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Proliferation, Metabolic Activity, and Adipogenic Differentiation of Human Preadipocytes Exposed to 2 Surfactants *In Vitro*

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ABSTRACT

Fat grafting is a pivotal technique for tissue repair. Adipose stromal cells, including preadipocytes, play a major role in the regenerative effects attributed to fat grafting. But the benefits are impaired by the low survival of the graft due to mechanical stress during harvesting, hypoxia, and nutrient deprivation. Nonionic surfactant molecules demonstrated their efficacy in preventing and repairing mechanical damage on the cellular membrane, but it is poorly understood if and how they affect cellular viability, proliferation, and differentiation. We investigated the influence of 2 nonionic surfactants, Kolliphor®P188 and Kolliphor®EL, on cultured human preadipocytes. We analyzed their effects on metabolic activity, cell number, adipogenic differentiation, and secretion of growth factors. Kolliphor®P188 increased metabolic activity, while it did not influence proliferation and differentiation as well as growth factors release. Kolliphor®EL confirmed its cytotoxic effect at the highest concentrations applied. Contrariwise, treatment with lower concentrations significantly raised metabolic activity, induced adipogenesis, and increased insulin-like growth factor-1 and vascular endothelial growth factor secretion. The effect on differentiation was inhibited by blocking peroxisome proliferator-activated receptor gamma. Our results revealed important effects of surfactants on preadipocytes' survival, proliferation, death, and the interplay with their environment. Particularly Kolliphor®EL provides modes of action, which could recommend it for novel treatment to improve fat graft viability.

Introduction

As soon as liposuction was developed about 30 years ago, it raised also interest in reusing the lipoaspirated adipose tissue. The pioneer approaches of clinical applications performed lipofilling to restore or increase volume in subcutaneous tissue,[1] whereas today it is used regularly for breast reconstruction after mastectomy[2] and also for functional reconstruction of the sole of the foot,[3] reconstruction of post-traumatic hemifacial atrophy,[4] and other applications.[5]

In 2001, Zuk et al.[6] demonstrated that adipose tissue is an assembly of various cell types containing not only adipocytes but also mesenchymal stem cells and adipose-derived stromal cells (ADSCs), including preadipocytes. This discovery significantly accelerated lipofilling as a regenerative therapy because many of the key benefits observed after lipofilling were attributed to ADSC effects.[7]

However, fat grafts underlie the limiting impact of unpredictable survival.[8,9] Depending on the harvesting and lipofilling technique, 40%-90% of the injected lipograft volume can remain,[10,11] while the rest is resorbed during the postgrafting period. The first advance came with fat harvesting followed by a subsequent centrifugation step before reinjection in the recipient site.[12] This performance improves fat graft survival by removing oil, fluid, and apoptotic and necrotic cells from the harvested tissue. For fat graft survival, the gauze-towel processing technique is found to be superior to centrifugation.[13] During this process, the aspirated fat is placed on a sterile cotton towel serving as a platform for concentrating fat particles and for separating them from fluids, oil, and debris.[14] However, if the focus lies on the number of ADSCs in adipose grafts, centrifugation improves the amount of cells that can be isolated.[15] On the recipient site, the assimilation of the graft depends on extensive (neo)vascularization. Decrease in fat graft volume appears to be caused primarily by mechanical stress during harvest, hypoxia, and nutrient deprivation.[8,16] These stressors can lead to cell destruction and induce apoptosis in severely damaged

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cells. Adipocytes are sensitive to hypoxia resulting in increased risk of apoptosis.[17-19] Furthermore, hypoxia inhibits lipogenesis and thus arrests preadipocytes in an undifferentiated state,[20] consequently inhibiting volume gain directly as well as indirectly by decreasing secretion of cytokines and necessary growth factors.[21,22]

Consequently, efforts to minor ischemic effects and to protect cells until sufficient vascularity have to be intensified. It was shown previously that decreasing apoptotic cell death results in a greater persistence of fat grafts over time.[23] It is known that nonionic surfactants are particularly effective for repairing damaged membranes and improving viability and survival of damaged cells,[24,25] while a comprehensive investigation of their effects on cell proliferation and the secretion of cytokines was not performed, yet.

In the present study, we investigated the effects of the 2 nonionic surfactants Kolliphor®P188 and Kolliphor®EL on primary human preadipocytes and highlighted their influence on viability by testing each substance on the cellular proliferation and metabolic activity. To do so, we added increasing concentrations of the surfactants and measured the number of cells and reducing activity at different time points. After 7 days of treatment, we measured the degree of adipogenic differentiation for each concentration tested. Further investigations were carried out to quantify the release of different growth factors (basic fibroblast growth factor [bFGF], vascular endothelial growth factor [VEGF], platelet-derived growth factor [PDGF-BB], matrix metallopeptidase 9 [MMP-9], and insulin-like growth factor-1 [IGF-1]) in the cell media.

