Acta Biomaterialia 20 (2015) 140–146

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Characterization of biomimetic calcium phosphate labeled with fluorescent dextran for quantification of osteoclastic activity

Salwa M. Maria [a,b], Christiane Prukner [c], Zeeshan Sheikh [a], Frank A. Müller [c], Svetlana V. Komarova [a,b], Jake E. Barralet [a,d,*]

[a] Faculty of Dentistry, McGill University, Canada
[b] Shriners Hospital for Children-Canada, Montreal, Canada
[c] Otto Schott Institute of Materials Research (OSIM), Friedrich Schiller University Jena, Löbdergraben 32, 07743 Jena, Germany
[d] Orthopaedics Division, Department of Surgery, Faculty of Medicine, Montreal General Hospital, McGill University, Canada

ARTICLE INFO

Article history:
Received 11 June 2014
Received in revised form 21 February 2015
Accepted 23 March 2015
Available online 28 March 2015

Keywords:
Osteoclast
Resorption
Calcium phosphate
Bone
Assay

ABSTRACT

Bone resorbing osteoclasts represent an important therapeutic target for diseases associated with bone and joint destruction, such as rheumatoid arthritis, periodontitis, and osteoporosis. The quantification of osteoclast resorptive activity in vitro is widely used for screening new anti-resorptive medications. The aim of this paper was to develop a simplified semi-automated method for the quantification of osteoclastic resorption using fluorescently labeled biomimetic mineral layers which can replace time intensive, often subjective and clearly non-sustainable use of translucent slices of tusks from vulnerable or endangered species such as the elephant. Osteoclasts were formed from RAW 264.7 mouse monocyte cell line using the pro-resorptive cytokine receptor activator of nuclear factor kappa-B ligand (RANKL). We confirmed that fluorescent labeling did not interfere with the biomimetic features of hydroxyapatite, and developed an automated method for quantifying osteoclastic resorption. Correlation between our assay and traditional manual measurement techniques was found to be very strong (R^2 = 0.99). In addition, we modified the technique to provide depth and volume data of the resorption pits by confocal imaging at defined depths. Thus, our method allows automatic quantification of total osteoclastic resorption as well as additional data not obtainable by the current tusk slice technique offering a better alternative for high throughput screening of potential antiresorptives.

1. Introduction

Osteoclasts are bone cells responsible for the resorption of the mineralized tissues in the normal bone remodeling process. The abnormal resorptive activity of osteoclasts occurring in serious disorders such as osteoporosis [1], rheumatoid arthritis [2–5], periodontitis [6] and cancer metastasis to bone [7]. Therefore, targeting osteoclastic resorption is an important therapeutic strategy. While a number of therapies including bisphosphonates [8] and anti-RANKL denosumab [9] are now successfully used to limit osteoclastic activity, intolerant and resistant cases necessitate the development of novel treatments. For this, the ability to rapidly screen the candidate chemical compounds is important.

We have previously validated a method of precipitating a thin layer of hydroxyapatite on tissue culture plates and glass coverslips [10] to study osteoclast resorptive activity [11]. Calcium phosphate substrates, including hydroxyapatite, can be fluorescently labeled, for example with calcein, which was used to quantify osteoblast mineralization [12], or fluoresceinamine labeled chondroitin polysulfate or calcein to study osteoclastic resorption [13].

Dextran is a nontoxic, water-soluble polysaccharide of a complex branched glucan, composed of many glucose molecules with variable chain lengths [14]. Dextran can be conjugated to a variety of fluorophores which expand the possibility of using these substrates for the fluorescence studies. Dextran has the ability to chemically bind to hydroxyapatite [15,16]. Dextran can also be internalized by a number of cells capable of endocytosis, including osteoclasts [17].

In this study we used dextran (10,000 Da), conjugated to Alexa Fluor488 or Rhodamine B to fluorescently label hydroxyapatite, and validated that the new fluorescent substrate is supportive of osteoclast formation and resorption and amenable for automated quantification.

* Corresponding author at: McGill University, Montreal, Quebec H3G IA6, Canada. Tel.: +1 514 398 3908.

http://dx.doi.org/10.1016/j.actbio.2015.03.026
1742-7061/

2. Materials and methods

2.1. Reagents

Chemicals used for mineral coating were purchased from Sigma and Fisher Scientific. Dulbecco's modification of Eagle's medium 1×, (DMEM), fetal bovine serum (FBS, 08050), penicillin–streptomycin (450-202-EL), sodium pyruvate (600110-EL) and 0.25% trypsin with 0.1% EDTA, (325-043-EL) were from Wisent Inc, Quebec, Canada. Fluorescein isothiocyanate-labeled-phalloidin (Phalloidin conjugate FITC) P5282, alendronate (A4978) were from Sigma–Aldrich, Ontario, Canada. Recombinant glutathione S-transferase-soluble RANKL was purified from the clones kindly provided by Dr. MF Manolson (University of Toronto, Canada) and freshly reconstituted before each experiment. Dextran Alexa Fluor® 488 (D-22910) and Rhodamine B (D-1824) of 10,000 Da were from Invitrogen, New York, USA. Lactate dehydrogenase activity assay kit^{plus} (LDH) was from Roche Applied Science, UK.

