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Sustained release of adipose-derived stem cells by thermosensitive chitosan/gelatin hydrogel for therapeutic angiogenesis

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ABSTRACT

Adipose-derived stem cells (ASCs) secrete several angiogenic growth factors and can be applied to treat ischemic tissue. However, transplantation of dissociated ASCs has frequently resulted in rapid cell death. Therefore, we aimed to develop a thermosensitive chitosan/gelatin hydrogel that is capable of ASC sustained release for therapeutic angiogenesis. By blending gelatin in the chitosan thermosensitive hydrogel, we significantly enhanced the viability of the encapsulated ASCs. During *in vitro* culturing, the gradual degradation of gelatin led to sustained release of ASCs from the chitosan/gelatin hydrogel. *In vitro* wound healing assays revealed significantly faster cell migration by co-culturing fibroblasts with ASCs encapsulated in chitosan/gelatin hydrogel compared to pure chitosan hydrogels. Additionally, significantly higher concentrations of vascular endothelial growth factor were found in the supernatant of ASC-encapsulated chitosan/gelatin hydrogels. Co-culturing SVEC4-10 endothelial cells with ASC-encapsulated chitosan/gelatin hydrogels resulted in significantly more tube-like structures, indicating the hydrogel's potential in promoting angiogenesis. Chick embryo chorioallantoic membrane assay and mice wound healing model showed significantly higher capillary density after applying ASC-encapsulated chitosan/gelatin hydrogel. Relative to ASC alone or ASC-encapsulated chitosan hydrogel, more ASCs were also found in the wound tissue on post-wounding day 5 after applying ASC-encapsulated chitosan/gelatin hydrogel. Therefore, chitosan/gelatin thermosensitive hydrogels not only maintain ASC survival, they also enable sustained release of ASCs for therapeutic angiogenesis applications, thereby exhibiting great clinical potential in treating ischemic diseases.

Statement of Significance

Adipose-derived stem cells (ASCs) exhibit great potential to treat ischemic diseases. However, poor delivery methods lead to low cellular survival or dispersal of cells from target sites. In this study, we developed a thermosensitive chitosan/gelatin hydrogel that not only enhances the viability of the encapsulated ASCs, the gradual degradation of gelatin also result in a more porous architecture, leading to sustained release of ASCs from the hydrogel. ASC-encapsulated hydrogel enhanced *in vitro* wound healing of fibroblasts and tube formation of endothelial cells. It also promoted in *vivo* angiogenesis in a chick embryo chorioallantoic membrane assay and a mice wound model. Therefore, chitosan/gelatin hydrogel represents an effective delivery system that allows for controlled release of viable ASCs for therapeutic angiogenesis.

1. Introduction

Adipose-derived stem cells (ASCs) have drawn attention in the field of regenerative medicine research because abundant ASCs can be easily accessible from subcutaneous adipose tissue using minimally invasive procedures like liposuction [1,2]. In addition to their capability of self-renewal and multi-lineage differentiation, ASCs secrete angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) [3]. This pro-angiogenic property, together with their ease of accessibility and low donor site morbidity, has made ASCs prime candidates for a broad range of cell-based therapeutics aimed at

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ischemic tissue repair and regeneration [4]. However, topically applied ASCs undergo cell death within 72 h of application, and the retention of the transplanted cells in the injection site is frequently limited [5]. For example, direct injection of cells into the myocardium has resulted in limited survival and retention of the transplanted cells [6,7]. Hence, overcoming the disadvantages of transplanting dissociated cells and choosing a suitable biomaterial to maintain the viability and angiogenic properties of ASCs during cell delivery is of paramount importance.

Several naturally occurring sea-derived biological materials, including chitosan, have been applied to enhance ASC survival for tissue engineering applications [8–10]. To improve the mechanical and biological properties of chitosan, blending chitosan with other polymers, such as gelatin, for tissue engineering purposes has been investigated [9,11,12]. Gelatin, the denatured type of collagen, has a high amino acid content of glycine, proline and hydroxyproline. When gelatin and chitosan are mixed, they form polyelectrolytic complexes in different gelated states [13]. Chitosan/gelatin composites have similar structures to glycosaminoglycans and collagen, and mimic the natural components of the extracellular matrix (ECM), thus providing a suitable environment for cell survival *in vitro* [12].

Hydrogels have been widely applied in drug delivery systems and cell-encapsulating biomaterials. Possible mechanisms used for *in situ* gel formation include solvent exchange, UV-irradiation, ionic cross-linkage, pH change, and temperature modulation [14,15]. Among these, the thermosensitive approach is considered advantageous for therapeutic applications since it does not require organic solvents, co-polymerization agents, or an externally applied trigger for gelation. These hydrogels remain liquid-like at room temperature or 4 °C, and undergo a gelling process when heated to body temperature *in vivo*. Chitosan-based thermosensitive hydrogels have been fabricated using a chitosan/glycerophosphate system [16]. These hydrogels have been successfully applied in drug release studies and tissue engineering purposes [17,18]. Previous studies also employed chitosan/glycerophosphate hydrogel to encapsulate ASCs [19–21]. However, whether this type of thermosensitive hydrogel can be modified to fulfill the sustained release of the encapsulated cells has not been well investigated.