Material and Methods

Materials

PrestoBlue, fetal calf serum, and Dulbecco's Modified Eagle's medium/F-12 were purchased from Life Technologies (Darmstadt, Germany). Collagenase (type I) was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Kolliphor®P188, Kolliphor®EL, paraformaldehyde, trypsin—ethylenediaminetetraacetic acid, penicillin—streptomycin, bovine serum albumin, and Tween®20 were obtained from Sigma (Taufkirchen, Germany). Acetic acid and crystal violet were from Roth (Karlsruhe, Germany). Isopropyl alcohol was bought from Applichem (Darmstadt, Germany). GW9662 was from R&D Systems (Wiesbaden-Nordenstadt, Germany). bFGF was obtained from PeproTech (Hamburg, Germany). Acetic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, Oil Red O was from Merck (Darmstadt, Germany). Phosphate-buffered saline (PBS) was bought from Biochrom (Berlin, Germany).

Cell Culture

Subcutaneous adipose samples were acquired by liposuction. The donors had been informed about the establishment of cellular models from their tissue and had given informed consent. The study protocol was approved by the regional ethics committee (Ethics Committee of the RWTH Aachen University Faculty of Medicine, Aachen, Germany; EK163/07). Adipose tissue was digested in collagenase solution (100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer containing 1% bovine serum albumin, 0.2% collagenase) with gentle agitation for 45 min at 37°C. The mature adipocyte fraction was separated from the stromal cell fraction by centrifugation at room temperature at 400 × *g* for 10 min. Subsequently, the stromal fraction was filtered through a 250 nm nylon mesh (neoLab, Heidelberg, Germany) and centrifuged at room temperature at 400 × *g* for 10 min. Pellets were resuspended in PBS and centrifuged again at room temperature at 400 × *g* for 10 min. Cells were cultured in cell culture medium Dulbecco's Modified Eagle's medium/F-12 supplemented with 0.1% bFGF, 10% fetal calf serum, penicillin, and streptomycin, each at 100 U/mL. From these primary cultures, experiments were conducted with cells from passage 4 seeded at a density of 3×10^4 cells per cm^2 in all experiments.

For the treatment of surfactants, Kolliphor®P188 (0.5%, 1%, 1.5%, 2%; taken from the dose-response curve of P188-treated fat grafts in the study by Medina et al.[24]) and Koliphor®EL (0.01%, 0.03%, 0.05%, 0.07%, 0.1%, 0.15%; taken from our own previous observations) were freshly added to the medium during the medium exchange on days of measurements (days 0, 2, and 4). For blocking PPARγ, the selective antagonist GW9662 was diluted at 10 μM in the medium 1 h before adding the surfactant, using the same treatment regime as described previously.

Crystal Violet Assay

Crystal violet staining was modified after the protocol of Gillies et al.[26] Briefly, preadipocytes were washed with PBS and fixed in 2-propanol for 10 min at room temperature. Following extensive washing with PBS containing 0.05% (v/v) Tween 20, cells were stained with a crystal violet solution (0.1% [w/v] crystal violet in bidest water) for 20 min. After removal of the crystal violet solution, plates were washed with bidest water. Afterwards, adsorbed crystal violet was washed out in 500 μL acetic acid (33%, v/v) per well and incubated for 15 min with gentle agitation. Finally, 70 μL per sample were transferred in triplets to an optical plate, and absorbance was quantified in a microplate reader (BMG Labtech, Ortenberg, Germany) at 620 nm. Measures of different concentrations were normalized by setting the control as 100%, and averaged afterwards. All data are presented as mean of the differences from the control values.

PrestoBlue Assay

Metabolic activity was measured on days 0 (prior to addition of surfactants), 2, 4, and 7 by PrestoBlue following the manufacturer's instructions. Briefly, after 45 min of incubation (100 μL PrestoBlue diluted in 900 μL medium), 100 μL of the medium was carefully transferred into a 96-well plate, and absorbance was measured in triplets at wavelength of 570 nm. Data were normalized by setting the day 0 measurement (before treatment beginning) as 100%, and averaged afterwards. All data are presented as mean of the differences from the control values.

Oil Red O Staining

Oil Red O staining was performed as described elsewhere.[27] Briefly, adipogenesis-induced preadipocytes were washed in PBS, fixed for 20 min in 4% paraformaldehyde solution in PBS. Fixed cells were washed in PBS and stained at room temperature with Oil Red O in 60% isopropyl alcohol. After 15 min, cells were gently washed in distilled water. To determine the adipogenic level, adsorbed Oil Red O was washed out with 500 μL of 100% isopropyl alcohol. Absorbance was measured in triplets (100 μL each) at 540 nm. Data of all measures were normalized by setting the control as 100%, and averaged afterwards.

Growth Factor Determination by Multiplex Immunoassay and ELISA

On days of measures, the media were collected for ELISA. The contents of bFGF, VEGF, PDGF-BB, and MMP-9 of conditioned media were analyzed by multiplex magnetic bead immunoassay using ProcartaPlex Sets (eBioscience, Inc., San Diego, CA) according to the manufacturer's guidelines. Detection was performed using a Luminex 200 multiplexing instrument (Luminex Corporation,