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Cell response to single-walled carbon nanotubes in hybrid porous collagen sponges

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ABSTRACT

Three-dimensional (3D) porous collagen sponges incorporated with single-walled carbon nanotubes (SWCNTs) were prepared and used for 3D culture of bovine articular chondrocytes (BACs). The pore structures of the sponges were controlled by using ice particulates as a porogen material. The responses of cells to SWCNTs were investigated in this 3D cell culture system by evaluation of cell functions and cellular uptake of SWCNTs. The results showed that cells adhered and spatially distributed in the porous sponges. The incorporation of SWCNTs in the porous sponges promoted cell proliferation and production of sulfated glycosaminoglycans (sGAG). Confocal Raman imaging revealed that SWCNTs could be internalized by cells. The hybrid porous sponges not only provided nanostructured pore surfaces to facilitate cell proliferation and extracellular matrix (ECM) secretion but also supplied nanomaterials for cellular uptake which may be useful for biomedical applications.

1. Introduction

Tissue engineering has been developed as a promising approach to improve or replace biological functions by the combination of cells, scaffolds and growth factors [1,2]. Among these three key factors, the scaffolds play a crucial role by furnishing a biomimetic microenvironment to control cell functions and to guide new tissue formation [3,4]. Scaffolds with ideal properties, such as good biocompatibility, appropriate mechanical property and inner pore structures are highly demanded for successful tissue regeneration. Thus far, a variety of scaffolds have been fabricated from either natural polymers or synthetic polymers [5,6]. Compared with other biodegradable polymers, collagen-based scaffolds are particularly attractive because collagen is one of the most abundant proteins in mammals and has regular helical structure, high hydrophilicity, excellent biocompatibility and moderate immunogenicity [7].

Except the microstructures of scaffolds, creation of nanostructures to mimic the native cellular microenvironments has been a great challenge. The extracellular matrix (ECM), as the main components of *in vivo* cellular microenvironments, is a hierarchically organized nanocomposite that provides structural and biochemical supports to the surrounding cells [8]. To address this challenge, researchers have made efforts on the design of advanced nanocomposite scaffolds that can better mimic the ECM by using existing nanotechnological tools [9–11]. One of the effective methods is to incorporate nanomaterials into porous scaffolds [12–14]. Nanomaterials may affect the functions of the cells not only through the interaction between their nanoscaled structures and cells, but also through the internalization. However, the effects of incorporated nanomaterials in porous scaffolds have not been well elucidated. Therefore, detailed interaction of nanomaterials with cells needs to be thoroughly investigated for their extensive biomedical applications.

In this study, 3D porous collagen sponges (Col sponges) with controlled pore structures were prepared by using ice particulates as a porogen material. Single-walled carbon nanotubes (SWCNTs), as one of the most attractive nanomaterials, were incorporated in Col sponges to prepare SWCNTs/Col hybrid sponges by immersion of Col sponges in SWCNTs-containing aqueous solution. The interaction of SWCNTs with cells was investigated by culturing bovine articular chondrocytes (BACs) in the hybrid porous scaffolds.

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2. Materials and methods

2.1. Preparation of porous Col and SWCNTs/Col sponges

3D porous collagen sponges (Col sponges) with controlled pore structure were prepared by using ice particulates as a porogen material as previously reported [15–18]. Ice particulates were prepared by spaying Milli-Q water into liquid nitrogen using a sprayer. The ice particulates were sieved by sieves with 355 and 425 μm mesh pores to obtain ice particulates having a diameter from 355 to 425 μm. The sieving process was conducted in a −15 °C low-temperature chamber (Espec, Osaka, Japan). The sieved ice particulates were stored in closed glass bottles in a −80 °C freezer until use. Collagen solution with a concentration of 2% (w/v) was prepared by dissolving freeze-dried porcine type I collagen (Nitta Gelatin, Osaka, Japan) in a mixture solution of 0.1 M acetic acid (pH 3.0) and ethanol at a ratio of 80:20 (v/v). The collagen solution and the sieved ice particulates were kept in the low-temperature chamber at −4 °C for 6 h for temperature balance. The temperature-balanced ice particulates and collagen solution were mixed homogeneously at a ratio of 50:50 (w/v) at the low-temperature chamber. The mixture was frozen in a deep freezer (−80 °C) for 6 h and freeze-dried in a freeze-dryer under a vacuum of 20 Pa (VirTis AdVantage Benchtop Freeze Dryer, SP Industries Inc.). After freeze-drying, Col sponges were cross-linked with glutaraldehyde vapor for 6 h, treated with 0.1 M glycine aqueous solution to block any unreacted aldehyde groups, washed with pure water for 6 times, frozen in the deep freezer (−80 °C) for 6 h and freeze-dried again as described above to obtain the cross-linked Col sponges.

