The Fate of Adipocytes after Nonvascularized Fat Grafting: Evidence of Early Death and Replacement of Adipocytes

Hitomi Eto, M.D.
Harunosuke Kato, M.D.
Hirotaka Suga, M.D.
Noriyuki Aoi, M.D.
Kentaro Doi, M.D.
Shinichiro Kuno, M.D.
Kotaro Yoshimura, M.D.

Tokyo, Japan

Background: Clinical outcomes following fat grafting are variable and technique dependent, and it is unknown how the graft is revascularized. The authors recently observed that living and dead adipocytes can be differentiated not with hematoxylin and eosin staining but with immunohistochemistry for perilipin.
Methods: The viability of cellular components (adipocytes, adipose stem/stromal/progenitor cells, vascular endothelial cells, and hematopoietic cells) in human adipose tissue was evaluated using (1) stored lipoaspirates, (2) cultured cells, and (3) organ-cultured adipose tissue. In addition, the groin fat pad (150 to 200 mg) in mice was transplanted under the scalp, and the graft was stained at 0, 1, 2, 3, 5, 7, or 14 days.
Results: In vitro studies revealed that adipocytes are most susceptible to death under ischemic conditions, although adipose-derived stromal cells can remain viable for 3 days. The in vivo study indicated that most adipocytes in the graft began to die on day 1, and only some of the adipocytes located within 300 μm of the tissue edge survived. The number of proliferating cells increased from day 3, and an increase in viable adipocyte area was detected from day 7, suggesting that repair/regeneration of the dead tissue had begun.
Conclusions: The authors show convincing evidence of very dynamic remodeling of adipose tissue after nonvascularized grafting. The authors observed three zones from the periphery to the center of the graft: the surviving area (adipocytes survived), the regenerating area (adipocytes died, adipose-derived stromal cells survived, and dead adipocytes were replaced with new ones), and the necrotic area (both adipocytes and adipose-derived stromal cells died). (*Plast. Reconstr. Surg.* 129: 1081, 2012.)

Free-fat grafting has become one of the standard procedures for soft-tissue augmentation/reconstruction and rejuvenation. However, fat grafting remains unpredictable and technique-dependent, and there are many aspects to elucidate, such as how the adipose grafts survive and retain their volume over long periods without microsurgical revascularization. Although infiltrated histiocytes (possibly meaning macrophages) were once proposed to acquire lipid material and eventually replace all adipose tissue of the graft that had disappeared ("host replacement theory"),[1] the "cell survival theory," which states that transplanted living cells partly survive and remain alive for a long time after grafting, has been generally accepted.[1–6] Based on the widespread idea that the number of viable adipocytes in a graft correlates with the ultimate volume of persisting fat, many

From the Department of Plastic Surgery, University of Tokyo School of Medicine.
Received for publication September 27, 2011; accepted November 17, 2011.

DOI: 10.1097/PRS.0b013e31824a2b19

Disclosure: *The authors have no conflicts of interest to disclose.*

Supplemental digital content is available for this article. Direct URL citations appear in the printed text; simply type the URL address into any Web browser to access this content. Clickable links to the material are provided in the HTML text of this article on the *Journal*'s Web site (www.PRSJournal.com).

efforts have been made to purify or maximize the number of viable adipocytes.[7–9]

There are various types of cells in subcutaneous adipose tissue, and adipocytes account for only 20 percent or fewer of the total number of cells.[10,11] We previously reported the orchestrated cell interactions caused by manipulations of adipose tissue such as mechanical injury, ischemia-reperfusion injury, and surgically induced ischemia.[12–15] In these studies, adipose stem/stromal/progenitor cells (adipose-derived stromal cells) were shown to play pivotal roles in adipose tissue repair/remodeling; they increase in number, contribute to angiogenesis/adipogenesis in response to primary signals released from the injured tissue, and release various secondary growth factors.[16,17] However, adipose tissue survival/remodeling after nonvascularized fat grafting should differ from that after both mechanical injury and ischemia (partly corresponding to vascularized transposition of the adipose flap) and that after ischemia-reperfusion injury (corresponding to a revascularized free flap with a microsurgical anastomosis).

Our current study was performed to clarify the fate of adipocytes and related cellular events after nonvascularized free-fat grafting. Therefore, we focused on the early phase (0 to 14 days) of the healing process. We analyzed the viability of each cellular component of human fat tissue with in vitro assays and organ culture (in vitro simulation of human fat grafting), and we very carefully evaluated adipocyte viability in fat grafts in mice (in vivo assay).

PATIENTS AND METHODS

Human Cell Isolation and Culture

Human liposuction aspirates were obtained from 10 healthy female donors aged 41.9 ± 10.4 years (mean ± SD) and with an average body mass index of 23.0 ± 2.5 kg/m^2 who underwent elective liposuction of the abdomen or thighs. Each patient provided written informed consent, and the research protocol was approved by the ethics committee of the University of Tokyo, School of Medicine. Stromal vascular fraction containing adipose-derived stromal cells was isolated from the aspirated fat tissue as described.[10] Briefly, the fatty portion of the aspirated fat tissue was washed with phosphate-buffered saline and digested in 0.075% collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in phosphate-buffered saline at 37°C for 30 minutes on a shaker. Mature adipocytes and connective tissue were separated from pellets by centrifugation (800 *g* for 10 minutes) and discarded. The pellets were resuspended in phosphate-buffered saline and filtered through a 100-μm mesh, followed by centrifugation (800 *g* for 10 minutes) to spin down stromal vascular fraction cell pellets. The stromal vascular fraction cells were plated at a density of 5×10^5 nucleated cells/100-mm dish and cultured at 37°C in an atmosphere of 5% carbon dioxide in humid air. Cells were grown in a control medium (Dulbecco's Modified Eagle's Medium; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal bovine serum. Primary cells were cultured for 7 days and were defined as passage 0. The medium was replaced every 3 days. Cells were passaged every week by trypsinization. Adipose-derived stromal cells at passages 1 to 3 were used in the following experiments.

To isolate human vascular endothelial cells from aspirated adipose tissue, the stromal vascular fraction cells were plated at a density of 2×10^6 nucleated cells/100-mm dish and cultured in endothelial growth medium-2 (Cambrex, Walkersville, Md.) with 2% fetal bovine serum for 2 days. Attached cells were harvested by trypsinization and incubated with phycoerythrin-conjugated mouse anti-human CD31 antibody (BD Biosciences, San Jose, Calif.), followed by incubation with anti-phycoerythrin magnetic microbeads (Miltenyi Biotec GmbH, Land Nordrhein-Westfalen, Germany). CD31$^+$ cells were isolated with a magnetic cell sorter (AutoMACS separator; Miltenyi Biotec). The primary cells were cultured in endothelial growth medium-2 for 7 days and were defined as passage 1. Endothelial cells at passages 3 to 5 were used in this study. To obtain adipocytes (experimentally differentiated adipose-derived stromal cells), adipose-derived stromal cells were incubated in adipogenic medium (control medium containing 0.5 mM isobutyl-methylxanthine, 1 μM dexamethasone, 10 μM insulin, and 200 μM indomethacin) for 3 days and then maintained in the control medium for another 10 days.

Flow Cytometry

The following monoclonal antibodies conjugated to fluorochromes were used: anti-CD31–phycoerythrin, anti-CD34–phycoerythrin-Cy7, and anti-CD45–fluorescein isothiocyanate (BD Biosciences). To distinguish dead cells from live cells, 7-aminoactinomycin D (7AAD) staining was used. Multicolor flow cytometry was performed with an LSR II (BD Biosciences), and cell composition percentages and cell numbers were calculated according to surface marker expression profiles.

In Vitro Cell Death Assay

Adipose-derived stromal cells, experimentally differentiated adipose-derived stromal cells (adipocytes), and endothelial cells were cultured under ischemia-mimicking conditions (1% oxygen with no serum), and apoptosis (annexin V) and necrosis (propidium iodide) were evaluated with a fluorescent microscope. All nuclei were counterstained with Hoechst 33342 (Dojindo, Kumamoto, Japan) ($n = 5$). Images acquired with a fluorescence microscope (Keyence, Osaka, Japan) were used to count apoptotic and necrotic cells.

