Micronized Cross-Linked Human Acellular Dermal Matrices: An Effective Scaffold for Collagen Synthesis and Promising Material for Tissue Augmentation

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Abstract Micronized cross-linked human acellular dermal matrices (ADMs) contain the extracellular components necessary for cell integration and tissue remodeling, and have high tensile strength and durability. We hypothesized that such material could serve as a scaffold to enhance the survival of adipocytes in grafted fat. Nude mice ($n = 15$) were randomly assigned to three groups, each receiving different subcutaneous injections into two dorsal paravertebral areas: fat and saline (control), fat and micronized ADM (E1), and fat and diluted micronized ADM (E2). Digital photographs were taken at 2-week intervals, and the grafted fat volumes and weights were examined after 10 weeks. A histological analysis of the grafted fat was performed, and the expression of vascular endothelial growth factor (VEGF) was examined. The grafted fat volumes decreased over time in all groups; however, at 10 weeks, the grafted fat was better preserved in both experimental groups, with significantly greater weights than in the group C (both, $p < 0.001$). In the experimental groups, there was more regular arrangement of collagen in the graft tissue, whereas relatively thin, disorderly collagen deposition was observed in the control group. In addition, VEGF expression was significantly greater in the experimental groups than in the control group ($p < 0.001$). These results are the first to show that micronized cross-linked human ADMs are an excellent scaffold for promoting adipocyte survival and may be an option for maintaining or promoting the *in vivo* survival of grafted fat.

Keywords Fat graft · Acellular dermal matrix · Scaffold · Adipocytes

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1 Introduction

Autologous fat grafting is a common aesthetic or reconstructive procedure for augmenting volume deficits or restoring various soft tissue defects. Autologous adipose tissue is also considered an ideal filling material because of its relative abundance, convenient access, and excellent host compatibility. Nevertheless, its inconsistent absorption yields unpredictable overall outcomes, resulting in challenges for many surgeons. Thus, diverse methods have been introduced to increase viability of the grafted fat and decrease its rate of resorption. In recent years, autologous fat grafts have been combined with numerous agents, including growth factors (platelet-derived growth factor, basic fibroblast growth factor, vascular endothelial growth factor [VEGF], insulin-like growth factor-1, and epidermal growth factor) and other agents (platelet-rich plasma,

activated M2 macrophages, polymer therapy, and matrix metalloproteinase-2) [1]. In addition, efforts to increase grafted fat survival have been made and are ongoing, including the use of botulinum toxin A, deferoxamine, oxygen-generating microspheres, *N*-acetylcysteine, and platelet-rich plasma [1–4].

Previously, we introduced a novel micronized cross-linked human acellular dermal matrix (micronized ADM; MegaDerm™), which allows adequate tissue ingrowth, provides long-term structural integrity, and results in prolonged remodeling [5]. These characteristics suggest that this material is suitable for soft tissue augmentation procedures, such as facial augmentation, abdominal hernia repair, and breast augmentation [6, 7].

Commercial micronized ADM (L&C Bio, Seongnam-Si, Gyeonggi-Do, Korea) is processed from donated human skin, supplied by US tissue banks following the guidelines of the American Association of Tissue Banks and the applicable rules and regulations of the US Food and Drug Administration. Briefly, micronized ADM is manufactured by micronizing lyophilized ADM using a Pilotina MC (IKA-Werke, Staufen, Germany) with a 500- μ m sieve. It is also composed of collagens, elastin, proteins and proteoglycans as with solid ADM. The particulate form of the tissue matrix is packed into medical device-grade syringes and sterilized using a 25-kGy electron beam. Currently, the material is used as a monotherapy for augmenting soft tissue, and is expected to be used as a scaffold for the ingrowth of native cellular elements in reconstructive surgeries [3, 6, 8].

