Biomaterials 30 (2009) 804–813

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The osteogenic differentiation of rat bone marrow stromal cells cultured with dexamethasone-loaded carboxymethylchitosan/poly(amidoamine) dendrimer nanoparticles

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ARTICLE INFO

Article history:
Received 1 September 2008
Accepted 21 October 2008
Available online 25 November 2008

Keywords:
Bone marrow stromal cells
Dexamethasone-loaded carboxymethylchitosan/poly(amidoamine) dendrimer nanoparticles
Hydroxyapatite
In vitro study
Starch–polycaprolactone
Osteogenic differentiation

ABSTRACT

There is an increasing interest in developing novel macromolecular vehicles for the intracellular and controlled delivery of bioactive molecules, since they can allow modulation of the cellular functions in a more effective manner *ex vivo*, and maintain the cellular phenotype *in vivo* upon re-implantation. The present study was designed to investigate the effect of combining novel dexamethasone-loaded carboxymethylchitosan/poly(amidoamine) dendrimer (Dex-loaded CMCht/PAMAM) nanoparticles and, both HA and SPCL scaffolds (3D system) on the proliferation and osteogenic differentiation of rat bone marrow stromal cells (RBMSCs) *in vitro*. A luminescent cell viability assay using RBMSCs was performed for screening cytotoxicity of the developed HA and SPCL scaffolds. Results corroborated previous ones which have demonstrated *in vitro*, the superior performance of the HA and SPCL scaffolds on supporting cells adhesion and proliferation. Furthermore, this work showed that RBMSCs seeded onto the surface of both HA and SPCL scaffolds differentiate into osteoblasts when cultured in the presence of 0.01 mg ml^{-1} Dex-loaded CMCht/PAMAM dendrimer nanoparticles. In addition, results demonstrated that Dex-loaded CMCht/PAMAM dendrimer nanoparticles combined with the HA enhance osteogenesis by increasing ALP activity and mineralization of the extra-cellular matrix. The pre-incubation of stem cells with these kinds of nanoparticles allows the delivery of Dex inside the cells and directly influences their cellular fate, being a promising new tool to be used in cells and tissue engineering strategies.

1. Introduction

Mesenchymal stem cells (MSCs) are a valuable therapeutic tool in tissue engineering (TE) [1], since they can proliferate and differentiate either *in vitro* or *in vivo* into a multitude of distinct cellular phenotypes [2–4]. It is well known that the process of stem cell maintenance and differentiation occurs *in vitro*, under optimal culture conditions [5,6]. For example, it has been shown that dexamethasone (Dex) delivered as a medium supplement promotes the osteogenic differentiation of marrow stromal cells [4,7]. Despite its use in clinical practice [8], Dex and other glucocorticoids have been restricted to few applications mainly due to adverse side-effects [9,10]. Therefore, the development of novel strategies that can stimulate stem cells to become osteoblasts *in vitro* and *in vivo*, and that provide a more effective treatment route with diminished complications is still regarded as a hot issue [11] that needs to be exploited further. In this context, the use of nanocarriers that possess high cellular uptake efficiency to deliver and target drugs can be seen as a possible and reliable solution [12]. Ultimately, these vehicles are expected to increase the solubility of drugs [13] and their bioavailability [14], and their delivery to the targets where they are required [15,16], thus suppressing harmful secondary effects on the patients during drug/cell-based treatments.

A huge portfolio of controlled-release vehicles is being investigated for their ability to process into nanocarriers [17,18], while several new emerging nanomaterials are being studied for their biodegradability [19,20] and for their controlled-release and stimuli–responsive properties [21,22]. In this context, we focus our present work on the surface engineering of poly(amidoamine)

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doi:10.1016/j.biomaterials.2008.10.024

(PAMAM) dendrimers with natural-based and biodegradable polymers such as the water-soluble carboxymethylchitosan (CMCht) [23]. Previously [24], we have clearly demonstrated that the CMCht/PAMAM dendrimer nanoparticles are efficiently internalized by different cell types. Since glucocorticoids bind to and activate a cytoplasmatic glucocorticoid receptor [25,26], our particular interest is in the loading of these novel macromolecular architectures with Dex in order to study their internalization and effects on the proliferation and osteogenic differentiation of stem cells, *in vitro* and *in vivo*.