Organ Culture of Aspirated Adipose Tissue and Measurement of Viable Adipocytes

Aspirated adipose tissue (1 g) was cultured in 1 ml of serum-free control medium under hypoxia (1% oxygen). A cover glass was placed over the fat to prevent exposure to the air. At each time point (days 0, 1, 2, 3, 5, and 7), the cultured adipose tissue was fixed (Zinc Fixative; BD Biosciences), embedded in paraffin, and sectioned at 6 μm for immunohistochemistry using guinea pig antiperilipin antibody (Progen Biotechnik GmbH, Heidelberg, Germany) and Alexa Fluor 488–conjugated secondary antibody (Molecular Probes, Eugene, Ore.). Perilipin-positive cells (viable adipocytes) were detected and counted under a fluorescence microscope.

Animal Models

Animals were cared for in accordance with our institutional guidelines. Six-week-old C57BL/6J mice were anesthetized with intraperitoneal pentobarbital (50 mg/kg), and the subcutaneous inguinal fat pad was harvested with a 1-cm incision. The fat pad (150 to 200 mg) was small like a fat injection material and transplanted by inserting the fat pad into a small pocket made under the scalp of the same mouse. After 0, 1, 2, 3, 5, 7, or 14 days, the adipose autografts were examined with immunohistochemistry.

Immunohistochemistry for Grafted Fat Tissue

Harvested adipose tissue was fixed (Zinc Fixative), embedded in paraffin, and sectioned at 6 μm for immunohistochemistry. The following primary antibodies were used: rabbit anti-Ki67 (Thermo Fisher Scientific, Fremont, Calif.) and guinea pig antiperilipin (Cedarlane Laboratories, Burlington, Ontario, Canada). Isotypic antibody was used as a negative control for each staining. For visualization, Alexa Fluor 488– or Alexa Fluor 568–conjugated secondary antibodies (Molecular Probes) were used. Nuclei were stained with Hoechst 33342.

Statistical Analysis

Data are expressed as mean ± SEM. Results were analyzed using Ekuseru-Toukei 2008 (SSRI, Tokyo, Japan). Comparisons of multiple groups were made using one-way analysis of variance with the Bonferroni multiple t test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Analyses of Stromal Vascular Fraction Cells from Lipoaspirates Stored in a Refrigerator for 0 to 7 Days

Lipoaspirates were stored in a refrigerator (4°C) for various periods (0, 1, 2, 3, 5, or 7 days), and the stromal vascular fraction was isolated and analyzed. Freshly isolated stromal vascular fraction from human aspirated adipose tissue contained all cell types in adipose tissue other than mature adipocytes. Flow cytometric analysis showed that stromal vascular fraction contained $CD45^+$ hematopoietic cells (circulating leukocytes and adipose-resident hematopoietic cells) and $CD45^-$ adipose-derived cells (Fig. 1, *above*). The $CD45^-$ component was further separated into $CD45^-/CD31^+/CD34^+$ endothelial cells, $CD45^-/CD31^-/CD34^+$ adipose-derived stromal cells, and $CD45^-/CD31^-/CD34^-$ other cells (Fig. 1, *above*). The viable cell composition of the stromal vascular fraction on day 1 differed considerably from that obtained from fresh adipose tissue (Fig. 1, *center*). The most remarkable difference was the percentage of endothelial cells, which were markedly decreased on the next day (Fig. 1, *center*). Analysis of total cell numbers (both viable and dead) in the stromal vascular fraction revealed that viable endothelial cells and viable hematopoietic cells were significantly decreased in number during the first 24 hours, whereas the number of viable adipose-derived stromal cells ($CD45^-/CD31^-/CD34^+$) was not significantly different (Fig. 1, *below*).

Flow cytometry was performed sequentially up to day 7. To compare the cell viability of endothelial cells, adipose-derived stromal cells, and other cells, only viable nonhematopoietic stromal vascular fraction cells ($7AAD^-/CD45^-$) were analyzed. [**See Figure, Supplemental Digital Content 1** (*above*) demonstrating flow cytometric analyses of the cellular composition of viable nonhematopoietic cells in the stromal vascular fraction from human aspirated adipose tissue, ***http://links.lww.com/PRS/A480***. Representative flow cytometric

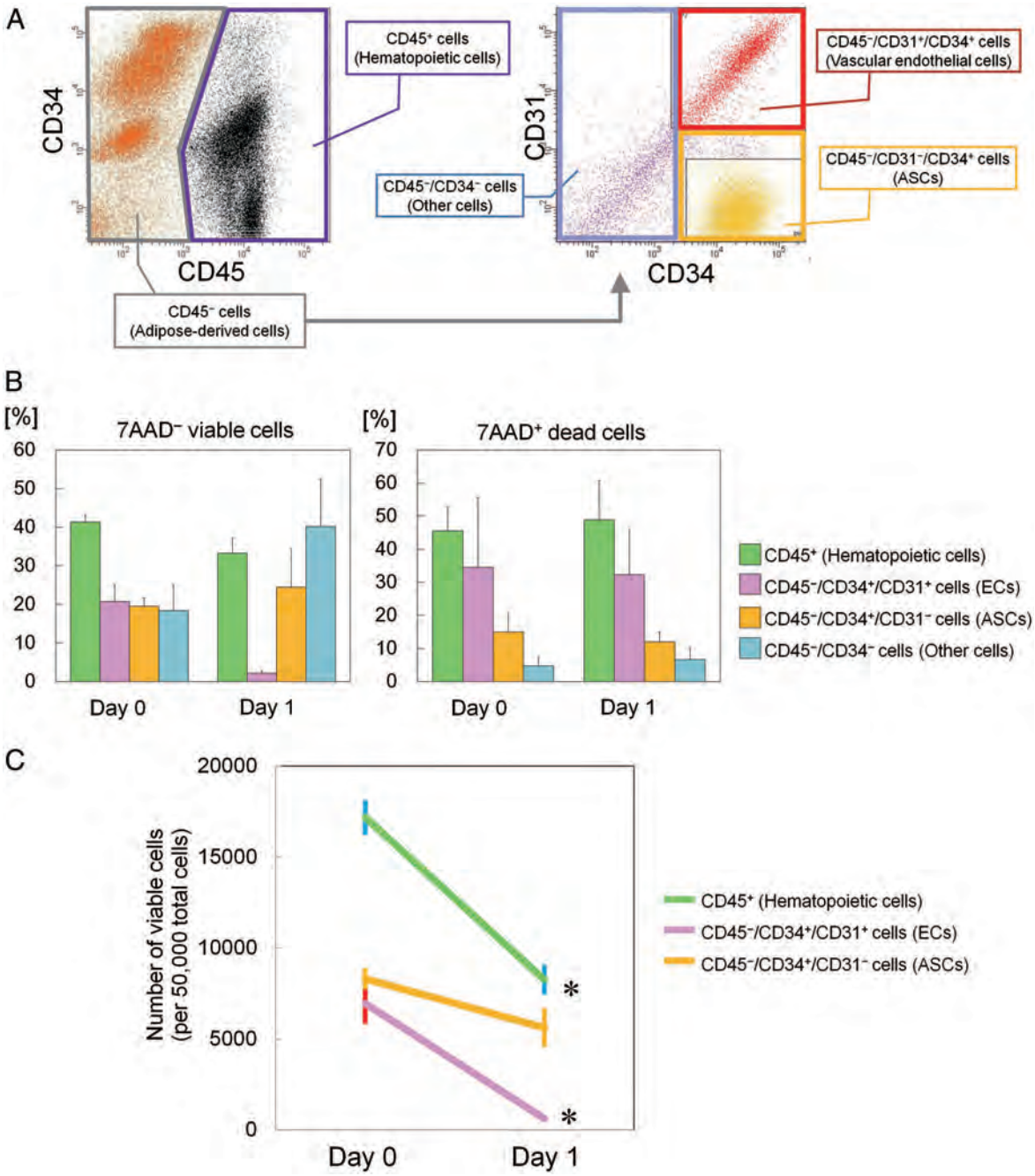

Fig. 1. Multicolor flow cytometric analysis of stromal vascular fraction cells derived from human aspirated adipose tissue. (*Above*) Representative plotted data. $CD45^+$ cells were hematopoietic cells (derived mainly from peripheral blood), whereas $CD45^-$ cells were adipose tissue-derived cells and were subjected to further analyses. $CD45^-/CD31^-/CD34^+$ cells, $CD45^-/CD31^+/CD34^+$ cells, and $CD45^-/CD34^-$ cells were regarded as adipose-derived stromal cells (*ASCs*), endothelial cells, and other cells (such as fibroblasts and mural cells), respectively. (*Center*) Cell composition of viable cells ($7AAD^-$) and dead cells ($7AAD^+$). The stromal vascular fraction composition of fresh fat samples (day 0) was compared with the day-1 samples, which were stored in a refrigerator without processing for 24 hours. The cellular composition of the viable cells was considerably different between day 0 and day 1. In particular, the ratio of viable endothelial cells was remarkably decreased on day 1 ($n = 4$). (*Below*) The number of viable cells detected from a total of 50,000 stromal vascular fraction cells containing both viable and dead cells. During the first postoperative day, a significant decrease in the number of viable cells was seen in hematopoietic cells and endothelial cells (*ECs*), but not in adipose-derived stromal cells (*ASCs*) ($n = 4$). Statistical analysis was performed between day 0 and day 1 for each cell type ($*p < 0.05$).

