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Innate immunity in human bone

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Abstract

Bone has exceptional regenerative properties. Oral bone appears to be particularly resistant to infection despite exposure to oral flora, even in circumstances such as oral surgery where the thin mucosal layer covering the bone is disrupted. The goal of this study was to determine whether the innate immune system of antimicrobial peptides exists inside bone.

Biopsies of non-infected and chronically infected mandibular bone were harvested from patients during maxillofacial surgical procedures. Bone biopsies from the iliac crest and fibula served as controls. Immunohistochemical staining was performed, directed against the human beta-defensin antimicrobial peptides (hBD) -1, -2 and -3. In addition, cultures of osteoblast-like cells were examined for the presence of each of the three beta-defensins and their mRNA transcripts.

All three human beta-defensins were detected within the mineralized bone matrix of chronically infected mandibular bone in the vicinity of the endosteum and osteocytes. hBD-1, -2 and -3 were also found in the cytoplasm of osteocytes. Expression of all three beta-defensins was detected in each of the non-infected bone types including the controls, however, to a lesser degree than that found in the chronically infected mandibular bone. This may reflect upregulation of antimicrobial peptide expression in the presence of chronic infection. Cultures of non-infected osteoblast-like cells were found to express mRNA for each of hBD-1, -2 and -3. Immunohistochemical staining of the cultures was positive for hBD-1 and -2, but not for hBD-3.

We provide the first evidence of a previously unrecognized innate immunological function of bone through the demonstration of the presence of the human beta-defensins hBD-1, -2 and -3 in bone.

Keywords: Antimicrobial peptides; Bone; Human beta defensins; hBD-1; hBD-2; hBD-3; Innate immunity; Endogenous antibiotics; Infection

Introduction

Bone is a highly adaptive tissue with exceptional regenerative properties. This permits procedures such as artificial joint replacement surgery, the setting of percutaneous osteosynthesis wires and the transplantation of large quantities of bone without a vascular pedicle [1–3].

We postulated that the success of these procedures requires that bone contributes to its immunological defense. Serious infections do occur occasionally as a consequence of these surgical procedures and prophylactic antibiotic cover is often required [4]. Our experience in procedures involving oral bone demonstrates that antibiotic cover is only infrequently required: in many circumstances including intraoral bone transplantation or even simple tooth extraction, oral bone is exposed to bacterial flora. Oral flora normally includes a wide spectrum of aerobic and anaerobic microorganisms separated from oral bone by only a thin mucosal layer over the alveolar crest. Despite this, infections of oral bone are rarely seen, even without prophylactic antibiotic cover in the course of intraoral surgery [3]. This observation led to our hypothesis that osteocytes in oral bone may be protected against infection through the expression and/or induction of antimicrobial peptides.

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Table 1
Primers used for PCR, their sequences, annealing temperatures and base pair fragments

Antimicrobial peptide	Primer sequence	Annealing temp [°C]	Base pair fragments
hBD-1	AGT-CGC-CAT-GAG-AAC-T ACA-GGT-GCC-TTG-AAT-TT	55	184
hBD-2	GAG-GGT-CTT-GTA-TCT-CCT CCT-TTC-TGA-ATC-CGC-ATC-A	60	235
hBD-3	AGC-CTA-GCA-GCT-ATG-AGG-ATC CTT-CGG-CAG-CAT-TTT-CGG-CCA	68	206

Antimicrobial peptides have been described to be primarily responsible for protecting against invasion by pathogenic microorganisms on intact epithelial surfaces [5]. They provide an additional layer of defense for the innate immune system.

Antimicrobial peptides were first reported and sequenced in 1981 and have since been described on epithelial cells of the skin and mucosa of humans and in a wide range of animal and plant species [6–11]. This includes the human beta-defensins, hBD-1, -2, and -3, which have been described on a variety of epithelial surfaces [12–15]. Neutrophils also utilize antimicrobial peptide weaponry in their role as a cellular regiment of the innate immune system [16]. As of November 1, 2004, 880 antimicrobial peptides, all of eukaryotic origin have been listed in a database in Trieste (http://www.bbcm.units.it/~tossi/pag1.htm).

The mechanism of action of antimicrobial peptides is still unclear; however, it is believed that many peptides operate by disrupting the integrity of bacterial membranes [5,6,17,18]. Such a system of antimicrobial peptide-based defense would be ideal for oral bone to ward off commensal or pathogenic flora and could explain its resistance to infection under normal healthy conditions.

