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ORIGINAL ARTICLE

Acellular Dermal Matrix Seeded with Adipose-Derived Stem Cells as a Subcutaneous Implant

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Abstract
Background Soft tissue augmentation using autologous materials usually is associated with high resorption rates. To obtain more durable soft tissue filler, acellular dermal matrix (ADM) was seeded with adipose-derived stem cells (ASCs) in this study.
Methods For ADM preparation, split-thickness skin was obtained from the dorsum of two Fischer rats. Harvested skin was acellularized to obtain ADM and subsequently seeded *in vitro* with ASCs isolated from the same rats. Subcutaneous soft tissue augmentation was carried out in the dorsal area of 20 Fischer rats. The implant materials were ADM (group 1), ADM with ASCs (group 2), collagen type 1 gel (group 3), and collagen type 1 gel with ASCs (group 4). Each specimen was harvested after 8 weeks for quantitative evaluation of thickness, vascular density, and collagen content.
Results Histologic analysis showed that ASCs were successfully seeded onto ADM. The thickness of the implanted material and the vascular density were highest 8 weeks postoperatively in group 2. The subpanniculus layer became significantly thicker in group 3 than in group 4. The collagen content was significantly higher in group 2 than in the other groups.
Conclusion These findings suggest that ADM seeded with ASCs forms the best implant material in terms of volume maintenance, vascular density, and collagen content.

Keywords Acellular dermal matrix · Adipose-derived stem cells · Augmentation · Soft tissue

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Soft tissue augmentation is sometimes required for both aesthetic [5, 22] and reconstructive purposes [19]. It can be performed by any method to be selected among a list of alternatives depending on the severity of the deformity. If the deformity is a complex three-dimensional defect, a free flap usually is used for augmentation [19], but in less severe cases or in aesthetic applications, a number of tissue fillers can be chosen [5, 28, 32, 33].

Ideal soft tissue filler should be safe with low immunogenicity, efficient with stable long-term results, and practical with low cost and ease of use [5]. The most commonly used fillers can be categorized currently into four different groups: autologous dermal fat/fat grafts, injectable xenogeneic collagen, biopolymers (e.g., hyaluronic acid), and acellular allogeneic dermal matrix (ADM) [5].

A commercially available form of ADM derived from human cadaver skin has been used successfully in the clinic for indications such as abdominal wall reconstruction [6], suggesting that ADM also might be a successful filler for aesthetic purposes. However, some literature shows that ADM is resorbed to some extent [29].

Since first described by Zuk et al. [35], adipose-derived stem cells (ASCs) have become increasingly popular due to ease of harvest and lack of significant comorbidity. Additionally, ASCs are known to secrete a variety of angiogenic growth factors presumed to be critical for their activity [4, 11, 24, 34].

Therefore, the purpose of this study was to determine whether ASCs can be seeded successfully onto ADM and to evaluate the effects of ASCs on ADM thickness, collagen quantity, and vascular density.

Materials and Methods

Isolation and Cultivation of ASCs

After general anesthesia with intraperitoneal injection of 50 mg/kg pentobarbitalnatrium, ASCs were obtained from the inguinal fat pads of male Fischer rats weighing 250–300 g (Saitama Experimental Animals Supply Cooperation Ltd., Saitama, Japan), as established previously [21]. In summary, the adipose tissue was digested using 0.15% (w/v) collagenase type 1 (Wako Pure Chemical Industries Ltd., Osaka, Japan) at 37°C with vigorous shaking. The effect of the enzyme was neutralized by an equal volume of Dulbecco modified Eagle medium (DMEM) with high glucose (Gibco-BRL, Grand Island, NY, USA) containing 10% (v/v) fetal bovine serum (FBS) (Gibco-BRL), and the suspension was centrifuged at 1,500 rpm for 5 min. The resultant cell pellet was plated on 100 mm^2 tissue culture plates (Becton-Dickinson, Franklin Lakes, NJ, USA) in control medium (DMEM, 10% [v/v] FBS and 1% antibiotic–antimycotic solution) (Gibco-BRL) and maintained at 37°C in an incubator with 5% carbon dioxide/air. The medium was changed every 3 to 4 days, and passages were carried out at 70% to 80% confluence. Cells from passage 3 were used for the subsequent experiments.

