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The cardiomyogenic differentiation of rat mesenchymal stem cells on silk fibroin–polysaccharide cardiac patches *in vitro*

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ABSTRACT

Polysaccharides and proteins profoundly impact the development and growth of tissues in the natural extra-cellular matrix (ECM). To mimic a natural ECM, polysaccharides were incorporated to/or co-sprayed with silk fibroin (SF) to produce SF/chitosan (CS) or SF/CS–hyaluronic acid (SF/CS–HA) microparticles that were further processed by mechanical pressing and genipin cross-linking to produce hybrid cardiac patches. The ATR–FTIR spectra confirm the co-existence of CS or CS–HA and SF in microparticles and patches. For evaluating the cellular responses of rMSCs to the SF/CS and SF/CS–HA cardiac patches, the growth of rMSCs and cardiomyogenic differentiation of 5-aza inducing rMSCs cultured on patches was examined. First, the isolated rMSCs were identified with various positive and negative surface markers such as CD 44 and CD 31 by a flow cytometric technique, respectively. For examining the growth of rMSCs on the patches, MTT viability assay was performed, and the results demonstrated that the growth of rMSCs on SF and SF-hybrid patches significantly exceeded ($P < 0.001$) that on culture wells after seven days of cultivation. Additionally, the relative growth rates of rMSCs on SF/CS and SF/CS–HA hybrid patches were significantly better ($P < 0.01$) than that on SF patches that were also observed by using vimentin stain to the cells. For instance, the relative cell growth rates (%) in cell culture wells, SF, SF/CS and SF/CS–HA patches were 100%, $282.9 \pm 6.5\%$, $337.0 \pm 8.0\%$ and $332.6 \pm 6.6\%$ ($n = 6$, for all), respectively. For investigating the effects of the hybrid patches on cardiomyogenic differentiation of 5-aza inducing rMSCs, the expressions of specific cardiac genes of cells such as Gata4 and Nkx2.5 were examined by real-time quantitative polymerase chain reaction (real-time PCR) analysis. The results of cardiomyogenic differentiation of induced rMSCs on SF/CS and SF/CS–HA hybrid patches significantly improved the expressions of cardiac genes of Gata4, Nkx2.5, Tnnt2 and Actc1 genes (all, $P < 0.01$ or better, $n = 3$) than those on SF patches and culture wells. Interestingly, the results of cardiac gene expressions of the cells on the SF/CS–HA hybrid patches were the most pronounced in promoting cardiomyogenic differentiations in this investigation. Furthermore, immunofluorescence staining of cardiac proteins such as cardiotin and connexin 43 for induced rMSCs cultured on SF/CS and SF/CS–HA hybrid patches were much pronounced compared with SF patches, indicating the improvements of cardiomyogenic differentiation on the hybrid patches. The results of this study demonstrate that the SF/CS and SF/CS–HA hybrid patches may be promising biomaterials for regenerating infarcted cardiac tissues.

1. Introduction

Silk fibroin (SF), a fibrous protein consisting of Glycine and Alanine as the main amino acid residues, has been extensively studied because of its favorable biological responses [1]. After sericin of SF is removed, the SF films with Arg-Gly-Asp (RGD) modifications have weaker antigenic effects and inflammatory responses *in vivo* than films fabricated using collagen or others [1,2]. Various SF-based membranes or scaffolds have recently been investigated for their potential applications in tissue engineering such as in repairing bone [3], ligament [4] and blood vessels [5], as well as wound dressing [6]. Moreover, positive responses of mesenchymal stem cells (MSCs) grown in gelatin or RGD modified

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SF-based scaffolds to the regeneration of bone, ligament and cartilage tissue engineering have been shown [3,4,7,8]. It is recognized that polysaccharides and proteins have significant roles in the organization of living cells and tissue growth. Interactions between these biopolymers in an extra-cellular cell matrix (ECM) lead to the formation of macromolecular structures by association [9]. Of interest, the responses of rMSCs to SF and SF/polysaccharide hybrid patches such as SF/CS and SF/CS–HA patches as are the question of whether new patches promote the growths of rat MSCs (rMSCs) and improve cardiomyogenic differentiation of 5-aza inducing rMSCs.

