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Specific interactions between human fibroblasts and particular chondroitin sulfate molecules for wound healing

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Abstract

The chondroitin sulfates (CSs) constitute an important group of biomacromolecules in the extracellular matrix. However, limited information is available about their specific biological functions. This study aimed to define the interactions between cells and various types of CS. The effects of CSs on cellular activities and the cell cycle were evaluated using cell culture, RNA interference, real-time polymerase chain reaction, flow cytometry, wound healing and contraction models. The results showed that C-6-S promoted both cell proliferation and adhesion, while C-4-S promoted proliferation but inhibited adhesion. Moreover, knockdown of chondroitin inhibited cell proliferation and migration, as well as arresting cells in the G_2/M phase. Also, both C-4-S and C-6-S promoted wound closure in a two-dimensional wound model, whereas only C-6-S inhibited wound contraction in a three-dimensional wound model. This study illustrates that the interaction between cells and different CSs are specific and sulfate-group-dependent. These findings provide useful information for better applications of CSs for wound healing.

Keywords: Chondroitin sulfate; Wound healing; Cell material interaction; Tissue engineering

1. Introduction

Extracellular matrix (ECM) components are valuable building blocks for the preparation of biomaterials used in tissue engineering, especially if their biological, chemical and physical characteristics can be controlled. The glycosaminoglycans (GAGs) are a family of native biopolymers which are widely present in the ECM. GAGs are negatively charged polysaccharides with biocharacteristics including the ability to hydrate the extracellular matrix [1] and bind effector molecules (e.g. growth factors and cytokines) [2,3]. The multiple biological functions of GAGs suggest great potential as biomaterials for tissue engineering and wound healing.

Chondroitin sulfates (CSs), which constitute a specific group of GAGs, consist of repeating sulfated disaccharides containing *N*-acetylgalactosamine. The CS chains are roughly classified into types A, C, D, E, K and H [4]. They are named chondroitin sulfates A (C-4-S), C (C-6-S), D (C-2,6-S), E (C-4,6-S), K (C-3,4-S) and H (IdoAα1–3GalNAc(4S,6S)), of which C-4-S and C-6-S are the most common. CSs play important roles in the wound healing process. Injection of glycylhistidyl-lysine-Cu^{2+}, an activator of wound healing, into full-thickness rat skin wounds results in the accumulation of CSs and stimulates wound tissue production [5]. Gilbert et al. found that CS hydrogel accelerates wound healing in sinonasal mucosa at a 4-day endpoint [6]. Together, these findings suggest that CSs have

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the potential to improve wound healing. Therefore, a number of recent studies have used CSs as biomaterial for cartilage repair [7], tissue engineering [8] and growth factor release [9]. Further, they can be manufactured into different formats, such as three-dimensional (3-D) matrix [10] and gel [11].

Although rapidly accumulating evidence strongly suggests that CSs have crucial biological functions in cellular activities and great potential for wound repair, the evidence is fragmentary and immature. On the other hand, limited information is available on the choice of different kinds of CS. Hence, we designed systematic experiments to investigate the interactions between cells and different CSs. We aimed to understand the specific roles of different CSs in regulating cellular activities and their efficiency in wound healing. The knowledge obtained from this analysis provided strong evidence for better application of CSs as biomaterials for tissue engineering and wound healing.

2. Materials and methods

2.1. Ethics statement for human cells

The dermal fibroblasts used in this study were obtained from spare skin tissues. The procedure was agreed to by patients and approved by the Hospital Ethics Committee.

2.2. Culture of human dermal fibroblasts

Human dermal fibroblasts were cultured in 75 cm^2 tissue culture flasks in 90% minimum essential medium (MEM; Invitrogen, Australia) with 2 mM L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g l^{-1} sodium bicarbonate, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate, and supplemented with 10% fetal bovine serum (Invitrogen, Australia), 50 U ml^{-1} penicillin G sodium and 50 μg ml^{-1} streptomycin sulfate (Sigma, USA). Cells between passages 3 and 5 were used.

2.3. The MTS assay

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega Corp. (Madison, WI, USA). MTS is a tetrazolium compound that is reduced to a colored formazan product by cell metabolism. The amount of formazan product is directly proportional to the number of living cells, which can be quantified by reading the culture plate at 490 nm. As cell division needs more than 12–24 h, we chose 8 h after cell seeding as the time point to evaluate cell adhesion and 7 days after cell seeding as the time point to evaluate cell proliferation.

2.4. Cell adhesion assay

The fibroblasts were seeded into 24-well plates at 6×10^4 cells per well and cultured for 8 h. The number of fibroblasts adhering to the culture plate was then determined by MTS assay. Briefly, the tetrazolium compound MTS was incubated with cultured cells for 1 h, during which metabolically active cells converted the MTS reagent to a soluble formazan dye. The amount of absorbance was then measured at 490 nm.

2.5. Cell proliferation assay

Cells were seeded into 24-well plates at 3×10^4 cells per well and cultured for 7 days. The number of fibroblasts after culture was measured at day 7 by MTS assay as described above.

2.6. Effects of extraneous CS on cell adhesion and proliferation

Pilot experiments compared the effects of low (100 ng ml^{-1}) and high concentrations (1000 ng ml^{-1}) of C-4-S and C-6-S on cell adhesion and proliferation. The 1000 ng ml^{-1} concentration of the CSs had a greater effect on cellular activities (data not shown), so this concentration was used in subsequent experiments.

Cells were seeded into 24-well plates at 3×10^4 cells per well in four groups (parallel samples of $n = 6$):

- *Group 1:* Complete culture medium (control group).
- *Group 2:* Complete culture medium with 0.5 U ml^{-1} chondroitinase ABC (Sigma, USA).
- *Group 3:* Complete culture medium with 1000 ng ml^{-1} C-4-S.
- *Group 4:* Complete culture medium with 1000 ng ml^{-1} C-6-S (Sigma, USA).

Plates were put into a humidified incubator (at 37 °C, 5% CO_2) for 8 h to evaluate cell adhesion, and for 7 days to evaluate cell proliferation. The numbers of cells were quantified by MTS assay.

2.7. Function of CS through CHSY1 knockdown by RNA interference

In order to illustrate the biological functions of chondroitin, three small interfering (si) RNAs (Table 1) were custom-designed and synthesized by Ambion. siRNAs were transfected into cultured cells to block the expression of chondroitin synthase 1 (CHSY1) and then the changes in cellular activities were evaluated. In the experiment, a Silencer™ siRNA transfection II kit (Ambion, USA) was used to transfer siRNAs into cells.

2.8. Real-time PCR for evaluating knockdown efficiency

Real-time polymerase chain reaction (PCR) was performed with a Light Cycler System (Roche, USA), using a QuantiTect™ SYBR Green PCR kit (Qiagen, USA). The primers of CHSY1 used were: forward: ccctccttcat-

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