In this work, we applied a TE strategy consisting of the combination of stem cells and either ceramic or polymeric scaffolds as a way to qualitatively and quantitatively evaluate their performance in supporting the osteogenic differentiation of rat bone marrow stromal cells (RBMSCs) exposed to the intracellular Dex-loaded CMCht/PAMAM dendrimer nanocarrier. The herein chosen scaffolds were the macroporous HA [27] and SPCL [28,29] scaffolds which have been found to exhibit a unique architecture and adequate physicochemical and biological properties to be used in bone related applications. However, the novelty of the present paper lies in deeper *in vitro* studies that illustrated the potential applications of combining nanoparticles and scaffolds for cells and tissue engineering. Therefore, we went a step further and reported on the osteogenic efficacy of this system, and how to make use of this potential in bone TE and regenerative medicine. *In vitro* studies were carried out in order to evaluate the viability of RBMSCs seeded onto the surface of the HA and SPCL scaffolds. In addition, RBMSCs-scaffold constructs' specimens were also examined using microscopic analyses to assess the cell adhesion and proliferation. Complementarily, scanning electron microscopy, LIVE/DEAD viability assay and DNA quantification were carried out after 14 d of culturing. Alizarin red and ALP stainings were also performed to qualitatively assess calcium deposition and ALP activity, which are parameters denoting osteogenic differentiation. Quantification of the alkaline phosphatase activity and osteocalcin content was carried out in order to evaluate the osteoblastic phenotype expression levels.

2. Materials and methods

2.1. Preparation of the hydroxyapatite (HA) and starch–polycaprolactone (SPCL) scaffolds

HA scaffolds were prepared by impregnating a polyurethane sacrifice sponge (PU, 5 mm diameter and 4 mm height, Eurospuma S.A., Portugal) with HA powders obtained as previously reported [27]. The elimination of the organic matrix consisted of burning the impregnated sponges in a furnace (Fornocerâmica-ATR 901, Portugal) at 900 °C for 24 h, then sintering at 1300 °C for 3 h.

Starch–polycaprolactone (SPCL) scaffolds were produced by adopting a two step procedure as previously reported elsewhere [28]. The material used was a blend of starch with approximately 70% by weight of poly-ε-caprolactone, SPCL. Briefly, fibres of SPCL were produced by melt spinning using a modular co-rotating twin screw extruder in two consecutive steps to a final draw ratio of approximately 1:100. Then, melt spun fibres were chopped. Fibre bundles were randomly displaced into the custom-designed mould cavities and subjected to thermal treatment at 60 °C for 30 min before compression and fibre bonding. SPCL scaffolds (5 mm diameter and 4 mm height) were obtained through cutting of fibre bonded meshes with a circular die. Standardized fabrication conditions enabled the production of scaffolds with 67.4 ± 1.3% porosity (as determined by micro-computed tomography). More details on SPCL scaffolds can be found elsewhere [30,31].

Prior to the cell culture studies HA and SPCL scaffolds were sterilized in an ethylene oxide gas atmosphere.

2.2. Synthesis of dexamethasone-loaded carboxymethylchitosan/poly(amidoamine) (Dex-loaded CMCht/PAMAM) dendrimer nanoparticles

Carboxymethylchitosan (CMCht) was synthesized by a chemical modification of chitin (Sigma, Germany) as described by Chen et al. [32]. Starburst® poly-(amidoamine)-carboxylic terminated dendrimers, PAMAM-CT (generation 1.5, 20% (w/v) methanolic solution) with an ethylenediamine core were purchased from Aldrich. CMCht/PAMAM dendrimer nanoparticles were prepared stepwise as previously reported [24]. Succinctly, Dex-loaded CMCht/PAMAM dendrimer nanoparticles were prepared by means of mixing CMCht/PAMAM dendrimer nanoparticles in an aqueous solution with a dexamethasone (Dex) solution with a final concentration of 5×10^{-4} M, under vigorous agitation. The mixture was then added to the precipitation media consisting of a saturated sodium carbonate (Aldrich, Germany) and acetone solution. Precipitates were collected and extensive dialysis (cellulose tubing, benzoylated for separating compounds with a cut-off of 1200 from Sigma, Germany) was carried out for 2 d. Dex-loaded CMCht/PAMAM dendrimer nanoparticles were obtained by freezing the solution at −80 °C and freeze-drying (Telstar-Cryodos-80, Spain).