2.2. Hydroxyapatite coating

The following solutions were prepared freshly before each coating as described in detail in [10]. Briefly, 2.5-fold simulated body fluid (SBF) was prepared by mixing 50% Tris buffer (50 mM Tris base, pH = 7.4 with 1 M HC1), 25% calcium stock solution (25 mM $CaCl_2{\cdot}H_2O$, 1.37 M NaCl, 15 mM $MgCl_2{\cdot}6H_2O$ in Tris buffer, pH 7.4), and 25% phosphate stock solution (11.1 mM $Na_2HPO_4{\cdot}H_2O$, 42 mM $NaHCO_3$ in Tris buffer, pH = 7.4). Calcium phosphate solution (CPS) was prepared by first adding 41 ml HCl (1 M) to 800 ml MilliQ water, then dissolving 2.25 mM $Na_2HPO_4{\cdot}H_2O$, 4 mM $CaCl_2{\cdot}2H_2O$, 0.14 M NaCl and 50 mM Tris, then bringing pH to 7.4 and the volume to 1 L. The solutions were sterilized by filtration with a 0.22 μm MillexGV, Millipore. Glass coverslips or tissue culture plates were incubated with SBF (0.5 ml/well) for 3 days at room temperature. SBF was aspirated and CPS (0.5 ml/well) was added for 1 day at room temperature, aspirated and 70% ethanol was added and evaporated to sterilize the surface. The

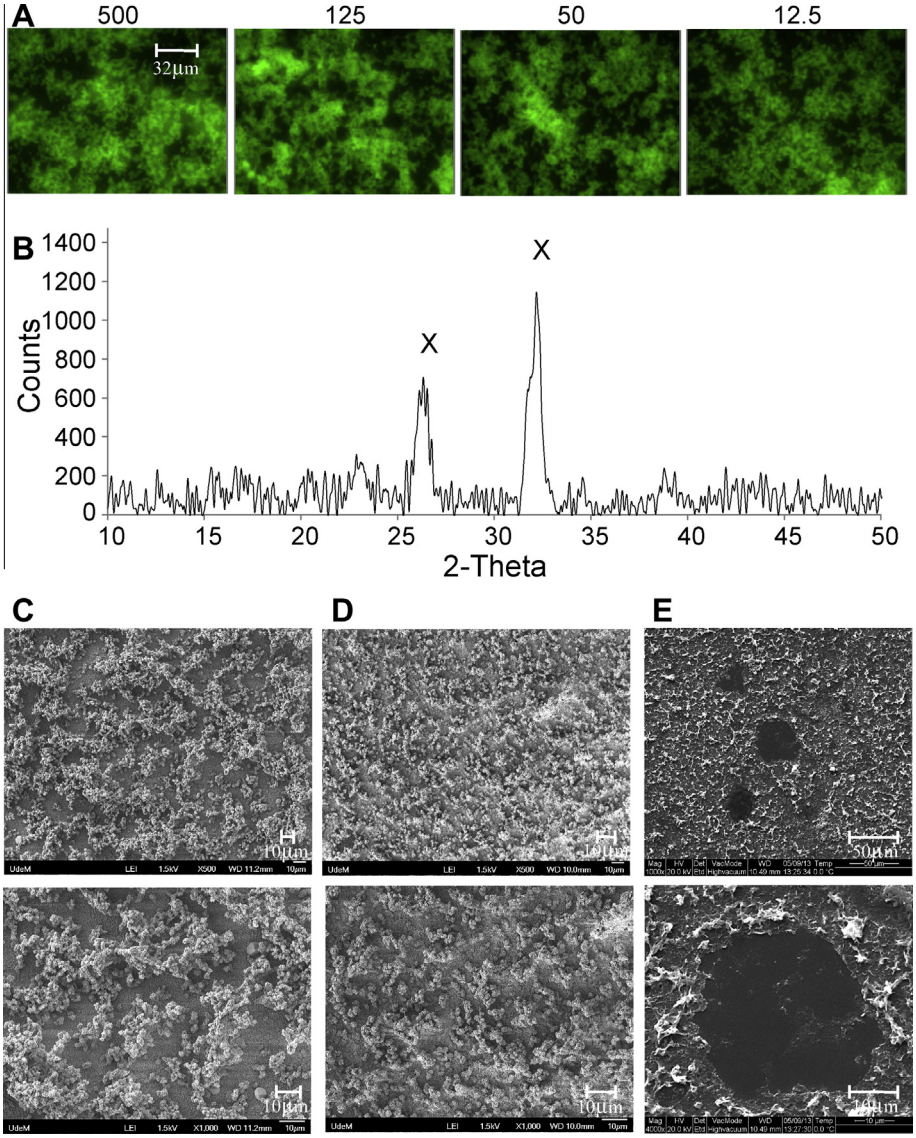

Fig. 1. Characterization of the fluorescent hydroxyapatite. (A) Representative images of hydroxyapatite surfaces labeled with 500, 125, 50, and 12.5 nM fluorescent dextran. Same exposure was used for all the images. (B) XRD patterns of the fluorescent dextran-labeled-calcium phosphate layer, crosses indicate standard hydroxyapatite peaks. (C–E) SEM images of fluorescent hydroxyapatite before and after culture, top and bottom rows are different magnifications of the same image. The fluorescent hydroxyapatite untreated; dry (C), the incubated fluorescent hydroxyapatite for 24 h with media with no cells (D), and with osteoclasts (E), then cells were removed, and resorption pits formed underneath osteoclasts were imaged.