGF-C (VEGF-C hydrogel) to the site of tissue injury in a lymphedema mouse model. Four weeks after the operation, we evaluated edema and determined lymphatic vessel density at various post-operative time points. Mice treated with hADSCs and VEGF-C hydrogel showed a significantly decreased dermal edema depth compared to the groups of mice that received hADSCs only or VEGF-C hydrogel only. Immunohistochemical analysis also revealed that the hADSC/VEGF-C hydrogel group showed significantly greater lymphatic vessel regeneration than all the other groups. hADSCs were detected in the implantation sites of all mice in the hADSC/VEGF-C group, and exhibited a lymphatic endothelial differentiation phenotype as determined by co-staining PKH-labeled hADSCs for the lymphatic marker LYVE-1. Our results suggest that co-administration of hADSCs and VEGF-C hydrogel has a substantial positive effect on lymphangiogenesis.

1. Introduction

Lymphedema is defined as the excessive accumulation of protein-rich fluid in the interstitial spaces; this condition commonly occurs following damage to the lymphatic vessels or surgical removal of lymph nodes during breast cancer and pelvic malignancy surgeries [1,2]. This disruption of the lymphatic system can result in chronic symptoms, including remodeling of the skin and the subcutaneous extracellular matrix (ECM), and leads to the accumulation of lipids and macrophages in the affected tissue [3,4]. Non-operative therapy including regular massage treatments and pressure applications has been used to treat this condition for quite some time [5]. However, care and management of this chronic condition require lifelong attention and good compliance as for other vascular diseases and diabetes. Therefore, new therapies to treat chronic lymphedema are in great demand.

Recent studies have identified that certain growth factors are key regulators of lymphatic development. Lymphangiogenesis (the growth of new lymphatic vessels) is a promising potential treatment option for lymphedema, and several studies have investigated the effects of lymphatic growth factors on the growth and development of lymphatic vessels [6,7]. VEGF-C is a lymphatic growth factor essential for lymphangiogenesis [8]. A recent study demonstrated that subcutaneous injection of an adenovirus vector encoding VEGF-C resulted in the generation of lymphatic vessels in the skin of normal mice and in a mouse model of primary lymphedema [9]. Building off of that idea, we developed a tissue-engineered hydrogel system that delivers VEGF-C to treat lymphedema. A number of gel-forming materials including alginate, collagen, heparin-sulfate, dextran, glycosaminoglycans, and poly (ethylene) glycol have been employed to develop controlled delivery vectors for growth factors to facilitate a sustained, localized, and effective dose response [10–12]. Gelatin is a natural and

* Corresponding author. Tel.: +82 02 2258 6227; fax: +82 02 599 7839.
E-mail address: uroljy@catholic.ac.kr (J.Y. Lee).

0142-9612/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2011.02.051

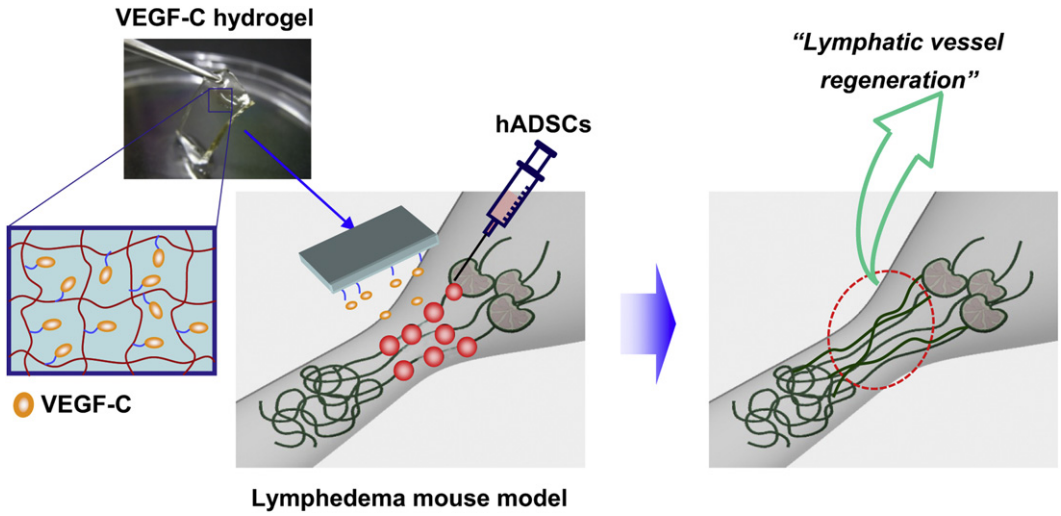

Fig. 1. Schematic diagram showing the mechanism by which co-application of VEGF-C gelatin hydrogel and hADSCs may induce lymphangiogenesis in a lymphedema mouse model.

abundant polymer that is considered a promising alternative candidate for tissue engineering. In addition, it can easily be induced to gelate through the addition of a crosslinking agent such as glutaraldehyde. Gelatin-based hydrogels have been shown to be biodegradable, non-immunogenic, and non-toxic, and thus are widely used as therapeutic scaffolds to encapsulate cells due to their high water content and physical properties emulating the native extracellular matrix (ECM) [13,14]. Tabata et al. demonstrated that an acidic gelatin hydrogel can release biologically active bFGF *in vitro* [15]. We have developed a VEGF-C gelatin hydrogel based on polyion complexation that is composed of acidic gelatin with an isoelectric point (IEP) of 5.0, which interacts ionically with the basic VEGF-C (IEP, 8.3) [16].

At present, adult stem cells are regarded as a promising treatment option for lymphedema. However, therapeutic lymphangiogenesis using adult stem cells is not yet well understood, although some headway has been made. Conrad et al. reported that mesenchymal stem cells showed a lymphatic phenotype *in vitro* and could regenerate the lymphatic vasculature after tissue injury *in vivo* [17]. Among the many types of adult stem cells, we focused on adipose-derived stem cells (ADSCs) in this study, because of the considerable mass of adipose tissue in the body [18,19].

In our study, we injected human ADSCs at the site of lymphatic vessel injury (mouse hindlimb model of lymphedema) and applied a gelatin hydrogel system to the site of injury to obtain the controlled release of VEGF-C. To examine whether VEGF-C and hADSCs had a synergistic effect on the regeneration of damaged lymphatic vessels, we examined the ability of the VEGF-C gelatin hydrogel (VEGF-C hydrogel) to create a suitable substrate for lymphangiogenesis of ADSCs. In addition, we evaluated changes in dermal edema depth and lymphatic vessel density *in vivo* using a lymphedema mouse model (Fig. 1).

