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Histological Skin Remodeling Following Autologous Fibroblast Application

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ABSTRACT The aim of this study was to quantify the effectiveness of intradermal application of autologous fibroblasts on lean tissue structures. The histological sections of the skin were analysed and evaluated for the expansion potential of autologous fibroblasts in the control skin patch area and the nearby pre-treated skin patch into which we had injected expanded autologous fibroblasts nine month earlier. The results show that the pre-injection of fibroblasts into the dermis leads to a long-term rejuvenation of the skin, as evaluated from the histological appearance and from the significantly increased density of fibroblasts in the pre-injected skin vs. controls, from around 60% to over 80%, determined as the percent of lean tissue by a novel image analysis approach. Interestingly, the rate of the *in vitro* fibroblast expansion from the pre-injected area of the skin was reduced in comparison with the controls, consistent with the view that fibroblasts exhibit a limited cell-division potential and that fibroblasts from the pre-injected skin already experienced expansion nine month earlier prior to the injection into the skin. We conclude that autologous fibroblast application results in a significant long-term augmentation of the lean tissue elements of the skin.

KEY WORDS: skin remodeling, autologous fibroblasts, rejuvenation

INTRODUCTION

Skin aging is a complex biological process influenced by a combination of intrinsic and extrinsic factors. Different modern anti-aging strategies that modern dermatology offers include preventive measurements, cosmetical strategies, topical and systemic therapeutic agents and invasive procedures. Three primary structural components of the dermis, collagen, elastin, and glycosaminoglycans (GAGs) have been the subject of the majority of anti-aging research. There are various procedures, most of which are intended to resurface the epidermis and especially to spur the formation of new collagen. These goals can be achieved by using chemical peels, visible light devices, radiofrequency, microneedling, or other invasive techniques which depend upon the use of injectable fillers. All have many limitations, such as being aggressive, the effects being transient, suffering from problems related to allergic reactions, and not sufficiently addressing and rectify the key issue, which is, at least in part, related to the reduced density of fibroblasts in the dermis (1).

Fibroblasts secrete collagen into the extracellular matrix, thereby contributing to the firmness of the skin (2). Thus, an ideal strategy would consist of using autologous fibroblasts as tissue fillers. Although such an approach was used in the past by Watson *et al*. (3), the treatment effectiveness is still under debate.

We designed a prospective pilot trial to test whether the application of autologous fibrobroblasts significantly alters the histological appearance of the skin. We examined whether the growth properties of the expanded fibroblasts from the skin biopsy, pre-treated nine months earlier by an injection of expanded autologous fibroblasts, differed from the growth properties of cells taken from a control biopsy of a nearby skin region. The results show a significant histological change of the cell-pretreated skin patch in comparison with the controls, indicating tissue rejuvenation. Although the expanded fibroblasts from the cell-pretreated area exhibited a slower growth rate vs. controls, the histological improvement was characterized by a significant increase in the density of fibroblasts in the dermis with a concomitant significant reduction of adipocyte density, both representing significant signs of rejuvenation.

PATIENTS AND METHODS

Sampling, cell isolation, cell culture and cryogenic preservation

Skin biopsies of a 67-year-old female volunteer in good general health were taken under local anesthesia. The first square biopsy of 4×4 mm was taken from behind the ear for *in vitro* cell propagation. The expanded cells were cryopreserved and used for autologous cell application (25×10^6cells at a concentration of 50×10^6 cells/mL) to the designated retroauricular area of the other ear. The second biopsy of the same size as the first one was taken nine months later from the pre-treated skin patch into which we pre-injected autologous fibroblasts. At the same time as the second biopsy, the third control biopsy of the same size as the second one was taken from the skin region adjacent to the pre-treated skin area. Each of the respective biopsies were cut longitudinally into two symmetrical pieces. One half of each biopsy was used for histological analyses and the other half for cell expansion *in vitro* for the evaluation of the cell growth in monolayer culture. The study was approved by the Ethical Commission of the Republic of Slovenia, and was performed following the patient's informed consent.

