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A new approach for adipose tissue regeneration based on human mesenchymal stem cells and hydrogels – An *in vitro* study

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Table of Contents: In this study an approach for adipose tissue regeneration based on human mesenchymal stem cells and hydrogels as supporting matrix was evaluated. The gelatin-based hydrogels developed in this study were cytocompatible and stem cell adhesion onto hydrogel surfaces was higher as compared to cell culture plastics. Furthermore, the adipogenic differentiation degree was increased. These results are promising for future applications of hydrogels in adipose tissue regeneration strategies.

Abstract: The aim of this study was to evaluate a new approach for adipose tissue regeneration based on the utilization of human adipose tissue-derived mesenchymal stem cells (ASC) and biodegradable hydrogels as supporting matrix. Adipose tissue provides large stem cell numbers and tissue harvesting can be performed minimally invasive. ASC exhibit multilineage differentiation potential (e.g. osteogenic and adipogenic differentiation capacity). To provide proof of applicability of ASC in adipose tissue regeneration strategies we have used hydrogels as adhesion matrix. Hydrogels are defined as hydrophilic polymer networks that are able to absorb a large amount of water. Gelatin-based hydrogels possess several interesting properties: they are biodegradable, show high biocompatibility, and offer possibilities for functionalization. In order to proof the adipogenesis-supporting potential of gelatin-based materials, hydrogel films were developed and compared regarding materials characteristics and behavior of stem cells seeded on them *in vitro*. ASC adhesion onto hydrogel surfaces was higher as compared to normal cell culture plastic. In addition the adipogenic differentiation degree (i.e. the extent of cytoplasmic lipid storage) was increased. These results are promising for future applications of hydrogels in tissue regeneration strategies.

Keywords – adipose tissue, hydrogels, adipose tissue-derived stem cells, adipogenic differentiation, tissue regeneration

Introduction

There is major clinical demand for adipose tissue surrogates in plastic and reconstructive surgery. However, there are no indisputable implant materials available at present. Non-degradable silicone implants are often used but complications like capsular contracture are numerous (reported variations from 10 to over 60 % incidence) and often require corrective surgery.[1] The aim of this study was to evaluate a new approach for adipose tissue regeneration based on the utilization of human adipose tissue-derived mesenchymal stem cells (ASC) and biodegradable hydrogels as supporting matrix.

The development of an autologous stem cell therapy aiming at tissue regeneration is a topical research area. Autologous grafts provide the advantage of low risk of immunogenic response. Adipose tissue is a

promising source for stem cells. Like bone marrow, adipose tissue contains a stromal fraction, which encloses a population of ASC that exhibits stem cell characteristics.[2, 3] Compared to bone marrow, adipose tissue has the advantage to provide large numbers of stem cells and the harvesting of tissue by liposuction is minimally invasive. ASC have been shown to possess multilineage potential and have the capacity to differentiate into adipocytes, osteoblasts, chondrocytes, myocytes, neuronal cells, and hepatocytes *in vitro*.[4] Therefore, ASC may have the potential to be used for various clinical applications. Recent studies revealed a cell population within freshly isolated ASC showing a distinct expression of CD34, formerly known as an exclusive marker of hematopoietic stem cells.[5] The CD34-positive subpopulation did not show endothelial cell-specific markers (e.g. von Willebrand factor, CD31) and was able to perform adipogenic differentiation after specific stimulation.[6]

To provide proof of applicability of ASC in regeneration strategies for adipose tissue we have used hydrogels as adhesion matrix. Hydrogels are defined as hydrophilic polymer networks, able to absorb a large amount of water (i.e. one tenth to thousand times their dry weight).[7] Hydrophilic groups or domains, which are hydrated in an aqueous environment, are present in the network, creating the hydrogel structure.[8] In order to avoid the dissolution of the hydrophilic polymer chains in the aqueous phase, crosslinks need to be incorporated. The introduced crosslinks can be of either physical or chemical type.[9] Unstable bonds are often introduced into the gels to make them biodegradable, which can be advantageous for many applications.[10] These bonds can be broken either enzymatically or chemically. In the latter case, this mostly occurs via hydrolysis.[11] Physical or reversible gels are formed by secondary forces such as ionic, hydrophobic interactions or hydrogen bonds. Irreversible or chemical hydrogels are covalently crosslinked networks.[12]

The degradation products of hydrogels, intended for medical device applications, should not be toxic. This means that the components formed after *in vivo*-utilization should be metabolized by the body into harmless products. Consequently, the starting products should be selected carefully.[10, 13] In this study, gelatin-based hydrogels were selected and applied as cell carriers because they possess several relevant properties, i.e. biodegradability, high biocompatibility, and the possibility to be functionalized.[14, 15] In order to proof the adipogenesis-supporting potential of gelatin-based materials,

hydrogel films with thicknesses of 1 and 5 mm were developed and compared regarding materials characteristics and stem cell behavior *in vitro*.

