Short Communication

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Regenerative Medicine

Micronized cellular adipose matrix as a therapeutic injectable for diabetic ulcer

Background: Despite the clinical potential of adipose-derived stem/stromal cells (ASCs), there are some clinical difficulties due to the regulation of cell therapies. **Materials & methods:** Micronized cellular adipose matrix (MCAM) injectable was prepared through selective extraction of connective tissue fractions in fat tissue only through mechanical minimal manipulation procedures. **Results:** It retained some capillaries and ASCs, but most adipocytes were removed. The presence of viable ASCs, vascular endothelial cells was confirmed and ASCs of MCAM kept intact mesenchymal differentiation capacity. In diabetic mice, skin wounds treated with MCAM showed significantly accelerated healing compared with phosphate-buffered saline-treated ones. **Conclusion:** The proven potential of MCAM to accelerate healing in ischemic diabetic ulcers may offer a simple, safe and minimally invasive means for tissue repair and revitalization.

Keywords: adipose stem cells • diabetic ulcer • extracellular matrix • flow cytometry • ischemia • lipoaspirates • minimal manipulation • tissue revitalization • vascular endothelial cells • wound healing

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Adipose tissue is structurally complex, harboring a variety of cells within its lobulated fibrous septal network. Through enzymatic digestion, this network can be disintegrated and its heterogeneous complement of indigenous cells, or so called stromal vascular fraction (SVF), may be isolated. Because adipose-derived stem/stromal cells (ASCs) are important SVF residents with mesenchymal multipotency [1,2], SVF has been strategically engaged as a supplement to enhance fat engraftment [1,3–4]. However, if dissociated SVF cells are injected separately, rather than properly integrated into grafted fat, unexpected migratory and/or phenotypic outcomes may result, creating adverse complications (i.e., ectopic fibrosis and lymphadenopathy) [5]. Currently, a number of vehicles for delivery of cells are available, such as injectable biomaterial scaffolds, 3D spheroidal cell cultures and engineered cell sheets [6–8]. These sophisticated constructs help prevent untoward cell migration, thus avoiding lost or undesired contributions by cellular and extracellular matrix (ECM) componentry.

Decellularized ECM of various tissues or organs may serve as bioactive scaffolding, thereby facilitating tissue remodeling and repair [9–11]. Although adipocytes account for >90% of fatty tissue by volume, the native ECM of fat provides a niche for other cellular subsets (e.g., ASCs, vascular endothelial cells and pericytes), enabling biologic functions that are shared in part with acellular dermal matrix.

As niche components, stem cells generally lie in wait for changes in microenvironment. Stem cells isolated from the tissue, however, are already activated in an unphysiological microenvironment, and thus extra care needs to be taken in controlling the fate and behaviors of those cells in clinical utilization. Since 2005, policies of the US FDA aimed at preventing potential contamination and genetic alteration of

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stem cells [12] and enzymatic isolation and cultural expansion of ASCs were not considered as 'minimal manipulation', although isolated or cultured ASCs are already extensively used in a number of clinical trials.

For this investigation, a bioactive injectable comprised of functional ECM and resident cells (including ASCs) was formulated through minimal manipulation of adipose tissue. Given the combination of micronized connective tissue, viable ASCs and vascular endothelial cells generated, we have applied the term micronized cellular adipose matrix (MCAM). In this study, injectable MCAM was tested for its therapeutic value for wound repair of diabetic skin ulcer. Wound healing impairment in diabetic patients is a significant clinical issue affecting millions of patients worldwide. The major underlying pathology is noted to be chronic inflammation and ischemia based on peripheral vascular dysfunction, where tissue-resident stem cells are considered to be depleted. Although numerous products for wound dressing with bioactive ECM or growth factors are available, the clinical effects on diabetic ulcer are very limited. Thus, we sought to characterize and evaluate MCAM, which contains human adipose derived stem cells and vascular endothelial cells in their original niche of ECM, as a potential therapeutic tool for stem cell-depleted pathological conditions.

Materials & methods

Mouse tissue preparation

C57BL/6JJcL mouse inguinal fat pads were harvested, washed and weighed. One gram of fat pads was cut into tiny pieces with surgical scissors (continuous fine mincing for 5 min). After transfer to a tube containing 2.5 ml cooled phosphate-buffered saline (PBS), the morcellated tissue was thoroughly shaken several times and then centrifuged (800 × *g*, 5 min). MCAM was extracted as tissue sediment, and floating fatty tissue was also sampled.

