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Modulation of the heterogeneous senescence of human mesenchymal stem cells on chemically-modified surfaces

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ABSTRACT

Human mesenchymal stem cells (hMSCs) are multipotent and have been recognized as a source for tissue engineering or cell therapy. It is, therefore, imperative to develop methods to acquire enough hMSCs that maintain self-renewal and differentiation potential. However, aged hMSCs are prone to have a gradual decline in differentiation and proliferation potential with continual cell cycle divisions during *in vitro* culture. The physiochemical properties of hMSCs are highly dependent on their micro-environment, i.e. the 'stem cell niche'. In this study, the heterogeneous aging of hMSC was examined on chemically defined self-assembly monolayer surfaces. Surface energy was shown to regulate aged hMSC morphology, survival, and proteoglycan expression. High surface energy supplied a preferable environment for hMSC survival and expression of proteoglycans. These results will prove valuable to the design of scaffolds for tissue engineering or for the modulation of implantation environments.

1. Introduction

hMSCs are self-renewed cells that can be differentiated into multi-lineages. These cells can be obtained from several tissues in an individual and has no ethical issues, unlike embryonic stem cells. Because of these benefits, hMSCs have recently been studied as potential sources for regenerative medicine. Several research and clinical reports have been published on potential therapeutic applications of hMSCs [1,2]. The level of hMSCs has been reported to be very low (0.01–0.001% [3] or a frequency of 1 in every 3.4×10^4 cells [4]). Thus, it's imperative to increase the number of hMSCs *in vitro* for uses in cell therapies or tissue engineering. However, the gradual decrease in telomere length or age-related changes in gene expressions after each division cycle causes cells to undergo a gradual senescence. The hMSC proliferation rate was also shown to decline with age [5]. In addition, late passage hMSCs displayed a decrease in differentiation potency [6]. Thus, to better understand the reason for such a decline in the overall quality of MSCs, there is a need to investigate hMSC aging during *in vitro* culture.

hMSCs occupy just a limited portion of the bone marrow, which contains several cells and extracellular matrix (ECM). The ECM is composed of collagen, fibronectin, and polysaccharides such as heparin sulfate, chondroitin sulfate, and hyaluronan [7]. Therefore, the behavior of MSCs is highly affected by the presence and interaction with these components [8]. Unlike cellular effects including endothelial cells, adipocytes or magakaryocytes, the effects of connective tissue proteins such as fibronectin or collagen, glycosaminoglycans and proteoglycans have rarely been studied although they are known to influence MSC proliferation, differentiation, and viability [9,10].

The ECM during *in vivo*/*vitro* cell culture exposes MSCs to their several chemical or mechanical cues such as stiffness, elasticity, charges, and morphologies. The properties of the ECM have been shown to affect self-renewal and differentiation of MSCs. There have been many studies that examined MSCs culture on scaffolds, including hydrogels [11], electrospun nanofiber mats [12] and sponges [13]. However, indefinite mechanochemical characteristics of ECM interfere with *in vitro*/*in vivo* studies of ECM's roles on MSCs. In addition, due to practical reasons, many studies have been performed in monolayer cultures of purified MSCs. Self-assembly monolayer (SAM) technology can deposit defined polymeric chains with functional end-groups on a flat surface. Therefore, SAM has

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Table 1
Surface energy comparison of self-assembly monolayer surfaces.

	Hydrophobic		Control	Hydrophilic		
	CF_3	CH_3	TCPS	Cl	SH	NH_2
Surface energy (mJ/m^2)[a]	14.9	23.6	45.8	52.7	51.2	50.8

[a] Calculated from contact angle measurements using distilled water and diiodomethane as a probe liquid and the geometric mean equation: $(1+\cos\theta)\gamma_{pl} = 2(\gamma_s^d\gamma_{pl}^d)^{1/2} + 2(\gamma_s^p\gamma_{pl}^p)^{1/2}$.

been widely used in cell biology studies to assess the effects of the chemico-mechanical properties on cellular biophysical behavior. Recently, stem cell studies have also utilized SAM as a substrate to culture MSCs [14–16].

Most MSC studies using SAMs have been done with early-passage cells. However, late-passage or long-term cultured MSCs on a defined surface must be investigated so that age-related changes or the behavior of long-living transplanted hMSCs could be better understood. In this study, senescence level of aged-hMSC was assessed and compared on SAM surfaces with each different chemical moiety and surface energy.

