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Biomaterials 26 (2005) 6185–6193
Biomaterials
www.elsevier.com/locate/biomaterials

The effect of RGD peptide-conjugated magnetite cationic liposomes on cell growth and cell sheet harvesting

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Received 21 December 2004; accepted 24 March 2005
Available online 17 May 2005

Abstract

Tissue engineering requires novel technologies for establishing 3D constructs, and the layered method of culturing cell sheets (cell sheet engineering) is one potentially useful approach. In the present study, we investigated whether coating the culture surface with RGD (Arg–Gly–Asp) peptide-conjugated magnetite cationic liposomes (RGD-MCLs) was able to facilitate cell growth, cell sheet construction and cell sheet harvest using magnetic force without enzymatic treatment. To promote cell attachment, an RGD-motif-containing peptide was coupled to the phospholipid of our original magnetite cationic liposomes (MCLs). The RGD-MCLs were added to a commercially available 24-well ultra-low-attachment plate the surface of which comprised a covalently bound hydrogel layer that was hydrophilic and neutrally charged. A magnet was placed on the underside of the well in order to attract the RGD-MCLs to the surface of the well, and then NIH/3T3 cells were seeded into the well. Cells adhered to the bottom of the culture surface, which was coated with RGD-MCLs, and the cells spread and proliferated to confluency. After incubation, the magnet was removed and the cells were detached from the bottom of the plates, forming a contiguous cell sheet. Because the sheets contained magnetite nanoparticles, they could be harvested using a magnet inserted into the well. These results suggest that this novel methodology using RGD-MCLs and magnetic force, which we have termed 'magnetic force-based tissue engineering (Mag-TE)', is a promising approach for tissue engineering.

Keywords: Magnetite nanoparticles; Liposomes; RGD peptide; Tissue engineering; Cell culture

1. Introduction

Tissue engineering is a promising technology for overcoming the organ transplantation crisis resulting from donor shortage. Currently, tissue engineering is based on seeding cells onto three-dimensional (3D) biodegradable scaffolds, which allow the cells to reform their original structure [1]. However, some problems remain with this approach, for example, insufficient cell migration into the scaffolds and inflammatory reactions due to scaffold biodegradation, and thus novel approaches for achieving 3D tissue-like constructs are desired. A major difficulty obstructing the fabrication of in vivo-like 3D constructs without the use of artificial 3D scaffolds is a lack of cell adherence in the vertical direction via cell–cell junctions. This non-adherence may be caused by enzymatic digestion of adhesive proteins. To overcome this, Okano and colleagues employed a thermo-responsive culture surface grafted to poly(*N*-isopropylacrylamide) (PIPAAm) [2–4]. Cells adhered to and proliferated on the thermo-responsive surface, as well as on tissue culture polystyrene dishes. Furthermore, confluent cells on the PIPAAm dishes were expelled as intact contiguous sheets by decreasing the temperature to below the lower critical solution temperature (LCST) of PIPAAm [5]. Kushida et al. recovered monolayer cell sheets from a surface grafted with PIPAAm and then deposited extracellular matrices

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doi:10.1016/j.biomaterials.2005.03.039

(ECMs); digestive enzymes were not used and ECMs remained to enhance cell–cell attachment [5]. These engineered cell sheets could be layered to construct multi-layered 3D cell sheets, and Okano et al. proposed the concept of 'cell sheet engineering' [4]. In the present study, we assessed a novel cell sheet engineering methodology using magnetic force and magnetite nanoparticles.