Human tissue preparation

Lipoaspirate was obtained from a healthy 23-year-old female donor (BMI = 24) submitting to abdominal liposuction under general anesthesia. The study protocol was approved by our Institutional Review Board. Once soft tissues were infiltrated with a solution of saline plus epinephrine (1:1,000,000), subcutaneous fat was suctioned (-500 to -700 mmHg) using a conventional liposuction machine equipped with a 2.5-mm (inner diameter) cannula. MCAM was extracted from the lipoaspirate with the above described micronization and centrifugation, and floating fat was also sampled.

Whole-mount staining

Whole-mount staining was performed with Wheat germ agglutinin (WGA) Alexa Fluor 488 (Life Technologies, CA, USA), lectin PNA Alexa Fluor 594 (Life Technologies) and Hoechst 33342 solution (Dojindo, Kumamoto, Japan) as instructed by manufacturers. Images were then acquired via confocal microscope (Leica DMIRE2; Leica Microsystems, Wetzlar, Germany).

Flow cytometry

Mouse MCAM and roughly excised inguinal fat were separately prepared. Each sample was digested in 0.1% collagenase (Wako, Osaka, Japan) Hank's balanced salt solution (HBSS) by incubation in a shaking water bath (37°C, 30 min). To remove the collagenase, SVF cells were washed with PBS for three-times. After filtration, SVF cells were washed and stained with the following antibodies and corresponding isotype controls: Anti-CD45-Viogreen (Miltenyi Biotec, Bergisch Gladbach, Germany), Anti-CD34-Biotin (eBioscience, Inc., CA, USA), Rat IgG2a Kappa Control Biotin (BD Biosciences, CA, USA), Streptavidin-APC (BD Biosciences), Anti-CD31-PE (BD Biosciences), Rat IgG2a Kappa Control PE (BD Biosciences). Samples and controls were then analyzed by flow cytometry (MACSQuant Analyzer 10; Miltenyi Biotec). Gating for each signal was set to eliminate 99.9% of the cells in corresponding isotype control. CD45 gating was applied first, and CD45-negative portion was further analyzed for CD34 and CD31 signals.

Cultured ASCs of human origin

Human ASCs in floating fat and in MCAM were cultured separately. Floating fat was digested with collagenase (as above) [12], and isolated SVF cells were seeded in Dulbecco's Modified Eagle Media (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 IU penicillin and 100 mg/ml streptomycin. Explant culture was performed for human MCAM to generate MCAM-derived ASCs.

Multilineage differentiation assay

Assays of adipogenic, osteogenic and chondrogenic lineages were conducted as follows: adipogenic differentiation: ASCs were incubated for 21 days in DMEM containing 10% FBS, 0.5 mM isobutyl-methyl-xanthine, 1 M dexamethasone, 10 μM insulin and 200 μM indomethacin; osteogenic differentiation: ASCs were incubated for 21 days in DMEM containing 10% FBS supplemented with 0.1 mM dexamethasone, 50 mM ascorbate-2-phosphate and 10 mM glycerophosphate (Nacalai Tesque Inc, Kyoto, Japan); and chondrogenic differentiation: a micromass culture system was

utilized, as previously reported [13], incubating ASCs in a 15 ml tube for 21 days in DMEM containing 1% FBS supplemented with 6.25 mg/ml insulin, 10 ng/ml TGFβ-1 and 50 nM of ascorbate-2-phosphate. Regular growth medium was used to plate controls. The three lineages were analyzed qualitatively via Nile red (adipogenic), von Kossa (osteogenic) and Alcian blue (chondrogenic) differential staining and gauged quantitatively by AdipoRed assay (Lonza, Basel, Switzerland), Calcium-E test (Wako Pure Chemical Industries Ltd, Osaka, Japan) and micromass diameter.

Healing of diabetic ulcers in mice models

Care of B6-db/db mice (BKS.Cg/Lepr^{db}/m/JCL, 8-week old male) was conducted in accordance with institutional guidelines, using a protocol approved by the Animal Experimental Committee of University of Tokyo. Under general anesthesia (isoflurane inhalation), the B6-db/db mice were depilated and two full-thickness cutaneous wounds (6 mm each) were created on both sides dorsally, using skin punch devices. A donut-shaped silicone splint was then placed to prevent wound contraction and secured by interrupted 6–0 nylon sutures. MCAM prepared from one inguinal fat pad of a wild-type B6 mouse was injected by 29-G needle into four differing points of subcutis at wound peripheries (n = 4). Injection of PBS served as control. Treated wounds and splints were covered by transparent sterile dressings. Wounds were photographed on days 0, 2, 4, 7, 9, 11 and 14, determining their areas by Photoshop CS5 (Adobe Systems, CA, USA).

Statistical analysis

Results were expressed as mean ± standard error of the mean. To compare capacity for multilineage differentiation, Student's *t*-test was applied; and paired *t*-test was invoked for comparing wound sizes. p-values < 0.05 were considered statistically significant.