Upregulation and induction of local expression of antimicrobial peptides in epithelial tissue have been demonstrated in response to contact with microorganisms [13] and specifically in response to interleukins (IL)-1α, IL-1β [13,19] and interferon-γ [13,14]. There is evidence showing that the process of induction occurs through Toll-like receptors −2 and −4 [20–24]. This mechanism may permit future therapeutic exploitation of antimicrobial peptides in bone surgery to reduce the use of standard antibiotics.

Despite being described on epithelial surfaces, neutrophils and Paneth cells [26–28], to our knowledge, no studies have yet examined whether human bone expresses antimicrobial peptides. The aim of this study was to examine whether the antimicrobial peptides human beta-defensins-1, -2 and -3 are found in human oral bone. The study is subdivided into 2 parts. Part A addresses the expression of antimicrobial peptide and their mRNA transcripts in cell cultures obtained from oral bone. Part B investigates the presence of antimicrobial peptides in histologic sections harvested from oral bone and compared to skeletal bone from other regions.

Material and methods

Part A: cell cultures from oral bone

Oral tissue samples for cell cultures

This study was approved by the Ethics Committee of the University of Kiel, Germany (Approval: AZ 309/00).

Ten biopsies of non-infected mandible cortical bone were obtained from a total of 10 different patients during routine surgical wisdom tooth extractions. Cortical bone chips were harvested (approximately 10 mm^3) from the retromolar region (wisdom tooth area). In order to prevent the simultaneous harvesting of gingival cells, the entire periosteum with any associated soft tissue was separated from the alveolar crest prior to the harvesting of bone. At the same time, biopsies from clinically non-inflamed gingival tissue (n = 10) and non-inflamed mucosa (n = 10) (each approximately 10 mm^3) were obtained from the same region before wound closure and served as controls.

Oral bone cell cultures

Human bone cells were isolated from oral cortical bone biopsies in the following manner. First, loosely adherent non-osseus tissue was mechanically removed manually under light microscopy. This was followed by extensive washing in phosphate-buffered saline (PBS) (pH 7.4). Thus prepared, the bone fragments were seeded into tissue culture flasks (50 ml Falcon tubes with filter; Sarstedt, Nümbrecht, Germany) and cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Culture medium was Dulbecco's modified eagle's medium (DMEM; Biochrom KG, Seromed, Berlin, Germany) supplemented with 10% fetal calf serum, 10^5 I.U./l penicillin, 100 mg/l streptomycin, 2.5 mg/l amphotericin B, 2 mM L-glutamine and 1 mM L-ascorbic acid 2-phosphate (Sigma, Deisenhofen, Germany). In addition, the cultures were supplemented with 100 nM/l dexamethasone (Biochrome, Berlin, Germany). Every 3–4 days, the culture media was exchanged and filtered (Filter pore size 0.2 mm) under sterile conditions and the cell cultures were inspected under a light microscope. Each new culture medium contained the same supplements as described above.

Cells were subcultured in a second passage at a density of $3.3 \times 10^6/cm^2$ after 14 days. For the second passage, a cell scraper was used to bring the cells into suspension. After a further 14 days cultivation, 6 ml of suspension was aspirated for RNA extraction and cDNA synthesis.

Additionally, small fragments of gingiva and oral mucosa were transferred into tissue culture wells in order to grow and isolate keratinocytes and fibroblasts. Oral keratinocytes but not oral fibroblasts express hBD-1, -2 and -3 as previously reported by our group [30,31]. As our laboratory standard, these two cell types serve as a control for detection of antimicrobial peptides in oral tissues [30,31].

Alkaline phosphatase (ALP) activity test and osteocalcin (OC) synthesis test in bone cell cultures

ALP and OC are markers associated with bone mineralization and their detection indicates the presence of osteoblast-like cells [32,33].

Osteoblast-like cells (6.0×10^5/ml) retrieved from the bone cell-cultures were seeded at a density of 1×10^3 cells/cm^2 in Lab/Tek chamber-slides (Nunc,

Table 2
Summary of test results of cell cultures (n = 10 patients)

Oral tissue cell cultures	hBD-1	hBD-2	hBD-3	ALP	OC immunohistochemistry	OC PCR
Osteoblast-like cells	+	+	+	+	+	+
Keratinocytes (control 1)	+	+	+	−	−	−
Fibroblasts (control 2)	−	−	−	−	−	−

Osteoblast-like cells were characterized and tested positive for mRNA transcripts of hBD-1, -2 and -3 (hBD = human beta-defensin, OC = osteocalcin, ALP = alkaline phosphatase activity).