



the REGISTRY

Newsletter of the NIDCD National Temporal Bone, Hearing and Balance Pathology Resource Registry

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The REGISTRY is published semiannually by the NIDCD National Temporal Bone, Hearing and Balance Pathology Resource Registry. The Registry was established in 1992 by the National Institute on Deafness and Other Communication Disorders (NIDCD) of the National Institutes of Health to continue and expand upon the former National Temporal Bone Banks (NTBB) Program. The Registry promotes research on hearing and balance disorders and serves as a resource for the public and the scientific community about research on the pathology of the human auditory and vestibular systems.

LACUNO-CANALICULAR PATHWAYS AND BARRIERS IN PERILABYRINTHINE BONE

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INTRODUCTION

The bony otic capsule forms a unique functional unit in which the spatial organization of postcartilaginous growth, modeling and remodeling suggest the existence of an inner ear mechanism that inhibits perilabyrinthine bone resorption *in vivo* (1,2,3). Recent studies suggest that a high ratio of the cytokines OPG (osteoprotegerin) to RANKL (receptor activator of nuclear factor kappa B ligand) competing for the osteoclastic RANK receptor is responsible for this effect, and that OPG produced in high levels in inner ear epithelia diffuses through the peri-osteocytic space of the lacuno-canalicular system into the surrounding bone (4).

Ultrastructural observations have identified large defects in the layer of cochlear bone lining cells, which expose a significant 15% of the inner periosteal bone surface to the OPG-rich perilymph (5) and provide an access route to the lacuno-canalicular system and its osteocytic syncytium (4). Cellular distortion and tissue disruptions are well known artifacts of the inner ear ultra structure, which are minimized by normal-pressure intravital perfusion with glutaraldehyde (GA) in combination with oxygenated fluorocarbon (6,7). For this reason cochlear bone lining cells and capsular osteocytes were re-examined in rats processed with this technique.

Fatigue microdamage (MDx) appears in bone tissue in response to physiological mechanical loading. MDx is constantly removed by bone remodeling and may accumulate when the remodeling rate is low. MDx is identified in undecalcified bone after bulk staining with 1% basic fuchsin in ethanol, which stains intravital cracks but not the mineralized bone matrix or artifactual cracks. Since MDx is expected to accumulate in perilabyrinthine bone, we have processed undecalcified human temporal bones with a modified bulk staining technique (8). In addition to MDx, the lacuno-canalicular system, the osteocytic syncytium and capsular vascular canals are clearly stained, and this material was used to study the canalicular network as well.

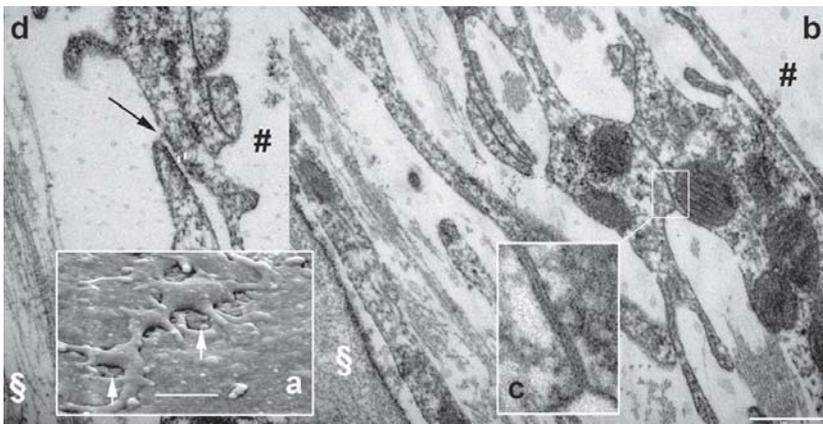
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MATERIALS AND METHODS

Six male Wistar rats (Pan:WIST) were fixed by vascular perfusion with 2% GA and 13.3% oxygenated fluorocarbon in 0.05 M sodium phosphate buffer (pH 7.4) at a steady pressure of 100 mm Hg as described in detail previously (6). Briefly, following induction of surgical anesthesia, the trachea was cannulated to simplify adequate artificial ventilation. A self-retaining cannula was inserted into the left ventricle of the heart and clamped tightly. The cannula consisted of two barrels: the outer barrel was connected to a peristaltic pump which delivered the fixative, the inner barrel was connected to a pressure transducer that monitored the perfusion pressure, directing the pump to deliver the perfusate at a steady pressure of 100 mm Hg. The caudal caval vein was severed immediately before the perfusion was initiated. Following vascular perfusion-fixation, the temporal bones were isolated, the bullae opened, followed by removal of the stapes and severing of the round window membrane.