Chitosan, an amino polysaccharide, is a biodegradable biomaterial containing numerous reactive groups that can be modified to accelerate cell growth in films or scaffolds [10,11]. In addition, the structure of CS resembles that of glycosamino-glycan in an ECM and may be a suitable complement to the aforementioned SF matrix used in tissue engineering. Hyaluronic acid (HA) is a non-sulfated glycosaminoglycan and distributed throughout the ECM of all connective tissues in humans and other mammals [12]. Furthermore, HA is of particular interest due to its ability to promote cell migration. Thus, HA is frequently applied to modify scaffolds for cartilage tissue engineering [13], wound healing and angiogenesis [12,14]. Since HA exhibits the aforementioned unique property, HA was adopted in this study as a complement to the SF matrix.

The efficacy of cell therapy by direct or intra-coronary injection of bone marrow cells has been shown in treating myocardial infarcted hearts or chronic ischemic heart diseases [15,16]. Moreover, using bone marrow-derived MSCs for generating cardiomyocytes in an infarcted area in animal models or *in vitro* by 5-azacytidine (5-aza) treatment have been demonstrated [16–18]. However, it is noticed that MSC therapy in recipient myocardium by direct myocardial injection or via the coronary artery shows several shortcomings, such as low efficiency in cell survival, and insufficient prevention of progressive left ventricular dilation. To improve these deficiencies, an alternative therapeutic approach used cells seeded and grown in bio-absorbable cardiac barriers or patches to repair infarcted cardiac tissues [19,20]. For examples, alginate/gelatin films and poly(glycolide-*co*-caprolactone) patches have been investigated *in vitro* and in animal models [19,20], respectively; each yielded positive results. However, patches designed for this goal from active biopolymers, such as SF, SF/CS or SF/CS-HA hybrid biomaterials are lacking reported.

In this investigation, SF, hybrid SF/CS and SF/CS–HA microparticles were first produced by spray-drying, fixed in alcohol, and then pressed to form patches, before they were crosslinked using genipin, a natural cross-linking agent which has a low cytotoxicity [21]. To evaluate cell responses to those patches, the growths of rMSCs, identified with various surface CD markers, on the patches were quantified, and their morphologies were observed by immunofluorescence stain. Moreover, to examine the effects of various patches on cardiomyogenic differentiation of 5-aza inducing rMSCs, the expressions of selected specific genes of cardiomyocytes were examined by real-time polymerization chain reactions (PCRs). The selected specific genes were Gata4 (GATA binding protein 4) and Nkx2.5 (Nkx2 transcription factor related, locus 5) genes of cardiomyocytes, that play essential roles in early heart development by regulating expressions of many genes which encode cardiac-specific proteins, and several cardiac muscle-specific marker genes such as troponin T expressed by Tnnt2 and α-cardiac actin expressed by Actc1 [24]. Furthermore, to observe the cellular responses of the patches to the myocardiogenic differentiations of rMSCs, the immunofluorescence stains of specific proteins of cardiomyocytes were examined. For this examination, troponin T [22], cardiotin [23] and connexin 43 [24] were selected since they are important for effective cardiomyocytes which contain contractile proteins, contraction-relaxation cycle of the cardiac muscle and propagation of electrical signals to induce coordinated contraction of the cardiac muscles for blood pumping in the heart, respectively. Through this study, the potentials of SF, SF/CS or SF/CS–HA cardiac patches for the cardiomyogenesis of rMSCs were examined.

2. Materials and methods

2.1. Preparation of SF-based patches

Silk cocoons were purchased from a silk center in Taiwan (ShihTan, Miao-Li, Taiwan). The SF solutions were prepared as described elsewhere [25,26]. Briefly, silk cocoons were boiled for 30 min in 0.02 M Na_2CO_3, and then rinsed thoroughly in distilled water to extract the glue-like sericin proteins. The extracted SFs were then dissolved in 9.3 M LiBr solution at 60 °C for 4 h, yielding a 20% (w/v) solution [1], which was then dialyzed against distilled water using a dialysis membrane (MWCO 6000) (Pierce, USA) at room temperature for 48 h to remove salt. The final concentration of the SF aqueous solution was 8% (w/v). This concentration was determined by weighing the residual solid in a known solution volume after drying at 60 °C for 24 h. Chitosan (96% de-acetylated; MW, 200 kDa) (Sigma–Aldrich, USA) and hyaluronic acid (HA) (MW, 15 kDa) (Lifecore, USA) were added and dissolved in the previously purified SF solution (1.5% w/v) to yield SF-to-CS-to-HA (w/w/w) ratios of 10:1:0 and 10:1:1, respectively.