2. Materials and Methods

2.1 Preparation of crosslinked gelatin hydrogels

Gelatin (type B), isolated from bovine skin by an alkaline process, was kindly supplied by Rousselot, Ghent, Belgium. Gelatin samples with an approximate iso-electric point of 5 and a Bloom strength of 257 were used. Methacrylic anhydride (MAA) was purchased from Aldrich (Bornem, Belgium) and was used as supplied. Dialysis membranes Spectra/Por® 4 (MWCO 12,000-14,000 Da) were obtained from Polylab (Antwerp, Belgium). 1-[4-(2-Hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one (Irgacure® 2959) was a kind gift from Ciba Speciality Chemicals N.V. (Groot-Bijgaarden, Belgium). A LWUV-lamp model VL-400L (Vilber Lourmat, Marne La Vallée, France) was used for sample curing.

Gelatin was chemically modified with methacrylamide side groups, resulting in a degree of substitution (DS) of 60 %, as described in a previous paper.[16] Next, 10 % (w/v) gelatin-methacrylamide (gel-MOD) was dissolved in double distilled water at 40°C, in the presence of 2 mol % photo-initiator Irgacure® 2959 as calculated to the methacrylamide side chains.

Using the 10 % (w/v) gel-MOD solutions, two hydrogel types possessing various thicknesses were prepared. Type I and II hydrogels were obtained after injection of the 10 % w/v gel-MOD solution between glass plates separated by silicone spacers of 1 mm and 5 mm thickness, respectively. In a subsequent step, the hydrogel films were stored overnight at 5°C followed by UV-irradiation (365 nm, 10 mW/cm^2) for 30 minutes and 2 hours for type I and II hydrogels respectively.

2.2 Characterization of gelatin hydrogels

The visco-elastic properties of the hydrogels were evaluated using dynamic oscillation measurements at small deformations using a plate-plate rheometer type Physica MCR-301 (Anton Paar, Sint-Martens-Latem, Belgium). The gelation of the polymer solution was studied by monitoring G’ and G’’

as a function of time and temperature. The frequency, the % strain and the normal force applied were kept constant at respectively 1 Hz, 0.1 % and 0.1 N.

In addition to rheology measurements, swelling experiments were also performed. After weighing, hydrogel films (Ø 20 mm) were incubated in 80 ml double distilled water, containing NaN_3 (to prevent bacterial growth), at 37°C. At regular time points, the swollen discs were removed, dipped gently with paper and weighed again.

After equilibrium swelling, the hydrogel discs were removed, freeze-dried and weighed again. The loss of dry mass of the hydrogel during incubation at 37°C, enabled the calculation of the polymer gel fraction. Equation 1 is based on the mass of the dry gel after incubation (= W_{de}) and the initial mass of the dry gel before incubation (= W_{d0}). Swelling experiments were performed in triplicate.

Equation 1 $$\text{gel fraction (\%)} = (W_{de} / W_{d0}) \times 100\ \%$$

Since the hydrogels were utilized directly from the cast and the swelling studies were performed using hydrated hydrogel films, extra discs taken from the different hydrogels, needed to be dried. The dry weight W_{d0} at time 0, needed for the calculation of the gel fraction, could be obtained based on this second hydrogel series.

2.3 Cell isolation and culture

Human ASC from liposuction-derived adipose tissue were isolated by collagenase digestion (collagenase type I-A, 6 mg/ml PBS, 0.25 ml collagenase solution/ml adipose tissue, Sigma-Aldrich, Munich, Germany). The enzymatic digestion was performed for 1.5 h at 37°C (slight shaking). Afterwards the homogenous solution was filtered through a 100 µm filter (nylon cell strainer, BD Falcon, Heidelberg, Germany) and the resulting cell suspension was washed 3 times with PBS containing 10 % fetal calf serum (FCS. PAN, Aidenbach, Germany) and repeated centrifugation at 400 x g. for 5 min. The final cell pellet was resuspended in DMEM (Gibco Invitrogen, Karlsruhe, Germany) containing 10 % FCS and antibiotics (final concentration: 100 U/ml penicillin, 100 µg/ml streptomycin, Invitrogen, Germany), seeded in cell culture flasks (Greiner, Frickenhausen, Germany) and cultivated at 37°C/5 % CO_2 in a humidified atmos-

