Acta Biomaterialia 10 (2014) 486–493

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Effects of clodronate and alendronate on osteoclast and osteoblast co-cultures on silk–hydroxyapatite films

Rebecca S. Hayden, Moritz Vollrath, David L. Kaplan *

Department of Biomedical Engineering, Tufts University, 4 Colby St., Medford, MA 02155, USA

ARTICLE INFO

Article history:
Received 15 June 2013
Received in revised form 20 September 2013
Accepted 24 September 2013
Available online 1 October 2013

Keywords:
Osteoporosis
Co-culture
Bisphosphonates
Silk

ABSTRACT

The goal of this study was to explore the effects of osteoporosis-related therapeutics on bone remodeling in vitro. A previously established bone-tissue mimetic system consisting of silk protein biomaterials in combination with hydroxyapatite and human cells was used for the study. Silk–hydroxyapatite films were pre-complexed with the non-nitrogenous bisphosphonate clodronate or the nitrogenous bisphosphonate alendronate and cultured with THP-1 human acute monocytic leukemia cell line-derived osteoclasts, human mesenchymal stem cell derived osteoblasts or a direct co-culture of the two cell types. Metabolic activity, calcium deposition and alkaline phosphatase activity were assessed over 12 weeks, and reconstructed remodeled biomaterial surfaces were also evaluated for quantitative morphological changes. Increased metabolic activity and increased roughness were found on the clodronate-complexed biomaterial substrates remodeled by osteoblasts and co-cultures of osteoblasts with osteoclasts, even at doses high enough to cause a 90% decrease in osteoclast metabolic activity. Films complexed with low doses of alendronate resulted in increased metabolic activity and calcium deposition by osteoblasts, while higher doses were similarly toxic among osteoclasts, osteoblasts and co-cultures. These results point to the utility of these well-defined bone-mimetic in vitro cultures as useful screens for therapeutics for bone-related diseases, particularly with the ability to conduct studies for extended duration (here for 12 weeks) and with pre-complexed drugs to mimic conditions found in vivo.

1. Introduction

Bisphosphonates are the current standard of care for osteoporosis, but debate remains regarding which patients should be treated. Bisphosphonates have positive effects on bone mineral density, as well as reducing wrist and spine fractures, but prevention of hip fractures has not been as clearly demonstrated [1]. Rare but serious adverse events such as jaw necrosis and atypical femoral fractures are associated with bisphosphonate use, and the mechanisms for these events are not well understood [2,3]. The benefits and drawbacks of long-term bisphosphonate use are particularly poorly understood, which is problematic for a chronic condition such as osteoporosis [4–6].

Because of their strong affinity for calcium, bisphosphonates efficiently bind to bone upon ingestion, where they are eventually taken up by osteoclasts during bone remodeling, resulting in reduced bone resorption. The primary mechanism of action differs between the two classes of bisphosphonates. Non-nitrogenous bisphosphonates are metabolized by osteoclasts, resulting in toxic adenosine triphosphate analogs and subsequent osteoclast apoptosis [7]. Nitrogenous bisphosphonates result in reduced osteoclast activity and osteoclast apoptosis through inhibition of the enzyme farnesyl diphosphate synthase (FPPS), which inhibits protein prenylation and interferes with the ruffled border that osteoclasts must maintain in order to resorb bone [8].

While most studies have focused on the effects of bisphosphonates on osteoclasts, some studies have investigated their effects on osteoblasts and osteoblast-like cells [9–13]. In a variety of systems, including animal models of osteogenesis and those with primary human osteoblasts and osteoblasts derived from human mesenchymal stem cells (hMSCs), bisphosphonates promoted the proliferation, differentiation and activity of osteoblasts at low doses, and had the opposite effect at higher doses [14]. The mechanism for increased osteoblast survival was extracellular signal-related kinase (ERK) activation, and was independent of bisphosphonate class and osteoclast inhibition [15]. However, in vitro studies of bisphosphonates have generally been of short duration (less than 2 weeks), and bisphosphonates have routinely been administered in cell culture media. While some studies have investigated the effects of bisphosphonates incorporated into mineral substrates of hydroxyapatite or octacalcium phosphate on co-cultures of osteoblasts and osteoclasts for 1–2 weeks, the long-term sustainability and cellular effects of such systems

* Corresponding author. Tel.: +1 617 627 3251; fax: +1 617 627 3231.
E-mail address: david.kaplan@tufts.edu (D.L. Kaplan).

