Intrinsics and Dynamics of Fat Grafts: An In Vitro Study

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Background: Despite a revived interest in fat grafting procedures, clinicians still fail to demonstrate clearly the in vivo behavior of fat grafts as a dynamic tissue substitute. However, the basic principles in cellular biology teach us that cells can survive and develop, provided that a structural matrix exists that directs their behavior. The purpose of this in vitro study was to analyze that behavior of crude fat grafts, cultured on a three-dimensional laminin-rich matrix.
Methods: Nonprocessed, human fat biopsy specimens (approximately 1 mm^3) were inoculated on Matrigel-coated wells to which culture medium was added. The control group consisted of fat biopsy specimens embedded in medium alone. The cellular proliferation pattern was followed over 6 weeks. Additional cultures of primary generated cellular spheroids were performed and eventually subjected to adipogenic differentiation media.
Results: A progressive outgrowth of fibroblast-like cells from the core fat biopsy specimen was observed in both groups. Within the Matrigel group, an interconnecting three-dimensional network of spindle-shaped cells was established. This new cell colony reproduced spheroids that functioned again as solitary sources of cellular proliferation. Addition of differentiation media resulted in lipid droplet deposition in the majority of generated cells, indicating the initial steps of adipogenic differentiation.
Conclusions: The authors noticed that crude, nonprocessed fat biopsy specimens do have considerable potential for future tissue engineering–based applications, provided that the basic principles of developmental, cellular biology are respected. Spontaneous in vitro expansion of the stromal cells present in fat grafts within autologous and injectable matrices could create "off-the-shelf" therapies for reconstructive procedures. (*Plast. Reconstr. Surg.* 126: 1155, 2010.)

Restoring form in soft-tissue deficiencies is achieved either by autologous tissue transfer or implantation of allogenic materials. However, the basic principle in tissue reconstruction is to "replace like with like," and allogenic devices will never fully duplicate the durability, strength, form, function, and biocompatibility of native tissues.[1,2] Ideally, the substitutive tissue not only needs to survive in a steady-state equilibrium within the adjacent tissue at the recipient site but also needs to function properly at a cellular and physiologic level.[2]

The charisma of the easily accessible adipose tissue as an autologous donor material stems from its intrinsic plasticity.[3–5] Still, the clinical and scientific debate as to whether fat grafts do have the potential to integrate completely as a viable tissue substitute within their recipient environment continues to divide the surgical community. Although several theories describe the fate of transplanted fat grafts—whether or not as a lipoaspirate graft—the consensus still dictates a considerable degree

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of resorption with partial replacement by a mainly acellular fibrotic tissue.[6]

The renewed interest in lipoaspirate material,[7] backed up by the expectations of adipose tissue engineering research, not only to repair soft-tissue defects but also to improve tissue quality,[3,8–12] burdens researchers with questions about the fate of transplanted fat grafts. The traumatic manipulation of the panniculus adiposus during the harvesting procedure results in a defragmentation of its cellular components and its structural integrity. It has been postulated that the grafted cells survive initially through the process of diffusion.[6] This basic cellular process has been hypothesized to contribute to the partial survival of the outer layer of cells within the graft.[6]

The main problem in fat grafting procedures is that the fundamental requirements to achieve cellular morphogenesis or even tissue homeostasis are not maintained.[13–15] The extracellular matrix is known to be the main vector in cellular behavior and development.[16,17] From a fundamentally scientific point of view, fat grafting—in particular, the lipofilling procedure—is essentially a reversed approach to restore tissue deficiency; adipocytes and their stromal cells, either committed, uncommitted, or terminally differentiated, are disrupted from their native extracellular matrix and are transplanted to a recipient site where a structural and nutritional matrix is lacking at the time of implantation.