phere. 24 h after isolation the CD34-positive subpopulation was isolated by the Dynal® CD34 progenitor cell isolation system (Invitrogen, Karlsruhe, Germany). For that, non-adherent cells were removed by washing the cell layer twice with PBS. Afterwards CD34-antibody coupled magnetic beads were added to cell culture medium (approx. 15 µl/75cm^2 flask) and incubated at 37°C for 15 min. Non-attached beads were removed by washing the cell layer twice with PBS. The subsequent cell detachment was achieved by trypsinization (0.25% trypsin, Invitrogen, Karlsruhe, Germany). The cell suspension was then purified for CD34-positive cells by repeated steps of magnet exposure and washing with a PBS/0.1 % bovine serum albumin-solution (Sigma-Aldrich, Munich, Germany) on a staggering mixer at 4°C (4 repeats). Afterwards cells were seeded adequately to the yield onto cell culture flasks and cultivated for 3 passages. Experiments were performed in passage 4.

2.4 Differentiation conditions and detection of differentiation

For testing the effects of hydrogel contact CD34-positive ASC in passage 3 (approx. 2 weeks after isolation) were seeded on top of the hydrogels (30.000 and 60.000 cells/cm^2). After reaching confluency ASC were stimulated with common adipogenic differentiation compounds (1 µM dexamethasone, 10 µg/ml insulin, 200 µM indomethacin, 0.5 mM 3-isobutyl-1-methylxanthine, Sigma-Aldrich, Munich, Germany) in DMEM containing 10 % FCS and antibiotics (see above). The differentiation characteristics of ASC on hydrogels (i.e. lipid accumulation) were compared with ASC seeded and stimulated on cell culture plastic.

The adipogenic differentiation was shown by the detection of cytoplasmic triglyceride storage by oil red O-staining. Therefore, cells were washed once with PBS and afterwards fixed with buffered 4 % paraformaldehyde (Sigma-Aldrich, Munich, Germany) for 10 min at room temperature. This was followed by PBS washing. Afterwards cells were incubated for 5 min with 60 % 2-propanol (Sigma-Aldrich, Munich, Germany). Staining was performed by adding oil red O-solution (12 mM in 60 % 2-propanol) for 10 min at room temperature. Excessive oil red O was removed by washing with 60 % 2-propanol. Stabilization of specifically bound oil red O was reached by adding distilled water.

In addition, alkaline phosphatase as a marker for osteogenic differentiation was tested. Therefore, cells were washed twice with PBS and fixed with buffered 4 % paraformaldehyde solution (see above). Fixation was followed by washing with PBS. Afterwards cells were incubated with alkaline phosphatase staining solution containing Amino-2-methyl-1,2-propane-diol (100 mM), Naphthol AS-MX phosphate (50 µg/mL) and Fast red violet LB salt (50 µg/mL) for 10 min (all compounds from Sigma-Aldrich, Munich, Germany). Finally, PBS irrigation was necessary to remove excessive staining solution.

3. Results

3.1 Preparation and characterization of crosslinked gelatin hydrogels

The formation of hydrogels based on gelatin-methacrylamide (gel-MOD) occurs in two steps. In a first step, gelatin is functionalized by the reaction with methacrylic anhydride, as already described earlier.[16] In a subsequent step, aqueous gel-MOD solutions containing Irgacure® 2959 were injected between glass plates separated by a spacer (1 mm and 5 mm thick) followed by a UV-induced chemical crosslinking.

The hydrogels developed this way were first characterized using rheometry to gain insight in the effect of both the physical and chemical crosslinking on the mechanical properties of the obtained materials. In a first part of the experiments, a temperature-induced physical crosslinking was applied by cooling a gel-MOD solution from 40°C to 20°C. The physical gelation was studied rheologically by monitoring both the storage and the loss modulus (i.e. G' and G'' respectively), as indicated in figure 1. Following this approach a characteristic 'cure' curve of log (G', G'') against time was obtained. Typically, this process possesses an initial lag time, and both G'' and G' increased, but with G' increasing faster than G''. At a given time a 'cross-over' (i.e. gelation point) is reached (indicated in figure 1 with an arrow).[17, 18] Subsequently, G' continued to increase until a plateau value of the modulus was reached. In a second part of the experiment, the physically crosslinked hydrogel was UV-irradiated to initiate the chemical crosslinking of the gel-MOD methacrylamide side groups. The observed increase in storage modulus upon UV-irradiation (i.e. 9,000 to 200,000 Pa) was higher compared to the response (i.e. increase from 0.2 to 9,000 Pa) of G' upon cooling below the transition temperature. It can thus be concluded that the chemical crosslinks contributed to a greater extent to the

resulting hydrogel properties compared to the physical entanglements. In addition, it should be mentioned that after UV-irradiation, G' and G'' still increased indicating an after-curing period during which the mechanical properties of the hydrogels developed further improved.