Results

Microstructure of micronized cellular adipose matrix

After mouse, adipose tissue was morcellated (100–400 μm maximum dimension), suspended in PBS and centrifuged, tissues were separated into yellowish floating adipose tissue (floating fat) and whitish bottom sedimentation (MCAM) (Figure 1A, left). Scanning electron microscopy (SEM) confirmed a scarcity of adipocytes in MCAM, whereas adipocytes were abundant in floating fat (Figure 1A, right). The lobular structure of floating fat was maintained, with MCAM consisting primarily of connective tissue and collagen bundles.

Whole-mount imaging highlighted functional features of MCAM (Figure 1B). As noted by scanning electron microscopy, MCAM was lacking in mature adipocytes (i.e., the large, round WGA$^+$ (wheat germ agglutinin) green cells seen in floating fat). However, Hoechst$^+$ nucleated cells persisted in MCAM at rather high density. In addition, MCAM retained branches and segments of vessels and identifiable capillaries.

Cellular content of SVF isolated from mouse MCAM

Fluorescence-activated cell sorting analyses were performed to delineate cellular composition, once SVFs were individually isolated from floating fat and MCAM through collagenase digestion (Figure 2). SVFs of fat and MCAM were characterized through a combination of surface markers and classified into four subpopulations; hematopoietic cells (mainly white blood cells; CD45$^+$), vascular endothelial cells (CD45$^-$/CD31$^+$/CD34$^+$), ASCs (CD45$^-$/CD31$^-$/CD34$^+$), and other cells (CD45$^-$/CD31$^-$/CD34$^-$). The SVF of floating fat consisted largely of CD45$^-$ nonhematopoietic cells (~75%), with ASCs accounting for half of the nonhematopoietic fraction. The SVF of MCAM contained all four subpopulations (including ASCs) albeit in differing ratios. ASCs accounted for 13% of MCAM-SVF cells, with hematopoietic cells, vascular endothelial cells and other cells constituting 57.9, 0.7 and 28.3%, respectively. The original protocol was designed for optimal adipose digestion, which may explain the less efficient digestion of MCAM connective tissue and its different composition of cells (ASCs and vascular endothelial cells).

Multilineage differentiation capacity of ASCs isolated from human MCAM

MCAM and floating fat were also extracted from the human lipoaspirate. Cultured ASCs derived from human floating fat and MCAM were compared in terms of capacity for differentiating into three mesenchymal lineages: adipogenic, osteogenic and chondrogenic. After 3 weeks of induction, ASCs of both floating fat and MCAM displayed similar degrees of multilineage differentiation. No morphologic differences in Nile red, von Kossa or Alcian blue staining were detected (Figure 3A), nor did quantification of lipid content, calcium deposition and micromass diameter (reflecting adipogenesis, osteogenesis and chondrogenesis, respectively) differ significantly (Figure 3B). Thus, it was indicated that MCAM contained ASCs with similar differentiating capability to those obtained from regular adipose tissue.