The purpose of this in vitro study was to examine whether minute amounts of fat graft do have the potential to act as a source of cellular expansion and morphogenesis if cultured on a supportive biological matrix. It was hoped that the observations would provide further insights into the in vitro behavior of the stromal cell population of white adipose tissue and would aid in the design of future strategies in the study of adipose cellular survival based on sustained control of cellular growth and differentiation.

MATERIALS AND METHODS

Adipose Tissue

Human fat tissue biopsy specimens were obtained following informed consent and ethics approval through the Department of Surgery at the St. Vincent's Hospital, Melbourne. Adipose tissue was excised (approximately 2 cm^3) from the lower midabdominal area in a 38-year-old female patient who underwent elective surgery for a scar revision (cesarean delivery). Her medical background was without specific antecedents. Tissue specimens were transported to the laboratory on ice in Dulbecco's Modified Eagle Medium (Invitrogen Australia Pty. Ltd., Mount Waverley, Victoria, Australia) enriched with antibiotics and antimycotics (100 U/ml penicillin sodium salt, 100 μg/ml streptomycin sulfate, and 25 μg/ml amphotericin B; Invitrogen Australia).

Culturing in Matrigel

Under sterile conditions, the fat tissue was cut into small cubes of approximately 1 mm^3. A 24-well plate (BD Biosciences, Franklin Lakes, N.J.) was used with each well coated with a layer of growth factor–reduced Matrigel (volume of 150 μl; BD Biosciences). The Matrigel was supplemented with 1 μg/ml of recombinant human fibroblast growth factor 2 (Pepro-Tech, Rocky Hill, N.J.) and 80 U/ml of heparin (Pfizer, New York, N.Y.). Once the Matrigel was aliquoted into the appropriate wells, it was allowed to semisolidify for a few minutes at room temperature before the human fat biopsy specimen (approximately 1 mm^3) was positioned in the center of the matrix-coated well. Fat chunks including a fibrous, stromal compartment were selected. This specific compartment tissue is the niche of the heterogenous precursor cell population within adipose tissue (Fig. 1). The matrix was then allowed to set for at least 5 minutes at 37°C to form a gel containing the biopsy specimen. After this, 1 ml of complete media [10% fetal calf serum (CSL Ltd., Melbourne, Victoria, Australia), 100 U/ml penicillin sodium salt, 100 μg/ml streptomycin sulfate, and 292 μg/ml glutamine (Sigma-Aldrich, St. Louis, Mo.)] was added on top of the Matrigel matrix. The plates were incubated in 5% carbon dioxide at 37°C over several weeks as indicated, with media changes twice weekly. The control group consisted of a 24-well plate with each well coated with 1 ml of complete media in which a fat biopsy specimen (approximately 1 mm^3) was inoculated. Over the course of the experiment, the biopsy specimens were observed every 2 days for cellular outgrowth and for morphologic and/or differentiation changes of the cells growing from the biopsy specimens. Photographs were taken at every stage to assess the above observations using a digital camera (DP10; Olympus, Centre Valley, Pa.) mounted onto an inverted microscope (CKX41; Olympus).

Subculture

To study their proliferative capacity, interconnecting spheroid islands of cells from the Matrigel group were isolated from the culture media at 6