Immediately after taking the biopsy, the tissue was washed and transferred into a sterile container with Dulbecco's Modified Eagle Medium (DMEM; Invitrogen,Carlsbad, CA) with antibiotics: penicillin (100 U/mL), streptomycin (100 µg/mL), and antimycotic amphotericin B (0.25 µg/mL, all from Invitrogen, Carlsbad, CA) and transported at room temperature in a transport container to the cell culture laboratory where the skin fibroblasts were expanded aseptically during the next 3-4 weeks in accordance with Good Manufacturing Practice and ISO/IEC 17025. The biopsy was washed with fresh medium and transferred into a petri dish with a mix of digestive enzymes in DMEM medium with antibiotics and antimycotics. The epidermis was detached from the underlying dermis after treatment with 2.5 mg/mL dispase (Invitrogen, Carlsbad, CA) for a few of hours at 37 °C, 5% CO2, and 90 % humidity. The dermal component was cut into small pieces and incubated with collagenase (Gibco BRL, Grand Island, NY) for several hours at 37 °C, 5% CO2, and 90 % humidity. Any cell clumps were mechanically disaggregated by trituration through 19 to 23 needles. After washing the cell suspension three times with DMEM medium, the cells were resuspended in DMEM supplemented with 10 % fetal bovine serum (Biochrom, Berlin, Germany) and antibiotics – antimycotic mix – and placed into a petri dish at 37 °C with 5 % CO2 and 90 % humidity. The medium was replaced every 2-3 days until the culture reached 80-90% confluency, then the cells were trypsinized (0.05 % trypsin, 0.02% EDTA; Sigma-Aldrich, Saint Louis, MO) for 5 min, and reseeded into tissue culture flasks at an initial density of 5000 cells/cm^2. Cell number and viability were determined at every passage by using a hemocytometer and trypan blue exclusion test. Cells were visualized under an inverted microscope, and the images of monolayer cell cultures were taken on an inverted microscope (Zeiss) equipped with an imaging acquisition system (Till Photonics, Munich, Germany).

The cells were expanded (in four passages) to sufficient numbers and prepared the intradermal application. Cells were washed several times in DMEM devoid of antibiotics and antimycotics and transferred to the injection solution (5% Glucose Ringer lactate, University Clinical Central Pharmacy, Ljubljana), then placed into a 1 mL syringe and sent in a sterile container to the site for application within 3 hours of filling the syringe in the laboratory.

Cytochemistry and confocal microscopy

We used collagen type 1 polyclonal primary antibodies (Abcam, Cambridge, UK). For immunolabeling; the cells were washed with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS for 10 min. Fixation was performed at room temperature.

After four rinses in PBS, the non-specific background staining was reduced by blocking buffer containing 3% bovine serum albumin (BSA) and 10% goat serum in PBS. The cells were then stained with antibodies, with primary staining followed by secondary (conjugated to the fluorescent dye Alexa Fluor 543 (red, Invitrogen, Carlsbad, CA)), diluted into 3% BSA in PBS and incubated at 37 °C. The cells were washed in PBS before and after applying the secondary antibody. They were then mounted using a SlowFade Gold antifade (Invitrogen, Carlsbad, CA). The fluorescence images were collected using an inverted Zeiss LSM 510 confocal microscope with a Plan-Apochromat DIC objective (63×, NA = 1.4). Conjugate Alexa Fluor 543 was excited by a He/Ne laser at 543 nm. The emission light was collected through the long pass filter, with the cutoff below 560 nm.

Histology and data analysis

Formalin-fixed paraffin-embedded biopsy tissue samples were routinely stained for hematoxylin and eosin. Manual and automatic analysis of micrographs of two skin samples were performed: skin pre-injected by autologous fibroblasts nine month prior to taking the biopsy, and the control sample taken from a nearby skin adjacent to the location with the autologous fibroblast pre-treatment.

Manual demarcation: Digital micrographs were printed on the ISO standard A4 size glossy paper and were overlaid with the transparency sheet. A pathologist traced and denoted all adipose tissue on the transparency sheet using a fine black felt-tip marker. Outlines of the samples were separately denoted on the same transparency. Markings and labels on transparencies were digitized by the flatbed optical scanner to produce a 150 dpi resolution computer image in a 1-bit tag image file format (TIFF). The next step was automatic filling of outlines and measurements of the surface area of the denoted foreground of the image. For this image processing task, we used a custom computer program running on a PC computer. 1-bit images were mapped into a two level scale, with 0 (black, marked regions) and 1 (white, background regions). The program summed up all pixels denoting marked area and separately also summed up all pixels denoting area of the corresponding sample. The surface area of the adipose tissue was normalized to the total surface area of the corresponding sample.