Prior to the preparation of porous SWCNTs/collagen sponges (SWCNTs/Col sponges), SWCNTs (purity > 90%, 0.7–1.3 nm in diameter, Sigma–Aldrich, USA) were coated with collagen using a simple non-covalent approach to improve the dispersibility in water as previously reported [19–21]. Briefly, 1000 μg SWCNTs were put in 10 mL 0.1 wt% collagen solution. The mixture was sonicated (135 W, Bransonic, Japan) in an ice bath for 2 h. The dispersion solution was then centrifuged (Tomy MX-301, Japan) at 5000 × *g* for 30 min to remove the aggregated and bundled SWCNTs and the supernatant was collected. To prepare SWCNTs/Col sponges, pure cross-linked Col sponges was immersed in the aqueous solution of collagen-treated SWCNTs at a concentration of ≈50 μg/mL over night, frozen in the deep freezer (−80 °C) for 6 h and freeze-dried again as described above.

2.2. Characterization of porous sponges

The inner structures of Col and SWCNTs/Col sponges were observed and imaged with a high resolution scanning electron microscope (SEM, S-4800, Hitachi, Japan). The freeze-dried specimens were plunged into liquid nitrogen and cut using a cold scalpel. The cross sections were taken out from liquid nitrogen and air-dried. They were then coated with platinum and observed by SEM at a 10 kV accelerating voltage. The mean pore size of the porous sponges was measured from the SEM images by a MetaVue Image System (Universal Imaging Corp., Buckinghamshire, UK). Six images taken from each scaffold were used for the mean pore size calculation.

2.3. Cell culture in the porous sponges

For cell culture use, the Col and SWCNTs/Col sponges were punched into cylindrical samples (Ø8 mm × *H*3 mm). The samples were sterilized with 70% ethanol, washed three times with sterile Milli-Q water and conditioned with cell culture medium at 37 °C for 30 min. The medium in the scaffolds was removed by being placed on sterilized dry Kimwipes paper before cell seeding. Bovine articular chondrocytes (BACs) were isolated from the articular cartilage derived from a 9-week old female calf. The cartilage dissected from the underlying bone were minced into 1–2 mm^3 pieces and digested with 0.2% collagenase II in Dulbecco's Modified Eagle Medium (DMEM) supplemented with penicillin (100 U/mL) and streptomycin (100 mg/mL) with shaking overnight at 37 °C. The digestion solution was filtered through a sterile 70-mm nylon mesh to remove any undigested fragments and the chondrocytes were then collected by centrifugation. Freshly isolated chondrocytes were defined as P0. BACs that were subcultured twice (P2) were used in this study. Cells were suspended in culture medium at a density of 5.0×10^7 cells/mL. The cell suspension was seeded into the cylindrical sponges (100 μL/sponge) that were placed in 24-well cell culture plates (non-treated). After 4 h, the sponges were transferred into 25-cm^2 flasks and cultured under an atmosphere of 5% CO_2 at 37 °C with shaking at 60 rpm for a designated time. The medium was changed twice per week. The cells in the medium and the cells adhered to each well of the cell culture plates after 4 h culture after cell seeding were collected, counted and taken as the number of cells leaked from the sponges during cells seeding. The number of captured cells in each sponge was obtained by subtracting the leaked cell number from the initial cell seeding number. The seeding efficiency of each sponge was calculated by dividing the number of captured cells by the number of initial seeded cells. Six samples were used for these measurements in order to obtain averages and standard deviations (SD). The culture medium was DMEM supplemented with 10% fetal bovine serum, 4500 mg/L glucose, 4 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mM nonessential amino acids, 0.4 mM proline, 1 mM sodium pyruvate and 50 μg/mL ascorbic acid.

After being cultured for another 2 h, the cell/sponge constructs were washed with PBS for three times and fixed with 0.25% glutaraldehyde solution at room temperature for 1 h. The fixed cell/sponge constructs were washed with pure water for three times and freeze-dried in a freeze-dryer under a vacuum of 20 Pa. The freeze-dried specimens were sliced using a scalpel. The cross sections were coated with platinum and the cell adhesion in the sponges was observed by an SEM at a 10 kV accelerating voltage. To visualize the cell distribution in the sponges, the cell/sponge constructs after 24 h cell culture were collected, washed with PBS for three times and sectioned with a scalpel. The as-prepared cross-sections were stained with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Inc.) and observed under a fluorescence microscope (Olympus, Tokyo, Japan).

2.4. Cell proliferation and sulfated glycosaminoglycans (sGAG) production

Cell proliferation in the porous sponges was investigated by the DNA content measurement. After being cultured for 6 h, 1 and 2 weeks, the cell/sponge constructs were washed with warm PBS, freeze-dried and digested with papain solution (Sigma–Aldrich, St. Louis, MO, USA, 400 μg/mL, with 5 mM L-cysteine and 5 mM EDTA in 0.1 M phosphate buffer at a pH of 6.0). An aliquot of the papain-digested solution was dyed with Hoechst 33258 dye (Sigma–Aldrich, St. Louis, MO, USA) and used for the measurement of DNA content ($n = 3$) with a spectrofluorometer (FP-6500, JASCO, Japan) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The fluorescence values were used to calculate DNA content based on a standard curve made from serial fluorescence values of known DNA concentration. The sGAG content was measured with the above papain digestion solution by a dimethylmethylene blue dye (Biocolor, Newtownabbey, UK) binding method and a microplate spectrophotometer (Benchmark Plus;