New protagonists of regenerative niches in different fat pads

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Conflict of interest: the authors declare that there is no conflict of interests regarding the publication of this paper.

Keywords: MUSE; Adipose tissue; pluripotency; SEEA3

Summary
Aims: New insights into the regenerative potential of adipose tissue, together with the success of fat grafting in reconstructive plastic surgery, have lead researchers to identify and investigate the role of regenerative niches in adipose tissue. In this context, the principal aim of the study was to investigate the cellular compartment of regenerative units in different fat pads. We describe not only regenerative niches isolated only from healthy patients, but also the presence and characteristics of niches isolated from specific types of adipose depots.
Materials and Methods: Adipose tissue was harvested by liposuction from 18 women classed as having obesity (n=5), radiotherapy (n=5) or subjected to breast reconstruction (n=8). Adipose tissue was digested, and mesenchymal stem cells were isolated from the vascular stromal fraction and placed in culture. Additionally, specimens of adipose tissue were processed for ultrastructural and morphological assessment using transmission electron and confocal microscopy in order to evaluate the cellular compartment of regenerative units of adipose tissue. We focused particularly on post-adipocytes, a novel cellular element recently described in stressed adipose tissue, and multilineage-differentiating stress-enduring (MUSE) cells, a subpopulation of mesenchymal stem cells characterised by high pluripotency and devoted to tissue regeneration.
Results: Both post-adipocytes and MUSE cells were detected in specimens harvested from radio-treated and obese patients, while in samples collected from patient subjected to lipofilling for breast reconstruction, even though the morphology of mature adipocytes was altered, there were fewer post-adipocytes and MUSE cells. This indicated that post-adipocyte and MUSE cell counts are correlated to the degree of tissue stress.
Conclusions: Although further evaluations at the molecular level are necessary, our findings could help surgeons and researchers better understand the cellular composition of regenerative niches, and the role of each cell in tissue regeneration.

Keywords: adipose tissue, regenerative unit, post-adipocyte, MUSE cells

Abbreviation list

SVF = stromal vascular fraction
MUSE = multilineage differentiating stress enduring
sWAT = subcutaneous adipose tissue
TEM = transmission electron microscopy

Introduction

We have known about the regenerative potential of adipose tissue for a decade now, and numerous studies regarding the application of autologous adipose tissue transplants in reconstructive and regenerative medicine have been published in that time (23,7,22). Although this research has told us much about the regenerative component of the adipose tissue, more specifically subcutaneous adipose tissue, several aspects of tissue regeneration after adipose tissue transplant require further clarification (18,23,10).

The first wave of studies on the regenerative potential of adipose tissue focused on adipose mesenchymal stem cells (7,15, 4), which are characterised by high multipotency and the capacity to differentiate into mature adipocytes, osteocytes and chondrocytes (19,20). In particular, research aimed to explain the morphological and biomolecular modifications occurring in adipose mesenchymal stem cells, and to describe their active role in tissue reconstruction and/or regeneration (17). In a second phase of research, attention began to shift to the other components of subcutaneous adipose tissue, i.e., the vascular stromal fraction (SVF), platelets and growth factors, in the attempt to comprehend whether these components also had the potential to promote tissue regeneration (13,28,3).

Then surgeons started to employ both adipose mesenchymal stem cells and SVF, growth factors and platelets (21), and the age of applied adipose tissue enrichment was begun, in which purified adipose tissue was enriched using different fractions isolated from fat, and co-injected (21). This was also the phase in which the niche theory was developed and described by Sbarbati et al. to explain the principle of tissue regeneration after adipose tissue transplant (25). According to this theory, regenerative capacity was attributable not only to mesenchymal stem cells, but also to the niche in which stem cells were enclosed. This niche has been described as composed of mesenchymal stem cells, mature adipocytes with active cytokine secretion, located near to blood vessels or capillaries (25). This regenerative unit, once implanted, causes the modification of subcutaneous tissue and the regeneration of different types of structures (fibrils, collagen, adipose tissue, blood vessels). So, it is possible to suppose that lipofilling could be used to provide regenerative units in situations where the regenerative potential of tissue had been damaged through radiotherapy, scars, burns or obesity.