Automatic demarcation: Images were first processed to obtain negative image. Since the predominant hue of the digital micrographs was magenta, the complement hue (green) was automatically thresholded using a custom computer program. The threshold level was set to 10% of the maximal intensity. The surface area above the threshold was normalized to the total surface area of the corresponding sample as described for the manual demarcation process.

RESULTS

Morphological properties of expanded fibroblasts

Autologous fibroblasts in culture are shown in Figure 1.

In comparison with controls (Figure 1, A), where cells were harvested from a site near the one into which cells had been pre-injected nine month earlier (Figure 1, B, pre-injected), the morphology of

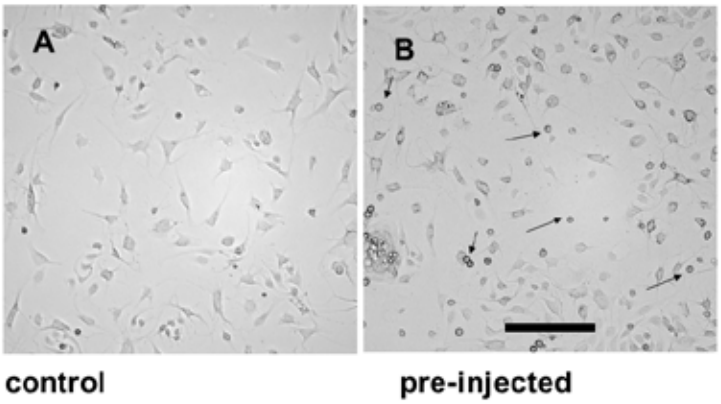

Figure 1. Micrograph of dermal autologous fibroblasts, isolated from postauricular skin punch biopsy. Cells were washeda in cell culture medium prior to making images using the Till Photonics Imaging System on a Zeiss Axiovert 200 inverted microscope. Magnification: ×10 objective, bar indicates 0.5 mm. A) Control cells were taken from the non-treated postauricular region (control), adjacent to the patch, which had been pre-injected with autologous fibroblasts 9 month earlier. B) Cells from the pre-injected site, where cells were harvested 9 months after the intradermal injection of expanded autologous fibroblasts. Biopsies from both regions were taken at the same time. The images were taken on post seeding day 2.

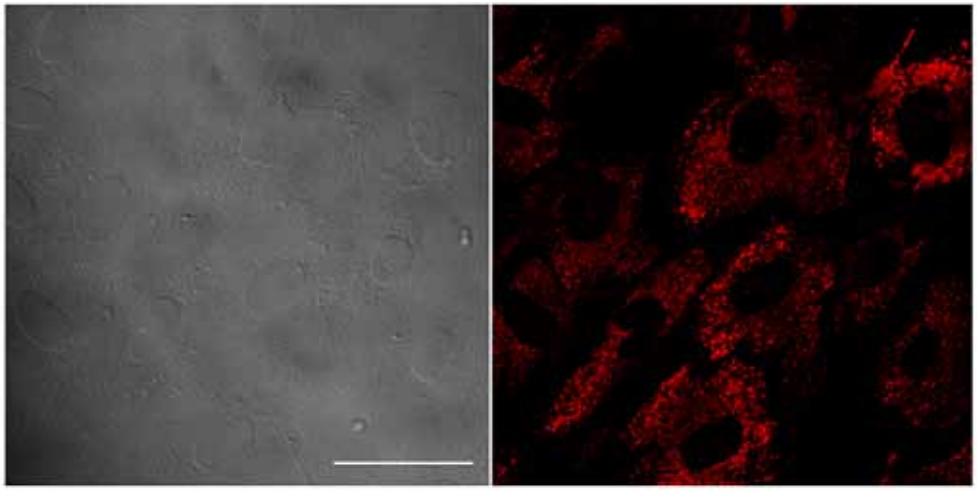

Figure 2. Cultured dermal cells are immunopositive for collagen I, a marker for skin fibroblasts. The panel on the left shows a DIC transparent image of the cell culture. The panel on the right shows confocal fluorescent micrograph of the same cells as in left panel, showing the immunochemical localization of anti-collagen I antibody, stained with the secondary antibody labelled by Alexa Fluor 543 fluorescent dye. The bar indicates a distance of 15 µm. Cells were taken from the passage 3. Note that the antibody stains subcellular puncta, likely representing structures enriched for collagen I.