Cell Labeling with 1,1′-Dioctadecyl-3,3,3′, 3′-Tetramethylindocarbocyanine

Before transplantation for in vivo tracking, ASCs were labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI; Molecular Probes, Eugene, OR, USA). The DiI was dissolved in 99% ethanol at a concentration of 25% and stored at −20°C until used. The cells were labeled according to the manufacturer's instructions. Briefly, cells in suspension were incubated with DiI at a concentration of 2.5 μg/ml in phosphate-buffered saline (PBS) (Gibco-BRL) for 5 min at 4°C, then washed with PBS and centrifuged at 1,300 rpm for 5 min to obtain a cell pellet.

Preparation of ADM

Two male Fischer rats were used as donors for skin to prepare ADM. The rats were killed with an overdose of pentobarbitalnatrium. After the dorsal hair had been shaved, split-thickness skin (0.5 mm thick) was harvested using an electric dermatome (Padgett electrodermatome; Padgett instruments, Inc., Kansans city, MO, USA) in a sterile manner. Preparation of ADM from the skin was according to the protocol previously published by Takami et al. [29]. Briefly, the skin pieces were incubated in povidone-iodine for 15 min for the purpose of disinfection, then washed in PBS. The skin pieces were treated with 0.25% trypsin/1 mmol/l ethylenediaminetetraacetic acid (EDTA) solution (Gibco-BRL) for 24 h at 4°C to remove the epidermal cells. The deepidermalized skin pieces then were treated with 0.05% trypsin/0.53 mmol/l EDTA solution for the following 5 days at room temperature to remove all the dermal cells. Next, the skin pieces, which had become ADM, were fully washed in PBS and stored in 1% antibiotic/antimycotic solution containing PBS at 4°C. Hematoxylin and eosin (H&E) staining and scanning electron microscopy (SEM) were performed to confirm the acellular nature of ADM before continuation of the procedure.

Seeding of ASCs Onto ADM

Pieces of ADM (five pieces measuring approximately 1 × 1 cm) were placed in 100-mm^2 tissue culture plates, papillary dermis sides facing upward. DiI-labeled ASCs were seeded onto ADM in control medium at a density of 1×10^6 cells/cm^2. Culture dishes were kept in a 37°C incubator with 5% carbon dioxide for 48 h. After 48 h, the medium was changed to remove the floating cells, and the DiI-positive cells attached to the ADM were counted directly under a fluorescence microscope. Briefly, photos of two different areas from each piece of ADM were taken with ×200 magnification. Five equal-sized squares (0.25 mm^2) were randomly selected in these photos, and the number of the cells in these squares were counted and averaged. The total number of cells was calculated by proportioning the total surface area of the squares to the total surface area of ADM (1 cm^2).

To further confirm attachment of the ASCs, SEM and fluorescence microscopy observation were performed. The ASCs were seeded onto an additional piece of ADM measuring 1 × 1 cm as described earlier and then cut into three pieces. One piece was used for SEM examination, and the other two pieces were used for frozen section.

To prepare the frozen sections for fluorescence microscopy, tissue specimens were frozen directly in Tissue-Tek OCT (Sakura Finetek USA Inc, Torrance, CA, USA) above cold acetone. Frozen tissue blocks were brought to −20°C, and 5-μm sections were obtained.

For SEM, specimens were fixed in 0.2 mol of cacodylate buffer containing 25% glutaraldehyte, osmicated (2% OsO_4), dehydrated, en bloc stained with 7% uranyl acetate,

and finally embedded in Durcupan. Ultrathin sections (50–70 nm) were prepared for electron microcopy.

Material Implantation

For this step, 20 male Fischer rats were used. The animals were kept under standard housing conditions and provided with water and standard dry diet ad libitum. All the animal procedures were approved by the Institutional Animal Care and Use Committee (approval no. 21-012), and the experiments were conducted in accordance with their guidelines. Operations were performed with the animals under general anesthesia using an intraperitoneal injection of 50 mg/kg pentobarbitalnatrium. The 20 rats were divided into the following four groups of 5 rats each: ADM (group 1), ADM seeded with ASCs (group 2), collagen gel (1 ml) (group 3), and collagen gel (1 ml) with ASCs (collagen gel: Cellmatrix type 1-C; Nitta Gelatin Inc. Osaka, Japan; ASCs: 1×10^6cells per rat) (group 4).