data of viable nonhematopoietic ($7AAD^-/CD45^-$) stromal vascular fraction cells. Lipoaspirates were refrigerated up to 7 days (for 0, 1, 2, 3, 5, or 7 days), and the stromal vascular fraction cells were isolated and analyzed. On day 2, the number of viable adipose-derived stromal cells ($7AAD^-/CD31^-/CD34^+/CD45^-$) observed was similar to that on day 0 or 1, whereas few viable endothelial cells ($7AAD^-/CD31^+/CD34^+/CD45^-$) were detected.] The number of viable endothelial cells was decreased during the first 2 days, and few were detected on day 2, whereas many adipose-derived stromal cells were still detected on day 2. The viability was calculated based on the number of $7AAD^-$ (viable) and $7AAD^+$ (dead) cells for each cell type. [**See Figure, Supplemental Digital Content 1** (*below*) demonstrating flow cytometric analyses of the cellular composition of viable nonhematopoietic cells in stromal vascular fraction from human aspirated adipose tissue, ***http://links.lww.com/PRS/A480***. Sequential changes in the number of viable endothelial cells, adipose-derived stromal cells, and hematopoietic cells ($7AAD^-/CD45^+$). Endothelial cells were the most susceptible to death under ischemia among the three cell types.] The viability of hematopoietic cells and endothelial cells was much lower than the viability of adipose-derived stromal cells throughout the experiment period, although adipose-derived stromal cell viability was also low from day 3. Thus, human endothelial cells and hematopoietic cells died easily under ischemic conditions, but more adipose-derived stromal cells remained viable for up to 3 days.

In Vitro Analyses of Human Adipose-Derived Stromal Cells, Adipocytes, and Endothelial Cells Cultured under Ischemia-Mimicking Conditions

To further examine the cellular response to ischemia (hypoxia and low nutrients), adipose-derived stromal cells, experimentally differentiated adipose-derived stromal cells (called adipocytes in this study), and endothelial cells were cultured under ischemia-mimicking conditions (1% oxygen with no serum) for 0, 12, 24, 48, or 72 hours, and cell apoptosis and necrosis were examined by staining with annexin V and propidium iodide, respectively (Fig. 2, *above*). Within 24 hours, adipocytes underwent apoptosis (annexin-positive/propidium iodide–negative) or necrosis (propidium iodide–positive), and endothelial cells lost their adherence to the culture dish. Only adipose-derived stromal cells remained intact up to 72 hours (Fig. 2, *below*). Thus, endothelial cells underwent apoptosis as early as 12 hours, and adipocytes most easily died under ischemia compared with endothelial cells and adipose-derived stromal cells. Adipose-derived stromal cells remained viable up to 72 hours under ischemic conditions.

Adipocyte Viability in Organ Culture of Human Adipose Tissue under Ischemia-Mimicking Conditions

Next, to further analyze the viability of human adipocytes under ischemia, organ culture of aspirated adipose tissue was performed. Adipose tissue cultured under ischemia-mimicking conditions (1% oxygen with no serum) was fixed at each time point (day 0, 1, 2, 3, 5, or 7) and immunostained for perilipin (Fig. 3, *above*). Because dead adipocytes were not stained with perilipin, we defined round-shaped cells that were strongly positive for perilipin as viable adipocytes. The number of viable adipocytes was significantly decreased during the first day, and most adipocytes lost their strong expression of perilipin by day 3, suggesting that mature adipocytes readily died under ischemic conditions (Fig. 3, *below*).

Viability of Adipocytes in Nonvascularized Grafted Fat Tissue in a Mouse Model

To evaluate adipocyte viability after fat grafting, we used a mouse model for nonvascularized fat grafting in which the inguinal fat pad was extracted en bloc (150 to 200 mg) and autotransplanted into the subcutaneous layer of the head. [**See Figure, Supplemental Digital Content 2**, which demonstratesevaluation of adipocyte viability after nonvascularized fat grafting using a mouse model, ***http://links.lww.com/PRS/A481***. (*Above*) Schematic of the fat graft model in mice. Inguinal adipose tissue (150 to 200 mg) was grafted under the scalp of the same mouse. (*Center*) Typical histology of regenerating zone on day 14. Dead adipocytes (*asterisk*) were not distinguished from living adipocytes in hematoxylin and eosin staining (*left*), whereas dead adipocytes (perilipin-negative) were easily distinguished from living adipocytes (perilipin-positive) in immunostaining for perilipin (*right*). (*Below*) Evaluation of perilipin expression by grafted adipocytes. The grafted adipose tissue and the overlying skin were harvested at each time point and immunostained for perilipin (*green*). Round adipocytes that stained strongly for perilipin as intact recipient adipocytes in the scalp were considered to be viable, whereas adipocytes weakly positive for perilipin or irregularly shaped, perilipin-positive adipocytes were re-

