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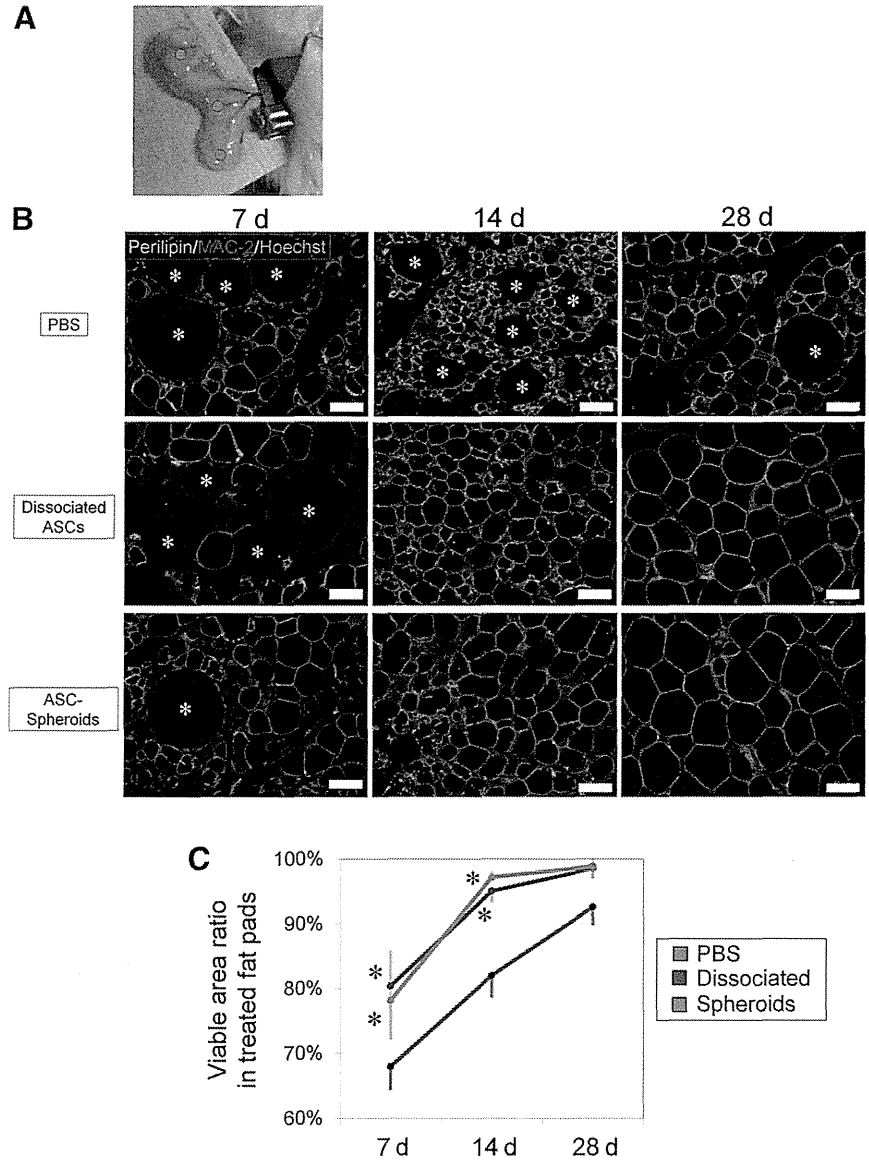

Figure 5. Histological evaluation of cell-based treatments for ischemia-reperfusion injury of inguinal fat pad in SCID mice. **(A):** The main nutrient vessels arising from the femoral vessels were clamped with a microclip for 4 hours and then allowed to perfuse. Mice models were divided into three groups, and each group was treated with an injection of PBS, monolayer-cultured ASCs, or ASC spheroids at three points (blue dots) of the fat pad. **(B):** Immunohistochemistry for perilipin (viable adipocytes) and MAC-2 (macrophages) revealed that both monolayer-cultured ASCs and ASC spheroids improved the adipose tissue repair and regeneration after ischemia-reperfusion injury. Many dead adipocytes (perilipin-negative) surrounded by infiltrated M1 macrophages were detected at 7 days, indicating substantial tissue damage by the injury. At 14 days, adipose regeneration (perilipin-positive small adipocytes) was seen, and the tissue repair was almost completed by 28 days in samples treated with monolayer-cultured or spheroid ASCs. Asterisks show dead adipocytes. Scale bars = 50 μm. **(C):** At 7 and 14 days, samples treated with monolayer-cultured or spheroid ASCs showed an increased viable area ratio compared with PBS-treated samples, suggesting that the injury repair process was accelerated by the cell treatments. *, $p < .05$ compared with control (PBS). Abbreviations: ASC, adipose-derived stem/stromal cell; d, days; PBS, phosphate-buffered saline.