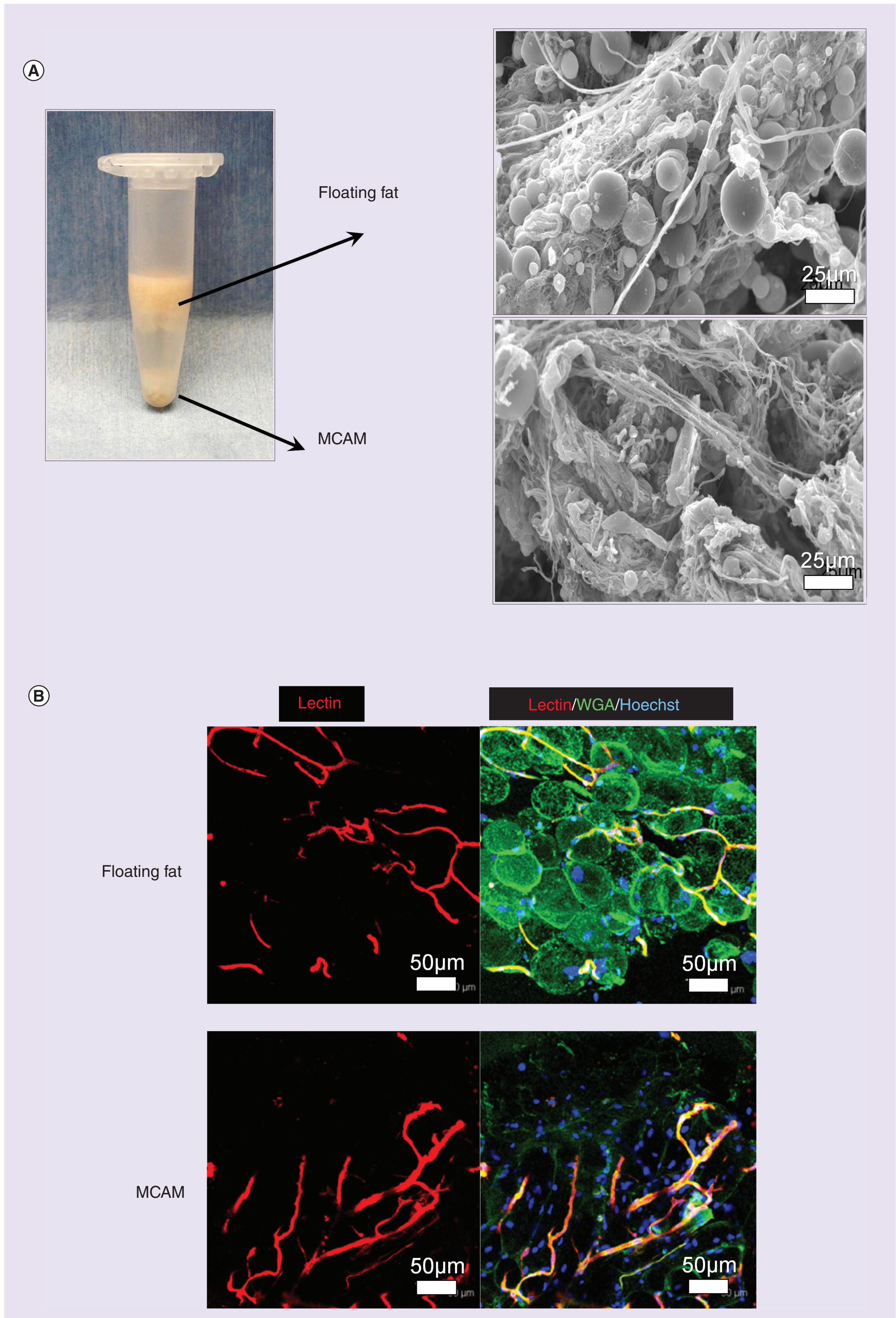
A
Floating fat
MCAM
25μm
25μm
B
Lectin
Lectin/WGA/Hoechst
Floating fat
MCAM
50μm
50μm
50μm
50μm

Figure 1. Microstructure of floating fat and micronized cellular adipose matrix (see facing page). (A) Once minced inguinal fat pad of mouse was centrifuged, micronized cellular adipose matrix (MCAM; whitish sediment) separated from floating fat. Scanning electron microscopy revealed highly fibrous nature of MCAM (vs floating fat), with few mature adipocytes. Bars = 25 μm. **(B)** Whole-mount staining of lectin (vascular endothelial cells: red), wheat germ agglutinin (cell membranes: green) and Hoechst (nuclei: blue); mature adipocytes scarce in MCAM, but microvascular structures in connective tissue remained intact. Bars = 50 μm.
MCAM: Micronized cellular adipose matrix; WGA: Wheat germ agglutinin.
For color images please see online at www.futuremedicine.com/doi/full/10.2217/RME.15.48

Therapeutic effects of mouse MCAM for diabetic ulcers

Subcutis of full-thickness dorsal skin ulcers inflicted in diabetic mice was injected at wound peripheries with MCAM prepared from wild-type mice, using pBS injections as control. Healing of diabetic ulcers was significantly more rapid with MCAM (vs pBS) injection, with 64% smaller wound size on day 4 ($p = 0.0082$) and 65% smaller size on day 7 ($p = 0.0043$; Figure 4). At the close of week 2, closure was essentially completed in MCAM-treated ulcers, whereas wound beds of pBS-treated ulcers remained hyperemic.

Discussion

For this investigation, our injectable MCAM by design was a formulation of bioactive ECM and functional ASCs, prepared by mechanical mincing and elimination of adipocytes from adipose tissue. Sharp-bladed instruments, such as scissors, easily micronized the connective tissue in fat without significantly altering its basic structure, and viability of cells was retained. Approximate specific gravities of human adipocyte and connective tissue obtained from human lipoaspirates were 0.85–0.87 and 1.1–1.2, respectively, underscoring that MCAM regularly sediments upon centrifugation of processed samples.