Itoi et al. compared the suitability of three scaffolds—type I collagen sponges, nonwoven polyglycolic acid (PGA), and hyaluronic acid gels—for *in vivo* adipogenesis involving adipose-derived stem cells; the type I collagen sponge was the most biocompatible scaffold [6]. Although the mechanism has not yet been fully described, the authors suggested that this result was due to the porous structure of the type I collagen sponge, which may promote easy migration, proliferation, and differentiation of adipose-derived stem cells. In the present study, we hypothesized that micronized ADMs could serve as a scaffold for enhancing survival of adipocytes during fat grafting.

2 Materials and methods

2.1 Ethical statement

All animal protocols used in this study were approved by the Institutional Animal Care and Use Committee of Yonsei University (IACUC No. 2013-0306-2). Additionally, all procedures were approved by our institutional review board (IRB No. 4-20130618).

2.2 Fat harvesting and preparation

Fat was obtained from healthy human subjects using Coleman's technique. First, a tumescent solution (normal saline, 1 L; 1% lidocaine, 50 mL; 0.1% epinephrine, 1 mL) was injected into the abdominal donor site. After approximately 20 min, fat was harvested using an appropriate instrument (Byron) and a 50-mL syringe. The extracted fat was centrifuged at 3000 rpm for 3 min. Subsequently, the oil layer was removed and the lower level of fluid, including blood and anesthetics, was decanted. The adipocytes in the residual middle layer were reserved.

2.3 Injection of harvested fat with or without micronized ADM

A total of 15 nude mice (BALB/cAnNCrjBgi-nu/nu; mean age, 9 weeks) were housed in a controlled-temperature animal facility, with a 12-h light/12-h dark cycle. The mice were randomly assigned, in groups of 5 each, to the three experimental groups: control group (C), experimental group 1 (E1), and experimental group 2 (E2) (Table 1).

After anesthetizing the animals with intraperitoneal zolazepam, the micronized ADM mixture was injected subcutaneously into two dorsal paravertebral areas, using an 18-gauge needle; consistent injection pressure was maintained to evenly spread the mixtures. Group C mice received 1 mL of fat and 0.5 mL of saline, those in the E1 group received a mixture of 1 mL of fat, 0.25 mL of ADM solution, and 0.25 mL of saline; those in the E2 group received a mixture of 1 mL of fat, 0.125 mL of ADM, and 0.375 mL of saline.

The fat and micronized ADM were mixed together immediately prior to injection. This was accomplished by placing the materials in separate, but connected, syringes. After removing any trapped air, the syringe contents were moved back and forth between the syringes until a homogenous mixture was obtained.

2.4 Gross evaluation

After injection, digital photographs were taken, at a consistent distance from the animals, at 2-week intervals. The approximate graft volume was calculated, at 2-week intervals, using the gross morphology (length, width, and height of the bulk graft volume). In addition, the length and width of each graft elevation was measured at 2-week intervals. The volume of the elevation was calculated as follows: volume (mm^3) = $(L \times W^2)/2$; “L” represents the longest of the measured lengths and “W” represents the shortest of the measured widths.

To determine long-term fat graft persistence, tissue samples were harvested, following careful dissection of the surrounding tissue, after 10 weeks (Fig. 1). The volumes of the harvested tissue samples were measured using the liquid overflow method, based on Archimede's principle of buoyancy.

2.5 Histologic evaluation

Tissue samples were fixed with 10% formaldehyde, embedded in paraffin blocks, sectioned to 3- μ m thicknesses, mounted on slides, and stained with hematoxylin and eosin (H&E) for histological examination. The slides were analyzed according to several histological parameters. Tissue sections were pretreated with 3% hydrogen peroxide for 10 min to block endogenous peroxidases, then treated with a protein block serum-free reagent (X0909; Dako, Cupertino, CA, USA;) for 30 min to prevent nonspecific reactions. Sections were incubated with primary antibody (rabbit anti-VEGF, RB-222-P; Laboratory Vision, Fremont, CA, USA), at 4 °C overnight, and then at room temperature for 20 min with the Envision Kit reagents (Dako) as secondary antibodies.