2. Experimental procedure

2.1. Preparation of gelatin hydrogels incorporating VEGF-C

Biodegradable gelatin hydrogels were prepared by ionic complexation of acidic gelatin with a crosslinking agent (glutaraldehyde) as described previously in Refs. [15,16]. The acidic gelatin powder (viscosity 32.0 mps; isoelectric point, 5.5), prepared from calfskin using an alkaline process, was supplied by Sammi Gelatin Co., Korea. Briefly, aqueous solutions of 10 wt.% acidic gelatin containing different concentrations of glutaraldehyde aqueous solution (Sigma, USA) were cast into polytetrafluoroethylene molds (10×10 cm^2, 2 mm depth) and left at 4 °C for 12 h to allow crosslinking to occur. The resulting hydrogels were shaped into a sheet (10×5 mm^2, thickness; 2 mm) by cutting them with a razor. These hydrogel sheets were placed in 100 mmol/L of glycine aqueous solution (biotechnology grade, Amresco Inc., USA), agitated at 37 °C for 2 h to chemically block the residual aldehyde groups, and then washed three times with water at 37 °C. The resulting hydrogel sheets were freeze-dried and sterilized with ethylene oxide gas.

An aqueous solution of human recombinant VEGF-C (MW = 15,500, IEP = 8.5, R&D Systems, USA) was diluted with phosphate-buffered saline solution (pH 7.4) to prepare VEGF-C solutions. To prepare a gelation hydrogel containing VEGF-C, solutions containing 100 μg of VEGF-C were dropped onto a freeze-dried hydrogel sheet and left at 4 °C for 12 h to allow VEGF-C to be homogeneously absorbed into the gelatin hydrogel.

2.2. Growth factor release study

In vitro VEGF-C release was investigated by placing cylindrical gelatin hydrogels with VEGF-C incorporated (diameter, 10 mm;

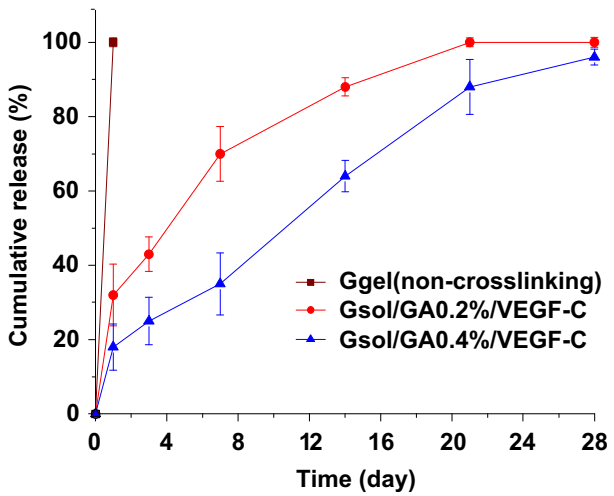

Fig. 2. Percent cumulative release of VEGF-C from a gelatin-based hydrogel incubated at 37 °C for 28 days. Error bars represent the standard deviation for $n = 3$ samples (Ggel: gelatin gel, Gsol: gelatin solution, GA: glutaraldehyde).

Please cite this article in press as: Hwang JH, et al., Therapeutic lymphangiogenesis using stem cell and VEGF-C hydrogel, Biomaterials (2011), doi:10.1016/j.biomaterials.2011.02.051

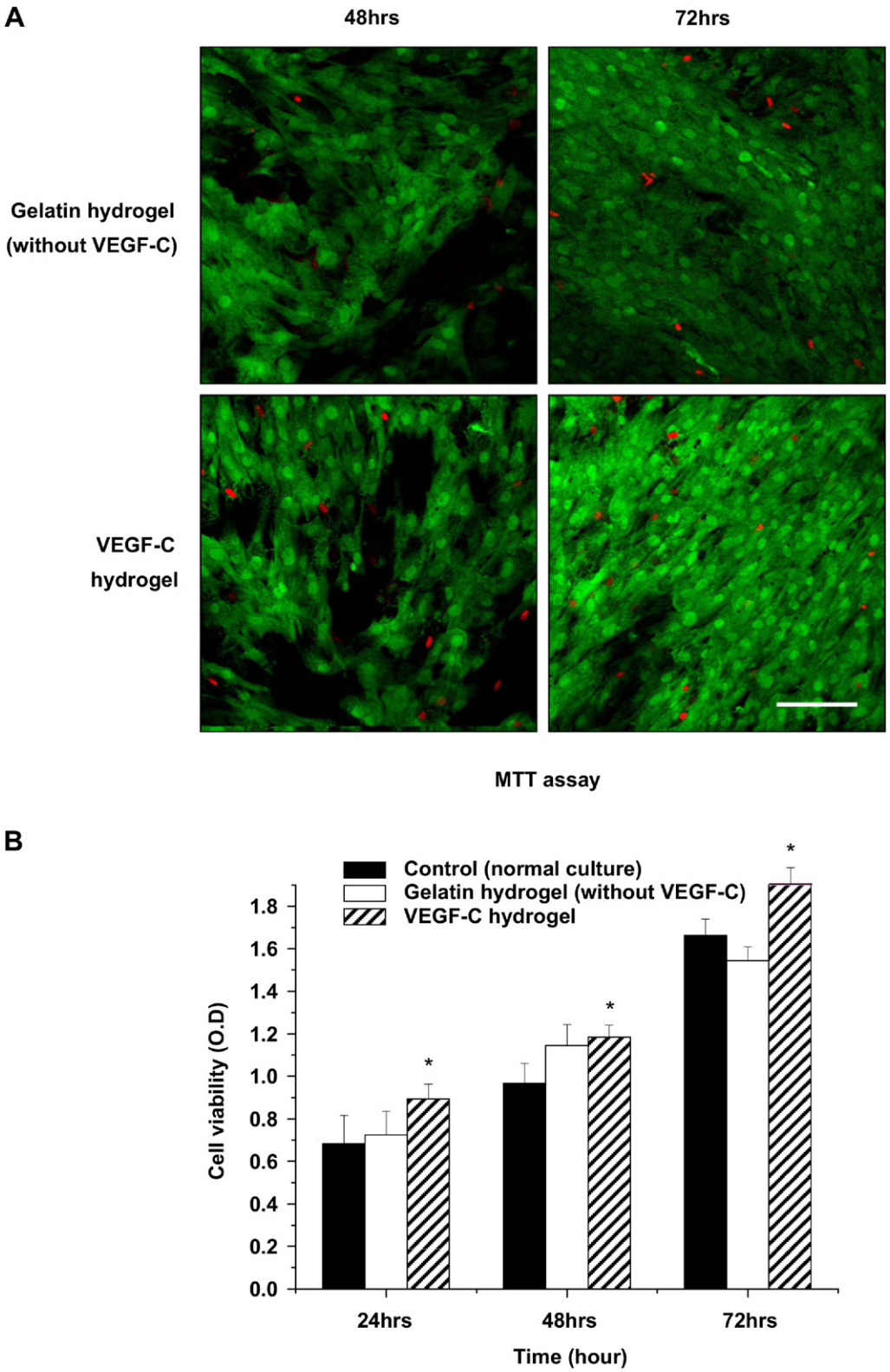

Fig. 3. A: Fluorescence micrographs of hADSCs grown on the surfaces of gelatin hydrogels with or without VEGF-C. Live cells are stained green, while dead cells are stained red. The scale bar represents 100 μm. B: The cell viability of hADSCs grown on gelatin hydrogels with or without VEGF-C at three time points (MTT assay; $n = 3$, $^*p < 0.05$ vs. control). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