After small skin incisions followed by minimal dissections under the panniculus carnosus, ADM and ADM seeded with ASCs were placed onto dorsal pockets (1 ADM per rat measuring 1×1 cm) and fixed at the four corners with 5/0 nylon sutures (Natsume Co, Tokyo, Japan). All the skin incisions were closed with 5/0 nylon sutures. Collagen gel was injected to the same plane in a 1×1-cm-sized rectangular area on the dorsum of the rats.

The animals were kept under standard housing conditions during the follow-up period. A povidone-iodine solution (Isodine, Meiji Seika Kaisha, Ltd., Tokyo, Japan) was applied to the wounds without any additional dressing.

Histologic Analysis

The animals were killed at postoperative week 8 by an overdose of pentobarbitalnatrium. Histologic analysis was performed by H&E staining, picrosirius red staining, and fluorescence microscopy for DiI-positive cell detection. Four biopsy specimens from each group were fixed with 10% formalin solution and paraffin embedded for H&E and picrosirius red stainings. One specimen from each group was used for postoperative frozen sections.

For H&E staining, 5-μm sections were obtained (3 slides from each specimen for each staining). The number of the vascular structures, the thickness of the implanted ADM in groups 1 and 2, and the thickness of the subpanniculus layer in groups 3 and 4 were recorded under $\times 200$ and $\times 100$ magnifications, respectively, using LuminaVision, V220 software (Olympus Co. Ltd, Tokyo, Japan).

Vascular structures were counted by marking them manually with imaging software. The thickness measurements were obtained at three randomly selected points on one slide, and the average value was recorded. The thickness of the ADM scaffolds in groups 1 and 2 were compared with each other and with the thickness before implantation to determine the resorption rates.

Picrosirius red staining was performed to quantify the total amount of collagen. The 5-μm-thick paraffin sections were deparaffinized and rehydrated with distilled water, then kept in Weigert's hematoxylin for 8 min and stained with a picrosirius red staining kit (Polysciences, Inc, Warrington, PA, USA) according to the manufacturer's instructions. Digital images were obtained under $\times 100$ magnification using polarized light microscopy (Olympus Optical Co. Ltd). The areas below the panniculus carnosus, stained in either red (type 1 collagen) or green (type 3 collagen), were quantified using LuminaVision, V220, imaging software (Olympus Co. Ltd., Tokyo, Japan).

Postoperative frozen sections were performed as described earlier to detect DiI-positive ASCs. Images were obtained using fluorescent microscopy (Olympus BX51, Olympus Optical Co. Ltd).

Statistical Analysis

Values are expressed as mean ± standard deviation. Statistical analysis was performed by one-way analysis of variance (ANOVA) and Tukey's HSD test if necessary using software SPSS for Windows (SPSS 16.0; SPSS Inc., Chicago, IL, USA). A p value less than 0.05 was considered significant.

Results

Morphologic Characterization of ASCs and DiI Labeling

The ASCs isolated from the inguinal fat pads of the Fischer rats showed an elongated fibroblast-like appearance in primary culture (Fig. 1, middle left). Red fluorescence with an excitation wavelength of 565 nm after DiI labeling was observed under the fluorescence microscope.

Preparation of ADM and Seeding with ASCs

No cells were detected in the ADM after the acellularization process and before seeding with ASCs. Only the normal appearance of the dermal collagen structure could be seen in H&E staining and SEM (Fig. 1, upper row).

Before and after frozen sections, fluorescence microscopy showed DiI-positive ASCs on the papillary dermis side of ADM (Fig. 1, middle and lower rows). Scanning electron microscopy visualized the organelles of the cells in a higher magnification, documenting the success of the seeding process (Fig. 1, middle right). The average number