Magnetite nanoparticles are being used in an increasing number of biological and medical applications, including cell sorting [6–9]. We previously developed magnetite cationic liposomes (MCLs), which are cationic liposomes containing 10-nm magnetite nanoparticles, in order to improve accumulation of magnetite nanoparticles in target cells by taking advantage of the electrostatic interaction between MCLs (positively charged) and the cell membrane (negatively charged) [10–12]. In addition, we recently developed a tissue engineering technique using MCLs [13–15]. Our technique using MCLs consisted of the following processes: (1) MCLs were added to the target cells, which were expanded to the required cell number before applying to Mag-TE in order to magnetically label the cells through uptake of magnetite nanoparticles; (2) magnetically labeled cells were harvested and reseeded onto an ultra-low-attachment plate, the surface of which comprised a covalently bound hydrogel layer that was hydrophilic and neutrally charged, and a magnet was set at the underside of the well in order to attract and accumulate the cells, allowing the formation of 3D cell constructs; (3) the magnet was removed in order to detach the 3D cell constructs from the well; and (4) the 3D cell constructs were harvested using a magnet. We reported that magnetically labeled keratinocytes, which we accumulated using a magnet, resulted in a multi-layered 3D construct of keratinocytes [15]. Thus, we developed a novel methodology for tissue engineering using magnetite nanoparticles and magnetic force, which we designated 'Mag-TE'.

In the present study, we developed a new biomaterial and methodology for Mag-TE. The RGD (Arg–Gly–Asp) sequence, an integrin recognition motif found in fibronectin [16,17] and one of the most extensively studied cell adhesion peptides, was conjugated with magnetite cationic liposomes (RGD-MCLs). Here, we investigate the process of Mag-TE using RGD-MCLs.

2. Materials and methods

2.1. Cells and culture

Mouse NIH/3T3 fibroblasts were obtained from American Tissue Culture Collection. Cells were cultured at 37 °C under a humidified atmosphere of 5% CO_2 and 95% air in minimum essential medium (MEM) supplemented with 10% fetal calf serum, 10 mM non-essential amino acids, 0.1 mg/ml streptomycin sulfate and 100 U/ml potassium penicillin G.

2.2. Preparation of MCLs and RGD-MCLs

The magnetite (Fe_3O_4; average particle size, 10 nm) used as the core of the MCLs and RGD-MCLs was kindly donated by Toda Kogyo (Hiroshima, Japan). MCLs were prepared from colloidal magnetite and a lipid mixture consisting of *N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (TMAG), dilauroylphosphatidyl-choline (DLPC), and dioleoylphosphatidylethanolamine (DOPE) in a 1:2:2 molar ratio, as described previously [10]. For RGD-MCLs, a lipid mixture consisting of TMAG, DLPC, and dioleoylphosphatidylethanolamine-*N*-[3-(2-pyridyldithio)-propionate] (PDP-DOPE, Sigma Chemical Co., St. Louis, MO) in a 1:2:2 molar ratio dissolved in chloroform was dried down by evaporation for a minimum of 30 min. Lipids were hydrated by vortexing in colloidal magnetite nanoparticles and the liposomes were sonicated for 30 min (28 W). Covalent coupling of RGDC (Arg–Gly–Asp–Cys) peptides with liposomes was carried out according to the method of Gyongyossy-Issa et al. [18]. Briefly, 2 ml of liposomes was mixed with RGDC peptides (Peninsula Laboratories Inc., Belmont, CA) to give an approximate molar ratio of 0.55 peptide to 1 PDP-DOPE which, in the case of liposomes, translates to a molar ratio of 1.1 peptides to 1 outside surface of PDP-DOPE. Coupling was carried out using gentle agitation at room temperature for 3.5 h in 2-morpholinoethanesulfonic acid (MES) buffer (pH 5.0). Peptide and magnetite concentrations were measured using the ninhydrin analysis method [19] and the potassium thiocyanate method [20], respectively. The size of RGD-MCLs was measured using a dynamic light scattering spectrophotometer (FRAR 1000, Otsuka Electronics, Osaka, Japan).

2.3. Cell culture on surface coated with RGD-MCLs

Media containing RGD-MCLs or MCLs at the indicated concentrations were added to a 24-well ultra-low-attachment plate (Corning, NY), the surface of which comprised a covalently bound hydrogel layer that was hydrophilic and neutrally charged. A cylindrical neodymium magnet (diameter, 30 mm; height, 15 mm; magnetic induction, 4000 G) was then placed on the underside of the low-attachment plate in order to provide magnetic force vertical to the plate, thus inducing the surface of the well to be coated with RGD-MCLs or MCLs. Approximately 2×10^4 NIH/3T3 cells were then seeded into the wells, and were cultured for the indicated periods. Viable cell number was measured using the dye-exclusion method with