thickness, 8 mm) in modified plastic cylinders (height, 50 mm; diameter, 12 mm) sealed at the bottom. PBS (2 ml, pH 7.4) supplemented with 1% BSA (Sigma–Aldrich) was then added immediately to each cylinder. Release studies were performed in an incubated shaker at 37 °C with constant shaking at 40 rpm. The cylinders were covered with parafilm to prevent medium evaporation. Medium was changed at preset times and the collected medium was placed in a 10 ml vial and frozen in a deep-freezer

Please cite this article in press as: Hwang JH, et al., Therapeutic lymphangiogenesis using stem cell and VEGF-C hydrogel, Biomaterials (2011), doi:10.1016/j.biomaterials.2011.02.051

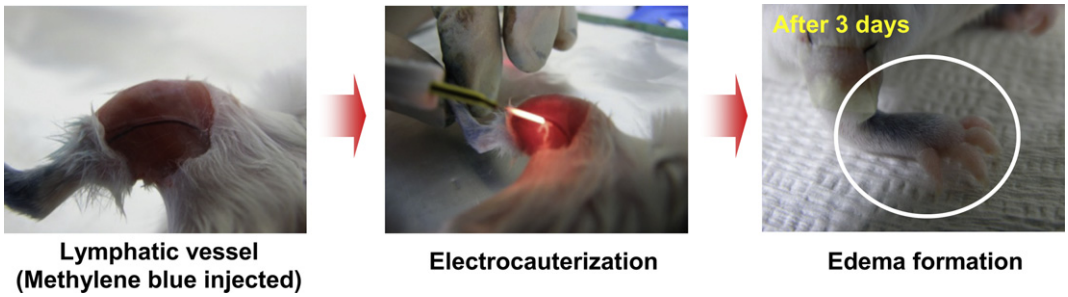

Fig. 4. Macroscopic images of the lymphedema mouse model; the electrocautery procedure used to induce edema formation in a hindlimb footpad is shown.

until ELISA analysis. The amount of VEGF-C released from the hydrogel was determined using a VEGF-C ELISA assay (Quantikine, R&D Systems). The optical density was determined at a wavelength of 450 nm with a reference wavelength of 570 nm.

2.3. Encapsulation of hADSCs within hydrogels (cell viability)

For the cell culture experiments, FDA-approved hADSCs were provided by RNL Bio Co., Korea. hADSCs at passage 4 were used for cell seeding. hADSCs were expanded in T-175 flasks supplemented with non-differentiation culture medium (DMEM containing 10% FBS, 1% penicillin/streptomycin). Hydrogel constructs were cut into 6-mm discs and placed in the wells of a 96-well tissue culture plate. To evaluate cell proliferation, hADSCs were seeded at a density of 1×10^4 cells/ml on the hydrogel discs. Following incubation for various periods, MTT reagent (thiazolyl blue tetrazolium bromide; Sigma, USA) was added to each well of the 96-well plates together with 200 μl fresh culture medium without FBS, and the plates were again incubated for an additional 4 h at 37 °C. After a predetermined time, the culture medium was removed and 200 μl of dimethyl sulfoxide was then added to the solution and mixed well to dissolve the formazan crystals. The absorbance of the formazan dye solution in culture medium at 440 nm was recorded using a 96-well microplate reader (Biorad).

The viability of the encapsulated cells was determined after 2 and 4 days of incubation. The medium was removed and the constructs were washed twice in PBS. The numbers of live and dead cells were assessed using the LIVE/DEAD® viability/cytotoxicity assay kit (Molecular Probes). Cultured gelatin hydrogels and VEGF-C gelatin hydrogels were incubated in the "Live/Dead" assay reagents containing 2 μM calcein AM (labeled live cells) and 4 μM EthD-1(labeled dead cells). These constructs were incubated in a covered dish to prevent drying of the samples for about 45 min at 37 °C. Live/dead cell images were taken using an LSM 510 confocal microscope (LSM510 META, Carl Zeiss, Germany).

2.4. Animal model and transplantation

We used 6- to 8-week-old female mice (BALB/c; Orient Bio Co., Korea), and assigned five mice to each group. All protocols were performed in accordance with the guidelines and regulations pertinent to animal experiments of the Institutional Animal Care and Use Committee, Catholic University Medical College. The hindlimb mouse model of lymphedema was obtained as follows. Normal mice were anaesthetized with a subcutaneous injection of ketamine (110 mg/kg). Methylene blue 0.5% was injected intradermally into the footpad of the hindlimb to visualize the lymphatic vessels. A circumferential incision of the limb (thigh) was made to access lymph vessels that were subsequently electrocauterized [20]. Then, PKH-26-labeled hADSCs were injected subcutaneously at the site of the damaged lymphatic vessels using a Hamilton syringe (25-gauge needle). A VEGF-C hydrogel sheet was then applied immediately to the injection site of the hADSCs and was sutured into the injured dermal junction. Experimental animals were randomly divided into five groups: a normal group (normal), a damaged lymphatic vessel group (control), a group that received hADSCs after lymphatic vessel injury (hADSC group), a group that received VEGF-C hydrogel only after lymphatic vessel injury (VEGF-C hydrogel group), and finally, a group that was injected with hADSCs after which a VEGF-C hydrogel was sutured at the site of lymphatic vessel injury (hADSC/VEGF-C hydrogel group).

2.5. Edema evaluation

To examine edema of the footpad in our lymphedema mouse model, the dermal edema depth of the afflicted footpad was measured using Vernier calipers. Measurements were performed every other day until the conclusion of the experiment. Both the operation site (thigh) and edema site (footpad) were removed, fixed in 4% paraformaldehyde, embedded with paraffin, and cut into 5 μm sections. The sections were mounted on positively charged slides and stained with H&E. H&E staining was used to visualize accumulation of interstitial fluid and the histology of the injury site.