Hence, the most recent phase of research has begun to investigate the molecular and biological pathways occurring in adipose tissue before and after fat grafting (5,9). The molecular signatures of mature adipocytes and mesenchymal stem cells have been revealed, shedding light on the events involved in tissue regeneration (19). For example, very recent studies have shown that the stem cell population in adipose tissue is not homogeneous (31,10,16,29,32). Specifically, a limited portion of the stem cell population is characterised by the expression of stress-enduring antigens, and has a high capacity to differentiate into numerous types of mature cells such as hepatocytes, myocytes, and neurons (pluripotency) (26). These cells were therefore named multilineage-differentiating stress-enduring cells (MUSE).

Simultaneously, studies on mature adipocytes were performed. They described a novel phase in the life cycle of mature adipocytes characterised by the loss of lipid charge consequent to mechanical, physical or chemical stress, which caused the formation of a newly discovered type of cell, the post-adipocyte (7,30,18). Due to the presence of both basal membrane and small lipid droplets, even though clustered in limited portions of the cytoplasm, this cell is classifiable as a mature adipocyte (7), which is able to restore its lipid charge when physiological tissue conditions are restored, switching back to a mature adipocyte.
Post-adipocytes and MUSE cells are undoubtedly the protagonists of this most recent phase of research into the regenerative capacity of adipose tissue (7). We set out to contribute by assessing the presence of these cells in different samples of adipose tissue harvested from women subjected to different types of stress, namely breast reconstruction, in which there is only the passage of tissue thought a cannula; radiotherapy, in which the radiation itself causes physical stress; and obesity, in which chemical stress causes an inflammatory state. Confocal microscopy and ultrastructural analysis were used to investigate each type of specimen in order to verify whether the formation of post-adipocytes and MUSE cells is really associated with stress endurance.

Materials and methods

Subcutaneous adipose tissue (sWAT) harvesting
sWAT samples were obtained from 18 women, 35-65 years old, subjected to outpatient liposuction for reconstructive purposes. All patients were invited to attend a liposuction planning meeting with a surgeon, and informed about the necessity of biopsy harvesting for scientific aims. All patients provided written consent during this preliminary meeting. Biopsies were anonymised immediately after liposuction, which was performed via the following method: donor adipose tissue was taken from the medial area of the knee or the abdominal or trochanteric regions using infiltration with a cold saline solution, with the addition of 1:400,000 epinephrine and 20 cc lidocaine 0.5% every 500 cc., and Coleman's instrumentation for removal. sWAT specimens were harvested from women who had undergone radiotherapy (n=5), had obesity (n=5) or were subjected to liposuction for breast reconstruction (n=8).

Ultrastructural analysis of adipose tissue
sWAT samples were processed for transmission electron microscopy (TEM). Specifically, specimens were fixed with 2% glutaraldehyde, and then post-?xed in 1% osmium tetroxide (OsO_4) in aqueous solution for 2h, dehydrated in graded concentrations of acetone, and embedded in Epon-Araldite mixture (Electron Microscopy Sciences, Fort Washington, PA, USA). Semi-thin sections (1 mm thick) were examined under light microscopy and stained with toluidine blue. Ultra-thin sections (70 nm thick) were cut and placed on Cu/Rh grids with Ultracut E (Reichert, Wien, Austria), stained with lead citrate, and examined using an FEI Morgagni 268D electron microscope (FEI Company, Eindhoven, Netherlands).