After three rinses in 0.15 M sodium cacodylate buffer (pH 7.4), the specimens for scanning electron microscopy (SEM) were processed by the osmium-thiocarbohydrazide (OTOTO) as described in detail previously (7). After a rinse in distilled water, the specimens were dehydrated to 100% ethanol according to standard procedures and were critical point dried (Balzers CPD 030, Lichtenstein) with CO₂. Subsequently, the specimens were mounted on stubs using colloidal silver as an adhesive, sputter coated with chromium (Edwards, Xenosput XE200, England) and investigated with a Philips FEG30 scanning electron microscope operated at 1–5 kV.

Fig. 1. a) Cochlear lining in SEM. Surface cavities – arrows. Bar = 2µm b) Multilayer of lining cells and fibrillar material in TEM, between # - perilymphatic space and § - mineralized bone matrix. Bar = 500nm c) Gap junction d) 20 nm wide gap between lining cells – arrow



For transmission electron microscopy (TEM), the specimens were demineralized in 4.13% EDTA with 1% glutaraldehyde in water for 6 weeks, postfixed in 1% OsO₄ in 0.12 M cacodylate buffer (pH 7.4) for 2 hours, subsequently dehydrated in graded series of ethanol, transferred to propylene oxide and finally infiltrated and embedded in Epon. Ultrathin sections were stained with lead acetate and/or uranyl acetate and examined and photographed in a Philips CM 100 electron microscope operated at an accelerating voltage of 80 kV. Human temporal bones removed at autopsy with a Schuknecht trephine were bulk stained by immersion in 62% ethanol with 1% basic fuchsin (Certistain fuchsin, Merck) for 2-4 months. Infiltration was enhanced by the use of vacuum and low-temperature microwave treatment. After dehydration in graded ethanols and defatting in ether-acetone 1:1, the bones were embedded undecalcified in methyl methacrylate (BDH, England) and sectioned horizontally at 50-150µm with an Accutom-2 milling machine (Struers, Denmark) or a KDG95 microslicer (BioScan BV, The Netherlands) as described previously (8). Selected sections representing various subject ages were studied with ordinary LM and with a SteREO Lumar V12 fluorescence microscope (Zeiss, Germany) equipped with a UV filter BP365/12 LP397 and a GFP filter BP470/40 BP525/50.

RESULTS

In the SEM, a continuous cell layer lined the inner periosteal capsule facing the cochlear spaces. A few artifactual linear tears were found. The surface displayed rounded cellular protrusions superimposed on a pattern of interdigitations between adjoining cells, where numerous rounded cavities 2-4µm wide and ½-1µm deep were seen. Inside these cavities, the exposed cell surfaces were clearly more granular, but no microfibrillar extracellular matrix was present.

In the TEM, the cochlear bone lining cells formed a continuous lining, which ranged from a single layer of flat cells to several layers of more irregular cells connected by large gap-junctions and occasionally separated by intercellular gaps of up to 20nm. A microfibrillar extracellular material was found between lining cells and around the capsular osteocytes inside the lacuno-canalicular space. No empty osteocytic lacunae were seen (Fig.1).

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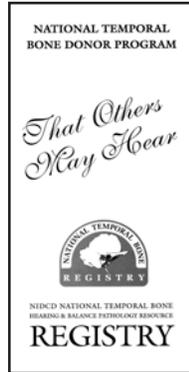
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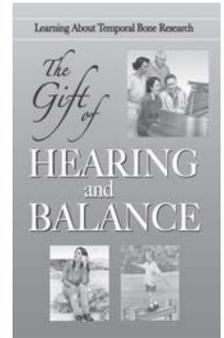
**Brochures about Temporal Bone Research and Donation
Order Free-of-Charge for Your Office, Clinic or Organization**

The NIDCD National Temporal Bone, Hearing and Balance Pathology Resource Registry, which is dedicated to promoting research on hearing and balance disorders through the study of temporal bones, has published two informational brochures, which you may request for display in your office and/or waiting rooms. Both brochures encourage individuals with hearing or balance disorders to bequeath their temporal bones to scientific research.



