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Research paper

The viability and spatial distribution of osteocytes in the human labyrinthine capsule: A quantitative study using vector-based stereology☆

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ABSTRACT

Background: Bone remodeling is highly inhibited around the labyrinthine space, most likely by the action of the anti-resorptive cytokine osteoprotegerin (OPG). Inner ear OPG may enter the bony otic capsule through the lacuno-canalicular porosity (LCP). The aim of the study was to investigate the patency of this extracellular signaling pathway by exploring the viability, age dependency and spatial distribution of capsular osteocytes.

Methods: Sixty-five bulk-stained undecalcified human temporal bones were selected to span the ages from 30th gestational week to 95 years. Osteocytes within 1000 μm wide iso-concentric perilabyrinthine zones of bone were identified by 3-D vector calculations and the number of cells per unit of bone volume estimated within each zone by optical dissectors.

Results: From a high initial numerical density and a centripetal distribution of viable osteocytes, the density declined over time. This effect was higher towards the inner ear space and shifted viable osteocytes into to a centrifugal distribution with age. Contrary to this, non-viable osteocytes accumulated centripetally around the inner ear space and accounted for 50% of all lacunae at 80 years of age. Non-viable osteocytes were heterogeneously distributed forming islets of varying size surrounded by the intact and viable parts of the LCP.

Conclusion: The simultaneous presence of high numbers of non-viable osteocytes within a dense network of viable osteocytes is unique for the bony otic capsule. Viable osteocytes may sustain a life-long anti-resorptive signaling pathway for inner ear OPG. Clustering of non-viable osteocytes may locally impede the effect of OPG leaving the ghost regions unprotected against focal bone remodeling, as in human otosclerosis.

1. Introduction

To understand the pathogenesis underlying impaired otic capsular fracture healing and otosclerosis, we must first recognize the unique kinetics of perilabyrinthine bone and understand how it differs from the rest of the skeleton.

The bony otic capsule forms a separate functional unit in which bone remodeling is progressively inhibited towards the labyrinthine space. From a sub-normal rate of 0.1% per year in perilabyrinthine bone, the bone turnover rate increase centrifugally towards a habitual level of 10% per year at the capsular periphery (Frisch et al., 1998, 2000). The candidate inhibitor of capsular bone resorption is the decoy receptor osteoprotegerin (OPG), which opposes perilabyrinthine osteoclast maturation and activation in competition with osteoblastic RANKL (receptor activator of nuclear factor-kappaB ligand) for the osteoclastic RANK receptor (Zehnder et al., 2005).

OPG knockout mice develop excessive capsular bone remodeling, stapes fixation and progressive hearing loss (Zehnder et al., 2006; Kanzaki et al., 2006). OPG is expressed in high levels inside the spiral ligament (1600 × normal bone levels) and the inner ear space (800 × normal bone levels) (Zehnder et al., 2005). OPG is assumed to enter the bony otic capsule through intercellular gaps of the inner ear lining and diffuse towards the capsular periphery via the lacuno-canalicular porosity (Zehnder et al., 2005; Sørensen et al., 2006). This extracellular signaling pathway is a branching fluid filled space accommodating the osteocytes and their connecting processes, and forms an "osseous functional network" involved in cellular metabolic traffic and signaling activity (Knothe Tate, 2003). The patency of the osseus functional network depends crucially on the viability of individual osteocytes (Holmbeck et al.,

Abbreviations: LCP, lacuno-canalicular porosity; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-kappaB ligand; RANK, receptor activator nuclear-kappaB; CAST, computer-assisted stereological toolbox.

☆ The work was done at Otopathological Laboratory, Department of Otorhinolaryngology Head and Neck Surgery, Rigshospitalet, University of Copenhagen, Denmark.

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2005; Inoue et al., 2006). On the other hand, bone cells and bone matrix are expected to deteriorate prematurely when these components are not constantly replenished by normal levels of bone remodeling (Sørensen, 1994).

The aim of the present study was to quantify the spatial distribution, viability and volume density of cochlear osteocytes to explore how the effect of ageing may affect the patency of the osseus functional network and the propagation of an endogenous OPG signal.

2. Materials and methods

2.1. Materials

Sixty-five human temporal bones procured during routine autopsies were selected to span the ages from 30th gestational week to 95 years of age. The temporal bones were divided into groups of 0–19 years ($n = 15$), 20–39 years ($n = 13$), 40–59 years ($n = 12$), 60–79 years ($n = 13$) and 80–99 years of age ($n = 12$). The series included 35 males and 20 females. No history of ear disease was indicated *a priori*.

2.2. Tissue preparation

Fresh tissue blocks of undecalcified human temporal bones were bulk-stained by immersion in 62% ethanol with 1% basic fushsin (Certistain fuchsin, Merck) for 2–4 months and embedded in methyl methacrylate. Thereafter, fiducial markers were created by vertical drilling, and the tissue blocks were sectioned horizontally with an Accutom-2 milling machine (Struers, Copenhagen, Denmark) into 50–80 μm thick sections. This produced 20–35 sections per specimen. The sections were slide-mounted, coverslipped and studied without any further staining.

2.3. Viability criteria

To evaluate the viability of osteocytes, we applied the same basic criteria as Frost (1969). (1) Osteocyte lacuna containing a living cell showed obvious recognition of basic fuchsin in a well-defined star-shaped lacuna. (2) Osteocyte lacuna containing no viable cell showed a lack of basic fuchsin inside the lacuna, or/a smooth lacuna with no canaliculi (which may occasionally contain some cellular debris).