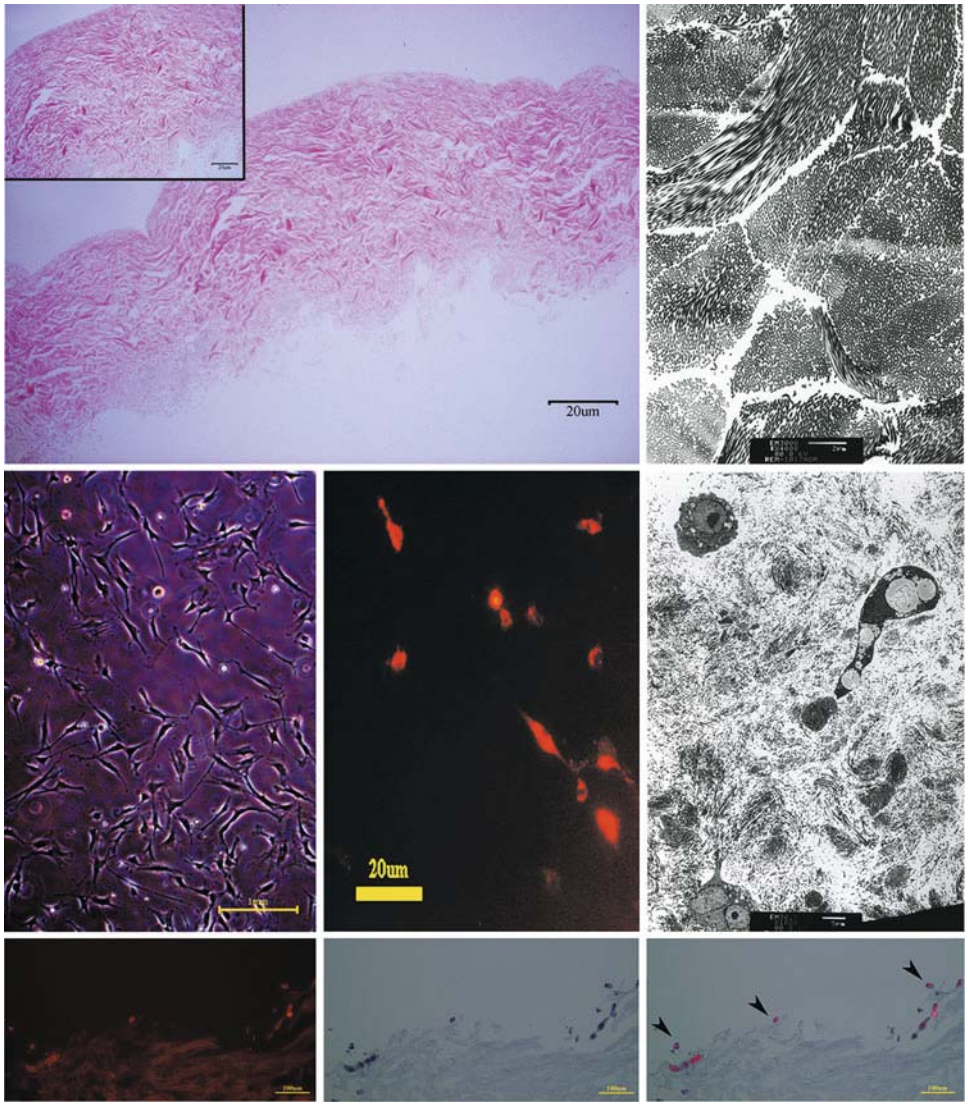

Fig. 1 Acellular dermal matrix (*ADM*) acellularization and seeding processes. The photos in the *upper row* show the acellular nature of ADM with hematoxylin and eosin (*H&E*) staining (*upper left,* microbar 20 μm) and scanning electron microscopy (*upper right*). The photos in the *middle row* show the adipose-derived stem cells (*ASCs*) in culture dishes with a fibroblast-like morphology (*middle left*) and ASCs attached onto ADM under fluorescence microscopy (*middle middle,* microbar 50 μm). Organelles of ASCs attached onto ADM can be seen in the higher-magnification scanning electron microscopy image (*middle right*). The photos in the *lower row* show the 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI)-positive ASCs attached onto ADM in frozen sections. *Lower left:* DiI image. *Lower middle:* hematoxylene image. *Lower right:* merged image (microbars 100 μm)

of attached cells on the surface of ADM was 7.1×10^5 cells/cm^2 (63% of cells added to the culture dish containing ADM).

Postoperative Histologic Examination

H&E Staining

No inflammatory findings of foreign body reactions against ADM were observed in H&E-stained sections (Fig. 2). The ADM implants were invaded by host cells and seemed to be integrated into the recipient site.

Group 2 differed significantly from the other groups in the thickness of the implanted materials ($p < 0.05$) (Fig. 3, left). The average thickness of ADM before implantation was 637.9 ± 67.5 μm. The ADM was resorbed by nearly 50% in group 1 (302.6 ± 76.9 μm), whereas a slight but nonsignificant increase in thickness occurred in group 2 (740.06 ± 57.09 μm) (Fig. 3, right).