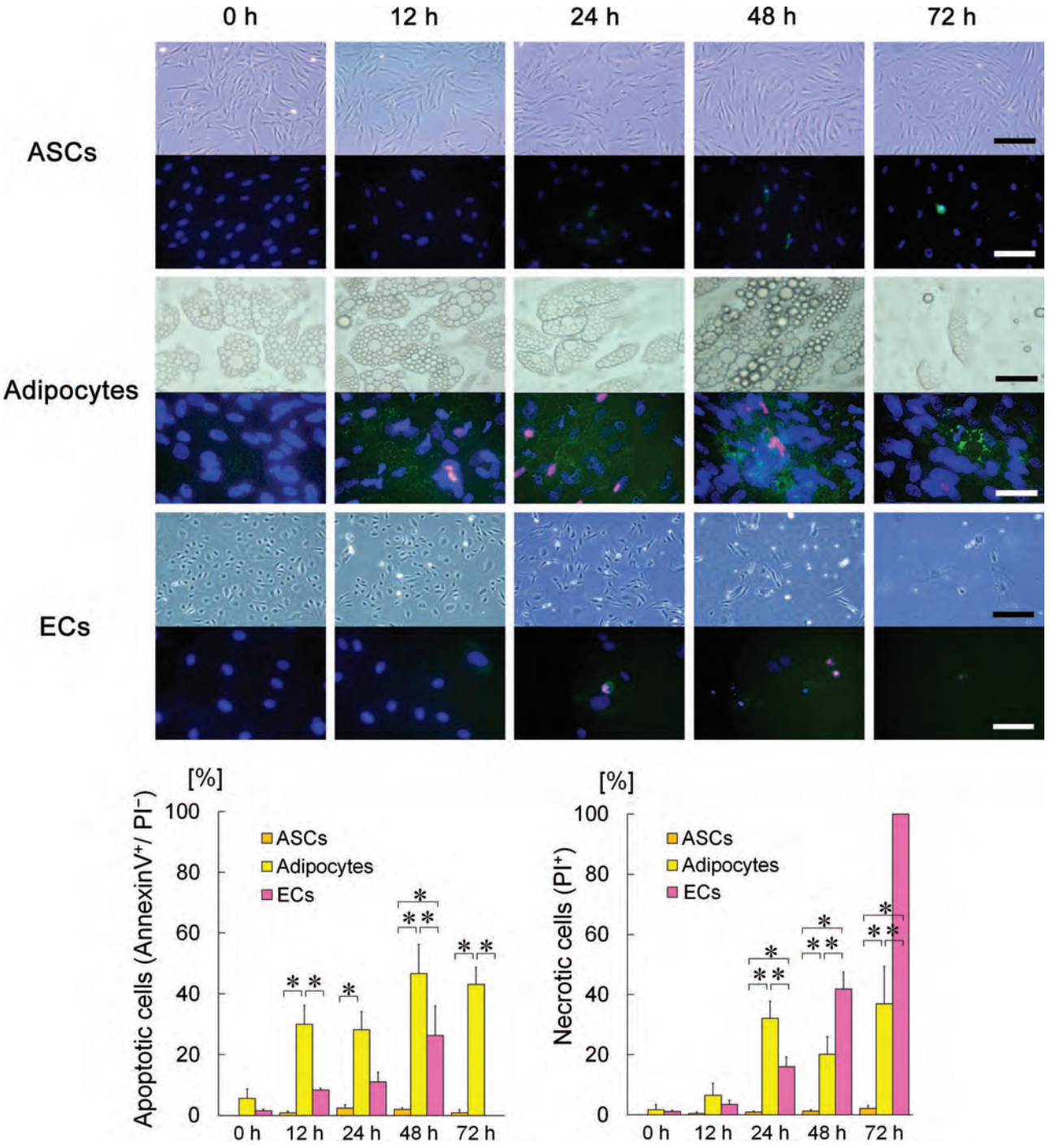

Fig. 2. In vitro cell death assay of human adipose-derived stromal cells (*ASCs*), adipocytes (differentiated adipose-derived stromal cells), and endothelial cells (*ECs*). (*Above*) Cytochemistry for apoptosis and necrosis. Adipose-derived stromal cells, adipocytes (differentiated adipose-derived stromal cells), and endothelial cells were cultured for 0, 12, 24, 48, or 72 hours under ischemic conditions, which were mimicked by low (1%) oxygen and serum-free culture medium, and stained with annexin V (apoptosis, *green*), propidium iodide (necrosis, *red*), and Hoechst 33342 (nuclei, *blue*). Adipocytes readily underwent apoptosis (annexin-positive) and necrosis (propidium iodide–positive), and endothelial cells lost their adhesion to the culture dish within 24 hours, whereas adipose-derived stromal cells remained intact for up to 72 hours. Scale bar = 50 μm. (*Below*) Quantification of annexin-positive/propidium iodide–negative apoptotic cells (*left*) and propidium iodide–positive necrotic cells (*right*) ($n = 5$). Adipocytes were the first to die under ischemic conditions, and endothelial cells were the second. Adipose-derived stromal cells remained alive for up to 72 hours. Statistical analysis was performed among the three groups at each time point (*$p < 0.05$).

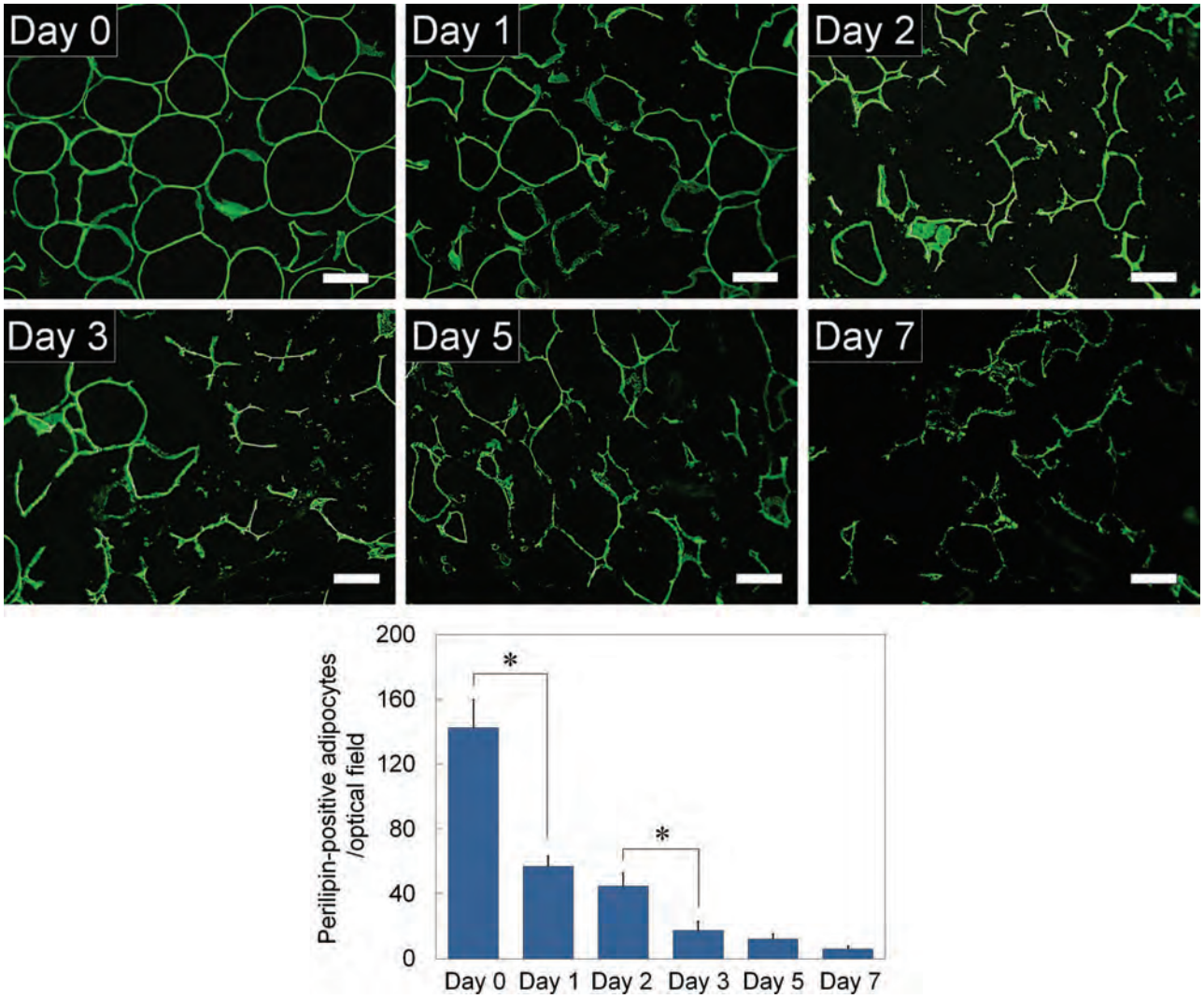

Fig. 3. Histologic assessment of viable adipocytes in organ culture of human aspirated adipose tissue. (*Above*) Immunohistology of organ-cultured aspirated adipose tissue. Aspirated adipose tissue was cultured under ischemic conditions (1% oxygen with no serum). The cultured adipose tissue was fixed at each time point and stained for perilipin (viable adipocytes; *green*). Scale bars = 50 μm. (*Below*) Quantification of viable (round, perilipin-positive) adipocytes ($n = 5$). Viable adipocytes decreased in number sharply on day 1, followed by a further decrease over time. Statistical analysis was performed among the six time points (*$p < 0.05$).

garded as dying adipocytes. Dead adipocytes had completely lost perilipin expression. *a*, recipient tissue; *b*, margin of the grafted donor adipose tissue; *c*, viable adipocyte area (strongly positive for perilipin); *d*, dying adipocyte area (weakly positive for perilipin); *e*, dead adipocyte area or connective tissue area (negative for perilipin). *Scale bar* = 100 μm.] The grafted adipose tissue and overlying skin were harvested and immunostained for perilipin to assess the viability of the grafted adipocytes because living adipocytes cannot be distinguished from dead adipocytes with hematoxylin and eosin staining. (**See Figure, Supplemental Digital Content 2**, *center*, ***http://links.lww.com/PRS/A481***). Our results indicated that adipocytes gradually lost perilipin expression after death and it took several days to completely lose perilipin expression. Healthy adipocytes in the scalp skin of the recipient mice were strongly stained for perilipin, whereas donor-derived grafted adipocytes were stained at varying degrees (**See Figure, Supplemental Digital Content 2**, *below*, ***http://links.lww.com/PRS/A481***). Therefore, we classified grafted adipocytes into three types according to the perilipin expression; strongly positive, round cells (viable adipocytes); weakly positive cells or strongly positive but irregularly shaped cells (dying adipocytes); and negative cells (dead adipocytes).

