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Characterization of human adipose tissue-derived stem cells in vitro culture and in vivo differentiation in a temperature-sensitive chitosan/β-glycerophosphate/collagen hybrid hydrogel

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ABSTRACT

In this study, the interaction of human adipose tissue-derived stem cells (ADSCs) with chitosan/β-glycerophosphate/collagen (C/GP/Co) hybrid hydrogel was test, followed by investigating the capability of engineered adipose tissue formation using this ADSCs seeded hydrogel. The ADSCs were harvested and mixed with a C/GP/Co hydrogel followed by a gelation at 37 °C and an in vitro culture. The results showed that the ADSCs within C/GP/Co hydrogels achieved a 30% of expansion over 7 days in culture medium and encapsulated cell in C/GP/Co hydrogel demonstrated a characteristic morphology with high viability over 5 days. C/GP/Co hydrogel were subcutaneous injected into SD-rats to assess the biocompatibility. The induced ADSCs-C/GP/Co hydrogel and non-induced ADSCs-C/GP/Co hydrogel were subcutaneously injected into nude mice for detecting potential of adipogenic differentiation. It has shown that C/GP/Co hydrogel were well tolerated in SD rats where they had persisted over 4 weeks post implantation. Histology analysis indicated that induced ADSCs-C/GP/Co hydrogel has a greater number of adipocytes and vascularized adipose tissues compared with non-induced ADSCs-C/GP/Co hydrogel.

1. Introduction

The adipose tissue-derived stem cells (ADSCs) obtained from adipose tissue has a strong proliferative capacity and multi-lineage differentiation potential as bone mesenchymal stem cells (BMSCs) [1,2]. Compared with BMSCs, the ADSCs are more available due to their large quantities with minimal possibility of morbidity and discomfort clinically [3,4]. In view of those practical advantages, ADSCs have recently received a widespread attention in tissue engineering.

In the tissue engineering research, stem cells must be combined with a scaffold material to form cell-material complex, cultured in vitro or in vivo under suitable conditions to allow stem cells grow, differentiate in the 3D space and eventually form desired functional tissues [5]. The ideal scaffold material should have good biodegradability, biocompatibility and 3D space structure as compared to natural extracellular matrix (ECM) [6,7]. The hydrogel is one of the favorable 3D materials which can mimic the native ECM. Chitosan and β-sodium glycerophosphate (GP) were mixed by Chenite [8] to produce chitosan/sodium glycerophosphate (C/GP) hydrogel—a kind of thermal sensitive hydrogel which does not gel until temperature rises to 37 °C or so. This characteristic of the synthetic hydrogel are probably due to the coordination of interaction forces including hydrogen bonding, electrostatic and hydrophobic interactions. Compared to normal process of implantation, thermal sensitive hydrogel shows huge advantages as it can be introduced into patients by injection and form gel which minimize pain and side-effects [9]. Due to its great property, thermal sensitive hydrogel is an ideal biological scaffold material for the tissue engineering. Chitosan materials have showed good biocompatibility [10,11], low immunogenicity and accepted biodegradable rate in previous studies [12]. The raw rubber materials are added to the system by Song et al. [13] to reduce the toxicity of C/GP/Co hydrogel plastic which has better biocompatibility. There are many reports demonstrating that ADSCs can be seeded into scaffolds followed by cultivation and cell differentiation [14–17]. However, few reports were on ADSCs growing in 3D space and differentiated in vivo.

In the present study, the ADSCs which harvested from human are composited with C/GP/Co hydrogels to investigate the cells growth, proliferation and spreading activity inside the material and to assess the possibility of engineered adipose tissue formation. ADSCs-hydrogel was then subcutaneously injected into nude mice for studying the

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potential of the adipogenic differentiation and the possibility of adipose tissue formation in vivo for further clinical application.

2. Materials and methods

2.1. Isolation and culture of human ADSCs

The adipose tissue was provided by Dr. Sha from the Cosmetic Plastic Surgery Clinic, Dalian, China with consent. In brief, approximately 2–3 g of human subcutaneous fat was washed for several times in D-hank's buffer, mincing and then incubated in digestion of collagenase and trypsin buffer(1:1 w/w) at 37 °Cfor 20 min. After incubation, the suspension was separated into three layers: upper layer containing yellow oily lipocytes, intermediate layer adipose tissue and the bottom layer of buffer and mononuclear cells. The buffer from the bottom layer was then carefully collected and the digestion was completed in a centrifuge tube containing h-Dulbecco's Modified Eagle Medium (h-DMEM, GIBCO, USA) with 10% fetal bovine serum (Sigma, USA). The mixture was centrifuged at 1500 rpm for 10 min prior to addition of trypsin-collagenase to the remnant adipose tissue for few times until adipose tissue was completely digested. Cell pellets were re-suspended in medium (DMEM + 10% FBS) and plated in tissue culture-flasks. To avoid the contamination of red blood cell, NH_4Cl or KRB (Krebs-Ringer-Bicarbonate) were not used. Cells were cultured at 37 °C and 5.0% Co_2 humidified incubator with media replaced every 3 days. Purified ADSCs at passage 4 were prepared for later use [18].