Vascular density was highest in group 2. The average number of vessels per unit area was 12.9 ± 1.3 in group 2, whereas it was 6.8 ± 1.2 in group 1, 0.7 ± 0.3 in group 3, and 1.7 ± 0.1 in group 4 ($p < 0.05$) (Fig. 4).

Picrosirius Red Staining

Type 3 collagen stained red in picrosirius red staining (Fig. 5) and was the most abundant type of collagen in all the groups. Type 1 collagen fibrils could be observed between the type 3 collagen fibers in groups 2 and 3 (stained green and marked by white arrowheads in Fig. 5). The highest total collagen content in terms of measured surface area was in group 2 (249.78 ± 8.9 μm^2) followed by group 1 (179.68 ± 8.3 μm^2) ($p < 0.01$). The collagen

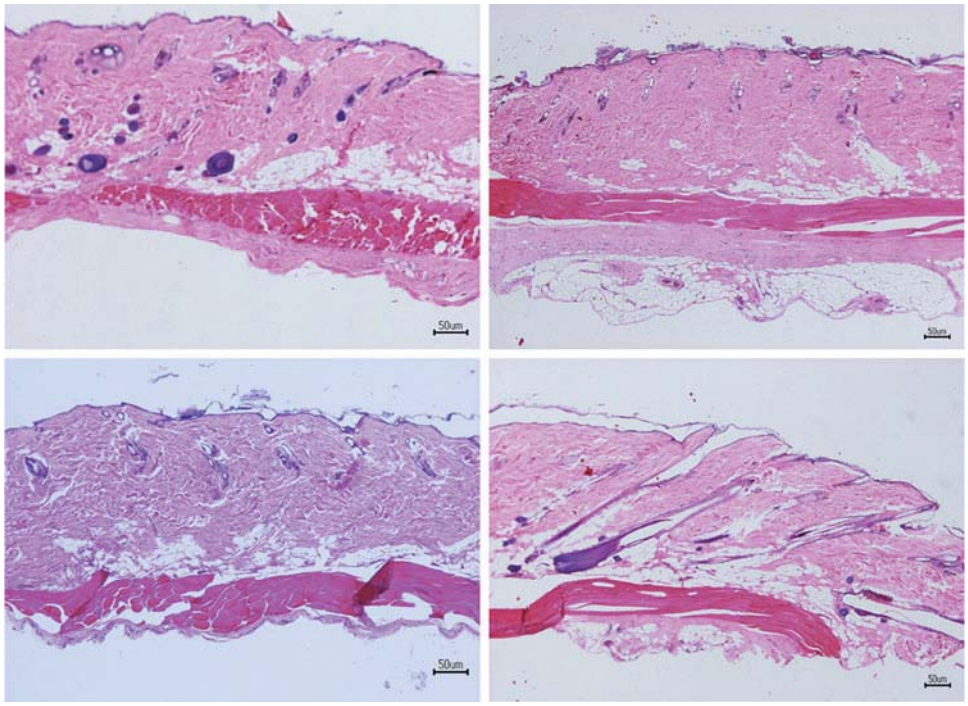

Fig. 2 Hematoxylin and eosin (*H&E*) staining sections of specimens obtained at 8 weeks in each group. *Upper left* group 1. *Upper right* group 2. *Lower left* group 3. *Lower right* group 4 (microbars 50 μm)