There is an abundance of evidence affirming the biologic utility of ECM, namely the capacity to regulate proliferation and differentiation of tissue-resident stem cells [14]. Acellular ECM products, whether dermal or fatty by nature, have proven therapeutic in clinical and experimental contexts [9–11,15–16], providing biocompatible substrates or scaffolding and trophic/growth factors needed to accommodate and recruit stem/progenitor cells. In addition to supplying ECM essentials, our MCAM injectable also incorporates an array of viable cells (ASCs, vascular endothelial cells and more) that engage in wound healing.

To date, such scaffolds are designed to mimic native environments, ensuring original cell functions are maintained for optimal cell expansion and tissue regeneration [17–19]. ASCs in MCAM occupy their original ECM niche, ready to assume original roles. As shown by whole-mount staining and flow cytometry, vessels and capillaries of MCAM, as well as ASCs and other stromal cells, retained their natural states and positions. Functional aspects of ASCs in MCAM were also well preserved, as partly indicated by differentiation assays.

ASCs are thought to be potent sources of trophic factors and to have multilineage differentiation capacity. Nonetheless, in transplantation of dissociated (suspended) ASCs, local retention was found to be poor (many disappearing in 1 week), thus nullifying therapeutic intent or resulting in unexpected stem cell behaviors [5,7,20]. As the size of injectable MCAM ranges 100–400 μm, the ECM of MCAM may also provide better mechanical support and anchorage for ASCs to avoid their rapid and seemingly detrimental migration.

Our *in vivo* results suggest that MCAM does impact the healing of diabetic ulcers by accelerating tissue repair. Although similar therapeutic effects have already been reported with use of isolated and cultured ASCs in diabetic or generic refractory ulcers [21–24], substantial procedural manipulations (i.e., enzymatic digestion and isolation or culture of cells) to possibly change biological properties of ASCs are not required for MCAM preparation, eliminating potential regulatory concerns. In skin wound healing models, significant differences are usually observed at a specific and limited time range [25,26], because the skin wound closes eventually in any model (thus no difference at later stages). Our objective was to design a safe and functional injectable, requiring little in the way of preparation. In this study, MCAM obtained from wild-type healthy mice was administered into diabetic mice and it is a limitation of this study that MCAM from diabetic mice was not evaluated. Although ASCs from diabetic patients may not have the same function as those from healthy subjects, ASCs are known to be relatively immunoprivileged and may work as a temporary drug to release trophic factors and help wound healing by allogeneic use.

Fat grafting is claimed to have comparable clinical effects, promoting wound healing of refractory ulcers, such as those seen postirradiation [27–29], and providing a remedy for stem-cell depleted conditions such as chronic ischemia [30,31], systemic sclerosis [32] and scar contracture [33–35]. Improvement of such problematic conditions suggests that the clinical benefits may be partly attributable to functional ASCs. However, if volumetric restoration is not desired, as in transplantations for tissue revitalization/fertilization, the volume occupied by adipocytes within the graft would signifi-