1742-7061/$ - see front matter
http://dx.doi.org/10.1016/j.actbio.2013.09.028

require further study [16–18]. The nature of osteoporosis as a chronic condition and bisphosphonates as agents with long half-lives in vivo particularly necessitate such long-term studies.

The objective of the present study was to develop and utilize an in vitro bone mimetic model to address the current minimal understanding of the effects of bisphosphonates on osteoblasts and other cell types in long-term culture. To address this objective, monocultures of bone marrow-derived hMSC osteoblasts and THP-1 acute monocytic leukemia cell-derived osteoclasts, as well as co-cultures of the two cell types, were maintained for 12 weeks on silk–hydroxyapatite (HA) biomaterial films with sequestered alendronate or clodronate. Standard measures of metabolic activity and differentiation were monitored throughout the experiment. Additionally, digital three-dimensional (3-D) images of remodeled film surfaces were reconstructed using surface metrology software and scanning electron microscopy (SEM) to quantify biomaterial remodeling (Fig. 1). This work points to the use of in vitro disease models for increased understanding of drug effects, here particularly focused on bone-related diseases in long-term culture, as well as appropriate sequestration of the drugs to provide more realistic systems to mimic physiological conditions.

2. Materials and methods

2.1. Cell culture

Unless otherwise noted, cell culture reagents were purchased from Life Technologies (Grand Island, NY). hMSCs were isolated from bone marrow aspirate (Lonza, Walkersville, MD) as described previously [19]. Briefly, aspirate from a male donor under 25 years old was combined with hMSC proliferation medium (MEM α with 10% fetal bovine serum (FBS), 1% antibiotic/antimycotic, 1% non-essential amino acids (NEAA)) and cultured at 37 °C with 5% CO_2 in a humidified environment. Flasks were rocked every day to allow hMSCs to adhere and medium was added every 3–4 days until hMSCs reached 80% confluence. hMSCs were used at passage 1 or 2. THP-1 cells (ATCC, Manassas, VA) were maintained in proliferation medium (RPMI 1640 supplemented with 10% FBS, 1% antibiotic/antimycotic, and 1% NEAA) prior to seeding. 15,000 cells cm^{-2} were seeded onto films (50% hMSCs and 50% THP-1 cells for co-cultures) in a 50 μl drop and incubated for 2 h to allow attachment. Following seeding, all cultures were maintained in the same medium, a half-and-half mixture of RPMI 1640 and MEM α supplemented with 10% FBS, 1% antibiotic/antimycotic, 1% NEAA, 100 nM dexamethasone (Sigma Aldrich, St Louis, MO), 10 mM B-glycerol phosphate (Sigma Aldrich, St Louis, MO), and 0.05 mM ascorbic acid (Sigma Aldrich, St Louis, MO) (for osteoblast differentiation, as described previously [20]), and 40 ng ml^{-1} phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, St Louis, MO) and 10 ng ml^{-1} receptor activator of nuclear factor kappa-B ligand (RANKL) (for osteoclast differentiation, as described previously [21]) with medium changes every 3–4 days.