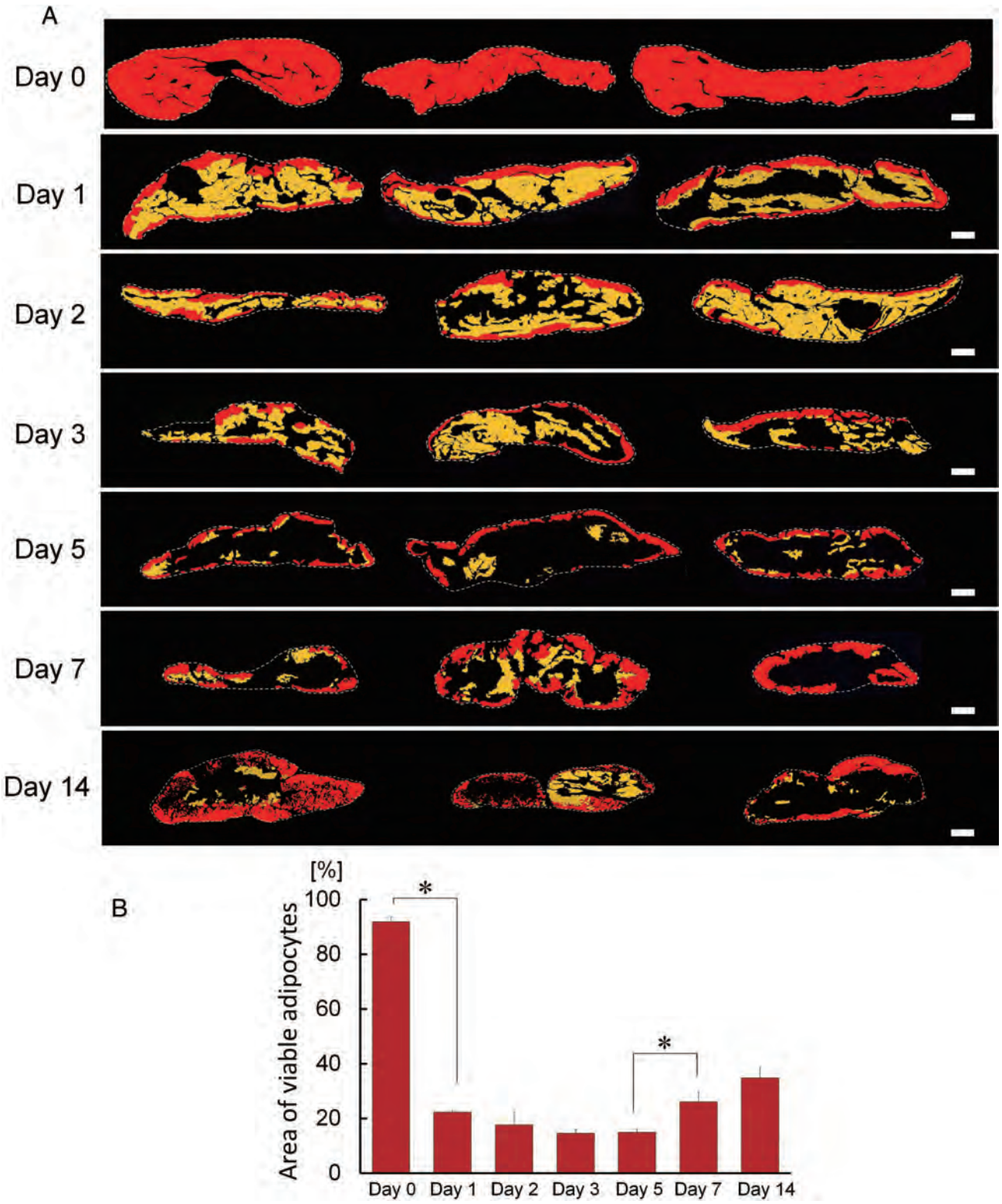

Fig. 4. Immunohistochemical analysis of adipocyte viability in grafted adipose tissue. (*Above*) Viability of grafted adipose tissue. Three samples seen in each row are obtained from three different mice. The area filled with viable adipocytes (strongly positive for perilipin) is colored in red, the area filled with dying adipocytes (weakly positive) is shown in yellow, and the area of dead adipocytes or connective tissue (negative area) is shown in black. The red area was dramatically reduced on day 1, and most of the yellow area had disappeared by day 5. It was revealed that only some adipocytes located less than 300 μm from the tissue surface remained alive, and all other adipocytes died within a few days after fat grafting. Scale bars = 1 mm. (*Below*) Sequential changes in the live adipocyte

Then, the grafted fat section was classified into three areas and coded in different colors: viable adipocyte area (red), dying adipocyte area (yellow), and dead adipocyte area or connective tissue area (black) (Fig. 4, *above*). Surprisingly, viable adipocytes were dramatically decreased as early as day 1, and they were detected only in the peripheral area (<300 μm from the surface) of the transplanted fat tissue, suggesting a better microenvironment in the periphery likely because of plasma diffusion from the surrounding intact tissue (Fig. 4). From day 2 to day 5, the number of viable adipocytes gradually decreased, and dying adipocytes completely lost perilipin expression, resulting in fewer adipocytes that were weakly positive for perilipin on day 5. Most adipocytes died, except for those on the peripheral edge. After day 7, however, resurgence was observed in the ratio of viable adipocyte area to the entire adipose tissue area (Fig. 4, *below*), suggesting that regenerative adipogenic changes, such as increasing numbers and size of new adipocytes, began from the peripheral dead area. Indeed, Ki67-positive proliferating cells were detected around the junction between the viable adipocyte area and the dead adipocyte area on days 5 and 7, suggesting proliferation of precursor cells (Fig. 5).

DISCUSSION

Over the years, we have performed flow cytometry to analyze the cellular composition of the stromal vascular fraction. We observed differences between the stromal vascular fraction examined on the day of surgery and the stromal vascular fraction on the next day. As shown in Figure 1 and **Supplemental Digital Content 1**, ***http://links.lww.com/PRS/A480***, our current experiments indeed showed that human adipose-derived stromal cells survived well under ischemic conditions compared with endothelial cells and hematopoietic cells, suggesting that each cell type has a different tolerance to ischemic stress. This was also demonstrated in mice in our previous study using surgically induced ischemic adipose tissue.[14] The cell death assay using adipocytes (differentiated adipose-derived stromal cells), and perilipin staining of organ-cultured adipose tissue, showed that adipocytes died as early as the first day of ischemia, endothelial cells died second, and finally adipose-derived stromal cells died on day 3. Thus, our in vitro results do not support the cell survival theory; the results suggested that a large proportion of adipocytes in a nonvascularized fat graft unlikely survive even when the graft is placed on a good recipient bed.

Nonvascularized fat grafts obtain nutrients and oxygen through plasmatic diffusion from surrounding tissues until vascularization is restored by ingrowth and reconnection of capillaries and vessels. Although numerous previous studies have demonstrated survival or neogenesis of adipose tissue, most studies determined whether adipocytes were viable only by their morphology.[7,18] In a previous study,[7] human aspirated fat tissue was grafted and histologically characterized into three zones at 3 to 4 weeks after grafting: the peripheral zone (viable adipocytes), the intermediate zone (inflammatory processes), and the central zone (necrotic zone). The study determined the graft viability based on the morphology of adipocytes and concluded that the viable region of the graft is approximately 1.5 ± 0.5 mm from the edge and that 60 percent of the adipocytes in the viable zone die. In other studies, cell viability and apoptosis assays were performed with the entire piece of adipose tissue, and adipocytes were not specifically evaluated.[19] However, we cannot discriminate viable adipocytes from dead adipocytes with standard stains such as hematoxylin and eosin; a dead adipocyte (a round lipid droplet) can be easily mistaken for a live adipocyte. Because an adipocyte has a large size (50 to 150 μm diameter), a single adipocyte is sliced into many histologic sections (with a regular thickness of 3 to 10 μm), and thus a single histologic section does not generally show most nuclei of healthy adipocytes. Therefore, we cannot confirm a healthy adipocyte by its shape or the presence of a nucleus. However, we recently found that we can very easily discriminate living and dead adipocytes by immunostaining for perilipin, which was used in this study. Perilipin is a protein that coats lipid droplets in adipocytes, and it has been demonstrated that dead adipocytes are negative for perilipin.[20–22]

We also evaluated adipocyte viability after fat grafting using a mouse model, which is the ultimate and most clinically relevant goal of this study. We very carefully evaluated adipocyte viability by killing the mice on almost every single postoperative day and meticulously evaluating the staining

Fig. 4. (*Continued*) area (ratio of the area strongly positive for perilipin to the whole cross-sectional area) ($n = 6$). The area filled with viable adipocytes was significantly decreased on day 1, suggesting that grafted adipocytes began to die within 1 day. However, the viable area began to increase from day 7, suggesting that adipogenesis had begun within the first week. Statistical analysis was performed among the seven time points (*$p < 0.05$).