2.6. LYVE-1 staining (identification and quantification of lymphatic vessels)

At 4 weeks after surgery, the mice were sacrificed and the whole thigh region, including the implanted site, was carefully dissected. The tissue was fixed immediately with 4% paraformaldehyde and embedded in paraffin. The embedded specimens were sectioned (5 μm) along the longitudinal axis of implantation. For antigen retrieval, the sections were incubated with citrate buffer (DAKO) at 120 °C for 10 min. After treatment with a protein blocker, the samples were incubated with an anti-lymphatic endothelial hyaluronan receptor-1 (LYVE-1) antibody (Abcam, Cambridge, UK) overnight at 4 °C. After washing with PBS-T (0.05% Tween 20 in PBS), the slides were incubated with Alexa Fluor 488 goat anti-rabbit secondary antibody (Invitrogen) for 1 h at room temperature in the dark. Cell nuclei were labeled with 4,6-diamino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA). Immunofluorescence was visualized using a Olympus BX51 fluorescence microscope. To identify lymphatic vessels, LECs were defined as cells with a blue (DAPI-stained) nucleus surrounded by green LYVE-1-stained cell membranes.

To quantify lymphatic vessels, sections were incubated with horseradish peroxidase-conjugated antibodies (DAKO), and then visualized with a DAB kit (Invitrogen, CA, USA). Cell nuclei were counterstained with hematoxylin. Color images were captured

Please cite this article in press as: Hwang JH, et al., Therapeutic lymphangiogenesis using stem cell and VEGF-C hydrogel, Biomaterials (2011), doi:10.1016/j.biomaterials.2011.02.051

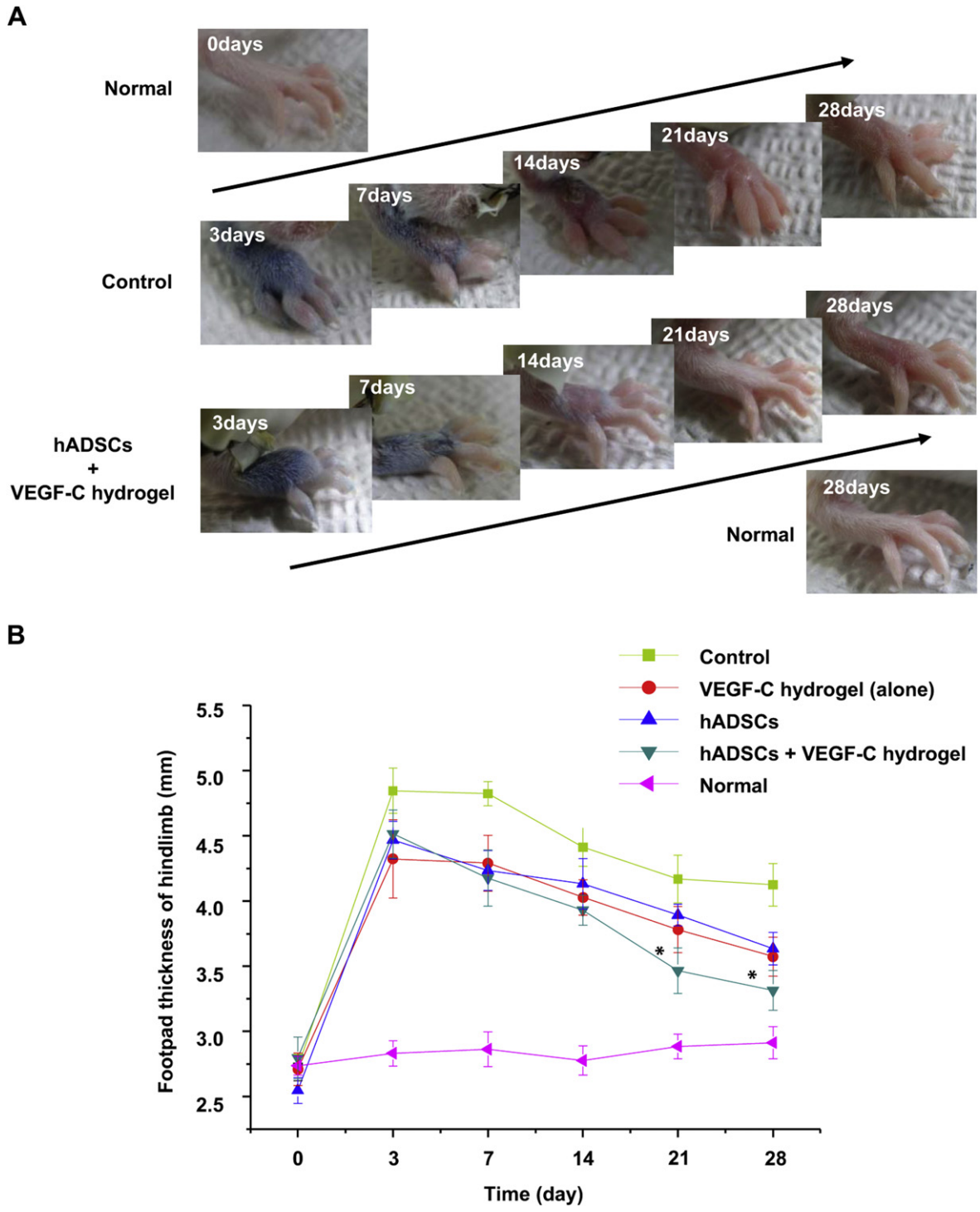

Fig. 5. Changes in dermal thickness of swollen footpads 28 days after surgery. A: Macroscopic images of changes in footpad thickness at various time points. B: Quantitative analysis demonstrating a significant decrease in footpad thickness in the VEGF-C gel group compared with the other groups at post-operative day 21 and 28 ($n = 5$, $^*p < 0.05$).

under a microscope and then counted in five fields per slide in triplicate by a blinded observer at 4 weeks.

2.7. PKH-26 staining

At 4 weeks after injection of PKH-26-labeled hADSCs, the dissected whole thigh region was fixed in 4% paraformaldehyde. The tissue was immersed in 0.5 M sucrose at 4 °C and then frozen in OCT medium. Sections (5 μm thick) were cut from each embedded specimen and placed onto coating slides. The procedure used for PKH-26/LYVE-1 co-staining was identical to that described above for LYVE-1 immunofluorescence staining. The cytoplasm of PKH-26-labeled cells fluoresced red under a fluorescence microscope, whereas the nucleus remained unstained. PKH-26-labeled cells are usually considered viable because this dye diffuses through the cell membrane after cell death [21].