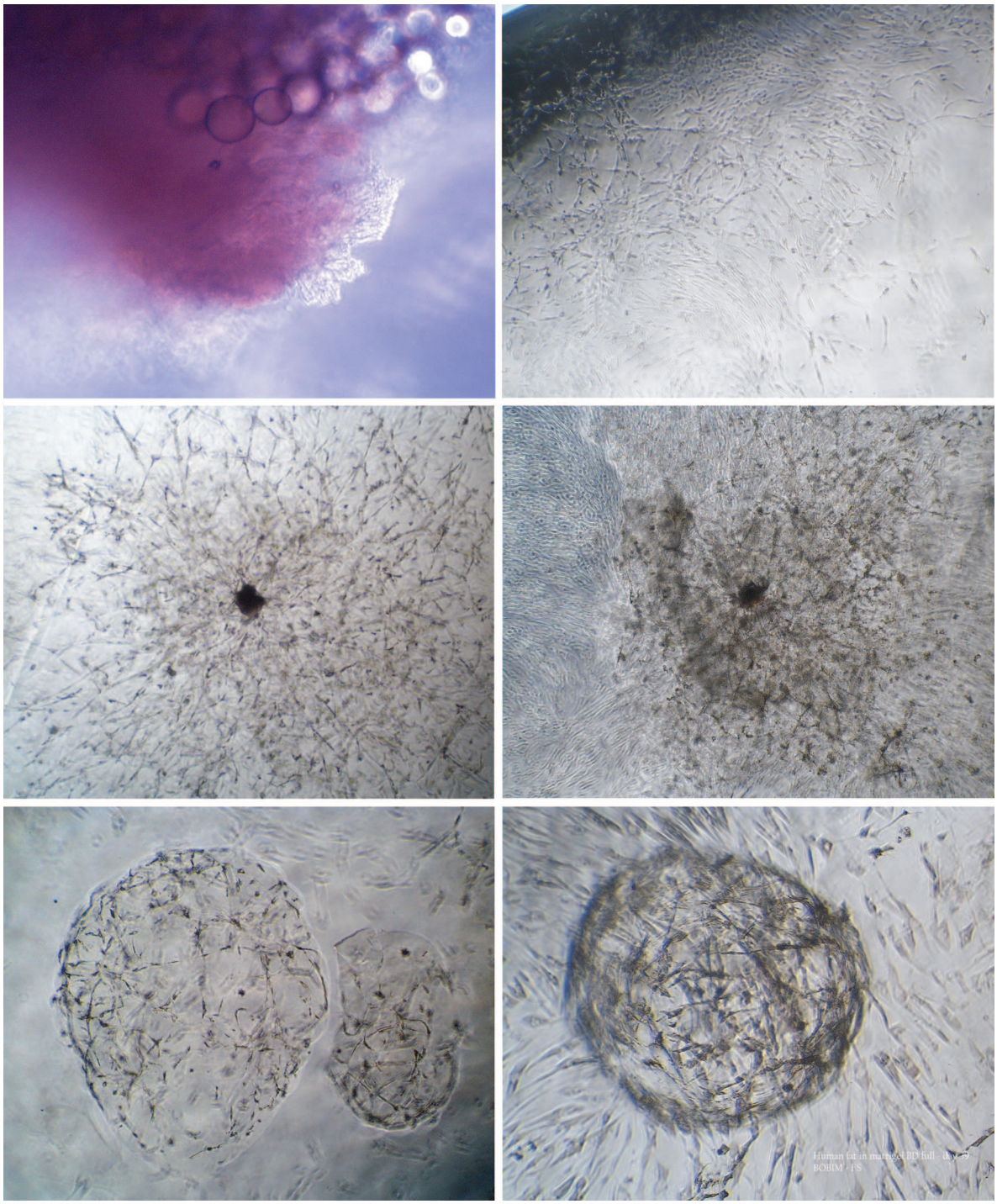

Fig. 1. Fat specimens including specifically a fibrous, stromal compartment were selected, as the stromal compartment is the source of the heterogenous cell population within white adipose tissue (*above*, *left*). In the control group (media alone), a two-dimensional outgrowth of fibroblast-like cells was observed, whereas the fat biopsy specimens migrated toward the periphery of the well (*above*, *right*). In the Matrigel group, a three-dimensional, expanding network of interconnecting cells originated from the core fat biopsy specimen (*center*, *left*). The in vitro culture is pictured at 30 days, showing ongoing cellular proliferation and the establishment of a denser network of interconnecting spindle-shaped cells (*center*, *right*). Self-limiting spheroids were formed and floated as independent islands of interconnecting cells within the culture media (*below*, *left*). Although their diameter remained fairly constant during further cultivation, the spheroids' skeleton became denser because of ongoing cellular proliferation. (*Below*, *right*) Photomicrograph of a spheroid seen at day 39.

weeks and transferred into new wells following the same protocol.