relative weight of the fat pad, and there was the least fat atrophy (2.0% ± 0.6%) in samples treated with ASC spheroids compared with the control (26.8% ± 11.0%) and ASC-treated groups (15.1% ± 7.7%; Fig. 6B).

Immunohistochemical Evaluation of Angiogenesis and Incorporation of Administered hASC Spheroids

Vascular endothelial cells were counted using lectin-stained sections of samples treated with hASC spheroids. Although the ASC spheroids group displayed the highest capillary density on day 7, the change was no longer significant on day 28 when angiogenesis/adipogenesis was almost completed (Fig. 7A). On day 7, human Golgi-positive cells (administered cells derived from hASC spheroids) were detected mainly in the degenerated area in the periphery of the injured inguinal fat pad in samples both treated with hASCs and hASC spheroids (Fig. 7B), suggesting that the injected hASCs migrated and homed selectively to the damaged area and were involved in the repair process. On day 28, immunostaining indicated that a number of

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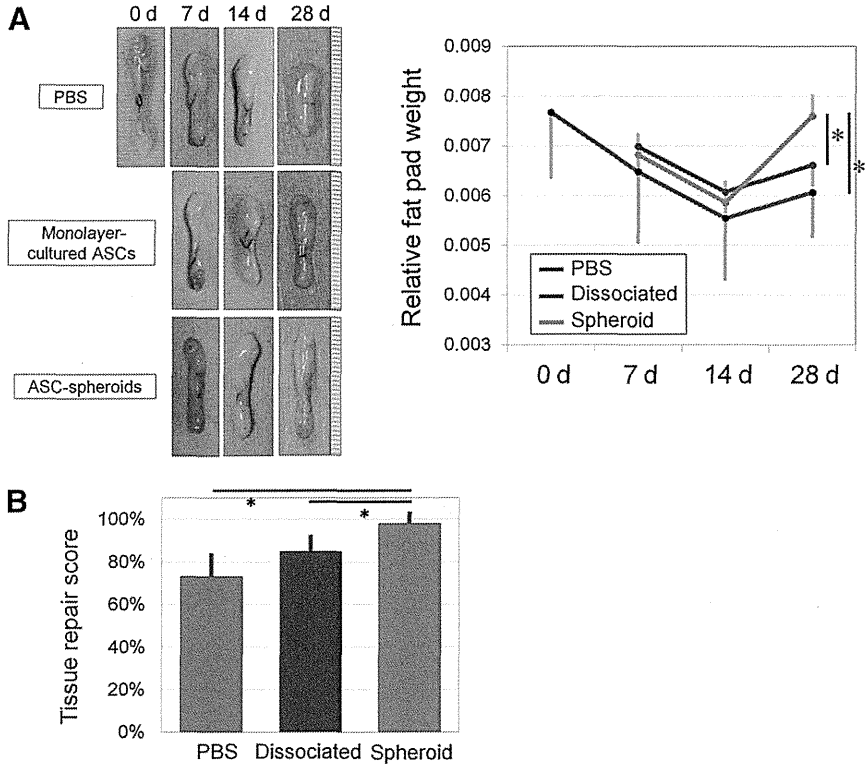

Figure 6. Treatment with human ASCs for ischemia-reperfusion injury. **(A):** Representative samples of treated inguinal fat pads harvested at 7, 14, and 28 days. PBS-treated samples showed injury-induced atrophy on day 28. The relative sample weight (sample weight/body weight) decreased after injury and reached its nadir on day 14 in each group, followed by weight recovery caused by adipose regeneration. The sample weights on day 28 were significantly greater in the spheroid-treated group compared with the other groups. *, $p < .05$. **(B):** Tissue recovery score was calculated by multiplying the relative weight and the viable area ratio of samples to show the gross recovery of damaged adipose tissue (100% means complete recovery to the level before injury). Nearly full recovery was seen in spheroid-treated samples, which was significantly superior to the other two groups. *, $p < .05$. Abbreviations: ASC, adipose-derived stem/stromal cell; d, days; PBS, phosphate-buffered saline.

