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Strategy for constructing vascularized adipose units in poly(L-glutamic acid) hydrogel porous scaffold through inducing in-situ formation of ASCs spheroids

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ABSTRACT

Vascularization is of great importance to adipose tissue regeneration. Here we introduced a paradigm that using scaffold to induce ASC spheroids, so to promote vascularized adipose tissue regeneration. Poly (L-glutamic acid) (PLGA) was activated by EDC, followed by being cross-linked by Adipic dihydrazide (ADH) to form a homogeneous hydrogel. Lyophilization was then carried out to create porous structure. The PLGA hydrogel scaffold possessed a significant swollen hydrophilic network to weaken cell-scaffold adhesion but drive ASCs to aggregate to form spheroids. Increase of seeding cell density was proved to result in the increase of spheroid size, upregulating angiogenic genes (VEGF and FGF-2) expression by enhancing the hypoxia-induced paracrine secretion. Also, the adipogenic differentiation of ASCs was achieved in spheroids in vitro. Moreover, the in vivo vascularized adipose tissue regeneration was evaluated in the dorsum of nude mice. After 12 weeks post-implantation, the significant angiogenesis was found in both adipogenic induced and non-induced engineered tissue. In adipogenic induced group, the clear ring-like morphology, the large vacuole in the middle of the cell and the Oil red O staining demonstrated adipose tissue formation.

Statement of Significance

Vascularization is of great importance to adipose tissue regeneration. Adipose derived stem cell (ASC) spheroids possessed not only the high efficiency of vascularization, but also the improved differentiation ability. Several research works have illustrated the advantage of ASC spheroids in vascularization. However, in adipose regeneration, ASC spheroid was rarely used. Even so, it is reasonable to believe that ASC spheroids hold a great promise in vascularized adipose tissue engineering. Thus in the present study, we introduced a method to create lots of ASC spheroids that acted as lots of individual adipogenesis and angiogenesis units inside of a porous hydrogel scaffold. Then, the scaffold carrying ASC spheroids was implanted subcutaneously in nude mice to preliminarily evaluate the adipose tissue generation and blood vessel formation.

1. Introduction

Currently practiced strategies in an attempt to develop adipose tissue engineering methods include scaffold guided tissue regeneration, injectable composite system, fragmented omentus based-tissue regeneration and *de novo* adipogenesis [1]. Various kinds of cells, associated with scaffold/hydrogel fabricated by collagen [2], hyaluronic acid [3] and polyester [4] have been studied to construct tissue-engineered adipose. However, since adipose is a highly vascularized tissue, the fate of adipogenic tissue reconstruction was recognized to be crucially dependent on the vascularization after implantation, which determines their long-term survival and function [1].

Towards the vascularized adipose tissue engineering, adipose derived stem cells (ASCs) have attracted the interest of researchers [5–7]. Because except their easy expansion ability and potential of adipogenesis, ASCs exhibit the advantage on therapeutic angiogenesis by paracrine secretion of angiogenic growth factors [8–11]. Furthermore, to enhance the regeneration of vascular network

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based on ASCs, the most commonly used method is the introduction of angiogenic growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF-2) into scaffold/hydrogel [12,13].

Several studies concerning to angiogenesis have reported that ASCs exhibit an improved angiogenic efficacy in vivo through hypoxia-induced paracrine secretion of higher amounts of VEGF and FGF-2 when cultured in aggregates [9,11,14,15]. According to S.H. Bhang [9], ASC spheroid cultures were more effective in preconditioning cells to the hypoxic environment, up-regulating hypoxia-adaptive signals, thus enhancing secretion of angiogenic factors. Thus, ASC spheroid was effectively applied in angiogenesis. But in adipose regeneration, ASC spheroid was rarely used. Even so, it is reasonable to believe that ASC spheroids hold a great promise in vascularized adipose tissue engineering. Because besides the improved angiogenic efficacy, ASC spheroids also have been proved to exhibit the enhanced differentiation potential [16] and advanced ability to resistant against apoptotic cell death [9].

Various techniques have been developed to achieve cellular spheroids, including hand drop technique [17], centrifugation [18], continuous agitation of suspension culture [19], fabricating cell repulsive substrates [16] and magnetic force [20]. In our previous study, we also designed to drive in-situ formation of spherical aggregates inside of three dimensional (3D) porous scaffolds, making the utilization of ASCs aggregates in tissue engineering more direct and effective [21]. Such kind of scaffold is on the basis of protein repulsive principle that swollen charged networks consisting of polyelectrolytes do not adsorb any proteins [22]. Poly (L-glutamic acid) (PLGA) is a synthetic polyelectrolyte with the hydrophilic nature. The carboxyl groups of PLGA make it available to be cross-linked to form significant swollen 3D structure, which may prevent proteins adsorption, thus weaken cell-to-material adhesion. Since cell-to-material adhesion was blocked, cell-to-cell interaction would drive cells to aggregate spontaneously. What is more, as a synthetic polypeptide implant, PLGA hydrogel could simulate protein matrix, while avoiding immunogenicity of protein.