Differentiation

Where indicated, the cultures were subjected to adipogenic differentiation media consisting of 0.5 mM isobutyl-methylxanthine, 1 μM dexamethasone, 10 μM insulin, 200 μM indomethacin, and 1% antibiotic/antimycotic, as described previously.[5]

RESULTS

During the first week, a consistent outgrowth of fibroblast-like cells from the core fat biopsy specimen was observed in both experimental groups (Fig. 1). Within the Matrigel group, the centrifugal cell proliferation pattern developed into a three-dimensional expanding network of interconnecting spindle-shaped cells (Fig. 1). This cellular network became progressively denser, indicating continuous cell proliferation (Fig. 1). From this proliferative event, three-dimensional spherical structures (spheroids) or "adiposphere" were subsequently generated from day 10 onward (Fig. 1). Those individual islands of cells consisted of interconnecting fibroblast-like cells and floated independently in the culture media (Fig. 1). The spheroids initially appeared to enlarge rapidly and then continued to grow steadily before reaching a critical diameter beyond which there appeared no further expansion. After further incubation, the spheroids became more spherical and dense because of ongoing cell proliferation (Fig. 1). Besides the standard, two-dimensional cellular expansion, no adiposphere were observed in the control group.

At 6 weeks, the adiposphere were removed and dispersed into new wells coated with growth factor–reduced Matrigel and complete media containing 10% fetal calf serum (Fig. 2). Throughout the apparently dormant phase when the primary spheroids did not expand further, the cell population in each spheroid remained viable. New spheroids or monolayers were produced by cells shed from dormant spheroids. After subculture, the spheroids functioned again as a source of outgrowing fibroblast-like cells with continuing cell proliferation (Fig. 2). Adipose differentiation media was added to some of the culture wells (Fig. 2). This resulted in the formation of lipid droplets within the fibroblast-like cells, indicating the initial steps of adipogenic differentiation (Fig. 2). Cells then began to accumulate lipid in the cytosol to the extent that the nucleus was eventually displaced from the center to the periphery of the cell. Ten days after the initial induction, the majority of the cells were converted into adipocytes, with the typical spherical cell and lipid-filled morphology (Fig. 2).

DISCUSSION

The adipose organ is essentially organized into a connective tissue composed of a heterogeneous cell population with multipotent characteristics and a microvascular system entwined within a highly organized extracellular matrix.[2–5] The enigma of whether fat grafts do survive, function as a source of either proliferating cells or vasoactive signals, or become replaced by an adynamic and unsubstantial host tissue is intricate because of the complex native environment in which mature adipocytes reside. It is clear that cells and their morphologic processes and interactions that accompany their further development need an instructive extracellular matrix that will function as a structural support and preserver of space, and as a depot for cytokine release.[18,19] Skepticism in recognizing the survival rates of those grafts is based on the rationality that considers fat grafting procedures as "reversed approaches" to restore tissue integrity. This evidence-based reasoning seems to be counteracted nowadays by vague, mostly operator-dependent interpretations, controversial and contradictorily data recovered from clinical assessments.