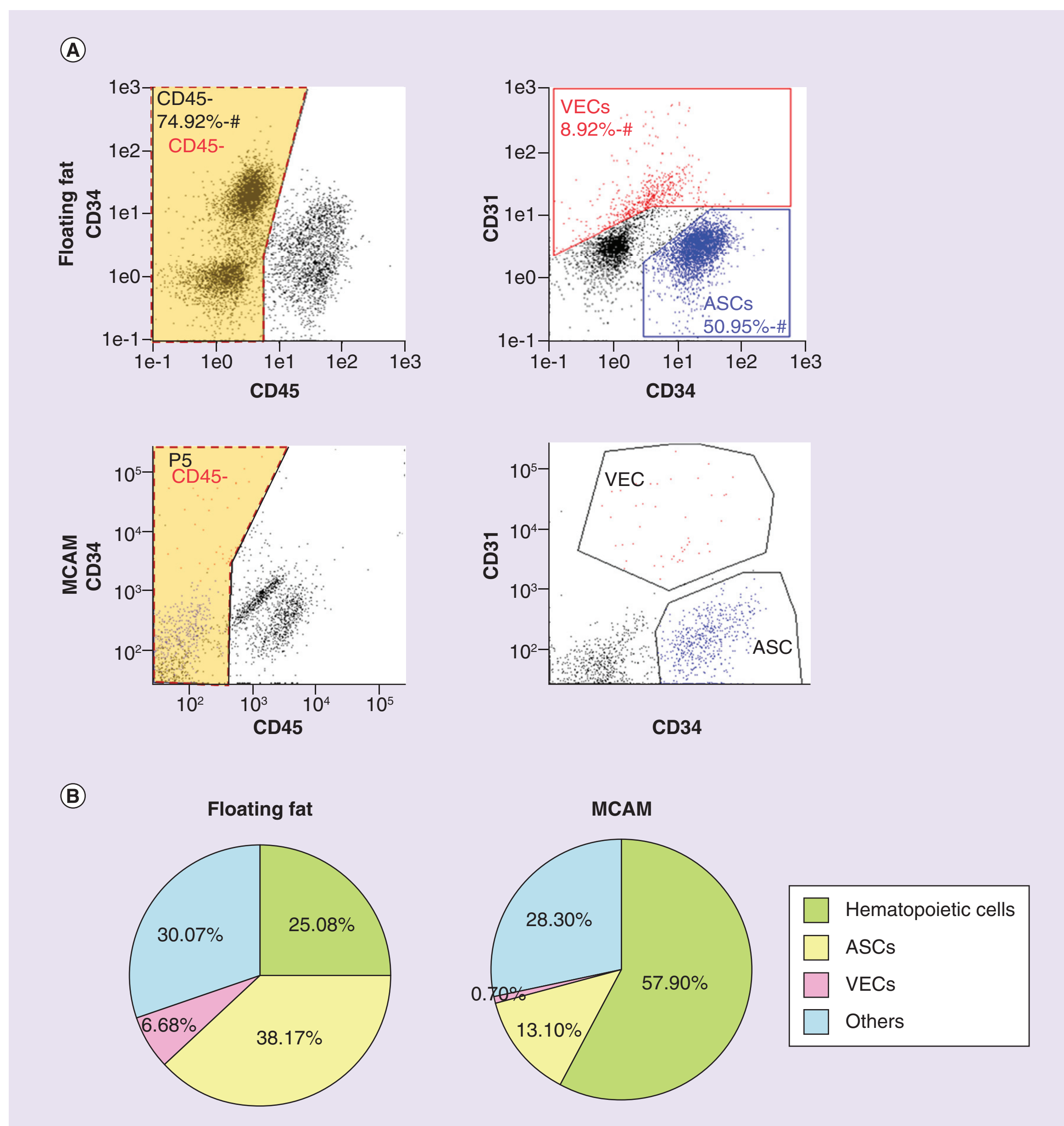

Figure 2. Cellular content of stromal vascular fraction isolated from mouse floating fat and micronized cellular adipose matrix. (A) SVFs from floating fat and that from MCAM were isolated through digestion by collagenase, identifying content as follows: hematopoietic cells (primarily WBCs; $CD45^+$), VECs ($CD45^-/CD31^+/CD34^+$), ASCs ($CD45^-/CD31^-/CD34^+$) and other cells ($CD45^-/CD31^-/CD34^-$). **(B)** ASCs in MCAM account for 13.1% of SVF cells. Hematopoietic cells, VECs and other cells comprise 57.9, 0.7 and 28.3%, respectively. More ASCs (38.2%) and VECs (6.7%) are evident in SVF of floating fat by comparison.
ASC: Adipose-derived stem/stromal cell; MCAM: Micronized cellular adipose matrix; SVF: Stromal vascular fraction; VEC: Vascular endothelial cell.

cantly decrease the density of ASCs and ECM. In these cases, ASCs alone or in conjunction with ECM (as in MCAM) may provide sufficient therapeutic effect, as we have shown.

Conclusion

MCAM is an autologous/allogeneic injectable of bioactive ECM and functional cellular components generated through minimal manipulation of adipose tissue. Smaller-sized and more homogeneous (50–100 μm) particles may be preferable as injectables, but as we already learned, such preparations may jeopardize the viability of cells. Further studies are needed to verify the hypothesized mechanism underlying the efficacy of MCAM and further optimization of preparation methods. Therapeutic effect of MCAM in other stem

cell-depleted states, including irradiation damage and fibrous diseases will also need to be tested. Besides, we still need to elucidate the specific functions of adipocytes in clinical fat grafting and to see what benefits of fat grafting will be lost when we use the product like MCAM, where mature adipocytes are virtually absent. Our efforts here attest to the therapeutic potential of MCAM in ischemic diabetic ulcers, offering a novel mode of tissue repair and revitalization with a minimally invasive approach.