2.6 Collagen fiber evaluation

The staining solution for Masson's trichrome stain was prepared using Bouin's solution (picric acid solution, 75 mL; 37% formalin, 25 mL; glacial acetic acid, 5 mL), Weigert's iron hematoxylin solution (hematoxylin, 1 g; 95% ethanol, 100 mL; ferric chloride, 2 g; concentrated HCl, 1 mL; distilled water, 95 mL), Biebrich scarlet-acid fuchsin solution (1% Biebrich scarlet, 90 mL; 1% acid fuchsin, 10 mL; glacial acetic acid, 1 mL), phosphomolybdic-phosphotungstic acid (phosphomolybdic acid, 2.5 g; phosphotungstic acid, 2.5 g), and an aniline blue solution (aniline blue, 2.5 g; distilled water, 100 mL; glacial acetic acid, 2 mL).

2.7 Neovascularization evaluation

VEGF expression was semi-quantitatively analyzed using MetaMorph image analysis software (Universal Image, West Chester, PA, USA), as previously described [7]. The results are expressed as the average optical density (OD) across five different digital images. OD quantifies the

opacity of objects when exposed to transmitted light, and is analogous to an inverse grayscale value pertaining to the amount of spectral, or reflected, light.

2.8 Statistical analysis

All data are presented as mean $\pm$ standard deviation (SD). Statistical analyses were conducted using SAS (version 9.2; SAS Institute, Cary, NC, USA). A linear mixed model was used to compare fat graft persistence among the three groups. Bonferroni's method for multiple comparisons was used in the post-hoc testing (adjusted significance level, $0.05/10 = 0.005$); a p value <0.05 was considered statistically significant.

3 Results

3.1 Grafted fat volume changes

To examine the *in vivo* volume changes for each grafted material, the mice in each group were subjected to volume measurements at four time points. Overall, the measured grafted fat volume decreased, over time, in all groups (Fig. 2). The volumes in both experimental groups (E1 and E2) were significantly greater than that for the control group animals, at multiple time points (Table 1). In addition, the fat graft volumes in the E1 group were significantly greater than those in the E2 group.

Fig. 1 Appearance of grafted fat from the dorsal area of a nude mouse. At 10 weeks postoperatively, the grafted fat was harvested from the dorsal area through a vertical midline incision

Table 1 Volume changes in the grafted fat (mean $\pm$ SD)

Group	2 weeks	4 weeks	8 weeks	10 weeks
Control group	0.832 $\pm$ 0.460	0.682 $\pm$ 0.107	0.408 $\pm$ 0.211	0.350 $\pm$ 0.183
E1 group	1.074 $\pm$ 0.230	0.979 $\pm$ 0.201	0.510 $\pm$ 0.180	0.488 $\pm$ 0.154
E2 group	0.958 $\pm$ 0.201	0.831 $\pm$ 0.354	0.671 $\pm$ 0.128	0.542 $\pm$ 0.146
p value	0.0019	0.0098	0.0004	0.0012

Fig. 2 Grafted fat volume changes at 2, 4, 8, and 10 weeks postoperatively. The mean volumes in both experimental groups (E1 and E2) were significantly greater than that in the Control group at multiple time-points (C)

3.2 Fat graft volumes and weights at 10 weeks

The grafted materials were harvested 10 weeks after the grafting procedure. Surgically dissected specimens from the experimental groups (E1 and E2) were grossly larger than those from group C (Fig. 3). The mean volumes of the grafted samples were 0.332 ± 0.1 mL (group C), 0.637 ± 0.05 mL (E1), and 0.618 ± 0.06 mL (E2). The mean graft volumes for groups E1 and E2 were also significantly greater than that for group C animals (* $p < 0.05$). The mean weights of the grafted samples were 173.8 ± 63.7 mg (C), 459.3 ± 41.3 mg (E1), and 370.9 ± 24.8 mg (E2). The graft weights in both experimental groups were significantly greater than that of group C (** $p < 0.01$). Although there was no significant difference in graft volumes between the E1 and E2 groups, there was a significant difference between the graft weights (Fig. 4).