2.3. Characterization of the HA and SPCL scaffolds, and Dex-loaded CMCht/PAMAM dendrimer nanoparticles

2.3.1. Surface topography characterization

The surface morphology and pore size of the HA scaffolds and SPCL scaffolds were examined under a scanning electron microscope, SEM (SM-300, Topcon Corporation, Tokyo, Japan). Prior to microstructure analysis, specimens were sputter coated with platinum using an Ion coater (IB-3, Eiko Engineering Ltd., Ibaraki, Japan) with a current set at 6 mA, for a coating time of 2–3 min.

2.3.2. Micro-computed tomography

The qualitative and quantitative information about the microstructure of the HA and SPCL scaffolds was obtained by micro-computed tomography, μ-CT 20 equipment (SCANCO Medicals, Switzerland). The X-ray scans were acquired in high resolution mode of 11 μm *x*/*y*/*z* [27]. Mimics® (Materialise, Belgium) was used as image processing software for CT reconstruction to create and visualize the 3D representations.

2.3.3. Transmission electron microscopy

The morphology of the Dex-loaded CMCht/PAMAM dendrimer nanoparticles was investigated by transmission electron microscopy, TEM (Philips CM-12, FEI Company, The Netherlands, equipped with a MEGA VIEW-II DOCU camera and Image Software Analyzer SIS NT DOCU). The nanoparticles were stained with 2% of phosphotungstic acid and placed on copper grids for observation.

2.4. In vitro cell culture studies

2.4.1. Isolation and culturing of rat bone marrow stromal cells (RBMSCs)

Seven-week-old Fischer 344/N male rats were purchased from Japan SLC Inc. (Shizuoka, Japan) and sacrificed in accordance with the Ethics Committee at the Tissue Engineering Research Center (Amagasaki, Japan). The epiphyseal regions of the femora were removed and marrow plugs in the femoral shafts were flushed out using Eagle's minimum essential medium (MEM, Nacalai Tesque, Japan) supplemented with 15% fetal bovine serum, FBS (JRH Biosciences, USA) and 1% antibiotic–antimycotic (Nacalai Tesque, Japan) solution. The RBMSCs isolation was performed under aseptic conditions. RBMSCs were transferred to a T75 cm^2 culture flask and expanded in the presence of complete MEM medium at 37 °C in a 5% CO_2 incubator. Then, the culture medium was changed every 2 or 3 d. After reaching confluency, the cells (passage 1, P1) were released from substratum with 1 ml of 0.05% trypsin–0.53 mM EDTA (Invitrogen, USA) and centrifuged at 900 rpm for 5 min. A cell suspension was prepared and cell concentration determined using an automatic cell counter (Cell Counter Sysmex F-520, Japan).

Viability of the RBMSCs was also analyzed with a NucleoCounter (ChemoMetec A/S, Denmark) as described elsewhere [33].

2.4.2. Cytotoxicity screening of the HA and SPCL scaffolds

A luminescent cell viability assay [24] based on the adenosine triphosphate (ATP) quantification was performed in order to evaluate the viability of RBMSCs seeded onto the surface of the HA and SPCL scaffolds. Prior to RBMSCs seeding, the HA and SPCL scaffolds were pretreated (de-airation) to prevent air bubble formation in the pores. Scaffolds were placed in 10 ml polystyrene tubes with ventilation caps. MEM medium was added and scaffolds were de-aired under vacuum using a 20 ml syringe with an attached 21G needle. Then, each scaffold was transferred into the respective well of a non-adherent 96-well tissue culture polystyrene (TCPS) plate. RBMSCs (P1) were seeded onto the surface of the HA and SPCL scaffolds at a cell density of 5×10^2 cells/scaffold, 1×10^3 cells/scaffold, 5×10^3 cells/scaffold and 1×10^4 cells/scaffold, and cultured in MEM medium under static conditions for 24 h, 3 and 7 d. After each time period, the ATP content which signals the presence of metabolically active cells was measured by means of performing a CellTiter-Glo® luminescent cell viability assay (Promega Corporation, USA). Luminescence was measured in a microplate reader (Wallac ARVOsx 1420, Perkin–Elmer Life and Analytical Sciences, USA), following the protocol provided by the supplier. All experiments were carried out 3 times using 3 replicates per experimental condition.

2.4.3. Assessment of proliferation and osteogenic differentiation of RBMSCs seeded onto the surface of the HA and SPCL scaffolds and cultured with Dex-loaded CMCht/PAMAM dendrimer nanoparticles

RBMSCs were isolated and expanded as mentioned above. HA and SPCL scaffolds were placed in each well of a 96-well TCPS plate. Then, 1×10^6 RBMSCs (P1) were added per each HA and SPCL scaffold, and the constructs were cultured in complete