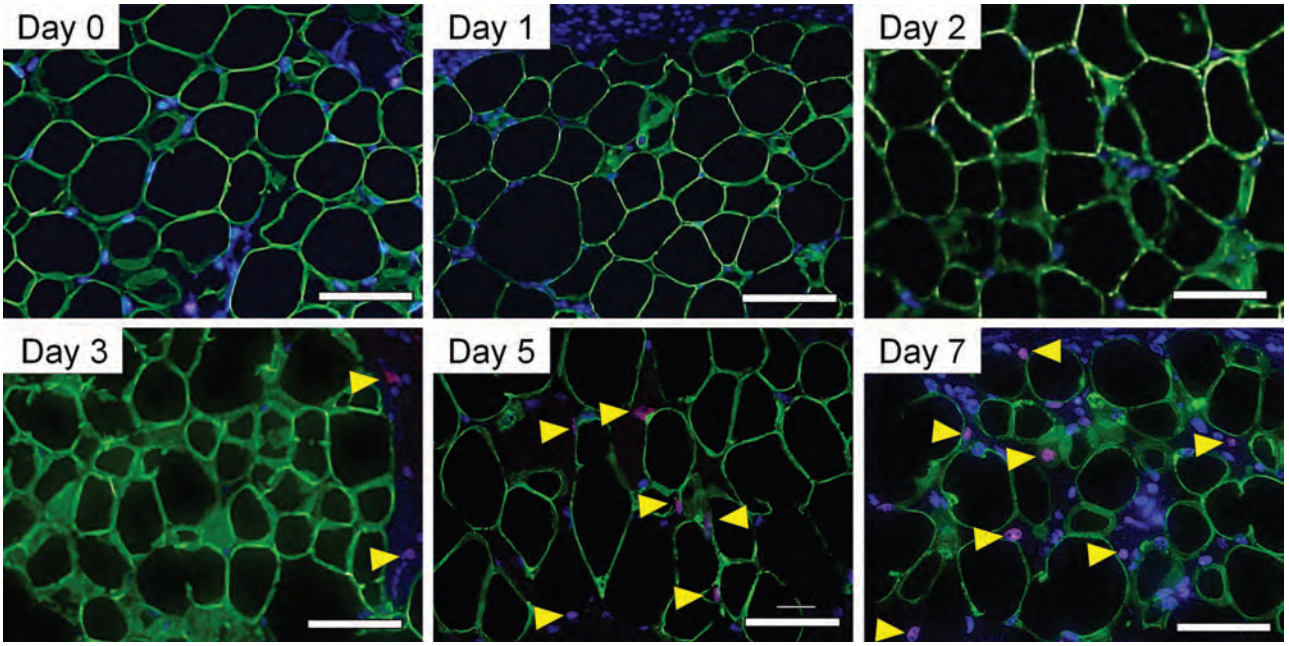

Fig. 5. Immunohistology of the surviving (perilipin-positive) area of grafted adipose tissue in mice. The most peripheral zone, in which most adipocytes were strongly stained for perilipin (*green*), was analyzed for Ki67 (*red*) and Hoechst 33342 (*blue*). A number of Ki67-positive proliferating cells (*yellow arrowheads*) were observed on days 5 and 7, suggesting that the regenerative process has already began in the periphery. Scale bars = 50 μm.

intensity of each adipocyte, because several days were required for an adipocyte to completely lose its perilipin staining after death. Adipocytes that were weakly positive for perilipin may have already been dead (but were called dying adipocytes in this study), because all such cells completely lost perilipin staining at a later stage, as seen on the histology performed on day 5. Our results clearly indicated that only peripherally located adipocytes survived and that the depth of the viable zone was approximately 300 μm from the graft edge: almost all adipocytes located deeper than 300 μm died within a few days after grafting. This means that we observed much fewer surviving cells compared with a previous report that used standard histology.[7]

From day 2 to day 5, no remarkable changes in the viable adipocyte zone were observed, but the dying adipocyte zone (weakly positive for perilipin) decreased in size day to day. Immunohistology on day 5 showed the percentage of adipocytes in the graft that actually survived, although the survival rate should theoretically differ depending on the size of the graft and the conditions (e.g., vascularity, tissue contact) of the surrounding tissue. Indeed, we noticed a difference in viable zone thickness between the overlying skin side and the underlying bone side of the graft; the viable zone thickness was frequently thicker on the skin side, suggesting better vascularity of the contacted tissue.

The in vivo experiment provided another intriguing insight into fat graft fate: regeneration. The number of proliferating cells increased after day 3, and the viable adipocyte area began to increase in size from day 7, suggesting regenerative changes to repair the tissue. Small (<50 μm in diameter) adipocytes strongly stained for perilipin—indicating newly born adipocytes—were observed in the inner border zone of the viable peripheral area after day 5. This suggests that tissue-resident stem/progenitor cells (such as adipose-derived stromal cells) did not die at least in the peripheral part of the graft and started to repair the tissue by adipogenesis and angiogenesis. Our in vitro studies indicating strong tolerance of adipose-derived stromal cells to severe ischemia support this notion. Bone marrow–derived precursors may also be involved in the repair process, but further analysis is needed to elucidate the type and origin of the proliferating cells.

Considered together, we now understand that adipocyte death does not mean adipose tissue necrosis, but can trigger a regeneration cascade; in contrast, death of both adipocytes and adipose precursors (adipose-derived stromal cells) theoretically results in adipose tissue necrosis. Conse-

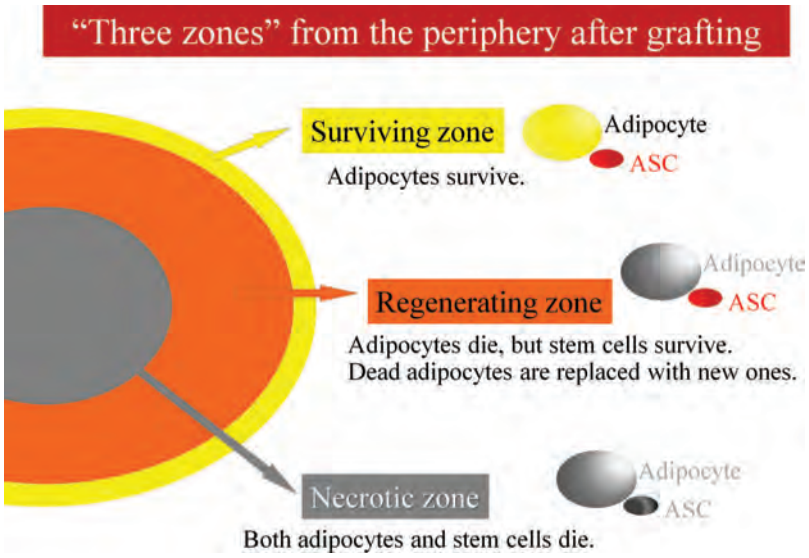

Fig. 6. Conclusive schema for three zones of the grafts. The most superficial zone is the "surviving zone," which is less than 300 μm thick. In the surviving zone, both adipocytes and adipose-derived stromal cells (*ASCs*) survive. The second zone is the "regenerating zone," the thickness of which varies depending on the microenvironmental conditions such as vascularity of and attachment to the surrounding tissue. In this zone, adipocytes die as early as day 1, but adipose-derived stromal cells survive and provide new adipocytes to replace the dead ones. The most central zone is the "necrotic zone," where both adipocytes and adipose-derived stromal cells die, no regeneration is expected, and the dead space will be absorbed or filled with scar formation.

quently, we observed three zones from the periphery to the center of the graft (Fig. 6): the surviving area (adipocytes survived), the regenerating area (adipocytes died, adipose-derived stromal cells survived, and dead adipocytes were replaced with new ones), and the necrotic area (both adipocytes and adipose-derived stromal cells died). Replacement of differentiated cells after grafting may be common in other nonvascularized composite tissue grafts in plastic surgery such as skin grafts or bone grafts. Many parts of the connective tissue can be preserved as a scaffold, but all differentiated cells (located more than 300 μm from the surface) may die and be replaced by those of the next generation derived from tissue-specific stem/progenitor cells, depending on the microenvironment. However, the distance of plasmatic diffusion is strictly limited because of a gradient of oxygen and nutrients, and thus regeneration is usually incomplete, leading to substantial tissue atrophy and/or dysfunction after nonvascularized grafting.