Thus, in the present study, adipic dihydrazide (ADH) was employed to cross-link PLGA to form a hydrogel, followed by freeze-drying to achieve porous structure. ASCs were seeded into PLGA scaffold to form multicellular spheroids, of which the process was monitored. Then the enhanced adipogenic differentiation and expression of angiogenic factors were detected. After in vitro adipogenic induction for 14 days, the ASCs spheroids-PLGA scaffolds were transplanted subcutaneously in nude mice to evaluate the adipose tissue generation and vascularization.

2. Materials and methods

2.1. Materials

Poly (L-glutamic acid) ($M\eta = 6.0 \times 10^4$) was prepared by removal of γ-benzyl protection groups of poly(γ-benzyl-L-glutamate) (PBLG), which was synthesized by the ring-opening polymerization of the N-carboxyanhydride (NCA) of γ-benzyl-L-glutamate in our laboratory. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Covalent Chemical Technology Co., Ltd (Shanghai, China). Adipic dihydrazide (ADH) was purchased from Shanghai Darui Finechemical Co., Ltd. (Shanghai, China). All other chemicals were of analytical grade and were used without further purification.

2.2. Preparation of PLGA hydrogel scaffold

Briefly, The PLGA was dissolved in deionized water to make a solution of an polymer concentration (from 5 to 7 wt%). Then, EDC were added to activate the γ-carboxyl groups of PLGA. ADH was employed to cross-link PLGA at room temperature. The molar ratio of $-NH_2$ of ADH to –COOH of PLGA was set at 1.5:1, 1:1, 0.85:1, 0.65:1, 0.45:1 and 0.25:1, respectively (EDC was 1:1). While the molar ratio of EDC to –COOH of PLGA was 0.5:1, 1:1, 2:1, respectively ($-NH_2$ of ADH to –COOH of PLGA was 0.65:1). The pH of the reaction mixture was adjusted to 4.8 by the addition of 0.1 M NaOH or HCl solution to yield the PLGA hydrogel. The PLGA hydrogel porous scaffolds were prepared by freeze-drying method. The gel was transferred into a refrigerator at −20 °C to solidify solvent and induce solid-liquid phase separation. Then the solidified hydrogel was transferred into a freeze-drying vessel to remove the solvent.

2.3. Characterization

FT-IR spectra were recorded on a Nicolet Model 380 Fourier transform infrared spectrometer.

X-ray diffraction patterns were analyzed using a diffactometer (D/MAX2550, Rigaku), with Cu Kα radiation at a voltage of 40 kV and 30 mA. The samples were scanned between $2\theta = 5–40°$ with a scanning speed of 5°/min.

The thermal gravimetric analysis was examined by means of thermogravimetry with a heating rate of 10 °C/min in nitrogen atmosphere on TA Q-500 instruments.

Morphology of PLGA hydrogel scaffold was examined by scanning electron microscopy (SEM). The cross-section of porous scaffold was gold coated and viewed using a microscope (JXA-840, JEOL).

For the swelling test, the scaffolds prepared at various conditions was immersed in PBS buffer solution and kept at 37 °C for 2 days until equilibrium of swelling had been reached. Fully swollen scaffolds were weighed (W_s). Dry scaffolds were weighed (W_d) after frozen at −80 °C and lyophilized. The experiments were performed in triplicate and the swelling ratio was expressed as $(W_s - W_d)/W_d$.

Rheological experiments were carried out with a rheometer (AR2000, TA instrument, USA) using parallel plates (diameter, 30 mm) configuration at 37 °C in the oscillatory mode. To study the visco-elastic behavior of the hydrogels, the frequency sweep test was performed, which covered a range of frequencies from 1 to 100 rad/s at controlled regular strain of $\gamma = 0.01$. The hydrogels and scaffolds were precured on the parallel plate at 37 °C for 30 min before testing. The storage modulus G′, loss modulus G″ were obtained with respect to frequency.

Degradation of the scaffolds was also examined with respect to weight loss. Initially dry scaffolds were weighed (W_0). Different scaffolds were incubated in PBS at 37 °C. At specified time intervals, hydrogels were transferred from the PBS and quickly frozen at −80 °C followed by lyophilization, then weighed (W_t). The weight remaining ratio was defined as $1\text{-}(W_0 - W_t)/W_0 \times 100\%$ (Fig. 1).

2.4. Isolation and culture of human ASCs

Fresh human lipoaspirates were obtained from 5 healthy patients (mean age 30 years ± 2.483) who had undergone abdominal liposuction in the Department of Cosmetic Plastic Surgery, Plastic Surgery Hospital of Chinese Academy Medical Sciences. All protocols for handing human tissue were approved by the patients themselves and the Research Ethics Committee of the hospital. Fresh lipoaspirates were with PBS and digested with 0.075% collagenase type I (Sigma, USA) at 37 °C for 70 min with intermittent shaking. After centrifugation at 1200*g* for 10 min, the resulting pellet was resuspended and cultured in basic growth medium, which was composed of LG-DMEM, 10% fetal bovine serum (FBS) and 1%