3.3 Histologic analysis

Histologic evaluations were performed 10 weeks postoperatively, just before the animals were sacrificed. Compared with the experimental groups, the control group

Fig. 3 Representative gross appearance of the samples, at 10 weeks postoperatively. The specimens from the E1 and E2 groups were grossly larger and denser than those from the Control group

Fig. 4 Bar chart showing the weights and volumes of the samples at 10 weeks postoperatively. **A** The fat graft volumes in both experimental groups (E1 and E2) were significantly greater than those in the Control group, at 10 weeks postoperatively (* $p < 0.05$). **B** In addition, the weights of the grafts in both experimental groups were significantly greater than those in the Control group, at the same time point (** $p < 0.01$)

showed diffuse inflammatory cell infiltrates and large, vacuolar degenerations. In the E1 group, there was mild vacuolar degeneration and intervening, haphazardly arranged collagen bundles; the E2 group displayed rare vacuolar degenerations, with relatively ordered collagen bundle arrangements. In addition, central necrosis was prominent, at low-power magnification, in the group C specimens (Fig. 5). The E2 group showed partial central necrosis, whereas relatively well-preserved central adipose tissue was typically seen in the E1 group.

Masson's trichrome staining revealed regular collagen arrangements in the graft tissue, which was uniformly stained in the experimental groups. Relatively thin, disordered collagen deposition was observed in the control group (100 $\times$; Fig. 6).

We also examined VEGF expression levels using the total integrated OD of immunohistochemically stained tissue specimens. A semi-quantitative analysis demonstrated that VEGF expression was significantly increased in the experimental groups ($213,525 \pm 63,314$ integrated OD units) compared with that in the control group ($108,736 \pm 34,453$ integrated OD units; $p < 0.001$).

Fig. 5 Diffuse inflammatory cell infiltrations and large vacuolar degenerations are evident in the Control group, but not in the experimental groups. **A** Representative microscopic findings of the Control group, at 10 weeks postoperatively (Hematoxylin and eosin stain, $\times 100$). **B** Representative microscopic findings of the E1 group, at 10 weeks postoperatively (Hematoxylin and eosin stain, $\times 100$). **C** Representative microscopic findings of the E2 group, at 10 weeks postoperatively (Hematoxylin and eosin stain, $\times 100$)

4 Discussion

Itoi et al. suggested that type I collagen sponges are bio-compatible scaffolds [6]. They also suggested that the relatively porous structure of the type I collagen sponges may promote the easy migration, proliferation, and differentiation of adipose-derived stem cells.

Micronized ADMs, containing type I collagen, elastin, proteins, and proteoglycans, are increasingly being used in reconstructive surgery, serving as scaffolds for the ingrowth of native cellular elements [8, 9]. Because micronized ADMs contain type I collagen, we hypothesized that this material would also enhance the survival of grafted fat. Among the available micronized ADMs, the current study utilized a micronized ADM that was processed using donated human skin.

In the current study, the calculated grafted fat volumes in both experimental groups were greater than that in the control group at multiple time points; the differences were especially prominent at 2 and 4 weeks, postoperatively. Furthermore, at 10 weeks postoperatively, the volumes and weights of the harvested samples were significantly greater for both experimental groups than were those from the control group. Based on these gross evaluations, micronized ADM has the potential for use as a supplement that increases fat graft survival.

Moreover, H&E staining indicated that the use of micronized ADM, during fat grafting, improves the survival of the grafted fat by reducing inflammatory cell

infiltrates. Additionally, VEGF immunohistochemistry revealed significantly upregulated new vessel growth in the experimental groups, compared with the control group. Masson's trichrome staining revealed a generally regular arrangement and a higher density of collagen fibers in the groups receiving fat and micronized ADMs, compared with the control animals. This positive effect on collagen production might be correlated with the decreased inflammatory cell infiltration observed in the H&E stained sections.