CONCLUSIONS

We show convincing evidence of very dynamic remodeling of adipose tissue after nonvascularized grafting. Most adipocytes in free grafts died shortly after transplantation, but resident stem/progenitor cells remained alive or became activated, and many adipocytes were regenerated (replaced dead cells with new adipocytes), depending on the microenvironment. Our observations may lead to a paradigm shift because it has been generally accepted that grafted adipocytes survive without replacement. Our body has a biological system termed "compensatory proliferation" for physiologic turnover of any tissue or organs[23]; in response to cell apoptosis, neighboring progenitor cells are activated for tissue homeostasis. Even for accidental cell death, our body likely has a similar system to properly respond to the emerging event, and we can explore new therapeutic approaches by dissecting and manipulating the underlying mechanisms of the self-repairing system.

Kotaro Yoshimura, M.D.
Department of Plastic Surgery
University of Tokyo School of Medicine
7-3-1, Hongo, Bunkyo-Ku
Tokyo 113-8655, Japan
kotaro-yoshimura@umin.ac.jp

ACKNOWLEDGMENT

This work was supported by a grant from the Japanese Ministry of Education, Culture, Sports, Science, and Technology (contact grant no. B2- 21390477).

REFERENCES

1. Billings E Jr, May JW Jr. Historical review and present status of free fat graft autotransplantation in plastic and reconstructive surgery. *Plast Reconstr Surg.* 1989;83:368–381.
2. Smahel J. Adipose tissue in plastic surgery. *Ann Plast Surg.* 1986;16:444–453.
3. Smahel J. Aspiration lipectomy and adipose tissue injection: Pathophysiologic commentary. *Eur J Plast Surg.* 1991; 14:126–131.
4. Cortese A, Savastano G, Felicetta L. Free fat transplantation for facial tissue augmentation. *J Oral Maxillofac Surg.* 2000; 58:164–169; discussion 169–170.
5. Rohrich RJ, Sorokin ES, Brown SA. In search of improved fat transfer viability: A quantitative analysis of the role of centrifugation and harvest site. *Plast Reconstr Surg.* 2004;113:391–395; discussion 396–397.
6. Piasecki JH, Gutowski KA, Lahvis GP, Moreno KI. An experimental model for improving fat graft viability and purity. *Plast Reconstr Surg.* 2007;119:1571–1583.
7. Carpaneda CA, Ribeiro MT. Study of the histologic alterations and viability of the adipose graft in humans. *Aesthetic Plast Surg.* 1993;17:43–47.
8. Boschert MT, Beckert BW, Puckett CL, Concannon MJ. Analysis of lipocyte viability after liposuction. *Plast Reconstr Surg.* 2002;109:761–765; discussion 766–767.
9. Crawford JL, Hubbard BA, Colbert SH, Puckett CL. Fine tuning lipoaspirate viability for fat grafting. *Plast Reconstr Surg.* 2010;126:1342–1348.
10. Yoshimura K, Shigeura T, Matsumoto D, et al. Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates. *J Cell Physiol.* 2006;208:64–76.
11. Eto H, Suga H, Matsumoto D, et al. Characterization of structure and cellular components of aspirated and excised adipose tissue. *Plast Reconstr Surg.* 2009;124:1087–1097.
12. Aiba-Kojima E, Tsuno NH, Inoue K, et al. Characterization of wound drainage fluids as a source of soluble factors associated with wound healing: Comparison with platelet-rich plasma and potential use in cell culture. *Wound Repair Regen.* 2007;15:511–520.
13. Suga H, Eto H, Shigeura T, et al. IFATS collection: Fibroblast growth factor-2-induced hepatocyte growth factor secretion by adipose-derived stromal cells inhibits postinjury fibrogenesis through a c-Jun N-terminal kinase-dependent mechanism. *Stem Cells* 2009;27:238–249.
14. Suga H, Eto H, Aoi N, et al. Adipose tissue remodeling under ischemia: Death of adipocytes and activation of stem/progenitor cells. *Plast Reconstr Surg.* 2010;126:1911–1923.
15. Eto H, Suga H, Inoue K, et al. Adipose injury-associated factors mitigate hypoxia in ischemic tissues through activation of adipose-derived stem/progenitor/stromal cells and induction of angiogenesis. *Am J Pathol.* 2011;178:2322–2332.
16. Yoshimura K, Suga H, Eto H. Adipose-derived stem/progenitor cells: Roles in adipose tissue remodeling and potential use for soft tissue augmentation. *Regen Med.* 2009;4:265–273.
17. Yoshimura K, Eto H, Kato H, Doi K, Aoi N. In vivo manipulation of stem cells for adipose tissue repair/reconstruction. *Regen Med.* 2011;6 (Suppl):33–41.
18. Dolderer JH, Thompson EW, Slavin J, et al. Long-term stability of adipose tissue generated from a vascularized pedicled fat flap inside a chamber. *Plast Reconstr Surg.* 2011;127: 2283–2292.
19. Medina MA III, Nguyen JT, Kirkham JC, et al. Polymer therapy: A novel treatment to improve fat graft viability. *Plast Reconstr Surg.* 2011;127:2270–2282.
20. Cinti S, Mitchell G, Barbatelli G, et al. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res.* 2005;46:2347–2355.
21. Westerbacka J, Kolak M, Kiviluoto T, et al. Genes involved in fatty acid partitioning and binding, lipolysis, monocyte/macrophage recruitment, and inflammation are overexpressed in the human fatty liver of insulin-resistant subjects. *Diabetes* 2007;56:2759–2765.
22. Tansey JT, Sztalryd C, Gruia-Gray J, et al. Perilipin ablation results in a lean mouse with aberrant adipocyte lipolysis, enhanced leptin production, and resistance to diet-induced obesity. *Proc Natl Acad Sci USA.* 2001;98:6494–6499.
23. Fan Y, Bergmann A. Apoptosis-induced compensatory proliferation: The cell is dead. Long live the cell! *Trends Cell Biol.* 2008;18:467–473.

Discussion: The Fate of Adipocytes after Nonvascularized Fat Grafting: Evidence of Early Death and Replacement of Adipocytes

Lee L. Q. Pu, M.D., Ph.D.
Sacramento, Calif.

Fat grafting remains one of the most controversial procedures in plastic surgery because there is no standardized technique used by plastic surgeons to harvest, process, and place fat grafts. In addition, and most importantly, how the fat grafts survive after they are transplanted in vivo remains unclear and needs to be determined. The present study was conducted by Dr. Kotaro Yoshimura's group from the University of Tokyo, one of the most active teams in the field of fat grafting and adipose stem/stromal/progenitor cell (adipose-derived stromal cell) research. The study, composed of two parts, is another example of an important and well-conducted study in understanding the mechanism of how fat grafts survive after transplantation. In the in vitro study, adipocytes from experimentally differentiated adipose-derived stromal cells and endothelial cells were cultured under ischemic conditions. The results show that adipocytes are most susceptible to death under ischemic conditions as early as 12 hours, but adipose-derived stromal cells would remain viable for 72 hours. The in vivo study shows that most adipocytes in the graft site begin to die on day 1 and only some of the adipocytes located within 300 μm of the tissue edge would survive. The number of proliferating cells increases from day 3, and increases in viable adipocyte areas can be detected from day 7. This would suggest that repair or regeneration of dead adipose tissue has begun after fat grafting in vivo. The in vivo study also shows three zones after fat grafting from the periphery to the center of the fat grafts: the surviving area (adipocytes survived), the regenerative area (adipocytes died, adipose-derived stromal cells survived, and dead adipocytes were replaced with new ones), and the necrotic area (both adipocytes and adipose-derived stromal cells died).[1]

From the Division of Plastic Surgery, University of California, Davis.
Received for publication December 21, 2011; accepted December 27, 2011.