Isolation of mesenchymal stem cells
For adipose tissue digestion, 10cc of sWAT was incubated at 37°C with 15 mL of collagenase type I for 40-45 min at 37°C. After digestion, the sample was centrifuged at 100g for 5 min, and the supernatant was discarded. The SVF pellet was re-suspended in NH_4Cl in a 50 ml vial to lyse blood cells, filtered, and then centrifuged at 100g for 10 min. The resulting SVF was re-suspended for 3 days in growth medium, specifically DMEM (Dulbecco's Modified Eagle Medium) + 10% Fetal Bovine Serum (FBS) + 1% of a mix of penicillin and streptomycin (Life Technologies), in a 25-cm^2 culture flask (FalconTM BD Medical, New Jersey, USA). Culture plates were kept in the incubator at 37°C, 5% CO_2 and 100% humidity.

Confocal microscopy

Cells cultured on 25 cm2 plates were treated with 1% trypsin for 5 minutes at 37°C, and then harvested and centrifuged at 400g for 5 minutes. Pellets were re-suspended in culture medium (DMEM+10%FBS+1% penicillin-streptomycin mix), and cells were counted in a Burker chamber in order to plate 2x104 cells in each well of multi-chamber slices for fluorescent microscopy (Sarsted, Italy). Each well was filled with 1 ml of culture medium, and the following day incubated with the antibody anti-SSEA3 (Bioloegend, San Diego, CA) functionalized with Alexa Fluor 488 (1:3000, NovusBio) and anti-CD105-conjugated with Alexa Fluor 700 (1:2000, BD bioscience); cells were incubated at 37°C and 5% CO2 for 1 hour and then washed three times with PBS. Coverslips were positioned on the glass and mounted using aqueous mounting medium containing DAPI for nuclei staining. Confocal images were acquired using a Leica TCS-SP5 confocal microscope at 40X magnification (Leica-Microsystem, Wezlar, Germany).

Results

Ultrastructural analysis and confocal microscopy of different sWAT samples revealed the presence of two cellular elements with differing characteristics and behaviours, depending on the source of fat harvesting. There were differences in sWAT morphology and ultrastructural appearance in samples from the radiotherapy, obesity and breast reconstruction groups. In particular, TEM analysis evidenced that radio-treated sWAT is characterised by large areas of mesenchymalization, and the loss of typical tissue organization. Mature adipocytes, identifiable by a unique lipid droplet and peripheral nucleus, were rare, but numerous wrinkled elements with lipid droplets clustered in small depots were detectable in the cytoplasm, often close to macrophages. These elements, described in our previous paper, may be called post-adipocytes; they have very thin cytoplasm, with wrinkled membranes and small clusters of lipid droplets (Fig. 1 panel A, black arrows). In some of these cells the nuclei were well visible, but in other cells the nucleus was thinner. In contrast, sWAT harvested from patients with obesity showed inflammation foci, and the preservation and presence of basal membrane only on a layer of adipocytes (Fig. 2 panel A white arrow), while the opposite side presented a very well defined membrane (Fig. 2 panel A black arrows). The cytoplasm of the adipocytes, or more specifically post-adipocytes, in these specimens was rich in small lipid droplets. The adipose tissue harvested from patients subjected to breast reconstruction, on the other hand, differed profoundly; mature adipocytes were characterised by typical ultrastructure, with a single lipid droplet and regular cytoplasmic membranes, as well as blood capillaries among the cells. Cytoplasm was almost absent, confined to a very thin layer adjacent to the membrane. All the remaining cellular space was occupied by the lipid droplet. The morphology of this type of biopsy appeared not substantially different to the sWAT generally described in the literature.

However, the most interesting aspect evidenced by this study is the presence of multilineage-differentiating stress-enduring (MUSE) cells, detectable by confocal microscopy due to the simultaneous expression of CD105, a typical mesenchymal stem cell marker, and SSEA-3, stress-enduring antigen-3. Double staining with the different antibodies revealed the concomitance of these markers in radio-treated sWAT (Fig. 1 panel B, CD195 red emission and SSEA-3 green emission) and in sWAT harvested from patients with obesity. SSEA-3 expression was greater than CD105, thereby indicating the greater degree of stress endured by adipose tissue in women with obesity and those undergoing radiotherapy. In the specimens harvested from patients subjected to breast reconstruction, on the other hand, the expression of SSEA-3 was lower, while the expression of CD105, the mesenchymal stem cell marker, was substantially unvaried.