2. Materials and methods

2.1. Monolayer preparation and characterization

3-Chloropropyltriethoxysilane (Cl), mercaptopropyltrimethoxysilane (SH), 3-aminoprophyltriethoxysilnane (NH_3), octyltrichlorosilane (CH_3), and trichloro(1H,1H,2H,2H-perfluorioctyl)silane (CF_3) were purchased from Aldrich. The glass substrates were cleaned in piranha solution for 30 min at 100 °C and then washed with copious amounts of distilled water. Vacuum-dried reaction flasks were charged with anhydrous toluene and cleaned glass substrates were placed under argon. Solutions of the alkylsilanes (10 mM) were then added to the flask and left to self-assemble on the glasses for 1 h under argon atmosphere. In the case of trichloro(1H,1H,2H,2H-perfluorioctyl)silane, anhydrous hexane was used and the reaction time was 2 min. The treated glasses were rinsed with toluene (hexane) and ethanol several times and then baked in an oven at 120 °C for 15 min. The samples were cleaned by ultrasonication in toluene (hexane) and then rinsed thoroughly with ethanol, followed by vacuum drying. The surface wettability of the various SAM treated substrates was measured with a contact angle analyzer (S-EO Phoenix touch, Korea) using distilled water and diiodomethane as probe liquids. The surface energy of the samples was calculated using the geometric mean equation, $(1+\cos\theta)\gamma_{pl} = 2(\gamma_s{}^d\gamma_{pl}{}^d)^{1/2} + 2(\gamma_s{}^p\gamma_{pl}{}^p)^{1/2}$, where γ_s and γ_{pl} are the surface energies of the sample and the probe liquid, respectively, and the superscripts d and p refer to the dispersion and polar (nondispersion) components of the surface energy, respectively.

2.2. hMSC culture

MSCs were maintained in 25 cm^2 tissue culture flasks (Corning). Pre-warmed DMEM complete medium was used and cells were incubated at 37 °C in a 5% CO_2 incubator. The media contained Dulbecco's Modified Eagle Medium (DMEM, Invitrogen); 10% (v/v) FBS (Invitrogen); and antibiotic–antimycotic (Invitrogen). Every two day, the supernatant was aspirated off and the cells were rinsed once and incubated with fresh DMEM complete medium for cell maintenance. The cells were passaged whenever a confluency of greater than 90% was obtained. The cells washed with PBS were incubated with 0.5% trypsin (Hyclone) for 7–15 min at 37 °C in a 5% CO_2 incubator and then at least an equal volume of DMEM medium was added to inactivate the trypsin activity. Harvested cells were triturated, centrifuged, counted and passaged.

For experiments, aged hMSCs were obtained by continuous culture of hMSCs to a passage number of 17. The prepared aged hMSCs were seeded in a number of 1×10^4 on 6 different SAM glasses and the control plate, polystyrene-treated culture dishes (tissue culture polystyrene, TCPS), all of which have area of 1.96 cm^2. All cells were maintained by exchanging medium every two days.

2.3. Immunostaining

For immunostaining cultured hMSCs with heparin sulfate proteoglycan (HSPG) and chondroitin sulfate proteogycan (CSPG), MSCs were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature. The cells were then washed twice with ice cold phosphate-buffered saline (PBS) for 5 min and permeabilized using PBST (pH 7.2) for 30 min. Non-specific binding was prevented by incubation with PBST containing 1% (w/v) BSA for 2 h at 37 °C. The primary antibodies, monoclonal HSPG and CSPG (Abcam) were incubated in PBST with 1% BSA at 4 °C overnight. The cultures were washed with PBS three times for 10 min each and incubated with secondary antibodies (FITC 495 or 528, Texas Red 596 or 620, 1:200) in PBST for 1 h. Finally, the samples were washed with PBS three times for 5 min each in the dark.

2.4. Image analysis for cell counting

We have taken phase-contrast images at 10th and 20th day after cell-seeding with an inverted microscope (Micros Austria) equipped with a CCD camera (Lumenera, Canada). Cells on each SAM-treated glass were stained with DAPI and counted by using the image analysis program (Image J). Cell numbers of all samples obtained at 10th and 20th day were normalized relative to cell number of TCPS at 10th day. All experiments were performed three times and cell number data was expressed as a mean value with standard deviation.

2.5. Statistical analysis

One-way ANOVA was performed on cell number data, yielding each average and its standard deviation value with confidence level of P-value <0.05. The Student t-test was carried out for statistical significance.

3. Results and discussion

3.1. SAM surface characterization

Six different moieties were self-assembled on a glass cover slip and their surface energies were determined by contact angle measurements using the geometric mean equation Table 1. SAM surfaces were grouped into hydrophobic (CF_3 and CH_3) and hydrophilic (NH_2, SH and Cl) categories, which was based on TCPS.