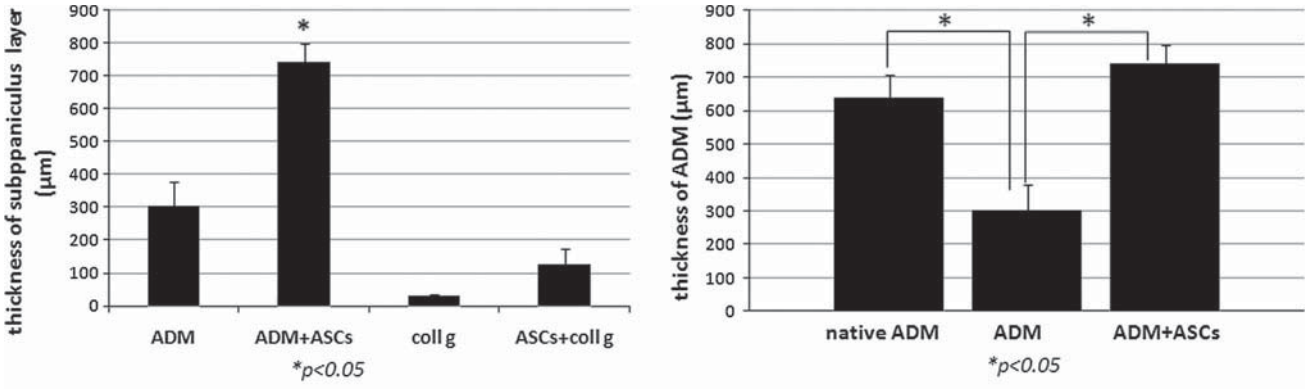

Fig. 3 *Left* Comparison of the subpanniculus layer thicknesses in which acellular dermal matrix (*ADM*) was placed between the experimental groups. *Right* Comparison of ADM thickness before implantation and thicknesses at the end of 8 weeks in groups 1 and 2

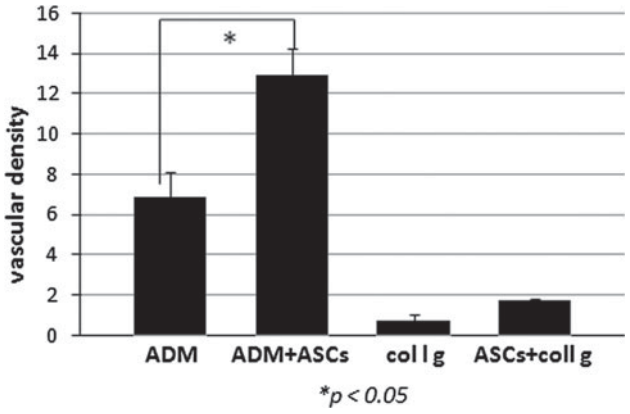

Fig. 4 The vascular density in each group

content in either group 3 or group 4 was less than in either group 1 or group 2 ($p < 0.05$) (Fig. 6).

Fluorescent Microscopy

Postoperative frozen sections showed DiI-positive ASCs at the subpanniculus layer in group 2 (Fig. 7). However, no DiI-positive cells were observed in group 3 (data not shown).

Conclusions

Because ASCs can attach to ADM and decrease its in vivo resorption, ADM seeded with ASCs may be a useful tool for soft tissue augmentation with stable long-term results.