Discussion

To our knowledge, lipofilling, or autologous fat grafting, is the best technique for regenerating cutaneous and subcutaneous tissues after injuries, radiotherapy or mastectomy (23,22,12). The power of lipofilling is granted by regenerative units, niches capable of regenerating damaged tissues in a relatively short time. Numerous papers have attributed the efficiency of autologous adipose

tissue implant exclusively to the number of stem cells present in the injected fat (20,14), but more recent research has focused on the role played by other cell factors in the regenerative niche (2). Indeed, the regenerative potential of lipofilling is likely not only due to the presence or abundance of stem cells, but also to the crosstalk between regenerative niches and damaged tissue, performed by growth factors, cytokines and differentiating stimuli. The crosstalk between damaged cells and mesenchymal stem cells is also considered important, and some researchers have stated that stem cells are not completely the same in tissues of mesenchymal origin.

We can now confirm that mesenchymal stem cells isolated from different adipose tissue specimens present different characteristics, in particular if the tissue is damaged or subjected to different forms of stress (26,27). The ultrastructure of adipose tissue harvested from radio-treated patients is completely different to that harvested from people with obesity (27). Similarly, evident differences are detectable in adipose tissue harvested from patients subjected to lipofilling for breast reconstruction. In the fat of patients with obesity and those having undergone radiotherapy we observed the concomitance of two novel types of cell: MUSE cells and post-adipocytes. The former, a subpopulation of mesenchymal stem cells, have been described as very sensitive to cytokine production and stress conditions. Recent manuscripts have reported the isolation of a subpopulation of mesenchymal stem cells expressing embryonic stress antigens (SEEA antigens) characterised by high affinity for lesioned tissues and strong pluripotency (8).

When the mesenchymal stem cells were isolated for the first time, at the origins of adipose stem cell research, it was possible to differentiate them into osteocytes, adipocytes and chondrocytes (19). Later, neuronal-like cells were also identified (1). MUSE cells, on the other hand, are also characterised by their capacity to differentiate into hepatocytes and myocytes. In addition, in our recently published manuscript we describe the post-adipocyte, a mature adipocyte that has lost its lipid charge after exposure to stress, such as radiotherapy or inflammation (7). These two novel types of cell belong to the regenerative unit of adipose tissue, and our experiments demonstrate, in the first instance, that the number of stem cells injected during lipofilling does not influence the efficacy of treatment and, in the second instance, that post-adipocytes and MUSE cells are physiological components of adipose tissue which the data suggest play an important role in tissue regeneration.

Conclusions

We provide a more specific description of the structure and cellular compartment of adipose tissue regenerative units. In particular, we show the presence of two new types of cell, MUSE cells and post-adipocytes, with regenerative potential present in adipose tissue subjected to different forms of stress. Alongside stem cells, these likely contribute to tissue repair and regeneration, being stimulated by chemical factors secreted in the stressed tissue. However, more detailed research will be required to fully comprehend the functional role of MUSE cells and post-adipocytes, and to explore their behaviour once transplanted into damaged tissue.

Captions to figures

FIG. 1. TEM and fluorescence microscopy of adipose tissue harvested from a radio-treated patient. In panel A the mesenchymalization of adipose tissue is appreciable, as is the loss of the typical morphology of adipose tissue. The arrows indicate cells with preserved nucleus and very wrinkled membrane, the post-adipocytes. In panel B the high expression of SEEA-3 (green colour) is observable, while the expression of CD105 (red colour) is lower, but in some cells it is possible to appreciate a co-localization of the two fluorescent probes. These data indicate the presence of MUSE cells in subcutaneous adipose tissue subjected to stress by radiation treatment.