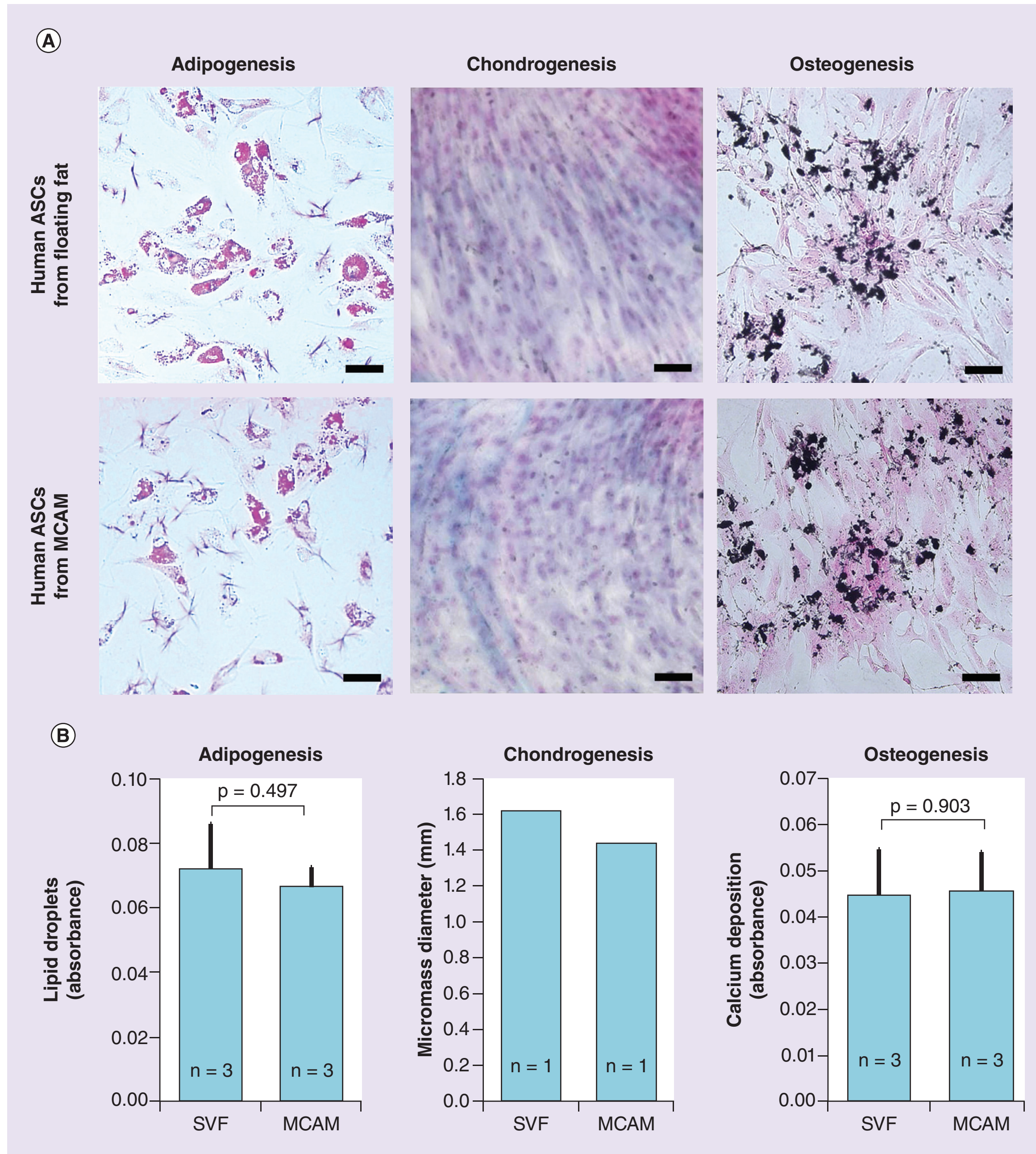

Figure 3. Multilineage differentiation capacity of cultured adipose-derived stem/stromal cells isolated from human floating fat and micronized cellular adipose matrix. (A) Microscopic images of differential induction: cultured human ASCs of floating fat and MCAM yielded similar adipogenic, chondrogenic and osteogenic differentiation; lineage-specific differentiation delineated by Nile red, Alcian blue and von Kossa stains, respectively. Scale bar = 100 μm. **(B)** Quantitative analysis of cellular differentiation: capacity for multilineage differentiation, as indicated by accumulated lipid (adipogenesis), micromass diameter (chondrogenesis) and calcium deposition (osteogenesis), showing no significant differences between fractions.
ASC: Adipose-derived stem/stromal cell; MCAM: Micronized cellular adipose matrix; SVF: Stromal vascular fraction.