2.2. Quantification of surface markers by flow cytometry

ADSCs at passage 5 were collected, and stained using the antibodies: *anti*-CD34-FITC, anti-CD44-FITC, anti-CD45-PE, anti-CD105-FITC and anti-HLA-DR-PE for 20 min in the dark with corresponding isotype and positive controls (BD, USA). After staining, the cells were washed twice in PBS and analyzed using a standard Becton-Dickinson FACS Aria instrument (BD, USA). The data were acquired and analyzed using the FACS Diva software (BD, USA).

2.3. Multiple differentiation potential of ADSCs

The cellular growth and morphology was observed under an inverted microscope in bright field and a confocal laser scanning microscopy (CLSM), respectively.

2.3.1. Osteogenic differentiation

ADSCs at passage 5 were seeded at cell density of 4×10^4 cells/mL. In the 24-well culture plate, 3 wells containing ADSCs and alkaline phosphatase (ALP) staining, another 3 wells for von-Kossa staining, and the remaining 6 wells were used as a negative control group. The culture medium was replaced with osteogenic differentiation media when the cells began to adhere to the wells followed by media replacement every 3 days. At 2 and 4 week's time point, cells were stained by ALP and von Kossa, respectively.

2.3.2. Chondrogenic and adipogenic differentiations

ADSCs collected at passage 5 were also seeded at density of 4×10^4 cells/mL for studying chondrogenic and adipogenic differentiations. ADSCs were cultured at standard condition, while media were changed to chondrogenic and adipogenic differentiation media when cell adhesion occurred. Morphology of induced cells were characterized, and attached and differentiated cell were also stained by toluidine blue and oil red (Sigma, USA) after 2 weeks of inductions, respectively.

2.4. Preparation of C/GP and C/GP/Co hydrogel

Chitosan (Deacetylation 93% v/v, Haidebei, Jinan) solution (2.2% v/v acetic acid solution) and β-glycerophosphate solution (50%*w*/w aqueous solution) were mixed in ratio of 5:1 v/v to prepare chitosan/glycerophosphate (C/GP) solution. Rat tail type I collagen (700 μL, Chuang'er Biotech, China) solution was prepared prior to mix with 30 μL of 0.1 mol/L NaOH solution and 100 mL of 10 × D-Hanks used as collagen solution. C/GP/Co hydrogel solution as the experimental group was then prepared by mixing equal volume of Rat tail collagen type I solution (1 mg/mL) with C/GP (C: GP = 5: 1, V/V) solution in ice bath [13,19]. A hydrogel solution without collagen was also prepared as control group. The feedstock used for hydrogel preparation had already been sterilized with a filter [19,20].

2.5. Osmolality of the culture media in contact with the hydrogels

C/GP/Co and C/GP hydrogel solution (30 μL) were added into 96-well culture plate, respectively, gelation for 1 h at 37 °C, following by adding 100 μL of culture media into each plate and remained at 37 °C overnight. The osmolality of the supernatant media in contact with each hydrogel was assessed through an osmometer (Wescor, USA), in comparison with standard culture medium.

2.6. Morphology of the hydrogels

Prepared solutions of the C/GP and C/GP/Co hydrogels were placed into separate wells of a 96-well plate, at a volume of 120 μL per well. Each well was then allowed to gelatinize at 37 °C for 15 min, pre-frozen for 2 h in a − 80 °C fridge prior to freeze-dry for 48 h. Dry samples were fixed with 2.5% glutaraldehyde (pH 7.2–7.4) for 3 h at room temperature, washed twice in PBS followed by dehydration using a series of ethanol dilutions(30–100%). Samples were vacuum dried, ion sputtered and scanned using scanning electron microscope (SEM) (S2520, Hitachi) [21,22].

The prepared C/GP hydrogel scaffold and C/GP/Co hybrid hydrogel scaffolds were placed on a 96-well culture plate with a dose of 120 μL each well respectively. After 15 min of gelation at 37 °C, the simple was put into −80°Crefrigerator for 2 h of refrigeration, then it was dried for 48 h in a freeze drier, the middle part of the scaffold was chosen to be observed under a SEM with an acceleration voltage at 40 kV.

2.7. Rheological measurement of hydrogel

The gelation temperature of C/GP and C/GP/Co thermosensitive hydrogel was measured through the temperature scanning of rheology using AR2000ex rheometer (TA Company, USA).

2.8. Determination of C/GP/Co hydrogel scaffold degradation in vitro

Degradation of C/GP/Co hybrid hydrogel scaffold was assessed via an established method. Briefly, 1 mL mixed solution at different ratio of C/GP and collagen was added into a tube. After 1 h of gelation at 37 °C, lysozyme and collagenase mixture in PBS (pH 7.4)(0.01 M) was added and then incubated at 37 °C with stirring at 50 rpm. Dry sample weight was measured at different time points. The degeneration rate was calculated by the following formulation.

$$\text{Degeneration rate}\%(t) = [Wd\,(0) - Wd\,(t)]/Wd\,(0)$$

Here, Wd (0) is initial weight of sample, Wd (t) is the weight of sample at time t.

2.9. 2.9 production of ADSCs-hydrogel constructs

ADSCs (passage 5) cell at density of 4×10^4 cells/mL suspension was prepared prior to mix with prepared collagen solution resulting in ADSCs-C/GP solution at 7×10^6 cells/mL. According to different requires of experiment, the solutions were added into separate well of 96-well culture plates (40 μL per well) and 24-well plates (250 μL per well),