hASC spheroid-derived cells (human Golgi-positive cells) were incorporated into the repaired tissue (Fig. 7C). Substantial parts of hASCs were lectin-positive and located between adipocytes, suggesting that they differentiated into vascular endothelial cells and contributed to the reconstructed capillary vessel network.

Discussion

HA is a glycosaminoglycan disaccharide composed of alternately repeating units of D-glucuronic acid and *N*-acetylglucosamine. HA is naturally found in the ECM of many human tissues/organs including the skin, joints, and eyes. In particular, the skin tissue contains the highest amount, which is approximately 50% of the total HA in the body [31]. Therefore, various preparations of HA are widely used as pharmaceutical and cosmetic products targeting the skin and subcutaneous tissue. In this study, we used the non-cross-linked HA powder (pharmaceutical grade), which was able to resolve rapidly with the culture medium. With this advantage, formed cell spheroids were isolated easily only by dilution of the gel and subsequent centrifugation without using an enzyme digestion. In the 3D floating culture in HA gel, hASC spheroids could be prepared over a comparative time interval to nonadherent culture system (as short as 48 hours) with relatively uniform size (approximately 30 μm in diameter) compared with 3D culture with a nonadhesive culture dish. For local injection of cell spheroids, a certain size such as 20 μm is required to avoid unfavorable local diffusion or migration into the circulation [32], but spheroids larger than 150 μm can induce central necrosis of spheroids/aggregation [33].

Cell spheroids/aggregates can be formed by various 3D culture methods including hanging drop, nonadherent culture flask, various biodegradable coatings, and scaffolds or gels of ECM components or synthetic materials [34, 35]. Furthermore, previous studies have suggested that spheroids produce growth factors to a greater degree than controls in vitro or in vivo and show therapeutic potential and that cell spheroids/aggregates may show pluripotent potential (perhaps because of cell selection or dedifferentiation). Our microarray data suggest that gene expression of some angiogenesis-related growth factors (such as *VEGFA*, *VEGFB*, *HGF*, *PDGFA*, and *PDGFC*) and pluripotent markers (such as *NANOG*, *OCT3/4*, and *STAT3*) are upregulated and that cell mitosis-related genes are down-regulated in HA-gel spheroid culture compared with monolayer culture. Increased secretion of cytokines such as HGF was also confirmed in ASC spheroids cultured under hypoxia. Immunohistology showed that ASC spheroids contained cells expressing some pluripotency markers such as NANOG, OCT3/4, and SOX-2. Additionally, approximately 40% of spheroids contained SSEA-3-positive cells, suggesting the efficient enrichment of muse cells by the 3D floating culture process. Because expanding the SSEA-3-positive cells by monolayer adherent culture was difficult (approximately 1%–2%; data not shown)