cells from the latter site exhibited less pronounced processes but a prominent cell soma and relatively increased cytoplasmic granularity in comparison with controls. Moreover, the starting cell culture from the pre-injected site contained a greater proportion of round, poorly attached cells (arrows, Figure 1, B). Both populations of cells, grown in monolayer, exhibited cell growth typical for fibroblasts, with a tendency to grow in bundles; however, this appearance was not present in regions occupied with round cells. In the population of control cells, we observed a relatively larger proportion of narrow spindle-shaped cells in comparison with the cultures of cells arising from the cell-pre-treated skin biopsy. In addition, these ranged from typical spindle-shaped cells with less pronounced nuclei to wider spindle-shaped cells with pronounced nuclei and granularity around the nuclei. However, in the sample of pre-injected cells the morphology appeared more heterogeneous in comparison with controls. Nonetheless, the viability in both cultures was over 90% (not shown). Cells from both cultures positively immunostained with the antibody against collagen I, a marker for skin fibroblasts (4), (Figure 2).

Expansion properties of fibroblasts in culture

We next examined the growth properties of expanded cells. Figure 3 plots the expansion properties of cells sampled from the control and from the pre-injected retroauricular skin areas. It is clear that the control cells displayed a higher expansion potential than the cells isolated from the pre-injected biopsy (Figure 3).

This is consistent with the view that the cells from the pre-injected area arise from a cell population that was already expanded prior to the injection nine month earlier and that cells underwent a higher number of cell-divisions than those from controls. Therefore, the latter cells are relatively more aged in terms of their divisions than the cells that resided in the control skin patch. It was believed that aging reduces the expansion potential of fibroblasts (5). However, if the results are presented in a semilogarithmic plot (Figure 3, bottom), it can be seen that the initial number of cells (isolated from the biopsy) that contribute to the expansion potential of the cells placed into culture is higher in the sample from the pre-injected patch of skin dermis. The initial estimated number of cells in control biopsies was about 7000, whereas it was over 9000 in the pre-injected sample. This result shows that the cells that have been pre-injected 9 month earlier contributed to the increased density of fibroblasts in the skin. Moreover, the pre-injection of autologous fibroblasts 9 months earlier augmented the number of cells in the dermis that contribute to the cell-propagation potential.

Histological analysis of skin biopsies

To further test this hypothesis, we examined the histological images of biopsies taken from the control and the pre-injected skin areas. Quantitative analysis revealed that the relative density of adipocytes in the pre-injected skin sample was significantly reduced