Please cite this article in press as: Hwang JH, et al., Therapeutic lymphangiogenesis using stem cell and VEGF-C hydrogel, Biomaterials (2011), doi:10.1016/j.biomaterials.2011.02.051

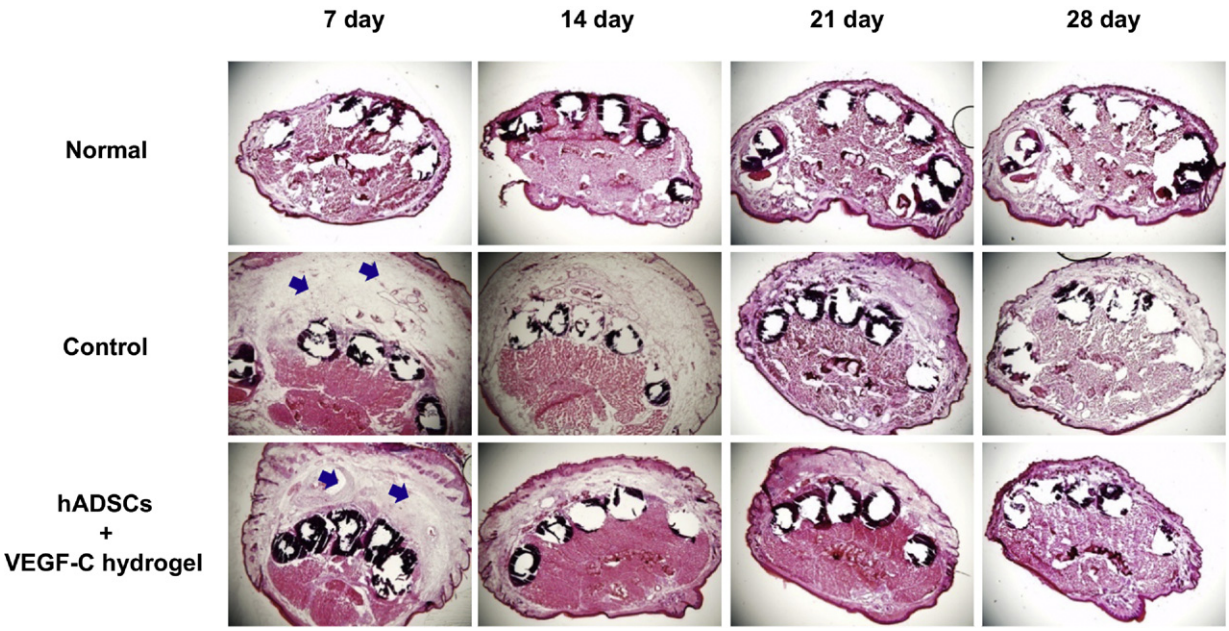

Fig. 6. Histological changes associated with fibrosis in the hindlimb footpad during the observation period. The arrow indicates fibrofatty tissue deposition. (H&E staining; magnification, ×40).

2.8. Statistical analysis

At least two sections were measured per specimen. At least five animals were used for each data point. Statistical analysis was carried out using Student's *t*-test. Mean values and standard deviations are presented. *P* values <0.05 were considered statistically significant.

3. Result

3.1. VEGF-C release behavior from gelatin hydrogels

VEGF-C was loaded into gelatin hydrogels to investigate whether these hydrogels have a positive effect on hADSC-mediated lymphangiogenesis *in vivo*. The concentration of VEGF-C in the collected release medium was measured using a VEGF-C ELISA kit. Fig. 2 shows that the release of VEGF-C from the gelatin hydrogel was sustained. VEGF-C was released at a more rapid rate from gelatin gels containing 0.2% glutaraldehyde than 0.4% glutaraldehyde over a 28-day release period. However, glutaraldehyde concentrations above 0.4% did not result in a significant change in the VEGF-C release rate (data not shown). We therefore used hydrogels cross-linked with 0.2% glutaraldehyde for all subsequent experiments.

3.2. hADSC attachment to VEGF-C gelatin hydrogels in vitro

To evaluate the ability of the gelatin hydrogel surface to support cell growth and differentiation, we cultured hADSCs on the gelatin hydrogels and monitored cell growth by Live/Dead staining and MTT assays. Fig. 3a shows hADSCs stained using the Live/Dead assay (live cells are stained green, dead cells red). hADSCs spread quite well on gelatin hydrogels with and without VEGF-C. The density of dead cells did not increase between 48 h and 72 h of culture. We also evaluated the proliferation of hADSCs on the gelatin hydrogels (with or without VEGF-C) over a 72-h culture period. As shown in Fig. 3b, the number of hADSCs generally increased on both the gelatin hydrogels (with and without VEGF-C) and the normal culture plate (control).

3.3. Edema evaluation in a hindlimb mouse model of lymphedema

At 3 days after electrocauterization of the lymphatic vessels for lymphedema mouse model, the footpad of the hindlimb becomes swollen (Fig. 4). The photograph shown in Fig. 5a revealed edema reduction of the hindlimb footpad. The hADSC/VEGF-C hydrogel group showed decreased lymphedema in the hindlimb. Mice in the hADSC/VEGF-C hydrogel group had a significantly greater dermal depth than the control group, the VEGF-C hydrogel group, and the hADSC group at both 3 and 4 weeks (Fig. 5b) (21 days: 3.45 ± 0.17 vs. 4.17 ± 0.18, 3.81 ± 0.19, and 3.91 ± 0.08 mm, $P < 0.05$; 28 days: 3.31 ± 0.15 vs. 4.12 ± 0.16, 3.57 ± 0.15, and 3.63 ± 0.12 mm, $P < 0.05$, respectively).

To investigate histological edema changes, we performed H&E staining over a 4-week period. Collagen deposition associated with edema was the greatest at the 7th post-operative day. Over this entire period, the hADSC/VEGF-C hydrogel group consistently showed decreased edema when compared with the control group (Fig. 6).

3.4. In vivo lymphatic regeneration

It was difficult to distinguish between blood vessels and lymphatic vessels due to the similar histological appearance of these vessels [22]. Lymphatic vessels in the injury site were therefore identified by staining with anti-LYVE-1 antibody. There were distinct differences in morphology between blood vessels and lymphatic vessels (Fig. 7a; left, ×200). As shown in the fluorescence (right, ×400) image, lymphatic vessels had irregular round shapes; the green and blue colors represent lymphatic endothelial cells and the nucleus, respectively. LYVE-1 DAB staining was conducted to quantify the number of LYVE-1-positive lymphatic vessels around the injury site at 4 weeks (Fig. 7b). A representative immunohistochemical image with lymphatic vessels shown in brown is shown in Fig. 7b. As shown in Fig. 7c, the hADSC/VEGF-C hydrogel group had a significantly higher lymphatic vessel density than the hADSC and VEGF-C hydrogel groups (49.12 ± 2.83, 35.24 ± 1.84, and 37.89 ± 1.93, respectively, $P < 0.05$).