That Others May Hear is a short form brochure which describes briefly the functions of the Registry, and answers commonly asked questions regarding the temporal bone donation process. (Dimensions: 9" x 4")

The Gift of Hearing and Balance: Learning about Temporal Bone Donation is a 16-page, full-color booklet which describes in more detail and with diagrams, the structure of the ear, types of auditory disorders, the microscopic



study of the temporal bone, and the benefits of temporal bone research. It also answers commonly asked questions regarding the temporal bone donation process. (Dimensions: 7" x 10")

If you are willing to display either or both of these brochures, please complete the form below and return it to the Registry by mail or fax. The brochures will be sent to you **free of charge**.

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Or submit your request using email: tbregistry@meei.harvard.edu

In the SteREO Lumar, the lacuno-canalicular system and the vascular spaces were seen as a dark blue 3-dimensional web suspended in the transparent mineralized bone matrix. Stained cells were clearly evident inside the majority of lacunae in bone tissues outside the otic capsule regardless of subject age. The otic capsular lacuno-canalicular system was clearly connected to inner periosteal lining cells of the inner ear spaces. In subjects younger than 16 years, the entire capsular canalicular network was intact and all the capsular lacunae contained

stained cellular material. In adult specimens, large areas of perilyabyrinthine bone contained very few if any stained osteocytes. The stained osteocytes in these areas generally displayed only one or two processes but all the vascular canals were stained. Otic capsular MDx was found only in adult bones, and with a clear relation to the acellular areas. The 3-D findings could not be photo documented at this time – the present images were captured in visible light with an Olympus BX50 at 40x magnification (Fig. 2).

DISCUSSION

The existence of large areas on the cochlear bony wall in which a cellular lining is missing and inner periosteal mineralized matrix is directly exposed to perilymph is not supported by the present observations. More likely, the structures found inside the rounded intercellular cavities represent lining cells or osteocytes, which were caught in the process of cellular differentiation and shedding from the superficial layer during ultimate apposition. This impression should be addressed by re-embedding the SEM specimens for sectioning and TEM of the cavities in a plane perpendicular to the surface.

Recent EM studies confirm the presence of a pericellular space surrounding the osteocyte process (9). This space contains extracellular fluid in a highly structured proteoglycan-rich fibrillar matrix that tethers the cell inside the lacuno-canalicular space (9), and forms a molecular sieve, which provides an interstitial pathway for fluid flow and diffusion of molecules smaller than 6nm, such as horseradish peroxidase, MW 44kD, introduced *in vivo* through an intact endosteal lining after iv injection (10).