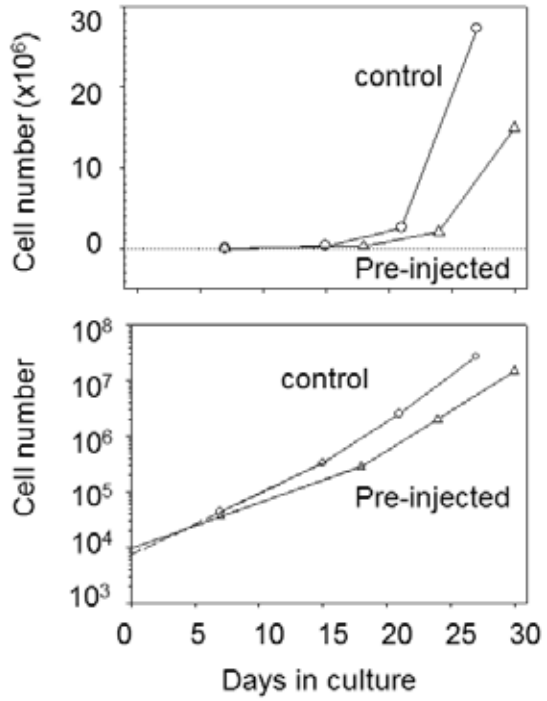

Figure 3. Time-dependent growth of fibroblasts. Upper panel: autologous fibroblasts from the control and the pre-injected skin region exhibited different growth rates. The rate of growth of cells from the pre-injected area (triangles) was smaller than the rate recorded in control cells (circles). Bottom panel: time-dependence of the growth of autologous fibroblasts from the control and the pre-injected skin regions. The ordinate shows the logarithm of cell numbers. The rate of cell growth from the pre-injected area (triangles) was smaller than the rate recorded in control cells (circles). However, the initial number of cells at time 0 was higher in samples from the pre-injected skin samples, as assessed by interpolation, indicated by the dashed lines.

and the relative density of fibroblasts was significantly increased (Figure 4). These changes are consistent with the view that pre-injection of autologous fibroblasts into the dermis reverts the histological aging of the skin.

DISCUSSION

The aim of this study was to quantify the effectiveness of intradermal application of autologous fibroblasts on lean tissue structure.

We examined whether the intradermal injection of expanded autologous fibroblasts affects the histological structure of the skin and whether the growth potential of cultured fibroblasts from the pre-injected area changes in comparison with cells isolated from a site adjacent to the pre-injected area.

The results of our approach show that the relative density of fibroblasts in the pre-injected skin sample was significantly increased from around 60 to 80%, whereas the relative density of adipocytes was significantly reduced. These changes support the view that pre-injection of autologous fibroblasts into the dermis reverts the histological aging of the skin.

Following cell isolation and subsequent seeding onto plasticware, we observed differences in cell morphology (shape and granularity) in the two groups of fibroblasts. Fibroblasts from the pre-injected skin sample seemed less attached, with a more rounded cell soma, less spindle-shaped, and polygonal, when cell culture medium was changed at day 2 post seeding (Figure 1). This likely reflects changes in the expression and abundance of the extracellular matrix (ECM) proteins (i.e. growth attachment factors, cell receptors) following the intradermal injection of cells and their subsequent placement in culture again. Moreover, reticular fibroblasts were abundant in the control culture, characterized by a more stellate morphology and resembling more senescent cells (6).

The morphological diversity of cells observed may also include multipotent cells. Targeted microdissection of the dermal papilla of adult human skin (facial dermis) samples also leads to efficient generation of multipotent neural crest-derived sphere-forming cells. Differentiation of human papilla spheres generated cells with flattened morphology of dermal fibroblasts that expressed fibronectin. Occasional cells with the characteristic morphology of adipocytes were seen in these conditions, but no cells with a neuronal- or glial-like morphology were observed (7,8).

Morphologic reasons for distinct growth rate: reticular vs. papillary

Different morphological properties of fibroblasts we observed may also be reflected in the growth rate. In controls, the proliferation kinetics exceeded that in cultures from the pretreated skin biopsies (Figure 3). These changes may reflect differences that have been observed in cultured papillary (Fp) and reticular (Fr) fibroblasts, which differ in their growth potential. Fibroblasts derived from the papillary dermis have greater *in vitro* growth potential and longer replicative lifespans than genomically identical fibroblasts derived from the reticular dermis. Adult papillary and reticular fibroblasts replicate at similar rates at low cell densities, but exponential growth of reticular fibroblasts slows at lower cell densities than in papillary fibroblasts, suggesting that they are more sensitive to density-dependent inhibition of replication (9).