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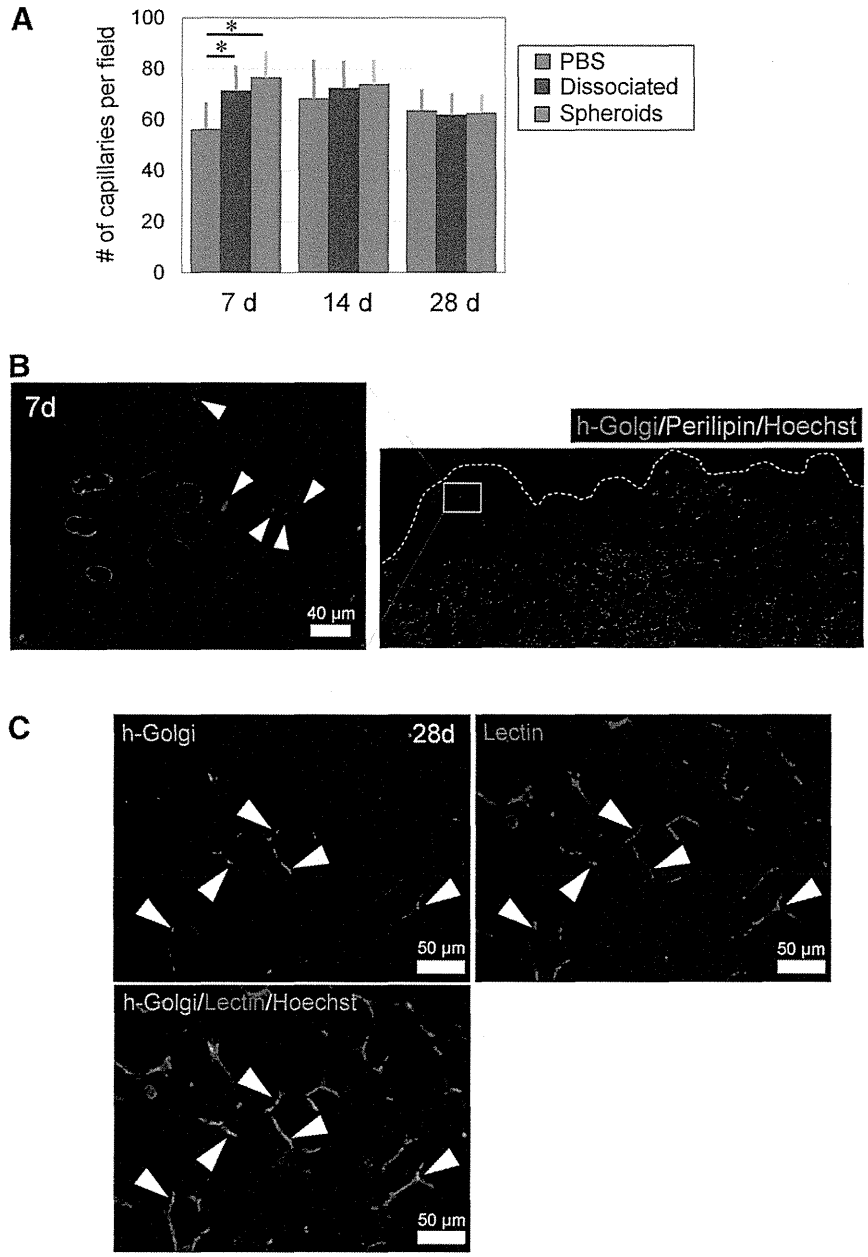

Figure 7. Histological assessment of vasculature and contribution of administered adipose-derived stem/stromal cells (ASCs). **(A):** The number of capillaries were counted using microsections stained with isolectin. Although the ASC spheroid group showed the highest capillary density at 7 days, this change was no longer significant at 4 weeks. **(B):** Immunostained section of spheroid-treated sample at 1 week. The human Golgi-positive cells (injected ASCs) were detected mainly in the degenerated adipose area, which showed less perilipin expression. Bar = 40 μm. **(C):** Immunostained section of spheroid-treated sample at 4 weeks. Some of the h-Golgi(+) cells (arrowheads) expressed isolectin, indicating that they were incorporated into the repaired adipose tissue as vascular endothelial cells. Scale bars = 50 μm. *, $p < .05$. Abbreviations: d, days; h-Golgi(+), human Golgi-positive; PBS, phosphate-buffered saline.

[8], a 3D floating culture in HA gel may selectively expand the SSEA-3-positive population, which we recently showed has greater therapeutic capacity for a refractory diabetic skin ulcer even without forming spheroids [36]. Indeed, numerous single cells were also observed at 48 hours in the HA gel, and thus only a specific ASC population may have expanded and/or aggregated under this condition.

HA gel culture has several specific characteristics. Although mesenchymal cells generally express the cell surface receptor for HA (CD44), HA appears not to alter cellular biological properties and functions compared with other major ECM components such as collagen, fibronectin, and laminin. Because spheroid formation did not occur when the HA gel was stiff at high concentrations, its mechanical properties may be key for the appropriate formation of spheroids. The results indicate that it is possible to efficiently obtain within 3 days a large number of ASC spheroids with a relatively uniform diameter using common cultureware and an incubator. HA is already clinically used as an injectable pharmaceutical in many formulations, and thus its safety and negligible immunoreaction have been established.