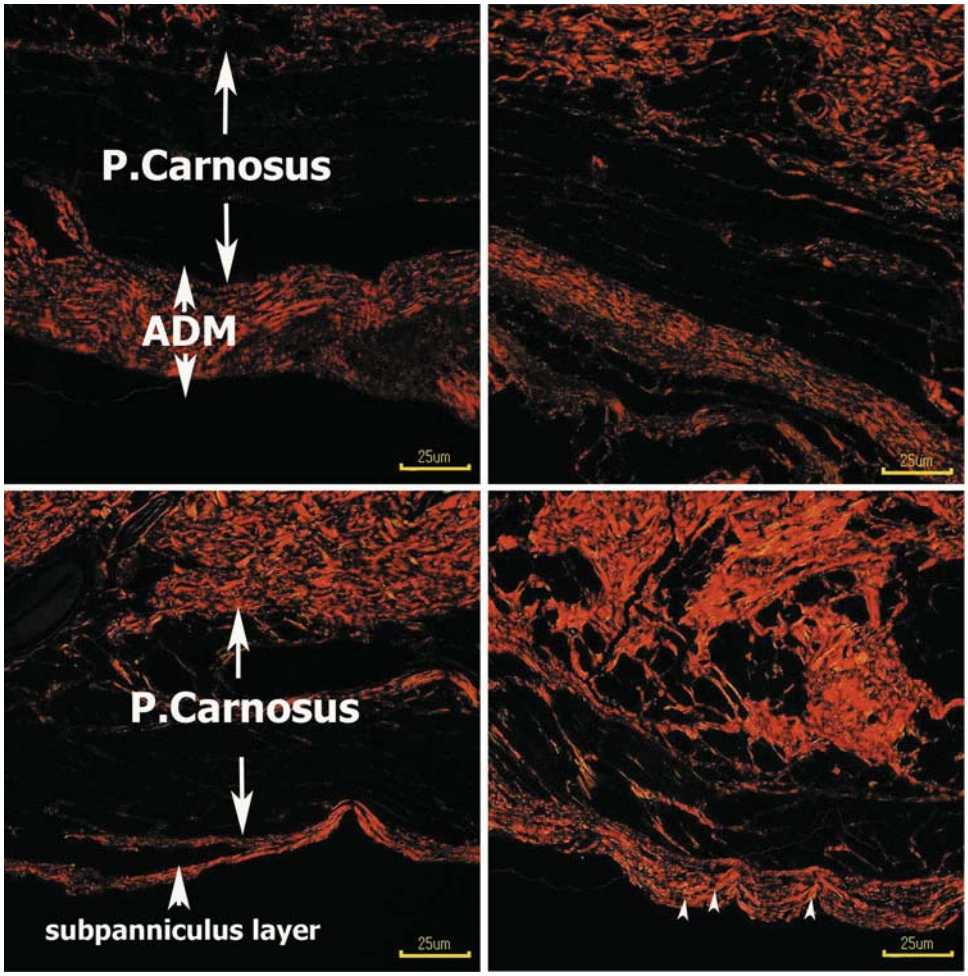

Fig. 5 Representative images of picrosirius *red* staining. *Upper left* group 1, *upper right* group 2, *lower left* group 3, *lower right* group 4. The small *white arrowheads* in the lower right photo mark the type 3 collagen fibers in *green* (microbars 25 μm)

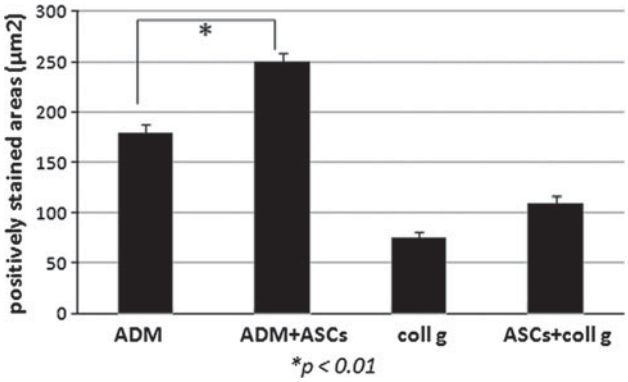

Fig. 6 Comparison of the collagen quantity in the subpanniculus layer (results given in μm^2)

Moreover, ASCs may improve the results in current clinical applications of ADM.

Discussion

The commercially available form of acellular dermal matrix (AlloDerm; LifeCell Corp, Branchburg, NJ, USA), derived from human cadaver skin, is a valuable alternative for soft tissue filling, eliminating problems such as implant rigidity, antigenicity, and donor-site morbidity [28]. Clinical application of AlloDerm in sheet form for the reconstruction of abdominal fascia defects after transverse rectus abdominis (TRAM) flap harvesting is already popular [3, 6]. Other clinical applications are burn reconstruction [6, 28, 31], cleft palate repair [13], construction of skin and mucosa equivalents [33], lip augmentation [27] and, recently, postmastectomy breast reconstruction [18].

Transplanted AlloDerm is well tolerated by the human body and quickly integrated into the recipient site [6, 8, 16, 28, 29], but it still is resorbed in vivo [29]. In this study, to decrease the resorption rates and increase the regenerating capacity of the ADM, we seeded ASCs onto ADM before implantation. Altman et al. [1, 2] successfully seeded ASCs onto ADM for the first time. Easily, ASCs can be obtained from subcutaneous adipose tissue without any type of highly invasive, painful procedures [9, 35]. Although we did not perform FACS analysis for characterization of ASCs in this experiment, data from the previous projects carried out in our lab showed that the cells obtained using