The objective in reconstructive surgery and in tissue engineering research is to recreate three-dimensional, living body parts or tissue substitutes that will fully integrate within the recipient's body.[11] Within this line of thought, transferred fat should prosper as a homeostatic tissue substitute by biochemical, physical, and cellular cues approximating the native tissue equivalent. Researchers are gaining better insights into those basic cellular and molecular events involved in neoadipogenesis, and recognize that adipose tissue is a complex, difficult organ to "engineer," requiring several crucial steps.[7,18–24]

There has been a relative lack of emphasis placed on understanding the principles of normal developmental biology of adipose tissue and the application of these principles to the ultimate objective of regenerating adipose tissue, or at least achieving a better survival rate of transplanted fat.[17] Cell growth in standard, flat tissue culture is two-dimensional and, apart from the constraints of the vessel surface area, is not self-regulating.[25] This in vitro growth is unrestricted, provided that medium is replenished and sufficient open space is available. It has been shown that cells removed

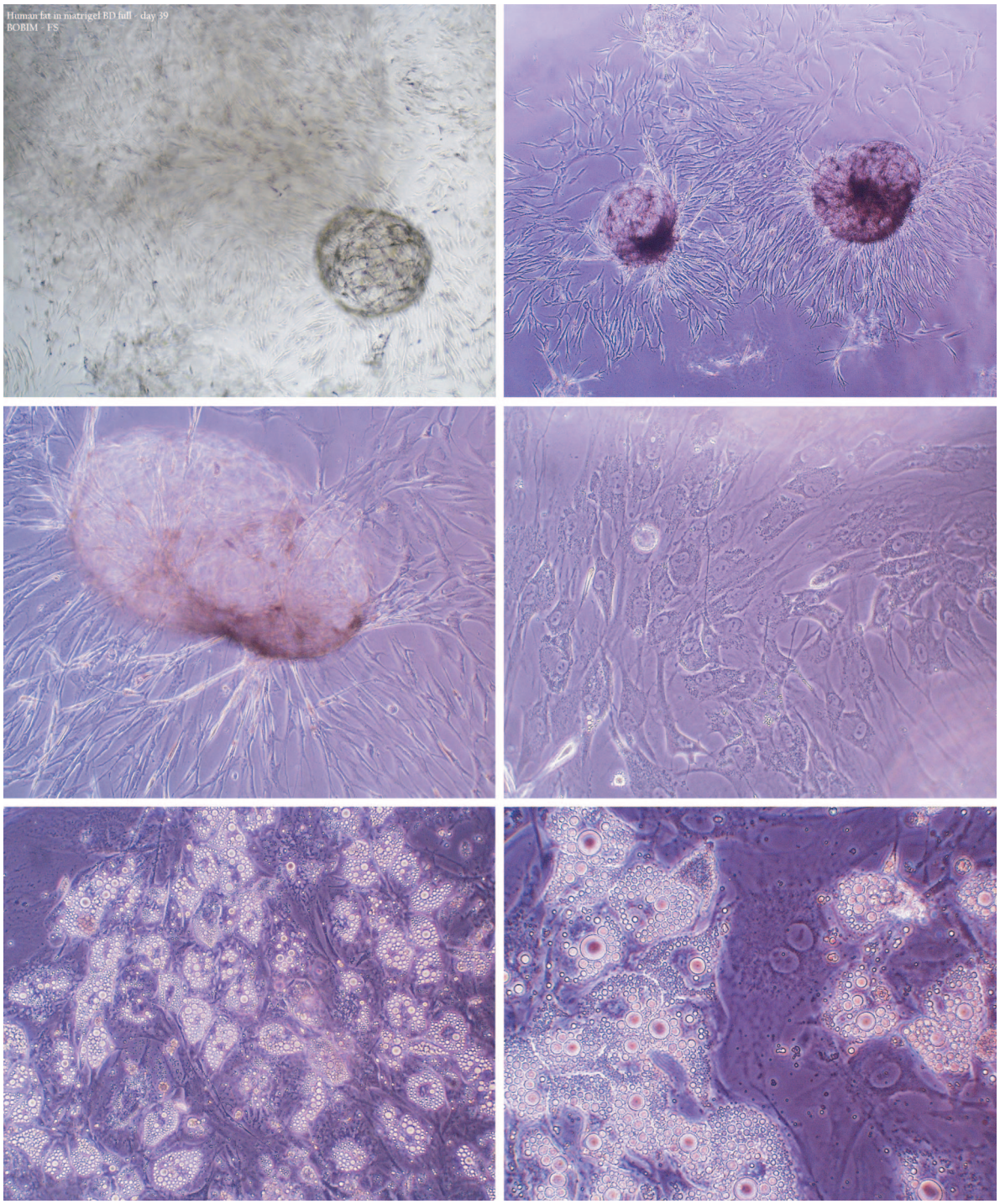

Fig. 2. At 6 weeks, the adipospheres (*above*, *left*) were isolated and transferred into new wells coated with Matrigel and 10% fetal calf serum (*above*, *right*). They functioned again as sources of outgrowing fibroblast-like cells with continuing cell proliferation (*above*, *right* and *center*, *left*). To induce differentiation, adipogenic medium was added to the culture wells containing an established monolayer of cells (*center*, *right*). Ten days after the initial induction, the majority of the cells were converted into adipocytes, with the typical spherical cell morphology (*below*).

from a planar surface and grown three-dimensionally into a spheroidal or ellipsoidal population are self-regulating and will not expand beyond a critical diameter and cell population regardless of how much new medium is provided or how much open space is made available.[26]