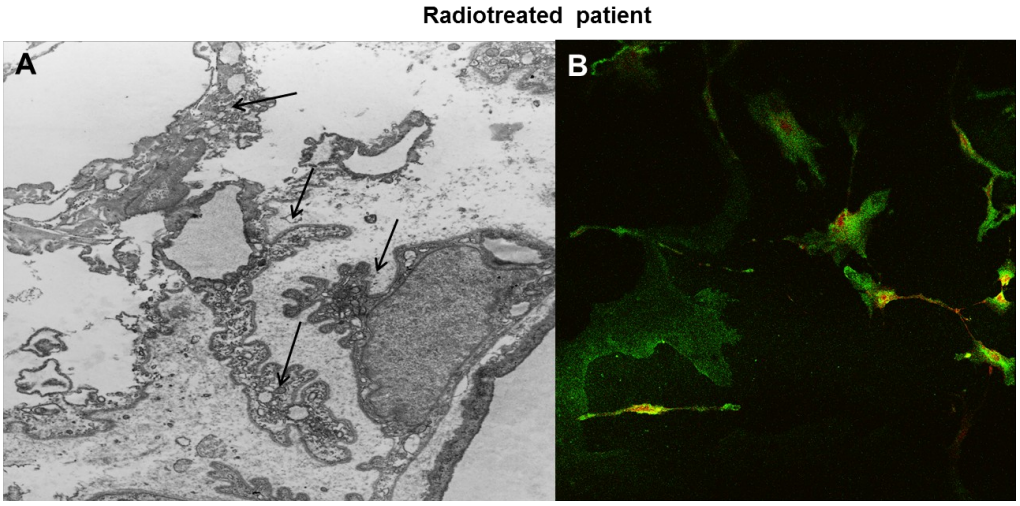


FIG. 2. TEM and fluorescence microscopy of adipose tissue harvested from a patient with obesity. In panel A the loss of typical adipose tissue compartmentalization is visible. The presence of post-adipocytes is detectable and indicated by black arrows. White arrows indicate the basal membrane, present on one side of post-adipocytes, the side characterised by a more regular shape. These data confirm the adipose origin of post-adipocytes, due to the simultaneous presence of lipid droplets and basal membrane. In the panel B the presence of MUSE cells is revealed by the co-expression of SEEA-3 (green colour) and CD105 (red colour). MUSE cells are markedly more abundant in specimens collected from patients with obesity in comparison to the other specimens.