2.4. Stereological setup

The computer-assisted setup consisted of an Olympus BX50 light microscope (Olympus, Japan) equipped with a motorized *X*–*Y* stage (Märzhäuser, Germany), a microcator device for accurate measurement of stage movement in the z-axis (Heidenheim, Germany), and a digital camera (ColorView II, Olympus, Japan) connected to a pc fitted with a Cintiq15x touch screen (Wacom, Saitama, Japan) and CAST-software (Visiopharm, Hørsholm, Denmark).

2.5. Counting osteocytes with the optical dissector

The numerical density of osteocytes was counted by an optical dissector, which is a "virtual" 3-D counting probe, based on optical sectioning through a thick section by the creation of focal planes with a thin depth-of-field, using an oil-immersion 100× objective. The volume of the 3-D counting probe is defined by the area of an

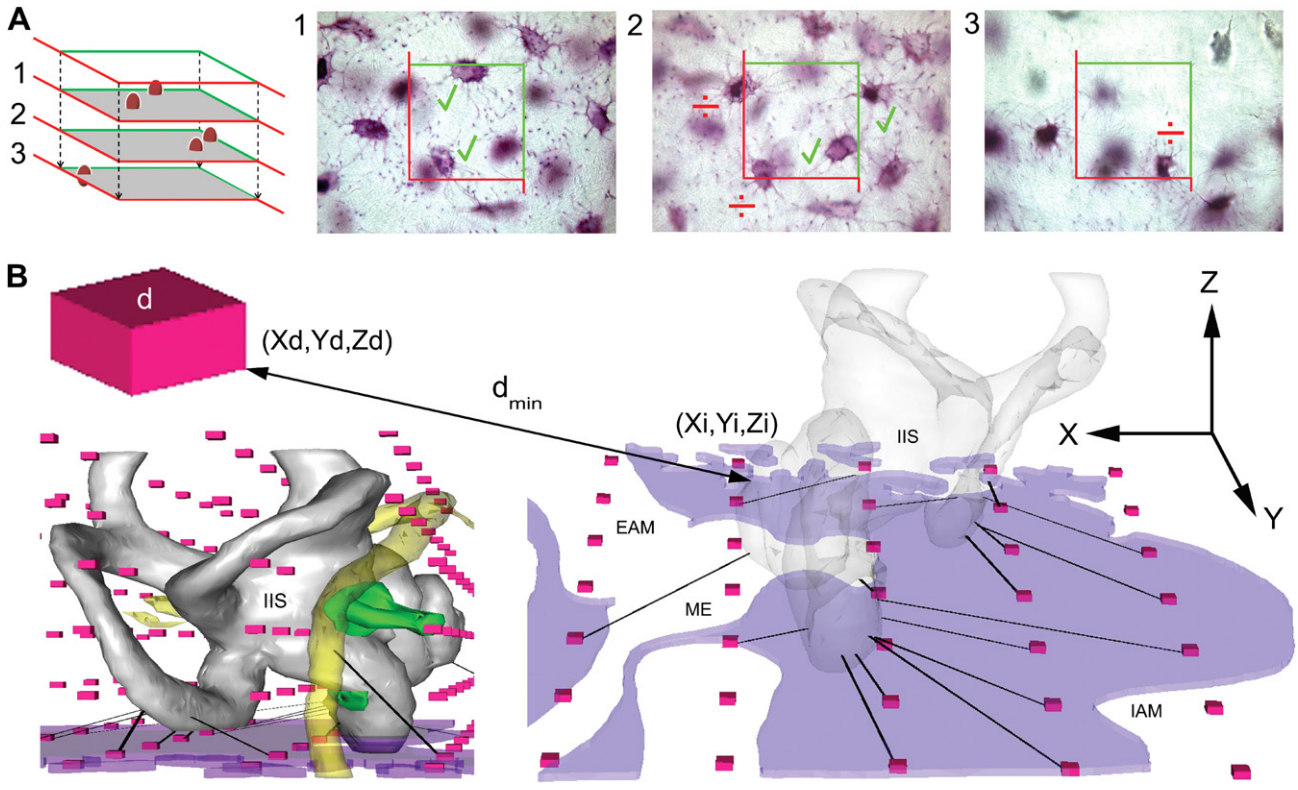

Fig. 1. (A) Osteocytes were counted within virtual 3-D dissector probes by optical scanning through thick bulk-stained sections. When an osteocyte or empty lacuna came into focus (1, 2 and 3), it was counted if it was inside the counting frame or touching the inclusion lines (green) and excluded if it was outside the counting frame or touching the exclusion lines (red). (B) The 3-D dissector probes were uniformly distributed over the total section area at random. By obtaining the spatial position of each dissector (d) probe (X_d, Y_d, Z_d) and the coordinate of the nearest inner (i) ear space boundary profile (X_i, Y_i, Z_i), the shortest distance (d_{min}) from each dissector probe to the inner ear space was estimated by: $d_{min} = [(X_i - X_d)^2 + (Y_i - Y_d)^2 + (Z_i - Z_d)^2]^{1/2}$. The average density of osteocytes at a given distance to the inner ear space was estimated from the multiple spatial density counts. IIS, inner ear space; EAM, external auditory meatus; ME, middle ear; IAM, internal auditory meatus.