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Culture of osteogenic cells from human alveolar bone: A useful source of alkaline phosphatase

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Abstract

The aim of this study was to obtain membrane-bound alkaline phosphatase from osteoblastic-like cells of human alveolar bone. Cells were obtained by enzymatic digestion and maintained in primary culture in osteogenic medium until subconfluence. First passage cells were cultured in the same medium and at 7, 14, and 21 days, total protein content, collagen content, and alkaline phosphatase activity were evaluated. Bone-like nodule formation was evaluated at 21 days. Cells in primary culture at day 14 were washed with Tris–HCl buffer, and used to extract the membrane-bound alkaline phosphatase. Cells expressed osteoblastic phenotype. The apparent optimum pH for PNPP hydrolysis by the enzyme was pH 10.0. This enzyme also hydrolyzes ATP, ADP, fructose-1-phosphate, fructose-6-phosphate, pyrophosphate and β-glycerophosphate. PNPPase activity was reduced by typical inhibitors of alkaline phosphatase. SDS-PAGE of membrane fraction showed a single band with activity of ∼120 kDa that could be solubilized by phospholipase C or Polidocanol.

Keywords: Alkaline phosphatase; Human alveolar bone; Cell culture; Osteogenic cells; Membrane solubilization; Kinetic data

1. Introduction

Biological calcification is a tightly regulated process in which different types of tissues, cells, organelles and biomolecules participate in the coordination and regulation of the metabolic events involved in accumulating large amounts of calcium phosphate (Anderson, 1995; Hsu and Anderson, 1995; Leone et al., 1997; Boyan et al., 2000). Understanding the role of each membrane component, such as membrane proteins, lipids, and carbohydrates will contribute to resolving the details of the calcification process (Anderson, 1995; Hsu and Anderson, 1995, 1996; Hsu et al., 1999, 2000; Kirsch and Claassen, 2000; Kirsch et al., 2000; Millán, 2006; Ciancaglini et al., 2006; Simão et al., 2007).

Bone marrow cells can be isolated, cultivated and induced to differentiate into cells involved in the calcification process, such as chondrocytes or osteoblasts (Phinney, 2002; Prockop et al., 2003; Osyczka and Leboy, 2005). Several studies have shown that the stages of differentiation to achieve the osteoblastic phenotype require the coordinated expression of many molecules (Cheng et al., 1996; Osyczka and Leboy, 2005). Additionally to the expression of collagen type I, osteopontin, bone sialoprotein and osteocalcin, high levels of tissue non-specific ecto-alkaline phosphatase, are also induced during the osteoblast differentiation for the mineralization process (Cheng et al., 1996; Osyczka and Leboy, 2005).

Histological and biochemical studies have shown that membrane of mineralizing cells or matrix vesicles (MV) are

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Nomenclature

ALP	alkaline phosphatase
AMPOL	2-amino-2-methyl-propan-1-ol
ATP	adenosine 5′-triphosphate
α-MEM	minimum essential medium
GPI	glycosylphosphatidylinositol
MV	matrix vesicles
PIPLC	specific phosphatidylinositol phospholipase C
PNPP	*p*-nitrophenyl phosphate
Polidocanol	polyoxyethylene-9-lauryl ether
PPi	pyrophosphate
SDS	sodium dodecylsulphate
TCA	trichloroacetic acid
TNAP	tissue non-specific alkaline phosphatase
Tris	tris hydroxymethyl-amino-methane

highly enriched in alkaline phosphatase (ALP), specially tissue non-specific alkaline phosphatase (TNAP), adenosine-5′-triphosphatase (ATPase), adenosine-5′-monophosphatase (AMPase), inorganic pyrophosphatase (PPiase) and other membrane associated enzymes (named nucleoside triphosphatase pyrophosphohydrolase, also called NTPPase, NPP1 or PC1) (Anderson, 1995; Hsu and Anderson, 1995, 1996; Anderson et al., 2004, 2005; Ciancaglini et al., 2006; Millán, 2006; Simão et al., 2007).

