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Ameloblastin expression and putative autoregulation in mesenchymal cells suggest a role in early bone formation and repair

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ABSTRACT

Ameloblastin is mainly known as a dental enamel protein, synthesized and secreted into developing enamel matrix by the enamel-forming ameloblasts. The function of ameloblastin in tooth development remains unclear, but it has been suggested to be involved in processes varying from regulating crystal growth to activity as a growth factor or partaking in cell signaling. Recent studies suggest that some enamel matrix proteins also might have important functions outside enamel formation. In this context ameloblastin has recently been reported to induce dentin and bone repair, as well as being present in the early bone and cartilage extracellular matrices during embryogenesis. However, what cells express ameloblastin in these tissues still remains unclear. Thus, the expression of ameloblastin was examined in cultured primary mesenchymal cells and *in vivo* during healing of bone defects in a "proof of concept" animal study. Real time RT-PCR analysis revealed human ameloblastin (AMBN) mRNA expression in human mesenchymal stem cells and primary osteoblasts and chondrocytes. Expression of AMBN mRNA was also confirmed in human CD34 positive cells and osteoclasts. Western and dot blot analysis of cell lysates and medium confirmed the expression and secretion of ameloblastin from mesenchymal stem cells, primary human osteoblasts and chondrocytes. Expression of ameloblastin was also detected in newly formed bone in experimental bone defects in adult rats. Together these findings suggest a role for this protein in early bone formation and repair.

Introduction

Formation of hard tissues generally follows a common scheme including mesenchymal cell recruitment, proliferation, differentiation and finally extracellular matrix secretion and biomineralization. Several of the involved matrix molecules, i.e. collagens and proteoglycans, are common to most hard tissues, regardless of their location or function. Enamel, on the other hand, is the only mineralized tissue formed by ectodermal cells in a highly specific process employing molecules such as amelogenin, enamelin and ameloblastin proteins to control the biomineral. The major role for these molecules is believed to be nucleating, templating and controlling hydroxyapatite crystal growth during enamel biomineralization. However, ameloblastin [1–3] and amelogenin [4–6] expression have also been detected in mesenchymal cells during embryogenesis or tissue repair, suggesting that at least some of the enamel matrix proteins also have roles outside enamel tissue and that these molecules could play a more general role during the formation of skeletal tissues. Ameloblastin, also known as amelin or sheathlin [7–9], is a well-conserved gene among species and present throughout evolution and ontogenesis of teeth [10]. It was originally identified as an enamel-specific protein [8,9,11,12] and its presence appeared to be critical for proper enamel prism formation [13,14]. Ameloblastin is suggested to be a two domain, intrinsically unstructured protein [15] that binds calcium [16] and is subject to intensive proteolysis by the matrix proteases enamelysin and kallikrein 4 [17,18].

Based to a large extent on localization by histochemical methods and transgenic experiments, ameloblastin has been proposed to be both a structural component of the enamel matrix, as well as functioning as a growth factor or signaling molecule during tooth growth [1–3,8,19–24]. The ameloblastin protein was first localized in ameloblast cells [7,11] that were shown to secrete it extracellularly when it accumulates into the sheath space between the enamel prisms of maturing enamel [25], as well as being expressed in

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Hertwig's root sheath cells during root formation [8]. Later, ameloblastin expression has been detected in early odontoblasts [1], in trauma-induced reparative dentin [2], and in osteoblast like cells during early stages of bone formation [3]. Genetic manipulations of ameloblastin show severe enamel hypoplasia [26] and reduced alveolar bone formation [27] in the $Ambn^{\Delta 5-6}$ mutant mouse model [27], and overproduction of ameloblastin in transgenic mice leads to formation of thinner and more porous enamel, with disrupted rod patterns and abnormal crystallites [28]. In the $Ambn^{\Delta 5-6}$ mutant mouse model, ameloblasts (and associated cell layers of the enamel organ) detach from the tooth surface as they enter the secretory stage. This directly or indirectly stops the typical differentiation sequence and terminates enamel formation [26]. The true function(s) of ameloblastin remains obscure, but the fact that ameloblastin expression is not restricted to enamel formation seems now to be well established. Recently, we reported that murine ameloblastin (Ambn) mRNA and protein are expressed during craniofacial bone development in rats at embryonic and early post-natal stages [3]. During intramembranous ossification, ameloblastin expression was detected in the superficial layer of the condensed vascularized connective tissue and in the cellular layer covering the surface of newly formed woven bone. In endochondral ossification, the protein was found in the extracellular matrix of the cartilage templates and in the perichondrium. Ameloblastin is expressed during ongoing growth in developmental and remodeling processes, however, the expression decreases at the end of the process, and is not detectable in mature bone [3].

The study presented here addresses the ubiquity of ameloblastin expression in precursor cells from blood (CD34+), mesenchymal stem cells, as well as primary human osteoblasts, chondrocytes and osteoclasts.

Materials and methods

Cell cultures

Human mesenchymal stem cells (MSC) were purchased from Cambrex Bio Science, Walkersville, MD, USA (BMC-1D) and cultured in Mesenchymal Stem Cell Growth Medium (MSCGM, Cambrex Bio Science). Bone marrow stem cells (BMSC) were isolated from bone marrow from the iliac crest from two different donors (BMSC). The mononuclear mesenchymal stem cells were isolated using Lymphoprep (AXIS-SHIELD, Oslo, Norway). The mononuclear cells isolated from the lymphoprep were subsequently cultured in MEM-α medium (Invitrogen Life Technologies, Grand Island, NY, USA) with 20% foetal calf serum (FCS; Biowest, Loire Valley, France), with 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Life Technologies).