Please cite this article in press as: Hwang JH, et al., Therapeutic lymphangiogenesis using stem cell and VEGF-C hydrogel, Biomaterials (2011), doi:10.1016/j.biomaterials.2011.02.051

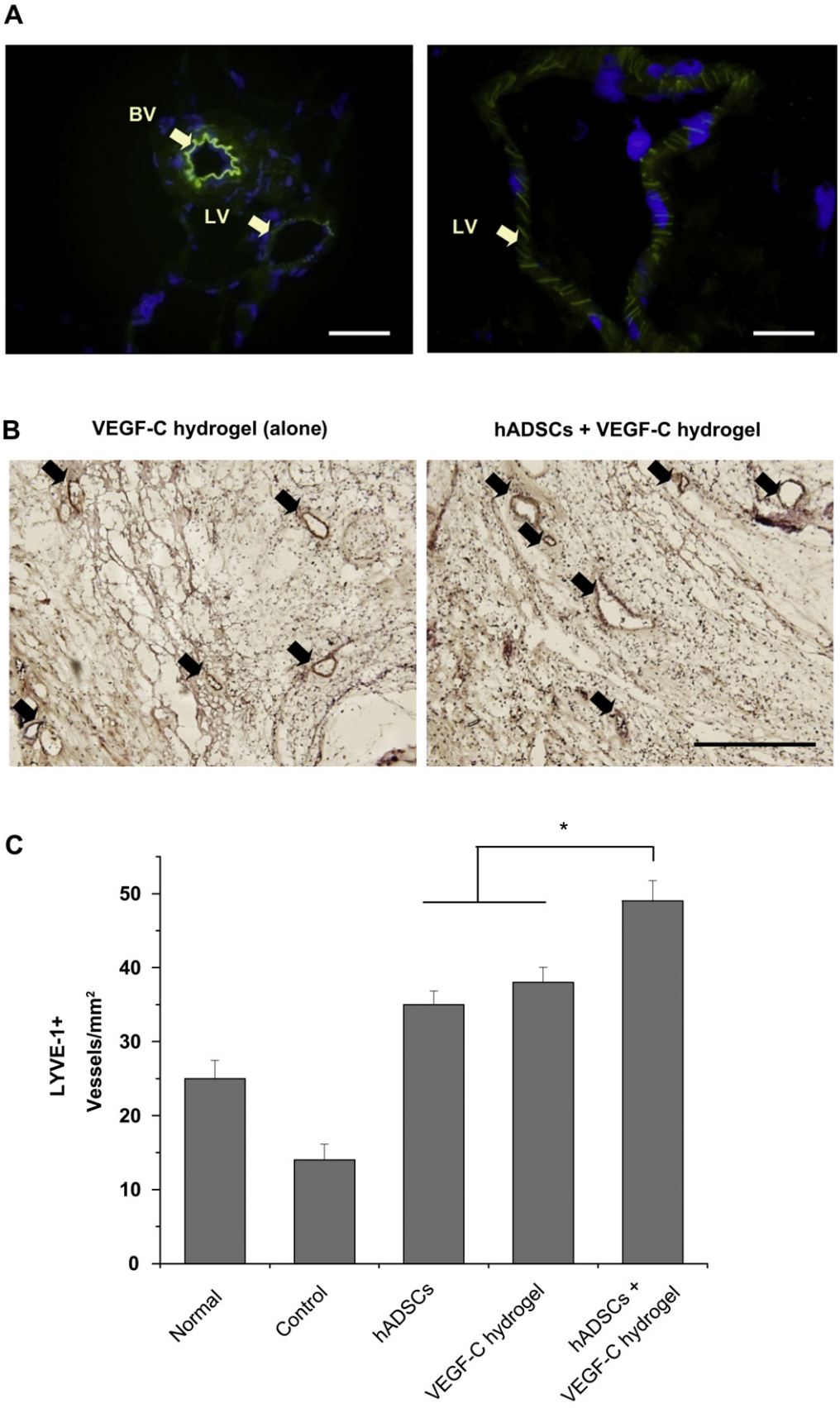

Fig. 7. Histological analyses of lymphangiogenesis. A: The LYVE-1 immunofluorescence image shows the cross-sectional morphology of blood vessels and lymph vessels (BV = blood vessel, LV = lymphatic vessel, scale bar (left) = 50 μm, scale bar (right) = 10 μm). B: Immunohistochemistry using the lymphatic endothelium marker, LYVE-1, in the VEGF-C gel and hADSC/VEGF-C groups. Lymphatic vessels are stained brown (black arrows). The scale bar represents 200 μm. C: Quantitative analysis of LYVE-1-positive lymphatic vessel density at 4 weeks. The hADSC/VEGF-C group demonstrated significantly higher vessel density than the other groups (*$P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Please cite this article in press as: Hwang JH, et al., Therapeutic lymphangiogenesis using stem cell and VEGF-C hydrogel, Biomaterials (2011), doi:10.1016/j.biomaterials.2011.02.051