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Therefore, ASC spheroids prepared in a HA gel can be easily injected without extraction (along with the HA gel as a safe cell carrier). The 4% non-cross-linked HA gel (0.1 ml) was absorbed in several days in the subcutis of mice (data not shown), and the degradation duration can be adjusted by modification of the molecular weight and cross-linking ratio of the HA products.

In the animal study, we used an ischemia-reperfusion injury to the fat pad in immunodeficient mice. Four hours of ischemia and subsequent reperfusion induced substantial damage to the adipose tissue, resulting in tissue atrophy (30% tissue loss) after recovery at 28 days. Acute tissue damage was greater in the periphery of the fat pad, leading to local tissue necrosis at 7 days, but damage was minimized in the spheroid-treated group, as indicated by an increased angiogenesis (increasing number of lectin-positive vascular endothelial cells). Regenerative changes, including adipogenesis (perilipin-positive small adipocytes between dead adipocytes), were observed at 7–14 days to a differential extent among the treatment groups. Samples treated with spheroid ASCs and monolayer-cultured ASCs clearly showed accelerated tissue repair at 14 days, when many unscavenged adipocytes remained, and newly grown small preadipocytes were seen in PBS-treated samples. Tissue repair was almost completed by 28 days, and spheroid-treated samples showed remarkable regeneration after 14 days. Cells finally recovered up to nearly an intact level (2.0% atrophy in viable adipose volume), which was significantly better than samples treated with monolayer-cultured ASCs showing 15.1% atrophy. Thus, although even monolayer-cultured ASCs indicated a therapeutic value in this model, ASC spheroids prepared in the HA gel clearly showed a superior capacity to enhance tissue regeneration and rescue the damaged tissue from irreversible atrophy.

Histological tracing of administered cells partly uncovered the fate of the administered spheroids. On day 7, injected human spheroids had already dissociated; they migrated and homed predominantly to the damaged peripheral region, suggesting that they were attracted by soluble factors derived from damaged tissue and were already involved in the tissue repair process. The sections of day 28 fat pads indicated that a number of injected spheroid ASCs were incorporated into the regenerated adipose tissue. Although it remains unknown whether they became adipocytes, ASCs definitively differentiated into vascular endothelial cells and contributed to the newly formed vascular network. ELISA assay showed upregulated HGF levels in HA spheroids compared with monolayer culture and elevated expression of EGF under hypoxic condition compared with normoxic condition in both monolayer culture and HA spheroids. EGF may have contributed to promoting healing in both monolayer and HA spheroid treatments, and HGF may further have helped neo-vascularization in HA spheroid-treated models. We also observed a substantial number of ASCs that were neither adipocytes nor endothelial cells and may be incorporated as perivascular ASCs for future remodeling of the tissue.

Conclusion

Human ASC spheroids enriched in undifferentiated cells were efficiently prepared by using 3D floating culture in non-cross-linked HA gel. The results suggest the superior plasticity and growth factor-secreting potential of the spheroids and clearly indicate their therapeutic power for promoting tissue regeneration. Unlike dissociated cells, their high engraftment efficiency and differentiation into vascular endothelial cells suggest the privileged therapeutic capacity of the spheroids. HA is a degradable ECM material, and its safety is well-established, indicating its practical value as a material for 3D culture and as an injectable cell carrier. Spheroid formation has various advantages other than enhancement of cell functionality; this process can avoid unfavorable migration of locally injected cells, leading to more consistent incorporation and therapeutic effects of the administered cells. As an easy local injectable, spheroids are expected to be a powerful tool to treat various stem cell-depleted conditions/organs through nonintravenous administration.

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Author Contributions

K.M.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; J.F., H.I., and H.T.: collection and/or assembly of data, data analysis and interpretation; K.D.: collection and/or assembly of data; S.K.: collection and/or assembly of data; K. Kinoshita, K. Kanayama, H.K., and T.M.: collection and/or assembly of data; I.H. and A.K.: data analysis and interpretation; H.N.: data analysis and interpretation, administrative support; K.Y.: conception and design, data analysis and interpretation, financial support, administrative support, final approval of manuscript, manuscript writing.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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