The Matrigel scaffold in this in vitro study is the "biological glue" that preserves the space where cells are constrained and orientated. We were able to demonstrate how minute, unprocessed fat grafts do have the ability to generate expanding colonies of progenitor cells that can be directed toward commitment to the adipogenic lineage. This in vitro microenvironment is specifically characterized by a spontaneous, homogenous distribution of tissue-specific precursor cells held securely within the matrix plug.

Secondary spheroids (adipospheres) were also generated. Those adipospheres reached a maximum or "dormant" diameter beyond which no further expansion was possible regardless of the frequency of media replenishment. Subsequent culturing of isolated adipospheres showed them to function as antecedents to reinduce the formation of new colonies of cells. Ongoing research needs to identify the cell phenotype within those colonies to determine whether some type of "purification process" of precursor cells occurred.

The mechanism of the dormant phase is most likely based on the findings of Folkman and Hochberg, where the ratio of surface to volume is diminishing while the spheroid is growing progressively.[26] When cell growth is two-dimensional in a plane, the ratio of surface area to volume remains constant in the face of unlimited fresh medium and space.[26]

Fat graft survival in vivo depends on diffusion for absorption of nutrients and release of catabolites before it is (partially) revascularized. The question regarding the in vivo manipulation of adipose-derived cells to generate an adipose-specific product for clinical applications remains to be addressed, as clinicians still rely on standard procedures, such as lipofilling, to address soft-tissue deficiencies. The addition of a three-dimensional, structural "niche" before or at the time of implantation could favor that cell survival by providing not only the space and orientation but also the nutrition for the cells to develop. The third dimension will also guide the generation of a stable neovascular plexus with subsequent deposition of newly formed connective tissue.

Our in vitro cultured, precursor cell–enriched, and injectable gel-like substratum can be considered as a precursor for clinical implementation. In a complementary, limited in vivo study, we injected the cell-enriched matrices in our tissue engineering chambers. The contents were harvested after 1 week and assessed histologically. We