In a subsequent part of the present work, swelling experiments were performed to obtain the gel fraction of both type I and II hydrogels. The gel fraction is defined as the percentage of dry polymer, remaining in the hydrogel after swelling. The insolubility of gel-MOD hydrogels is caused by three-dimensional network formation, via interchain crosslinking. The gelatin fraction, which is not anchored chemically, will dissolve at 37°C by the melting of the physical entanglements. The insoluble weight fraction of the initial hydrogel is the gel fraction. A higher gel fraction is thus related to improved crosslinking. Type I hydrogels possess a significantly higher gel fraction compared to type II hydrogels (i.e. 99.95 ± 1.31 % versus 86.82 ± 2.32 %). The latter can be explained by a depth-dependent UV-penetration since type II hydrogels possess a higher thickness compared to type I hydrogels (i.e. 5 mm versus 1 mm). This phenomenon was reported previously for dextran-methacrylate hydrogels.[19] Kim et al observed that a dextran-methacrylate hydrogel possessed heterogeneities in crosslink degree after photopolymerization. At locations closer to the surface the crosslinking was more efficient. Although the thickness of the dextran-methacrylate hydrogels studied appeared to be thin (i.e. 3 mm), it was thick enough to be affected by differential UV-penetration through the material.[19] Since type II hydrogels applied in the present work possess a thickness of 5 mm, we anticipated that the conversion of methacrylamide side groups in type II hydrogels would be lower compared to type I hydrogels. Both for type I hydrogels as well as type II materials, equilibrium swelling was reached after incubation overnight (data not shown).

3.2 Testing ASC compatibility of hydrogels

For testing cytocompatibility human ASC were seeded onto hydrogels of two thicknesses. Prior to cell seeding the hydrogels were pre-incubated for 2 h with cell culture medium which was discarded before cell seeding. Standard cell culture plastic was used as control adhesion surface. Phase contrast microscopy revealed that 24 h after seeding more adhered ASC were detectable on hydrogels compared to cell culture

plastic (Fig. 2). No differences in ASC adhesion were detectable in dependency on hydrogel thickness (Fig. 2 b, c).

After two weeks of stimulation with pro-adipogenic compounds, ASC showed an adipogenically differentiated phenotype by accumulating lipid droplets in the cytoplasm (Fig. 3). The degree of adipogenic differentiation (i.e. increased cytoplasmic lipid accumulation) was higher in ASC adhered to hydrogels compared to ASC on standard cell culture plastic (Fig. 3). Again the differences in thickness of the applied hydrogels did not induce apparent differences in the differentiation degree (Fig. 3 b/c).

Furthermore, we tested the protein expression of alkaline phosphatase, an early marker for osteogenic differentiation. We could show that by adhesion to both adhesion substrates, cell culture plastic and hydrogels, ASC expressed alkaline phosphatase. Expression was restricted to the non-adipogenically differentiated cells, which did not store lipid droplets. However, the individual differences in the extent of alkaline phosphatase protein expression were high (one exemplary individual shown in Fig. 4). Again no differences in the effects of hydrogel thicknesses were detectable.

Figure 1: Physical crosslinking, followed by UV-irradiation of 10 % (w/v) gel-MOD hydrogels (1 Hz, 0.1 % strain, $F_N = 0.1$ N). G' and G'' are plotted logarithmically.

Figure 2: Phase contrast microscopy of ASC adhered to cell culture plastic (a) and to hydrogels 24 h after seeding (b: 1 mm thickness – type I, and c: 5 mm thickness – type II, scale bar: 200 µm).