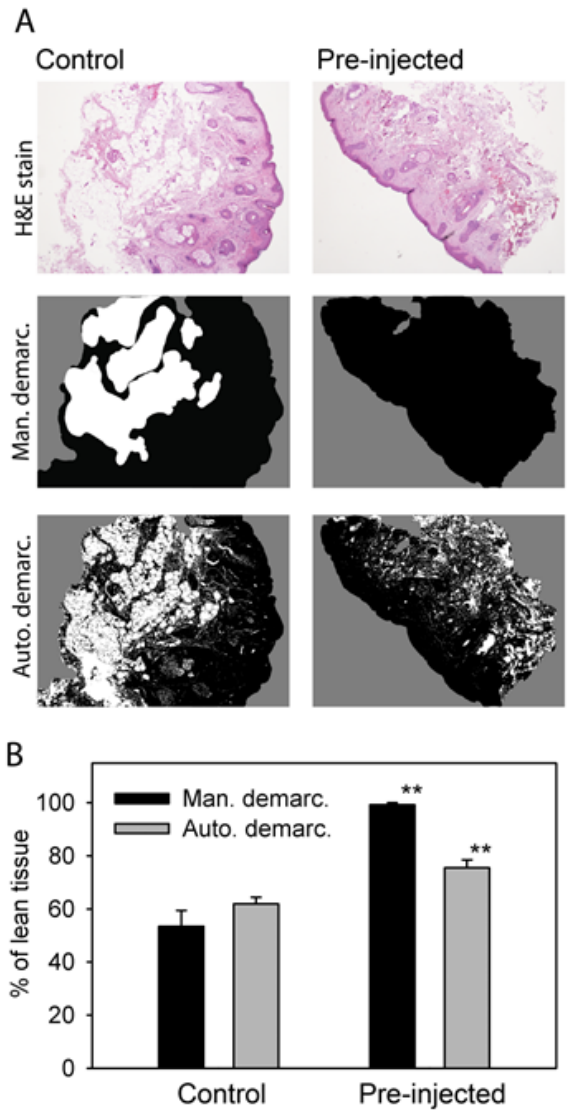

Figure 4. Analysis of biopsy samples of the skin pre-injected by autologous fibroblasts compared with controls. A) Micrographs showing typical histological properties of the skin biopsy pre-injected by autologous fibroblasts nine month prior to taking the biopsy (right), whereas the left panel shows the histology of the control sample, taken from nearby skin adjacent to the location with the autologous fibroblast pre-treatment. Upper panels show histological sections stained by a hematoxylin and eosin stain (H&E stain), the middle panels show the same sections with manually demarked (Man. demarc.) lean tissue (black) versus adipose tissue (white), the lower panel shows automatic demarcation (Auto. demarc.) of lean tissue (black) vs. adipose tissue (white; see Methods section). Original magnification: ×5. B) Summary of the analysis of the percentage of lean tissue in control skin sample and skin pre-injected by autologous fibroblasts using manual (Man. demarc.) and automatic demarcation (Auto. demarc.) approach. Six images were analysed in each group (** $P<0.01$, Student's t-test).

The surface morphologies of reticular fibroblasts and papillary fibroblasts at confluence correlate with their growth kinetics. The decreased cell yields of reticular fibroblasts appears related to the spreading behaviors of individual cells which stretch and occupy more area of the growth surface than in papillary fibroblasts. These data and the reports cited clearly show that one must account for the presence of at least two distinct populations of dermal fibroblasts when examining their biological properties *in vitro* (10).

Limited cell division capability may be related to aging

It is likely that the relatively slow growth rate of fibroblasts from the pre-injected biopsies we observed (Figure 3) is due to an age-related effect, since these fibroblasts were exposed to an additional expansion stage required for the intradermal injection nine month earlier, which is consistent with a limited span of cell division of somatic cells (5). Furthermore, a study by Maier *et al*. demonstrated that fibroblast cultures from skin biopsies of participants aged 90 years retain high and reproducible replicative capacity, but with huge variability in a decline in mitotic activity which was observed between 26 and 81 population doublings (11). An increased variability in cell morphology was also observed in cultures from pre-injected biopsies (Figure 1, B).

CONCLUSIONS

In summary, the present results indicate that intradermal injection of expanded autologous fibroblast leads to histological remodeling of the skin, resembling rejuvenation. We used a novel quantitative method of evaluating histological skin sections (Figure 4). However, the growth properties of fibroblasts from these regions reflected the aging-related slowing. Finally, these results provide a clear answer that the use of expanded autologous fibroblasts can be used for rejuvenation that outlasts any of the methods currently used for this purpose.

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Ethics:

This study was approved by the Republic of Slovenia National Medical Ethics Committee.

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