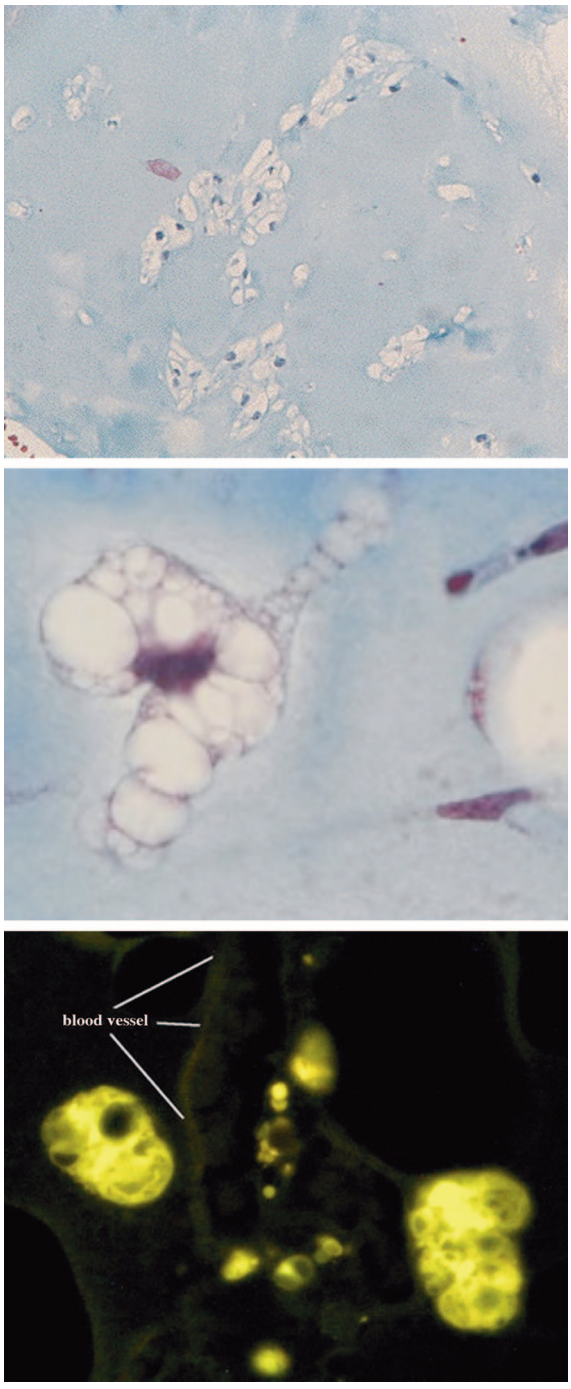

Fig. 3. Cell-enriched Matrigel matrices were injected in our tissue engineering chambers in nude mice and harvested after 1 week to assess their viability. Histologic analysis showed further differentiation of injected cells acquiring the morphologic characteristics of an adipocyte (*above* and *center*). Arterial injection of a fluorescent contrast substrate shows developing adipocytes adjacent to blood vessels (*below*).

observed survival and development of the homogenously distributed cell population within the matrix plug (Fig. 3). The latter acts as a supportive three-dimensional setting before any revascularization occurs (Fig. 3). The gel-like matrix "embraces" the in vitro expanded cell colonies, preventing migration from the recipient site and favoring ingrowth of neocapillaries (Fig. 3). Adding the third dimension to the lipoaspirate specimens could bridge the critical, ischemic time delay.

Our previous work studied fat graft behavior in vivo using an experimental model that incorporates the basic principles of tissue engineering.[11] However, this in vivo study concluded that there was no cellular contribution from the graft. We hypothesized the stromal-vascular fraction within the adipose tissue graft to be crucial to induce the host-derived adipogenic and angiogenic responses. This stromal-vascular fraction appeared to function as a trigger to induce a host-derived cellular morphogenesis, confirming the host-replacement theory.[6] Both cellular and humoral contributions are likely to be relevant in the integration of fat grafts within adjacent tissue in vivo.

The redirected interest in fat tissue as a source of multipotent progenitor cells and as a tissue strongly influenced by its microenvironment is just a prelude toward the development of more sophisticated, preferably autologous, matrices not only to nourish grafted lipoaspirate material but ultimately to induce nonneoplastic and controlled adipose tissue growth in vivo (Fig. 4).

