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Patterned carbon nanotubes as a new three-dimensional scaffold for mesenchymal stem cells

Verda Ceylan Bitirim [a,1], Gokce Kucukayan-Dogu [b,1], Erman Bengu [c], Kamil Can Akcali [a,d,*]

[a] Department of Molecular Biology and Genetics, Bilkent University, 06800, Ankara, Turkey
[b] Institute of Engineering and Science, Material Science and Nanotechnology Graduate Program, Bilkent University, 06800, Ankara, Turkey
[c] Department of Chemistry, Bilkent University, 06800, Ankara, Turkey
[d] Bilgen, Bilkent University Genetics and Biotechnology Research Center, 06800, Ankara, Turkey

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ABSTRACT

We investigated the cellular adhesive features of mesenchymal stem cells (MSC) on non-coated and collagen coated patterned and vertically aligned carbon nanotube (CNT) structures mimicking the natural extra cellular matrix (ECM). Patterning was achieved using the elasto-capillary induced by water treatment on the CNT arrays. After confirmation with specific markers both at transcript and protein levels, MSCs from different passages were seeded on either collagen coated or non-coated patterned CNTs. Adhesion and growth of MSCs on the patterned CNT arrays were examined using scanning electron microscopy image analysis and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) assays. The highest MSC count was observed on the non-coated patterned CNTs at passage zero, while decreasing numbers of MSCs were found at the later passages. Similarly, MTT assay results also revealed a decrease in the viability of the MSCs for the later passages. Overall, the cell count and viability experiments indicated that MSCs were able to better attach to non-coated patterned CNTs compared to those coated with collagen. Therefore, the patterned CNT surfaces can be potentially used as a scaffold mimicking the ECM environment for MSC growth which presents an alternative approach to MSC-based transplantation therapy applications.

1. Introduction

Stem cell research has gained tremendous pace in the last four decades and a wealth of information on their physiology has been gathered since then. Mesenchymal stem cells (MSCs), which are also called as bone marrow stromal cells, are a subset of adult progenitor cells and have the ability to differentiate into adipocytes, chondrocytes, osteocytes, and cardiomyocytes [1]. They are characterized by their morphological features and the expression of a number of cell surface marker genes. In recent years, owing to their multiple-lineage potentials and immune-privileged properties, MSCs have become a feasible and potential source for the cell-based therapy and tissue engineering applications due to their proliferative and differentiation capabilities [2]. Moreover, these cells do not induce immune reaction in the host which allows their usage in allogeneic transplantation [2].

A key issue in MSC based tissue engineering is to control the growth and differentiation of cells. Extracellular matrix (ECM) plays a crucial role in proliferation and differentiation of MSCs. In recent years, by using different scaffold proteins the transplantation of differentiated MSCs into damaged tissue such as bone and chondrocytes was accomplished [3]. However, use of natural ECM extracted from animal tissues as scaffold is limited by the dimension, form of the original tissue and the potential pathogen risk [4]. Therefore, there has been a tremendous demand to develop better materials and scaffolds which can closely mimic the surrounding native tissue in the last decade [5]. Surface properties of scaffolds were given a high priority due to the potential of developing new environments capable of stimulating the adhesion and proliferation of cells. Recently, numerous studies reported on the effects of topographical patterns on cell viability [6,7]. In this regard, patterning of scaffold surfaces at a nanometer scale is considered as a promising tool [8,9]. It is reported that gene expressions of fibroblast cells were found to be enhanced on relatively rougher surfaces [10]. Nevertheless, conflicting reports in the literature for the relationship between cell adhesion and surface topography do exist such as those reported by Kunzler et al. [11] and Gentile and co-workers [12].