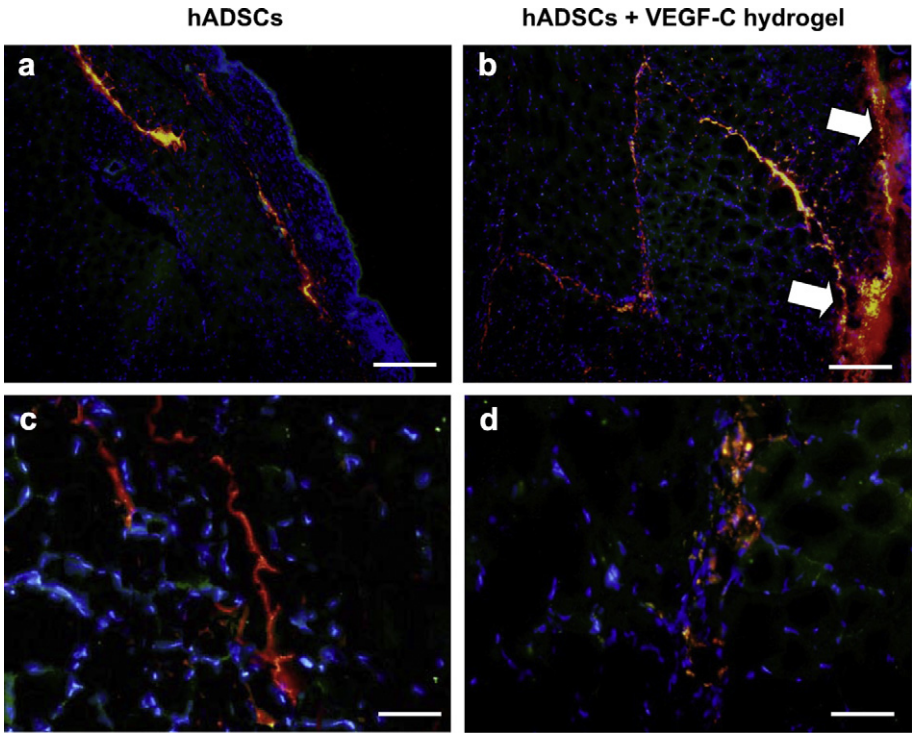

Fig. 8. Double immunohistochemistry using LYVE-1 and PKH-26 for tissue sections obtained from around the operation site at 4 weeks. In a and b, the spread of hADSCs (PKH-26-labeled) was detected in the dermis. Arrow indicates numerous hADSCs around the site at which the VEGF-C hydrogel was applied. The scale bar represents 500 μm c and d are enlargements of a and b, respectively. Note that the hADSCs shown in the left panel did not have a lymphatic phenotype, while in the right panel, co-localization (yellow) of LYVE-1 and PKH-26 was observed in hADSCs, indicating a lymphatic phenotype. The scale bar represents 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

To investigate whether sustained release of VEGF-C induced the hADSCs to differentiate into lymphatic endothelial cells, we performed PKH-26 and LYVE-1 staining. Fig. 8(a,b) shows intradermal spreading of PKH-26-labeled hADSCs after 4 weeks. Numerous hADSCs were detected in and around the VEGF-C hydrogel implantation site (indicated by the arrow) (Fig. 8b). These results indicate that gelatin hydrogels have a positive effect on the proliferation of hADSCs as well as growth factor delivery. There was no histological evidence of lymphatic differentiation of hADSCs, as shown in Fig. 8c. However, co-localization (yellow) of hADSCs (red) and LYVE-1 (green) staining was observed in the hADSC/VEGF-C hydrogel group (Fig. 8d). These results confirm that hADSCs acquired a lymphatic phenotype due to the sustained release of VEGF-C.

4. Discussion

Lymphangiogenesis is the formation of lymphatic vessels from pre-existing lymphatic vessels, similar to angiogenesis [6,23]. Recently, lymphangiogenesis has been investigated as a promising treatment option for lymphedema. Several studies have investigated the ability of lymphatic growth factors to accelerate lymphangiogenesis. Results from several investigations have demonstrated that exogenous VEGF-C administration significantly improves edema resolution by increasing the growth of functional capillaries [7,8,24,25]. However it has also been reported that an overdose of VEGF-C may induce lymphatic hyperplasia (i.e. the proliferation of lymphatic endothelial cells (LECs) within existing lymphatic vessels) without increasing lymphatic vessel density [26,27], which is undesirable, because hyperplastic vessels are poorly functional. We used a gelatin hydrogel protein delivery system to overcome the complications of excess VEGF-C. Moreover, we investigated whether sustained release of VEGF-C facilitated ADSC-based lymphatic regeneration in a lymphedema mouse model.

The gelatin hydrogel were able to maintain the sustained release of VEGF-C during the 4-week release period (Fig. 2). This hydrogel provides matrices for hADSCs to anchor, thereby reducing apoptosis. In addition, the gelatin hydrogel protects hADSCs from direct attack by fibrous connective tissue infiltration, provides a microenvironment for hADSC differentiation, and provides a mechanical support for diseased tissue [28,29]. These characteristics of gelatin hydrogels indicate that they are non-toxic substrates for the proliferation and differentiation of hADSCs for the treatment of lymphedema.

Tomer and colleagues demonstrated that soft-tissue fibrosis and edema are associated with impairment of lymphatic regeneration and lymphatic function [30]. Fibrosis and edema are key inhibitors of lymphatic regeneration. Accordingly, we used a mouse hindlimb model for *in vivo* evaluation of lymphatic edema. Total blockage of lymphatic drainage from the mouse hindlimb is a useful model of severe lymphedema. As shown in Fig. 4, edema was induced after cautery injury to the lymphatic vessels in the hindlimb footpad. Experimental deviation of edema symptoms, an important parameter associated with lymphedema, was also low. Using this lymphedema model, we investigated whether the therapeutic efficacy of autologous hADSCs could be improved by co-application of a VEGF-C-containing gelatin hydrogel. We found that dermal depth was significantly decreased in the hADSC/VEGF-C hydrogel group compared to the other groups. Furthermore, lymphatic regeneration was significantly greater in the hADSC/VEGF-C hydrogel group than the other groups.

Currently, the contribution of hADSCs to lymphangiogenesis is not well understood, and the molecular mechanisms underlying the differentiation of hADSCs at the implant site are largely unclear.

Please cite this article in press as: Hwang JH, et al., Therapeutic lymphangiogenesis using stem cell and VEGF-C hydrogel, Biomaterials (2011), doi:10.1016/j.biomaterials.2011.02.051

However, several studies have demonstrated conclusively that stem cells can acquire a lymphatic phenotype *in vitro* [17,31]. Furthermore, our immunohistochemical results revealed that PKH-labeled hADSCs underwent lymphatic endothelial differentiation as they co-stained with the lymphatic marker LYVE-1 [Fig. 8]. These data, however, do not address the mechanisms underlying the lymphatic endothelial differentiation of hADSCs; it remains unclear whether autografting hADSCs would restore damaged lymphatic vessels. We can only speculate that hADSCs may affect lymphatic function via the paracrine release of cytokines and growth factors [32,33]. However, we are able to draw the following conclusions from our results. First, sustained release of VEGF-C can directly promote the survival and proliferation of hADSCs *in vitro*. Second, VEGF-C may act as an indirect activator of lymphatic regeneration by stimulating the release of other lymphatic factors [7,25] as well as increasing the lymphangiogenesis potential of hADSCs.

5. Conclusions

In this study, we examined the effect of the controlled release of VEGF-C from gelatin-based hydrogels on the proliferation of hADSCs *in vitro*. Our *in vivo* lymphedema mouse model revealed that co-administration of hADSCs and VEGF-C hydrogel significantly decreased dermal edema depth and increased lymphatic vessel density compared with other groups at various post-treatment time points. Our results indicate that VEGF-C-containing hydrogels are suitable non-toxic tissue-engineered substrates for lymphatic regeneration and can be used as vectors to improve lymphangiogenesis in conjunction with hADSCs.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (No. 2010-0027-578).