DOI: 10.1097/PRS.0b013e31824a2b4b

The study appears to show convincing evidence that there is a dynamic remodeling of adipose tissue after conventional fat grafting. Based on the present study, and studies from the same group published previously, the authors have proposed a new theory of how fat grafts survive, the so-called host replacement theory, because it is indeed the case that adipose-derived stromal cells play an important role in fat graft survival. Such a new theory would at least partially explain some of the promising clinical results after cell-assisted lipotransfer proposed by Dr. Yoshimura's group and used by some of our colleagues worldwide.[2,3]

For the past decade or so, the "cell survival theory" has been well accepted by most plastic surgeons to describe how fat grafts survive after they are transplanted in vivo. This theory states that transplanted living adipocytes can survive once they receive adequate nutrients and are incorporated into adjacent vascularized tissue in the recipient site.[4] One previous study shows that human fat may survive after it is grafted to a recipient site and can histologically be characterized into three zones approximately 3 to 4 weeks after grafting. These zones are the peripheral zone (viable adipocytes), the intermediate zone (inflammatory area), and the central zone (necrotic adipocytes). The viable region of the fat grafts is approximately 1.5 ± 1 .5 mm from the adjacent tissue edge, and approximately 60 percent of the adipocytes in the viable zones would eventually die.[5] Based on this theory, the fat grafts should be viable before they are transplanted in vivo and should be placed in a unique fashion that allows fat grafts to have a maximal amount of contact with adjacent living tissue. Therefore, the surgeons who perform fat grafting have refined their technique to harvest and process fat grafts, and to place fat grafts in an

Disclosure: *The author has no financial interest to declare in relation to the content of this Discussion or of the associated article.*

optimal way to maximize the survival of the fat grafts.[6]

The present study, although based on a mouse model, has demonstrated that only peripherally located adipocytes survive, that the depth of a viable zone is approximately 300 μm from the graft edge, and that almost all adipocytes located farther than 300 μm would die within a few days after grafting. This well-conducted animal study clearly indicates that a lower percentage of fat grafts would survive when they are located more than 300 μm away from the viable tissue edge in the recipient site. However, locally resident adipose-derived stromal cells remain viable and then become activated, and many dead adipocytes are gradually replaced by new ones through tissue remodeling or regeneration. Therefore, the current study proposes a new theory, the "host replacement theory," to describe how fat grafts survive after they are transplanted in vivo. Furthermore, the authors observe three zones from the periphery to the center of the fat grafts that are well illustrated in Figure 6: the surviving area (adipocytes survive), the regenerating area (adipocytes die, adipose-derived stromal cells survive, and dead adipocytes are replaced with new ones), and the necrotic area (both adipocytes and adipose-derived stromal cells die).[1] This can certainly be a very important finding from the present study, and I believe that if the same finding can be confirmed by future studies from others or even from humans, such a new theory would revolutionize our thinking in terms of how fat grafts survive after autologous fat grafting. In addition, the adipose-derived stromal cell–enriched fat grafting technique, proposed by Dr. Yoshimura's group, has been proven to have scientific merit, and such a technique may have great potential to improve overall clinical outcome of fat grafting for our patients if the patient's own adipose-derived stromal cells are safe for use in conjunction with conventional fat grafting without detrimental effects, such as induction of cancer.[7,8]

Several advances have been made in basic fat grafting research lately. I believe the reasons can be summarized as follows. Many active research teams, including one from the University of Tokyo, are challenged by some amazing clinical results after fat grafting and unanswered basic fundamental questions (e.g., How do fat grafts survive after they are transplanted in vivo?). Much of the methodology used in stem cell research or in molecular biology research in general is routinely used in basic fat grafting research.[9] Several new immunohistochemical staining methods have recently been used to accurately determine living or dead adipocytes or fat grafts in basic fat grafting research. For example, the immunohistochemical staining for perilipin to differentiate between living and dead adipocytes or the whole-mount staining to determine living or dead adipose tissue.[1,10] The above new histologic techniques, championed by Dr. Yoshimura's group, also greatly improve the scientific quality of basic fat grafting research because now the detailed structure of living adipose tissue can be analyzed histologically. In addition, a few good animal models have been developed and used to study the fate of fat grafts after they are transplanted in vivo. The animal model described by the authors in the present study and another by the New York University group have clearly added new ways of evaluating the survival of fat grafts after they are transplanted in vivo.[1,11] I believe all of the above have contributed to the recent development in basic fat grafting research.

Although the present study is well conducted, with sound and contemporary methodology, I would encourage the authors to perform a long-term in vivo study, such as 2 to 3 months in the same animal model, which may be equivalent to approximately 6 to 12 months in humans. In addition, the authors may consider conducting a similar study in a large-animal model to confirm the findings from the present study. I believe that results from further studies conducted long term and in a large-animal model may be more convincing because they would more closely resemble a clinical setting than does the present study.

Dr. Yoshimura's group should be congratulated for conducting another important study in basic fat grafting research along with previous studies from the same group.[1,2,7–10] Their tireless efforts will help us to further understand the mechanisms of fat survival after grafting and the role of adipose-derived stromal cells to improve the clinical outcome of autologous fat grafting. The host replacement after fat grafting, if confirmed by future studies, will guide plastic surgeons to achieve better outcome after autologous fat grafting, with emphasis on adipocyte remodeling or regeneration once adipose-derived stromal cells are proven to be safe without the potential for inducing tumor growth in patients. Cell-assisted lipotransfer or adipose-derived stromal cell–enriched fat grafting may have great potential for improving the clinical outcome of fat grafting once we better understand its science, technique, and safety.[12,13] We are all looking forward to this exciting future of plastic surgery,

when fat grafting will become a true cell-directed therapy for soft-tissue augmentation.

Lee L. Q. Pu, M.D., Ph.D.
Division of Plastic Surgery
University of California, Davis
2221 Stockton Boulevard, Suite 2123
Sacramento, Calif. 95817
lee.pu@ucdmc.ucdavis.edu

ACKNOWLEDGMENT

The author expresses his appreciation to Amy Dykstra for secretarial support during the preparation of this Discussion.

REFERENCES

1. Eto H, Kato H, Suga H, et al. The fate of adipocytes after non-vascularized fat grafting: Evidence of early death and replacement of adipocytes. *Plast Reconstr Surg.* 2012;129:1081–1092.
2. Yoshimura K, Sato K, Aoi N, Kurita M, Hirohi T, Harli K. Cell-assisted lipotransfer for cosmetic breast augmentation: Supportive use of adipose-derived stem/stromal cells. *Aesthetic Plast Surg.* 2008;32:48–55; discussion 56–57.
3. Sterodimas A, de Faria J, Nicaretta B, Boriani F. Autologous fat transplantation versus adipose-derived stem cell-enriched lipografts: A study. *Aesthet Surg J.* 2011;31:682–693.
4. Billings E Jr, May JW Jr. Historical review and present status of free fat graft autotransplantation in plastic and reconstructive surgery. *Plast Reconstr Surg.* 1989;83:368–381.
5. Carpaneda CA, Ribeiro MT. Study of the histologic alternations and viability of the adipose graft in humans. *Aesthetic Plast Surg.* 1993;17:43–47.
6. Pu LLQ. Towards more rationalized approach to autologous fat grafting. *J Plast Recontr Aesthet Surg.* 2012;65:413–419.
7. Yoshimura K, Sato K, Aoi N, et al. Cell-assisted lipotransfer of facial lipoatrophy: Efficacy of clinical use of adipose-derived stem cells. *Dermatol Surg.* 2008;34:1178–1185.
8. Yoshimura K, Asano Y, Aoi N, et al. Progenitor-enriched adipose tissue transplantation as rescue for breast implant complications. *Breast* 2010;16:169–175.
9. Suga H, Eto H, Aoi N, et al. Adipose tissue remodeling under ischemia: Death of adipocytes and activation of stem/progenitor cells. *Plast Reconstr Surg.* 2010;126:1911–1923.
10. Eto H, Suga H, Matsumoto D, et al. Characterization of structure and cellular components of aspirated and excised adipose tissue. *Plast Reconstr Surg.* 2009;124:1087–1097.
11. Thanik VD, Chang CC, Lerman OZ, et al. A murine model for studying diffusely injected human fat. *Plast Reconstr Surg.* 2009;124:74–81.
12. Yoshimura K, Suga H, Eto H. Adipose-derived stem/progenitor cells: Role in adipose tissue remodeling and potential use for soft tissue augmentation. *Regen Med.* 2009;4:265–273.
13. Yoshimura K, Eto H, Kato H, Doi K, Aoi N. In vivo manipulation of stem cells for adipose tissue repair/reconstruction. *Regen Med.* 2011;6(Suppl):33–41.