Carbon nanotubes (CNT) have been proposed for many potential application areas due to their chemical stability, good electrical conductivity and mechanical strength. In recent years, CNTs have been also studied in biotechnology as a support material for cell growth in tissue engineering and bio-sensors [13,14]. However, there are extensive studies on the biocompatibility and cytotoxicity of CNTs. While cytotoxicity of CNTs [15,16] was not a direct consideration in this work, it is worth mentioning that evidence for cytotoxicity of CNTs has not been reported for cases

* Corresponding author at: Department of Molecular Biology and Genetics, Bilkent University, 06800, Ankara, Turkey. Tel.: +90 312 2902418; fax: +90 312 2665097.
E-mail address: akcali@fen.bilkent.edu.tr (K.C. Akcali).
[1] Equal contributions of authors.

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where CNTs attached to a substrate or limited concentrations of CNTs were used [17,18]. There are also similar reports on the cytotoxicity of CNTs on stem cells. Zhu et al. demonstrated that powdered multi-walled carbon nanotubes (MWCNT) induced DNA damage in mouse embryonic stem cells [19]. On the other hand, Lobo et al. [20] and Giannona et al. [21] have shown the adhesion and growth of fibroblasts on CNT arrays without any toxic effects if they are firmly attached to a substrate. Furthermore, CNTs have also been reported as a suitable scaffold material for the growth of cells due to their superb electrical conductivity and chemical stability [22]. Finally, patterned surfaces made by CNTs were found to be guiding the growth of MSCs and neural cells [23,24].

The fundamental criterion for growth and differentiation of stem cells on ECM surfaces is their adhesion. Cell adhesion sets off the cell growth, survival and migration. The common media used for cell growth consists of protein fibers such as collagen and elastin which are roughly 10 to 300 nm in diameter. In biotechnology, MWCNTs can be considered as an alternative for collagen fibers due to their similar sizes [13]. The present study focuses on the growth and viability of MSCs on patterned CNT arrays. The cell counts and viability experiments show that MSCs preferred non-collagen coated CNT surfaces which can have a significant impact in the design and preparation of advanced CNT-based scaffolds.

2. Materials

2.1. Synthesis and patterning of CNT arrays

The vertically aligned CNT arrays were grown by the alcohol catalyzed chemical vapor deposition (ACCVD) method on oxidized Si (100) surfaces. The catalyst layers were applied on the oxidized Si surface before the synthesis of CNTs. First, 10 nm layer of Al was evaporated on Si surface as the diffusion barrier, and then this was followed by the electron beam evaporation of 1 nm Co layer. Finally, Co catalyst layer was capped by another 0.5 nm thick Al layer. Si substrates with the aforementioned layers were introduced into the ACCVD furnace for the growth of CNT arrays through reduction and reaction steps. The reduction step was conducted at 625 °C for 15 min under flowing H_2 and Ar gases (20 sccm and 100 sccm, respectively). Then, the growth of CNT array was achieved through the reaction step in where ethanol was used as a carbon source under flowing H_2 and Ar gases (20 sccm and 100 sccm, respectively) for 30 min. Patterning was induced to the vertically aligned CNT arrays by using a dropper filled with deionized water. Following this step, some of the patterned CNT arrays were treated with 1 μg/μl sterilized collagen solution for every cm^2 (approximately 10:1 weight ratio of collagen to CNT) resulting in two separate groups of patterned CNT arrays: one non-coated and the other collagen coated. It has been shown in an earlier study that, a collagen/MWCNT composite scaffold was used for bone cells, and researchers observed improved cell attachment [13].

2.2. Isolation and culture of MSCs

MSCs were obtained from male 9-week old, 280–300 g Sprague–Dawley rats. The animals were permitted unlimited access to food and water at all times and were housed under controlled environmental conditions (22 °C) with a 12 h light and 12 h dark cycle in the animal holding facility of the Department of Molecular Biology and Genetics at the Bilkent University. This study protocol complied with Bilkent University's guidelines on humane care and use of laboratory animals. Bone marrow heterogeneous cell population was collected from the femur and tibia by flushing with a 5 ml syringe containing 10% fetal bovine serum (Hyclone) in Dulbecco's modified Eagle medium (Invitrogen) after the rats were sacrificed by cervical dislocation. The cells were cultured in plastic culture dishes with Mesencult Media (StemCell Technology) with 20% supplement (StemCell Technology) and 1% penicillin–streptomycin solution (Hyclone) in a 5% CO_2 incubator at 37 °C. Twenty-four hours after plating, media of the tissue culture plates were changed and the non-adherent cells were removed. Thereafter, their media were changed every 4 days, after washing with sterile 1× phosphate buffered saline (PBS) prior to the change. At the 14th day, 3×10^5 cells were seeded on CNT surfaces and stated as passage zero (P0) group. When MSCs became confluent, they were passaged for the second and third time and then they were seeded on patterned CNT surfaces. In the rest of the manuscript, these were stated as the first passage (P1) and the second passage (P2) groups, respectively.