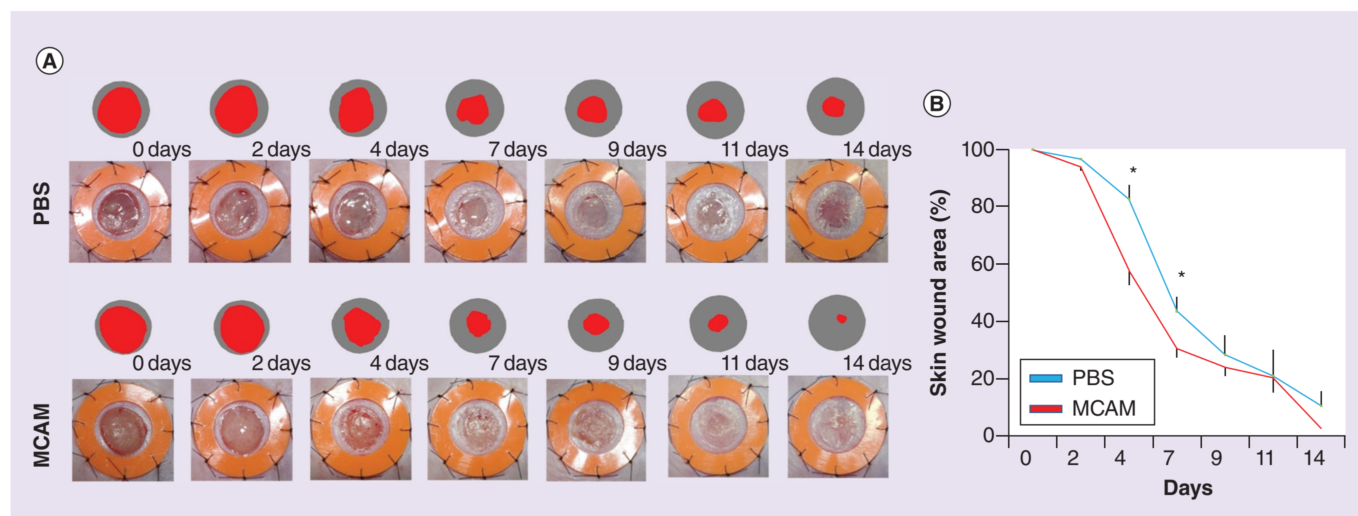

Figure 4. Therapeutic mouse micronized cellular adipose matrix injection of diabetic ulcers. (A) Representative photos of cutaneous wounds (6 mm) created on dorsal areas of diabetic (db/db) mice (n = 4) and treated by local injection of PBS or MCAM on day 0, with 2-week follow-up; ulcerated surface areas determined via software. **(B)** Comparison of wound surface areas (determined digitally): significantly reduced ulcer size in MCAM-injected mice (vs PBS-injected controls), day 4 ($p = 0.0082$) and day 7 ($p = 0.0432$).
MCAM: Micronized cellular adipose matrix; PBS: Phosphate-buffered saline.

Future perspective

Regulatory organizations in many countries regard SVF isolated from adipose tissue as a 'more than minimal manipulated' biological drug, of which uses are strictly regulated due to its associated safety issues. Although therapeutic potential of ASCs in the SVF was extensively studied, cell suspension of ASCs may not be the optimal means in order to maximize its therapeutic effects and avoid unfavorable migration. If injectable tools containing ASCs can be prepared through minimal manipulation and show comparable therapeutic effects, it would be a potential alternative. Also, as ASCs are relatively immunoprivileged, allogeneic ASCs may be clinically used, for example, as a temporarily-working drug to release cytokines. It may be a good news for diseased patients whose ASCs cannot function as those of healthy patients. In this study, we focused on the characterization and therapeutic effects of MCAM as the first step to develop a new cellular/tissue product. MCAM accommodates viable ASCs within their natural niche and may be valuable in

Executive summary

Micronized cellular adipose matrix definition and preparation

- MCAM is the abbreviation for micronized cellular adipose matrix. It is an injectable cellular matrice product extracted from the adipose tissue.
- MCAM is obtained as the pelleted material from centrifugation after finely mincing and fragmentation of fat tissue.

Microstructure of MCAM

- Comparing with the intact fat tissue, MCAM includes few adipocytes but a substantial number of adipose derived stem cells (ASCs) within its abundant connective tissue.
- Microvascular structures in extracellular connective tissue of MCAM remained intact.

Analysis of cellular composition

- MCAM retained ASCs and vascular endothelial cells.
- ASCs within MCAM were viable and maintained similar mesenchymal differentiation capacity compared to those ASCs obtained through the standard collagenase digestion method.

Regenerative potential

- In diabetic mice model, excisional skin wounds treated with MCAM showed accelerated healing compared to phosphate-buffered saline-treated wounds.

Conclusion

- MCAM is an injectable tissue product contains both functional stem cells and extracellular matrix. MCAM is prepared through minimal manipulation and thus can be readily used without regulatory issues. MCAM may offer a safe and effective option in chronic wound repair and tissue revitalization.

treating stem cell-depleted conditions such as chronically inflammated tissues/ulcers and radiated tissue damage. Another future study is needed to explore the effectiveness of MCAM compared with other cellular formulations such as freshly isolated SVF and cultured ASCs.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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