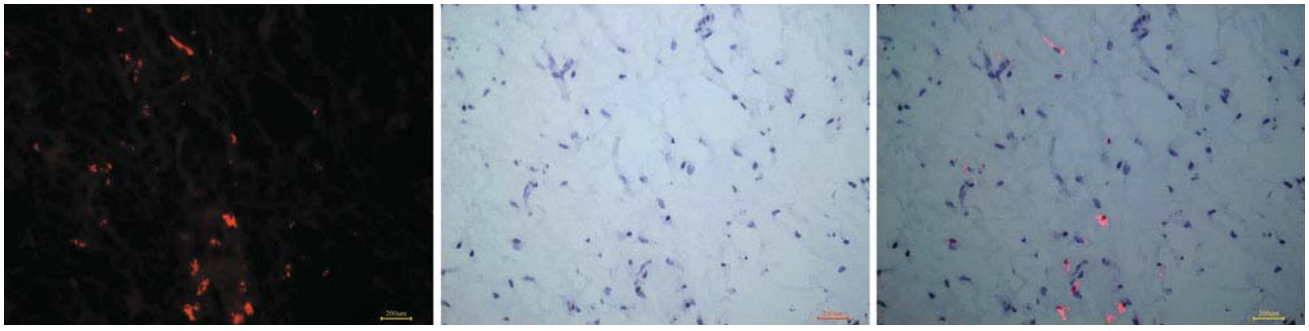

Fig. 7 Frozen sections 8 weeks after surgery (photos taken from group 2). The 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI)-labeled ASCs are still detectable. *Left* DiI image, *middle* hematoxylene image, *right* merged image (microbars 200 μm)

our established protocol expressed CD 11−/31−/34+/44+/ 45−/45R+/49d−/49e+/54+/81+/90− 2+/106+/161c−/184+/c-kit-/Sca-1+ and thus can be called pure ASCs [7].

Several reports indicate the clinical usefulness of ADM seeded with ASCs. Nie et al. [20] hypothesized that local delivery of stem cells via ADM scaffold could effectively accelerate wound healing by secretion of paracrine factors such as vascular endothelial growth factor (VEGF), transforming growth factor β (TGFβ), PDGF, insulin-like growth factor (IGF), and human growth factor (HGF). In a recent study, Kim et al. [10, 12] documented the stimulating effects of ASCs on collagen secretion from fibroblasts. Park et al. [23] reported in 2008 that intradermal injection of ASCs leads to a small increase in dermal thickness but to a distinctive increase in collagen gene expression.

A potential use for this combination is in postmastectomy breast reconstruction with silicone implants for patients who have received radiation therapy. The postoperative results for these patients may not be aesthetically satisfying due to the poor soft tissue coverage of the implants and disturbed integration of AlloDerm [18]. We speculate that if ASCs are used together with AlloDerm, they may help restore AlloDerm integration via paracrine factor secretion. Growing evidence in the literature supports this theory [3, 10, 12, 15, 23, 24].

Acellular dermal matrix with ASCs also can be used for some selected cases of aesthetic soft tissue augmentation in which improvement in the quality of overlying skin also is a goal. Klinger et al. [14] and Mojallal et al. [17] reported an improvement in the overlying skin after fat injection and speculated that ASCs in the fat tissue led to this improvement.

In current clinical practice, bovine collagen or hyaluronic acid usually is used for aesthetic soft tissue augmentation, but both of these materials have high resorption rates and long-term instability [5, 26, 28]. However, it should be mentioned as a drawback of this study that the ADM used was not the commercially available form. Thus, our results should be confirmed using AlloDerm and additional animal models.

Any type of avascular organic material, although autologous, will be resorbed to a certain extent due to the lack of nourishment and progressive loss of viability. If by any means, early vascularization and nourishment of the transplanted organic tissue could be induced, then the extent of resorption might be decreased. In our opinion, this may be a possible explanation for the decrease in absorption of ADM in our experiment. Angiogenic growth factors secreted by ASCs [4, 11, 24, 34] may have promoted the early vascularization, thus improving the nourishment and preventing the absorption of implanted ADM. In group 1, the natural resorption process took place, leading to the difference between this group and group 2 in terms of the thickness of explanted ADM. The increase in subcutaneous thickness in group 3 may be attributed to stimulatory effects of ASCs on fibroblasts and to an indirect increase in the synthesis of collagen and extracellular matrix components, as mentioned previously by Park et al. [23].

Although ASCs also can be applied via the intravenous route, local transplantation has unquestionable advantages over injection, and ADM is a very good alternative as a scaffold for local delivery of the stem cells because it is resistant to infection, nonimmunogenic, and integrated rapidly into the recipient tissues [20]. Based on these results, we have concluded that seeding ADM with ASCs may further enhance the aforementioned properties of ADM and may help the regeneration of new connective tissue at the recipient site. Scaffolds seeded with ASCs may be used to improve the results for other clinical applications of ADM including treatment of abdominal fascia defects [3], burn reconstruction [6, 30, 31] cleft palate repair [13] construction of skin and mucosa equivalents [33], lip augmentation [27], postmastectomy breast reconstruction [18], and wound repair [15].

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Conflict of interest None.

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