Alkaline phosphatase (E.C.3.1.3.1) from cartilage and bone is a phosphatidylinositol-anchored membrane ectoprotein in contact with extracellular cartilage fluid, in which natural putative substrates are present at nanomolar or micromolar concentrations (Matsuzawa and Anderson, 1971; Hunter et al., 1993; Pizauro et al., 1995; Millán, 2006; Simão et al., 2007; Garimella et al., 2006). In the phosphatidylinositol structure, a phosphatidylinositol-glycolipid anchor is covalently attached to the carboxyl terminus (C-terminus) of the protein through an amide linkage. This anchor structure of ALP results in lateral mobility in the membrane and allows the release of the protein from the membrane through the action of phospholipases (Harrison et al., 1995; Pizauro et al., 1995; Leone et al., 1997; Ciancaglini et al., 2006; Millán, 2006; Simão et al., 2007).

Osteogenic cells produce high levels of TNAP during the process of differentiation and therefore can provide a good source of this enzyme (Cheng et al., 1996; Osyczka and Leboy, 2005). Osteoblasts can be obtained from the periosteum, bone marrow, and bone explants (Breitbart et al., 1998; Krupnick et al., 2002; Xiao et al., 2003). Alveolar bone is one of the most active bones in human body and, accordingly, may be a useful site for harvesting bone cells in order to obtain TNAP (Xiao et al., 2003).

ALP is a glycosylated membrane-bound enzyme that catalyses the hydrolysis of phosphomonoester bonds and may also play a physiological role in the metabolism of phosphoethanolamine, inorganic pyrophosphate, and pyridoxal 5′-phosphate (Whyte, 1996). Besides providing inorganic phosphate, it has been suggested that ALP play a role in the degradation of pyrophosphate, a naturally occurring inhibitor of mineralization (Whyte, 1994). ALP is though to play a primary role in mineralization and, because it is present early in osteoblast development, has been proposed to be a progression factor in osteoblast differentiation (Aubin et al., 1993).

The best evidence that ALP actually is involved in bone mineralization has resulted from the study of the human hereditary disease hypophosphatasia (Whyte, 1996). These patients have subnormal serum levels of ALP, and the main clinical feature is defective bone mineralization, manifested as rickets or osteomalacia. More recently, two mouse ALP knockout models showed that, when the gene for ALP was specifically deleted, the mice had normal bone at birth but developed defects in bone mineralization thereafter (Waymire et al., 1995; Narisawa et al., 1997). Beck et al. (1998) showed that subbasal levels of ALP activity are sufficient to support mineralization in MC3T3-E1 cells. Using the same cell lineage, it was not observed bone-like nodule formation when ALP activity was inhibited (Sugawara et al., 2002). Despite these results, it is not clear if there is a positive correlation between the level of ALP activity and the amount of matrix mineralization.

The present study addresses an improved technique to obtain membrane-bound alkaline phosphatase from cultures of human alveolar bone derived-cells without the use of organic solvents, collagenase or another protease treatment, and the biochemical characterization of this membrane fraction is also described.

2. Materials and methods

2.1. Culture of osteogenic cells derived from human alveolar bone

Human alveolar bone fragments (explants) were obtained from healthy donors, using the research protocols approved by the Committee of Ethics in Research of the University of Sao Paulo for human tissue specimens. Osteogenic cells were obtained from these explants by enzymatic digestion using collagenase type II as described by Mailhot and Borke (1998). Alveolar bone explants were transferred into a sterile centrifuge tube and collagenase type II (Gibco, Grand Island, NY, USA) was added at a concentration of 1 mg/ml to start digestion and then placed in a 37 °C water bath under constant agitation. After 30 min of digestion, the supernatant was extracted and transferred to another centrifuge tube containing an equal amount of culture medium. Fresh collagenase was added to the remaining explants and the digestion process was repeated six times. The supernatant fractions numbers one and two were discarded and fractions numbers three to six were then centrifuged at $200 \times g$ for 5 min. The bone-derived cells and remaining explants were combined and cultured until subconfluence in α-minimum essential medium, supplemented with 10% fetal bovine serum, 50 μg/ml vancomycin, 20 μg/ml de ampicillin, 0.3 μg/ml fungizone, 10^{-7} M dexamethasone, 5 μg/ml ascorbic acid, and 7 mM β-glycerophosphate. Such culture conditions favored the development of the osteoblast phenotype (Rosa and Beloti, 2003; Coelho and Fernandes, 2000). During the culture period, cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air and the medium was changed every 3 or 4 days.

2.2. Characterization of osteogenic cells derived from human alveolar bone

At 7, 14 and 21 days total protein content, collagen content, and alkaline phosphatase activity were evaluated. Total protein content was measured according to Lowry et al. (1951) method and expressed as μg protein/10^4 cells.