Mesenchymal stem cells from human liposuction waste material from two different donors; Adipose derived adult stem cells (ADAS) were cultured in Dulbecco's Modified Eagle's Medium/ Nutrient Mixture F-12 Ham (1:1 v/v; Sigma-Aldrich, St.Louis, MO, USA) supplemented with 20% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin, and MSC were sorted by flow cytometry. The BMSC and ADAS cells were subcultured in MEM-α medium (PAA Laboratories, Pasching Austria) with 10% FCS (PAA) and 100 U/ml penicillin, 0,1 mg/ml streptomycin (Sigma) and Dulbecco's Modified Eagle's Medium (DMEM; PAA Laboratories with 10% FCS (PAA Laboratories), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma), respectively. The immunophenotype of the undifferentiated MSCs from bone marrow and adipose tissue were characterized by flow cytometry to be CD45 poor or negative, and CD10, CD13 and CD90 positive. Furthermore, their ability to differentiate in osteogenic and adipose directions, as well as clonogenic growth in a low density CFU-f (colony forming unit-fibroblast) assay was tested [29,30].

Human peripheral mononuclear cells (PBMC) were isolated from blood by Ficoll-Hypaque gradient centrifugation (Lymphoprep; Nyegaard, Norway), and CD34+ cells were positively selected using anti-CD34 coated magnetic beads (Dynal, Oslo, Norway) as described by Smeland et al. [31]. Osteoclasts (Ocl) were differentiated from human PBMC by adding macrophage-colony-stimulating factor (M-CSF) and receptor for activation of NF-κB (RANK) ligand to the medium (50 ng/ml each). The cells were stained for tartrate resistant acid phosphatase (TRAP) activity using naphtol AS-BI phosphate and Fast Garnet in the presence of sodium tartrate, as described by the manufacturer (Sigma). Cells that were TRAP positive and contained three or more nuclei were counted as osteoclasts. All donor recruitment and cell sampling were performed in accordance with a protocol approved by the local ethical committee, and made anonymous according to the rules of the Norwegian bio-bank laws and health register authorities.

Commercially available human primary osteoblasts (NHO) from tibia (NHOst cell system; Cambrex Bio Science) were grown in Osteoblast Growth Media (OGM) (Cambrex Bio Science). Osteoblasts cultured to facilitate differentiation were exposed to hydrocortisone hemisuccinate (200 nM) and β-glycerophosphate (10 mM) (Cambrex Bio Science) in the OGM medium. Commercially available human primary chondrocytes (NHAC) from knee joints were purchased from two different companies (hCa, Cell Applications, CA, San Diego, USA and NHAC-kn, Cambrex Bio Science, respectively). These cells were grown in Chondrocyte Growth Medium (Cell Applications and Cambrex Bio Science, respectively). Chondrocyte Differentiation Medium (CDM, containing FBS 5%, gentamicin sulfate-amphotericin B 0.1%, TGFβ-1 0.5%, R3-IGF-1 0.2%, insulin 0.2%, transferrin 0.2% and ascorbic acid 2.5%) was added to chondrocyte cell cultures to facilitate differentiation.

Human dental pulp cells (Dominon Pharmakine, Derio, Spain) and human periodontal ligament (PDL) cells (ATCC, Manassas, VA, USA) were cultured in DMEM (PAA Laboratories) supplemented with 10% FCS (PAA Laboratories), 100 U/ml penicillin and 0,1 mg/ ml streptomycin (Sigma). Dental pulp cells have previously been found to express AMBN [32] and were used as positive controls.

The mouse enamel organ epithelial cell line, LS8, is an immortalized ameloblast-like cell line that expresses enamel-specific genes such as Ambn [33–35], was grown in DMEM (PAA Laboratories), 10% FCS (PAA Laboratories), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma).

The mouse osteoblastic cell line MC3T3-E1 (No ACC 210) was obtained from Deutsche Sammlung von Microorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and maintained in α-MEM (PAA Laboratories) containing 20 mM HEPES, 10% FCS (PAA Laboratories), and 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma).

To maintain cell viability half of the medium was changed twice weekly in the stem cell cultures, whereas the entire medium was changed every second day in the other cell cultures. The cells were grown in a humidified 95% air, 5% CO^2 atmosphere at 37 °C throughout the experiments.

mRNA isolation

The cells were harvested 3, 12, 24, 48, 72 or 168 hours after confluence, and lysed in lysis/binding buffer (100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, pH 8.0, 0.5 mM dithiothreitol (DTT), and 1% sodium dodecyl sulfate (SDS). mRNA was isolated using magnetic beads (oligo (dT)25 as described by the manufacturer (Dynal AS, Oslo, Norway). Beads containing mRNA were re-suspended in 10 mM Tris-HCl, pH 8.0, and stored at -70 °C until use. One microliter of the mRNA-containing solution was applied directly to obtain a first-strand complementary DNA (cDNA) using the iScript cDNA Synthesis Kit which contains both oligo (dT) and random hexamer primers (BioRad, Hercules, CA, USA).

Real-time reverse transcriptase –PCR

Real time RT-PCR reactions were performed and monitored using iCycler iQ (BioRad). The 2X iQ SYBR Green Supermix was based on