To prepare the patches, the SF-based hybrid microparticles were initially fabricated using a spray-drying machine (EYERS SD-1000) (Tokyo Rikakikai Co., Tokyo, Japan) at 120 °C, under a 20 KPa tip pressure, airflow rate of 0.65 m^3/min and solution flow rate of 20 ml/min. The SF-based particles formed had diameters of 3–8 μm. The particles were immersed in 95% alcohol for several hours and then air dried for subsequent applications. These microparticles were pressed using with a pressing machine at a pressure of 10 GPa for 5 s at room temperature to produce three different patches. These SF, SF–CS and SF/CS–HA patches were then cross-linked in 1% genipin (Challenge Bioproducts Co., Ltd., Taipei, Taiwan) solution for 12 h at 45 °C; the cross-linking reactions were terminated by adding 3% glycine at 25 °C for more than 12 h. After treatment with glycine, the SF-based patches were washed in distilled water to remove residual glycine via stirring for in excess of 1 h. These dark-blue patches had a diameter of 13 mm, thickness of 200 μm, and mass of 20–25 mg, and swelling ratio of 15–20% (swelling ratio (%) = (wet weight − dry weight)/dry weight × 100%).

2.2. Surface characterization of SF-hybrid patches

The sizes and zeta potentials of SF, SF/CS and SF/CS–HA microparticles in the aqueous solution were determined at 25 °C using a dynamic light-scattering (DLS) analyzer equipped with a device for measuring zeta potential (Zeta Plus 90 Particle Sizer) (Brookhaven Instruments Co., USA) with a 5 mW He–Ne laser ($\lambda = 633$ nm). The transmission spectra of the SF, SF/CS and SF/CS–HA microparticles were determined using an ATR–FTIR analyzer with a resolution of 2 cm^{-1}, and analyzed utilizing the built-in standard software package Perkin–Elmer Spectrum One (Perkin–Elmer Co., Norwalk, CT, USA). The surface morphology of the SF and SF/CS hybrid patches was examined via scanning electron microscopy (SEM) following the procedure in this group research [27].

2.3. rMSC culture and differentiation of cardiomyocyte-like cells on various SF-based hybrid patches

Bone marrow from Wistar rats was aspirated from the anterior iliac crest following anesthesia. The marrow was then centrifuged in a 1.077 g/ml Percoll (Sigma–Aldrich, USA) density gradient at 600 g for 10 min. The enriched cells were collected from the interphase and then re-suspended in culture medium. The cells were cultured in a 10 cm dish (Cellstar, Germany) containing alpha-minimum essential medium (α-MEM) (Gibco, USA) of 10% fetal bovine serum (FBS) (Gibco, USA), 100 μg/ml penicillin (Sigma–Aldrich, USA), and 100 μg/ml streptomycin (Sigma–Aldrich, USA) at 37 °C with 5% CO_2/95% air, and 90% relative humidity; the medium was changed every 2 days. Non-adherent hematopoietic cells were removed, and the culture medium was changed 3 times per week. The adherent, spindle-shaped rMSCs at 90% confluence were trypsinized using 0.25% trypsin–EDTA (Sigma–Aldrich, USA) and transferred to fresh dishes [18].

The SF and SF/CS hybrid patches one-by-one were put on a well in a 24-well plate for cell culture. Surface markers of cell passages 3–5 were detected by flow cytometric technique (see Section 2.4) to verify the phenotypes of the rMSCs. After verification, 2×10^4 rMSCs were seeded onto a 24-well culture plate and SF-based hybrid patches, respectively. The viability of rMSCs on various SF-based hybrid patches and culture plates after seven days of culturing were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (see Section 2.5).