3. Methods

3.1. Characterization of CNT arrays

Synthesized CNT arrays were displayed by scanning electron microscope (SEM; Carl Zeiss Evo 40) using variable vacuum mode around 40 kPa (under water vapor) and transmission electron microscope (TEM; JEOL 2100 F) using holey-carbon film supported grids. Raman analysis was carried out using a Jobin Yvon microscope with an Ar ion excitation laser (λ; 532 nm). A 50× microscope objective was used to focus the laser beam and to collect the scattered light. Atomic force microscope (AFM; Nanomagnetics) was utilized for the characterization of surfaces. The surface roughness of vertically aligned CNT arrays was calculated using 10 μm × 10 μm AFM image taken in the tapping mode.

3.2. Total RNA isolation and reverse transcription

MSCs were trypsinized and the total cellular RNA was isolated from the precipitate using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol with additional DNase treatment. The cDNAs were synthesized from the total RNA samples with the DyNAmo cDNA synthesis kit (Finnzymes) according to the manufacturer's protocol.

3.3. RT-PCR

cDNA amplification for *CD90*, *CD71*, *CD45*, *CD34*, *CD29* and *β-actin* were performed using DyNAzyme II (Finnzymes). The primers and product sizes were listed in Table 1. The initial denaturation step was at 95 °C for 5 min, followed by 30 (for *CD90* and *CD34*), 35 (for *CD71*), 26 (for *CD29* and *CD45*) and 25 (for *β-actin*) cycles of denaturation for 30 s for all genes at 94 °C, annealing for 30 s at 55 °C (for *CD90*, *CD34*), 60 s at 66 °C (for *CD71*), 45 s at 60 °C (for *β-actin*) and 30 s at 60 °C (for *CD29* and *CD45*), followed by extension for 30 s (for *CD90*, *CD34*, *DC29*, *CD45*), 40 s (for *β-actin*) and 45 s (for *CD71*) at 72 °C. A final extension at 72 °C for 5 min was applied to all the reactions.

3.4. Protein isolation and quantification

MSCs were scraped from the cell culture plates in 1× PBS and the precipitate was treated for 30 min on ice with a lysis buffer containing 0.05 M Tris–HCl, 1× protease inhibitor, 0.25 M sodium chloride and

Table 1
Primer sequences used in PCR amplification to characterize MSCs.

Gene	Sequence
CD90	(F) 5′-CCAGTCATCAGCATCACTCT-3′
	(R) 5′-AGCTTGTCTCTGATCACATT-3′
CD34	(F) 5′-TGTCTGCTCCTTGAATCT-3′
	(R) 5′-CCTGTGGGACTCCAACT-3′
CD71	(F) 5′-ATGGTTCGTACAGCAGCAGA-3′
	(R) 5′-CGAGCAGAATACAGCCATTG-3′
CD29	(F) 5′-ACTTCAGACTTCCGCATTGG-3′
	(R) 5′-GCTGCTGACCAACAAGTTCA-3′
CD45	(F) 5′-ATGTTATTGGGAGGGTGCAA-3′
	(R) 5′-AAAATGTAACGCGCTTCAGG-3′
β-actin	(F) 5′-CTGGCCTCACTGTCCACCTT-3′
	(R) 5′-GGGCCGGACTCATCGTACT-3′