Further in vivo studies will investigate the ability of those injectable, homogenized precursor cell–enriched culture media to generate tissue-specific substitutes. Potential clinical strategies based on tissue engineering methods should be based on rational, simple, and straightforward procedures with minimal discomfort for the surgeon and the patient. Extrapolation of our findings to future, "from bench to bedside" applications could result in a search for autologous matrices that could be enriched, in an in vitro setting, with autologous, stromal-derived cells. Simple addition of crude fat to the matrices could overcome the complex, multistep process of cell isolation and expansion. Thus, the fabricated or engineered off-the-shelf product could be reinjected into the host to address soft-tissue deficiencies.

CONCLUSIONS

Basic principles in developmental biology teach us that cells do have the potential to survive and undergo morphogenesis, provided that a structural extracellular matrix exists that guides, directs, and coordinates cellular behavior. Adding

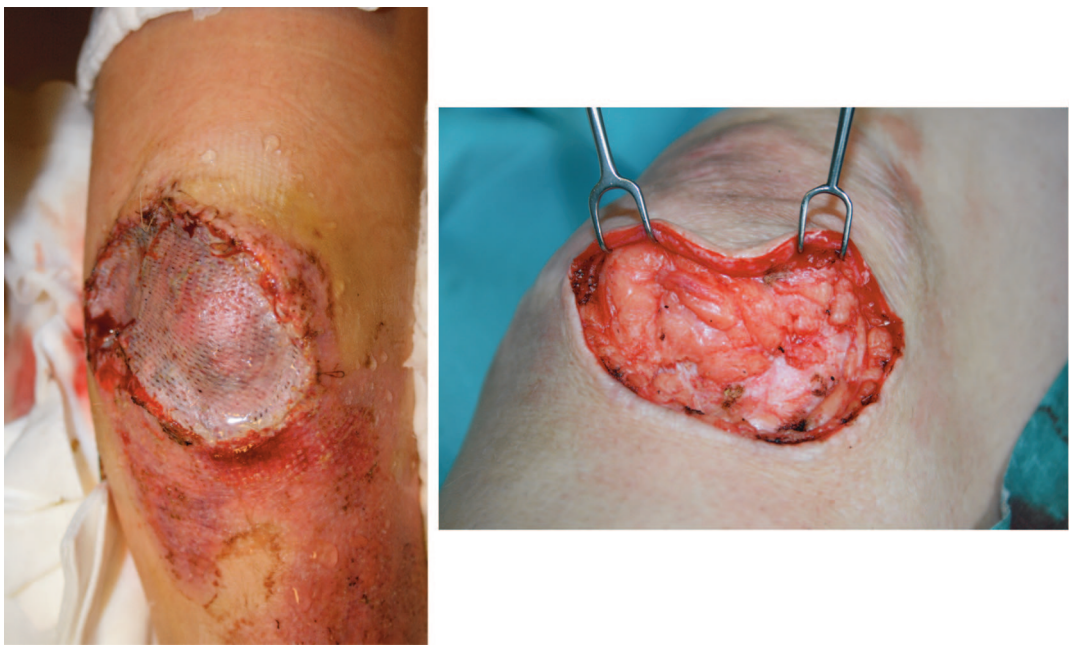

Fig. 4. Addition of supportive matrices or biomaterials to fat grafts could enhance their survival. Migration of fat grafts in areas of great motion (such as the knee joint) could be prevented, as the matrix will act as "biological glue." This patient sustained a severe degloving injury in the prepatellar region (*left*). The wound was skin grafted and additional lipofilling, enriched with commercially available hyaluronic acid filler, resulted in optimal survival of the lipoaspirate material (*right*).

the third dimension to an in vitro inoculated, minute fat biopsy specimen induced not only an expandable, interconnecting framework of stromal cells but also three-dimensional isolated cell colonies or spheroids. Those self-regulating spheroids or adipospheres again functioned as sources of cellular proliferation. This work opens new directions for adipose tissue engineering research by adding the hypothesis that simple, nonprocessed fat biopsy specimens could be simply cultured to obtain a precursor cell–enriched substrate that could easily be injected to address soft-tissue deficiencies.

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