According to Kato et al. superficially located adipocytes are more likely to survive within a fat particle than those located in the deepest portions [10]. Our histological findings showed that both experimental groups, regardless of the degree of ADM dilution, had more adipocytes and higher collagen densities than did the control animal grafts. Thus, we believe that micronized ADM has an adipogenic effect and acts as an effective scaffold, keeping adipocytes in contact with the surrounding tissue.

The cell survival theory states that the cellular population of grafts consists of adipocytes that are able to diffuse into the tissue to initially survive transplantation. This is followed by angiogenesis within the recipient tissue. Thus, we assumed that micronized ADM might help grafted adipocytes to effectively diffuse into the surrounding microenvironment. Additionally, we believe that micronized ADM contributes to long-term volume maintenance, based on the 10-week postoperative volume and weight measurements reported in the present study.

Fig. 6 Representative microscopic findings, at 10 weeks postoperatively (Masson's trichrome stain, $\times 400$) Masson's trichrome staining was performed to evaluate and compare collagen deposition in the treatment groups. Animals in groups E1 and E2 show higher collagen density than animals in the control group. A significantly higher density of well-arranged collagen bundles is evident in group E1 animals compared with group E2 animals. **A** Representative

microscopic findings of the control group, at 10 weeks postoperatively (Masson's trichrome stain, $\times 400$). **B** Representative microscopic findings of the E1 group, at 10 weeks postoperatively (Masson's trichrome stain, $\times 400$). **C** Representative microscopic findings of the E2 group, at 10 weeks postoperatively (Masson's trichrome stain, $\times 400$)

In addition to volume maintenance, the properties of the grafted fat are also important. Although viscoelastic properties were not objectively evaluated in the present study, the grafted fat samples in both experimental groups remained soft. Yoshimura previously reported on the fate of adipocytes in grafted adipose tissues. Three months after fat grafting, the transplanted adipocytes were categorized as being in surviving, regenerating, or necrotizing zones [10]. During regeneration, adipocyte absorption and replacement with fibrosis occurs. Moreover, Nishimura et al. reported that fibrosis occurs in fat cells at 90 and 180 days after transplantation [11]. They also reported that fibrosis in grafted fat is dependent on the characteristics of the microenvironment, such as the extent of microvascular angiogenesis.

In the present study, the experimental group animals, receiving fat mixed with micronized ADM, showed more numerous vessels and less inflammatory or fibrotic reactions than did the control animals. These results suggest that the micronized ADM facilitates angiogenesis and maintains fat volume. Further studies are warranted to determine the optimal fat volume for maximizing the effects of micronized ADM. In addition, the experimental

group animals showed more homogenous collagen fiber arrangements than did the control animals. We believe that this type of collagen arrangement resulted from improved microvascular angiogenesis, leading to favorable properties in the grafted fat.

Clinically, micronized ADM has the advantage of being injectable and compatible with available dermal fillers. This characteristic makes the material very easy for many clinicians to use. Additionally, the non-solid nature of the material ensures that three-dimensional soft tissue defects can be camouflaged without difficulty.

The current study has several limitations. First, considering the importance of fat particle size in fat graft survival, further studies are necessary to optimize the fat particle size for supporting micronized dermal matrices. In addition, dosage optimization of both the fat and the micronized ADM would be another interesting topic for a future study. Lastly, as we grafted human abdominal fat into immunocompromised animals, the actual performance of micronized ADM in the context of human fat grafting will require investigation.

To the best of our knowledge, the current investigation is the first to show that micronized ADM acts as an

excellent scaffold for promoting adipocyte survival. We suggested that our micronized ADMs could serve as a scaffold for enhancing survival of adipocytes by promoting easy migration, proliferation, and differentiation of adipose-derived stem cells. Therefore, this material may be an excellent option for maintaining or even increasing the *in vivo* survival of grafted fat.

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Compliance with ethical standards

Conflict of interest No authors have conflicts of interest that could potentially influence the described research.

Ethical statement All animal protocols used in this study were approved by the Institutional Animal Care and Use Committee of Yonsei University (IACUC No. 2013-0306-2). Additionally, all procedures were approved by our institutional review board (IRB No. 4-20130618).

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