Figure 3: Adipogenic differentiation of ASC adhered to cell culture plastic (a) and to hydrogels (b: 1 mm thickness – type I, c: 5 mm thickness – type II, oil red O-staining of ASC after 14 d of adipogenic stimulation, scale bar 200 µm)

Figure 4: Alkaline phosphatase protein staining (red) of ASC adhered to cell culture plastic (a) and to hydrogels (b: 1 mm thickness – type I, c: 5 mm thickness – type II, after 14 d of adipogenic stimulation, scale bar 200 µm)

4. Discussion

It is known that the commitment of mesenchymal stem cells (MSC) to different lineages is not only dependent on the differentiation inducing soluble compounds; it is also dependent on the adhesion surface and surface induced cell shape modification. McBeath et al. (2004) showed that cell shape regulated commitment of MSC to adipocyte and osteoblast lineages. MSC in a flattened and spread phenotype underwent osteogenesis, while unspread, rounded cells became adipocytes. The study of McBeath et al. demonstrated that mechanical cues experienced in developmental and adult contexts, embodied by cell shape, cytoskeletal tension, and specific intracellular signaling (i.e. RhoA), are integral to the commitment of stem cell fate.[20] Deductively, a material that allows modulations of cell shape by modulating its chemical and mechanical characteristics could be utilized for targeted stem cell commitment. Thus, a topical research area is the development of materials that allow modifications in adhesion substrate composition (e.g. extracellular matrix proteins) and also mechanical characteristics (i.e. softness, flexibility and chemical stability).

Since gelatin-based hydrogels have several interesting properties (among them variability of mechanical properties) [21] we examined the adipogenesis-supporting potential of hydrogel films *in vitro* by use of a human stem cell population (ASC). We could show that ASC attached and spread more effective after seeding onto hydrogel surfaces compared to cell culture plastic. Furthermore, adipogenic differentiation of ASC was more pronounced by the contact to hydrogels. No differences in cellular behavior were observed between the two hydrogel thicknesses.

Higher ASC numbers on hydrogels 24 h after seeding could be caused by high binding capacity of gelatin for fibronectin [22]. Fibronectin is present in abundant amounts in FCS [22] of the culture medium and is a well known high-molecular weight extracellular matrix glycoprotein that binds to membrane-spanning receptor proteins called integrins via RGD-domains [23].

Hydrogels allow variations in their mechanical properties by chemical and physical modifications. For example, the variation of the hydrogel thickness can lead to variations in the conversion of the methacrylamide side groups upon UV-irradiation, as reflected by the gel fraction. Consequently, this can then influence the mechanical properties of the crosslinked networks obtained. Swelling experiments indeed indicated that type I hydrogels possessed a higher gel fraction and a lower swelling degree compared to type II hydrogels. Consequently, we anticipated differences in mechanical properties of the materials developed. Since the cellular behavior does not indicate such differences in response to the hydrogels thicknesses (i.e. 1 and 5 mm) despite the variations in gel fraction/conversion, it can be assumed that the cells do not sense thus induced differences in mechanical properties related to depth-dependent UV-penetration.

In order to explore possible mechanical property/cell behavior correlations, further studies will elaborate on applying gelatins with a higher content of crosslinkable groups. We have indeed shown that this leads to an increase in mechanical strength (i.e. storage modulus).[16] For example, upon increasing the modification degree of gel-MOD from 7 % to 40 %, the storage modulus at 37°C increased from approximately 2,500 Pa to 17,500 Pa for 15 w/v % gel-MOD hydrogels. In addition to modulation of the modification degree, variations in the constitution can also be considered. As an example, the introduction of other extracellular matrix molecules including chondroitin sulphate leads to significant changes in physico-chemical properties.[24] Also, the induction of porosity could be helpful for improved cellular colonization. In a previous paper, we have reported on the development of porous gelatin-based hydrogel scaffolds which were prepared by a cryogenic treatment and subsequent freeze-drying.[14] These porous scaffolds showed good cytocompatibility for different human cell types (e.g. endothelial, epithelial, osteoblasts) [15] and could thus be a supportive scaffold for increased cellular colonization and also stem cell differentiation. These aspects, which are currently under study, will be the topic of forthcoming papers.

One limitation in tissue engineering approaches and tissue regeneration is an insufficient vascularization (blood vessel formation) within the affected region.[25] Vascularization allows the supply with oxygen and nutrients and the removal of metabolic degradation products. An insufficient or impaired vascularization leads to an insufficient oxygen and nutrient supply and this impairs cellular colonization and survival.[26, 27]

Vascular endothelial cells (EC) built up the inner layer of all blood vessels and are the main players in vacularization. The hydrogels used in this study demonstrated EC compatibility.[15]

Thus, we have shown that hydrogels meet the basic requirements for future applications of hydrogels in tissue regeneration strategies, because they are compatible for ASC and allow adipogenic differentiation *in vitro*. Autologous stem cell approaches based on the use of ASC together with highly sophisticated materials are promising and may have the potential for various clinical applications.

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