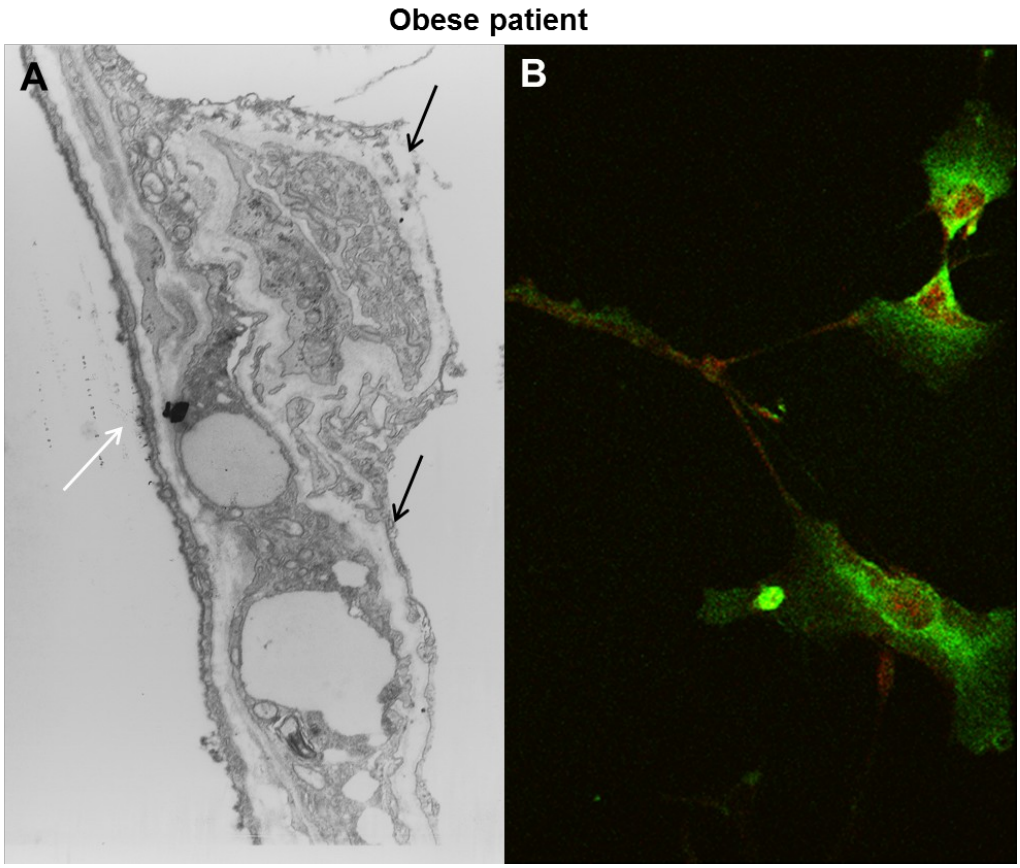
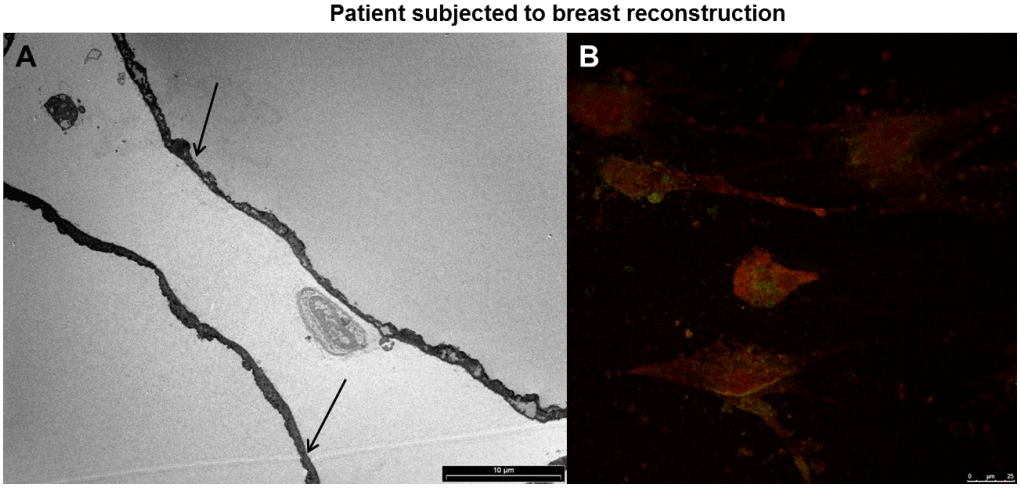


FIG. 3. TEM and fluorescence microscopy of adipose tissue harvested from a patient subjected to breast reconstruction. In panel A two adipocytes are visible. They are located near to a blood vessel and characterised by the presence of a regular membrane, even though in some tracts it seems

thickened. Note the single lipid droplet that occupies the entire cytoplasm; this resembles the typical structure of sWAT. In panel B it is possible to observe a marked expression of CD105 (red colour), but a very reduced expression of SEEA-3, indicating a very low presence of MUSE cells, whose presence is correlated with the conditions of stress; in this specimen stress is reduced, and there is a corresponding lack of post-adipocytes and MUSE cells.



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