We have therefore aimed to blend gelatin in the chitosan/glycerophosphate hydrogel to make it more biocompatible in this study. Moreover, we took advantage of the different degradation properties of chitosan and gelatin, thus allowing sustained cell release from the composite hydrogel when placed *in vivo*. These unique characteristics can facilitate efficient ASC delivery from the injected thermosensitive hydrogel to promote angiogenesis in ischemic tissues.

2. Materials and methods

2.1. Preparation of the chitosan/gelatin hydrogel

The temperature-sensitive chitosan/glycerophosphate hydrogel was prepared by a protocol modified from previous reports [21]. Briefly, 2% chitosan (w/v, in distilled water, Protasan, Oslo, Norway) with 2% or 4% gelatin (Sigma, St. Louis, MO) was prepared and sterilized using a regular liquid autoclave. Then, 40% β-glycerophosphate (w/v, in distilled water, Sigma) was prepared and filter sterilized. The glycerophosphate solution was added drop-wise into the chitosan/gelatin solution under stirring, and the pH value was adjusted to 7.4. Chitosan/gelatin hydrogel was formed by mixing 8 ml of chitosan/gelatin solution with 2 ml of filter-sterilized glycerophosphate solution and incubating it at 37 °C.

2.2. Swelling ratio measurement

To measure the weight-to-swelling ratio, hydrogel samples were immersed in deionized water for 24 h at room temperature. The swollen samples were weighed by an electronic balance, and the hydrogels were then lyophilized and weighed again. The swelling ratio was calculated as follows: *Ws* and *Wd* are the weights of swollen and dried hydrogels, respectively. The $Swelling\ ratio = (Ws - Wd)/Wd$.

2.3. Release of gelatin from the chitosan/gelatin hydrogel

The enzymatic degradation properties of chitosan/gelatin hydrogels were determined at 37 °C in phosphate-buffered saline (PBS; Omics Biotechnology, Taipei, Taiwan) containing 0.25 mg/ml of collagenase type I (Sigma). At day 1, 3, 5 and 7, PBS in each well was aspirated, and each well was replenished with fresh PBS. Protein concentration of the aspired solution was determined by a bicinchoninic acid assay (Pierce, Rockford, IL) according to the manufacturer's protocol. The percentage of gelatin released from a chitosan/gelatin hydrogel was calculated by normalizing to the initial gelatin content in the gel.

The chitosan/gelatin hydrogel samples were enzymatically digested for seven days. Samples of the gels before and after digestion were harvested for electronic microscopic examination. After lyophilization, the gel samples were fixed on studs and then sputter coated with platinum. The prepared scaffolds were then viewed with a scanning electron microscope (JSM-6700F, JOEL, Tokyo, Japan). We selected pores with a diameter greater than 1 μm and a long-to-short axis ratio of not more than 1.5, and the pore number and pore size on the surface of the hydrogel was estimated using Image J software.

2.4. Culturing of human ASCs

The study protocol was approved by the Internal Ethics Committee of National Taiwan University Hospital. Informed consents were obtained from all patients involved in this study. The ASCs were obtained from a combination of four nondiabetic donors with an average age of 45 y (range 32–57 y) and an average BMI of 24.6 (range 21.0–26.6). The ASCs were isolated as described previously [22]. Briefly, subcutaneous adipose tissue was collected and finely minced. The scraped adipose tissue was then placed in a digestion solution containing 1 mg/ml collagenase type I (Gibco, Carlsbad, CA). Following digestion, the cell suspension was filtered and cultured in an expansion medium comprising Dulbecco's modified Eagle's medium (DMEM)/F-12 (Hyclone, Logan, UT), 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel), 1% penicillin-streptomycin (Biological Industries), and 1 ng/ml of basic fibroblast growth factor (bFGF; R&D systems, Minneapolis, MN). The cells were cultured at 37 °C in a 5% CO_2 incubator, and the medium was changed every 2–3 days. On reaching 90% confluence, the cells were detached with 0.05% trypsin-EDTA (Biological Industries) and replated, and the third passage ASCs were used for different experiments. For ASC encapsulation in chitosan/gelatin hydrogels, cells were suspended in hydrogels at an initial concentration of 10^6 cells/ml. The mixture was then allowed to gel at 37 °C. After gelling, ASC growth medium (DMEM supplemented with 10% FBS and 1% penicillin-streptomycin) was added for cell culture.

2.5. Cell viability and proliferation assay

Cell viability in the hydrogel was assessed using a Live/Dead kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. On day 5 of ASC culture, the cell-encapsulated hydrogels were