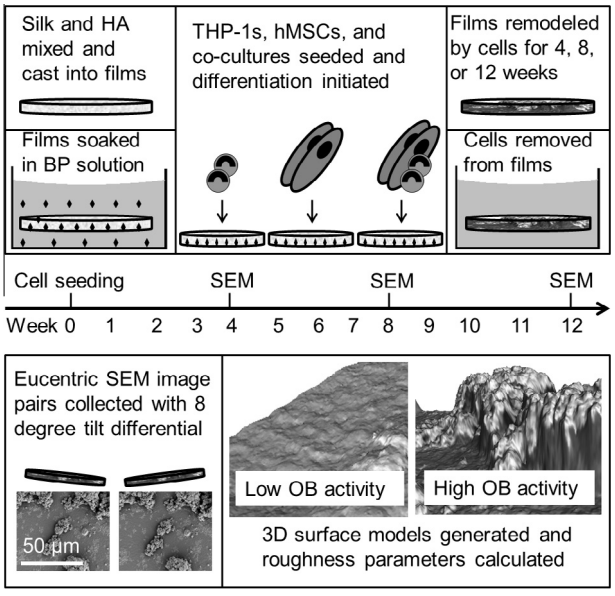

Fig. 1. Schematic of 12 week studies. Top: films were cast from a dispersion of HA in aqueous silk solution. Following drying and water annealing, films were soaked in solutions of clodronate or alendronate, which bound to the HA. Following autoclaving, films were seeded with hMSCs, THP-1s or a co-culture of the two cell types in equal number. Differentiation was then initiated, and films were remodeled by cells for 4, 8 or 12 weeks. For surface metrology analysis cells were removed from films by soaking in water overnight at 4 °C and films were dried and sputter coated. Bottom: eucentric SEM images were taken with an 8° difference in tilt angle. 3-D surface models were generated and roughness parameters were calculated. Example surfaces from low and high osteoblast activity (~600 μm^2) are shown.

2.2. Silk film preparation and drug loading

Aqueous silk solution was prepared as described previously [22]. Briefly, cocoons of *Bombyx mori* were cut to pieces ~1.5 cm^2 and boiled for 30 min in water containing 0.02 M Na_2CO_3, and then rinsed thoroughly with water to remove sericin. The remaining silk fibroin was then dried and dissolved in 9.3 M LiBr (Sigma Aldrich, St Louis, MO) solution at 60 °C for 4 h. This solution was dialyzed in distilled water using a Slide-A-Lyzer dialysis cassette (MWCO 3,500, Thermo Fisher Scientific, Rockford, IL) for 2 days, resulting in an 8% silk solution. Silk–HA films were prepared using a 5.0% (w/v) silk solution mixed with 5.47 mg ml^{-1} synthetic HA powder (Sigma Aldrich, St Louis, MO). For each film 100 μl of this freshly prepared dispersion was cast into a well in the lid of a 96 well plate. The silk–HA dispersion was mixed periodically to maintain a homogenous dispersion and the same HA content in each film. The films were covered and dried for 24 h at room temperature and then water annealed for 24 h using a desiccator as described previously [23]. The silk–HA films were then soaked in solutions of alendronate sodium trihydrate or clodronic acid disodium salt (Sigma Aldrich, St Louis, MO) for 48 h at 37 °C. Target quantities of drugs to be loaded on the silk–HA films were selected based on the literature, and pilot studies were carried out to determine the percentage of bisphosphonate that bound to the films. For the long-term cultures, higher targets were selected for osteoclast cultures and co-cultures, while lower targets were selected for mono-cultures of osteoblasts. Drug loading is reported as μg per silk–HA film (8 mm diameter). Following loading, films were sterilized by autoclaving and incubated overnight in medium prior to cell seeding.

2.3. Measurement of calcium release

Silk–HA films were incubated in phosphate buffered saline (PBS) at 37 °C. Every 24 h the films were transferred to fresh PBS and calcium release was quantified using the Stanbio (Boerne, TX) calcium cresolphthalein complexone assay according to the manufacturer's protocol. Absorbance was measured at 550 nm.

2.4. Quantification of bisphosphonate concentrations

Following soaking, the concentrations of bisphosphonates remaining in solution were measured to calculate the amount of drug sequestered on the films. Clodronate concentration was determined by taking 100 μl of the reserved soaking solution, adding 30 μl of 50 mM $FeCl_3$ solution in 2 M $HClO_4$, and measuring the