References

[1] Clodius L. Minimizing secondary arm lymphedema from axillary dissection. Lymphology 2001;34(3):106–10.
[2] Goffman TE, Laronga C, Wilson L, Elkins D. Lymphedema of the arm and breast in irradiated breast cancer patients: risks in an era of dramatically changing axillary surgery. Breast J 2004;10(5):405–11.
[3] Olszewski W. On the pathomechanism of development of postsurgical lymphedema. Lymphology 1973;6(1):35–51.
[4] Daróczy J. Pathology of lymphedema. Clin Dermatol 1995;13(5):433–44.
[5] Földi E. The treatment of lymphedema. Cancer 1998;83(12):2833–4.
[6] Tammela T, Alitalo K. Lymphangiogenesis: molecular mechanisms and future promise. Cell 2010;140(4):460–76.
[7] Yoon YS, Murayama T, Gravereaux E, Tkebuchava T, Silver M, Curry C, et al. VEGF-C gene therapy augments postnatal lymphangiogenesis and ameliorates secondary lymphedema. J Clin Invest 2003;111(5):717–25.
[8] Szuba A, Skobe M, Karkkainen MJ, Shin WS, Beynet DP, Rockson NB, et al. Therapeutic lymphangiogenesis with human recombinant VEGF-C. FASEB J 2002;16(14):1985–7.
[9] Enholm B, Karpanen T, Jeltsch M, Kubo H, Stenback F, Prevo R, et al. Adenoviral expression of vascular endothelial growth factor-C induces lymphangiogenesis in the skin. Circ Res 2001;88(6):623–9.
[10] Liu Y, Chan-Park MB. Hydrogel based on interpenetrating polymer networks of dextran and gelatin for vascular tissue engineering. Biomaterials 2009;30(2):196–207.
[11] Ferreira LS, Gerecht S, Fuller J, Shieh HF, Vunjak-Novakovic G, Langer R. Bioactive hydrogel scaffolds for controllable vascular differentiation of human embryonic stem cells. Biomaterials 2007;28(17):2706–17.
[12] Lim SM, Oh SH, Lee HH, Yuk SH, Im GI, Lee JH. Dual growth factor-releasing nanoparticle/hydrogel system for cartilage tissue engineering. J Mater Sci Mater Med 2010;21(9):2593–600.
[13] Balakrishnan B, Jayakrishnan A. Self-cross-linking biopolymers as injectable in situ forming biodegradable scaffolds. Biomaterials 2005;26(18):3941–51.
[14] Kuijpers AJ, van Wachem PB, van Luyn MJ, Plantinga JA, Engbers GH, Krijgsveld J, et al. In vivo compatibility and degradation of crosslinked gelatin gels incorporated in knitted Dacron. J Biomed Mater Res 2000;51(1):136–45.
[15] Tabata Y, Ikada Y. Vascularization effect of basic fibroblast growth factor released from gelatin hydrogels with different biodegradabilities. Biomaterials 1999;20(22):2169–75.
[16] Tabata Y, Nagano A, Muniruzzaman M, Ikada Y. In vitro sorption and desorption of basic fibroblast growth factor from biodegradable hydrogels. Biomaterials 1998;19(19):1781–9.
[17] Conrad C, Niess H, Huss R, Huber S, von Luettichau I, Nelson PJ, et al. Multipotent mesenchymal stem cells acquire a lymphendothelial phenotype and enhance lymphatic regeneration in vivo. Circulation 2009;119(2):281–9.
[18] Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 2001;7(2):211–28.
[19] Kuhbier JW, Weyand B, Sorg H, Radtke C, Vogt PM, Reimers K. Stem cells from fatty tissue: a new resource for regenerative medicine? Chirurg 2010;81(9):826–32.
[20] Shin WS, Szuba A, Rockson SG. Animal models for the study of lymphatic insufficiency. Lymphat Res Biol 2003;1(2):159–69.
[21] Gulbins H, Pritisanac A, Anderson I, Uhlig A, Goldemund A, Daebritz S, et al. Myoblasts for survive 16 weeks after intracardiac transfer and start differentiation. Thorac Cardiovasc Surg 2003;51(6):295–300.
[22] Yamauchi Y, Michitaka K, Onji M. Morphometric analysis of lymphatic and blood vessels in human chronic viral liver diseases. Am J Pathol 1998;153(4):1131–7.
[23] Bruyère F, Noël A. Lymphangiogenesis: in vitro and in vivo models. FASEB J 2010;24(1):8–21.
[24] Tammela T, Saaristo A, Holopainen T, Lyytikkä J, Kotronen A, Pitkonen M, et al. Therapeutic differentiation and maturation of lymphatic vessels after lymph node dissection and transplantation. Nat Med 2007;13(12):1458–66.
[25] Uzarski J, Drelles MB, Gibbs SE, Ongstad EL, Goral JC, McKeown KK, et al. The resolution of lymphedema by interstitial flow in the mouse tail skin. Am J Physiol Heart Circ Physiol 2008;294(3):1326–34.
[26] Goldman J, Le TX, Skobe M, Swartz MA. Overexpression of VEGF-C causes transient lymphatic hyperplasia but not increased lymphangiogenesis in regenerating skin. Circ Res 2005;96(11):1193–9.
[27] Jeltsch M, Kaipainen A, Joukov V, Meng X, Lakso M, Rauvala H, et al. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science 1997;276(5317):1423–5.
[28] Li Z, Wang F, Roy S, Sen CK, Guan J. Injectable, highly flexible, and thermosensitive hydrogels capable of delivering superoxide dismutase. Biomacromolecules 2009;10(12):3306–16.
[29] Yang KC, Wu CC, Lin FH, Qi Z, Kuo TF, Cheng YH, et al. Chitosan/gelatin hydrogel as immunoisolative matrix for injectable bioartificial pancreas. Xenotransplantation 2008;15(6):407–16.
[30] Avraham T, Clavin NW, Daluvoy SV, Fernandez J, Soares MA, Cordeiro AP, et al. Fibrosis is a key inhibitor of lymphatic regeneration. Plast Reconstr Surg 2009;124(2):438–50.
[31] Kerjaschki D, Regele HM, Moosberger I, Nagy-Bojarski K, Watschinger B, Soleiman A, et al. Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates. J Am Soc Nephrol 2004;15(3):603–12.
[32] Albersen M, Fandel TM, Lin G, Wang G, Banie L, Lin CS, et al. Injections of adipose tissue-derived stem cells and stem cell lysate improve recovery of erectile function in a rat model of cavernous nerve injury. J Sex Med 2010;7(10):3331–40.
[33] Huang YC, Shindel AW, Ning H, Lin G, Harraz AM, Wang G, et al. Adipose derived stem cells ameliorate hyperlipidemia associated detrusor overactivity in a rat model. J Urol 2010;183(3):1232–40.

Please cite this article in press as: Hwang JH, et al., Therapeutic lymphangiogenesis using stem cell and VEGF-C hydrogel, Biomaterials (2011), doi:10.1016/j.biomaterials.2011.02.051