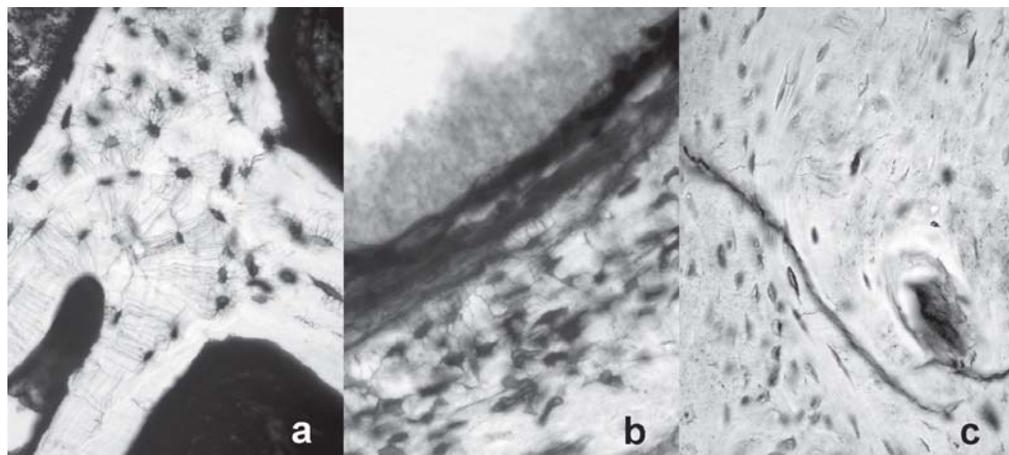


Fig. 2. Bulk stained human bone, subject age 69 years, basic fuchsin, LM at 40x. a) Osteocytes in lamellar mastoid bone b) Osteocytes in cochlear inner periosteal bone c) Pericochlear endochondral bone with a vascular canal, an MDx and osteocyte deficiency

With a MW of 20kD, OPG may well diffuse from the cochlea through lining cell gaps 20nm wide into the perilyabyrinthine lacuno-canalicular system as recently suggested (4) even without cellular defects to expose the surface of capsular inner periosteal bone matrix directly to the inner ear fluid compartments. Evidently, basic fuchsin with a MW of 300D is able to diffuse easily via the lacuno-canalicular as well as the vascular system but fails to penetrate the mineralized matrix, except when MDx is present. This demonstrates the extension of the capsular lacuno-canalicular system from the inner periosteal lining throughout the entire volume of the bony otic capsule in young individuals as a possible route for OPG mediated restriction of perilyabyrinthine bone remodeling. The findings of adult perilyabyrinthine bone areas devoid of stained lacunae and canaliculi confirm previous observations on devitalized capsular bone (11,12). However, since all the vascular canals are stained even in these areas, vascular obstruction cannot account for the missing lacunar stain. The preservation of a patent lacuno-canalicular porosity may depend on constant trimming of the surrounding mineralized matrix by cellular enzymatic activity (13). With increasing age, capsular osteocyte degeneration or death by apoptosis may cancel this activity and cause the obstruction of the lacunae and canaliculi despite vascular sufficiency. Studies on human bone biopsy and autopsy materials indicate that bone remodeling (14) and MDx (15,16) increase in relation to osteocyte deficiency. Animal studies indicate that the lacuno-canalicular patency and osteocyte viability suffer in response to experimentally induced MDx (17,18), but

the general mechanism that links osteocyte death, MDx and bone remodeling together is unknown at present.

Normally, capsular bone remodeling is low (2), possibly due to the inhibition of osteoclasts by OPG produced by inner ear tissues and distributed via the lacuno-canalicular system (4). Gap junctions connecting the capsular bone cells, which have been reported previously (5), may offer another perilabyrinthine signaling pathway by wiring up the capsular osteocytic syncytium for signal transduction through the intercellular exchange of small molecules or the propagation of transmembranous calcium fluxes (19). In adult perilabyrinthine bone, MDx as well as empty lacunae are abundant compared to other bone tissues, a condition normally in favor of enhanced bone remodeling. Certainly, MDx and osteocyte deficiency may well obstruct a perilabyrinthine signaling pathway through the lacuno-canalicular porosity or via the osteocytic syncytium, interfere with OPG mediated inhibition of resorption and consequently have a role in pathological capsular bone remodeling.

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LABORATORY SPOTLIGHT

Scientific study of the human temporal bone and related brain tissue is a time-consuming process performed in highly specialized otopathology laboratories by researchers who are dedicated to enhancing our understanding of the pathology underlying disorders of hearing and balance. "Laboratory Spotlight" is a continuing series of articles offering a glimpse inside the laboratories in the United States and abroad conducting temporal bone research.

Temporal Bone Research at the Otopathological Laboratory at Rigshospitalet and the Panum Institute at the University of Copenhagen, Denmark (1955 - 2005)

Mads Sølvsten Sørensen, M.D. and Klaus Qvortrup, M.D.

The Otopathological Laboratory at Rigshospitalet was established in 1955 by Professor H. K. Kristensen. M. Balslev Jørgensen headed the laboratory from 1965-1992, with Professor P. Bretlau becoming Co-Director in 1985. The collection currently contains 674 decalcified temporal bones serially sectioned in a horizontal plane at 22 microns, including 95 fetal bones, 76 specimens from children aged 0-2 years, 49 specimens from children aged 2-18 years and 454 adult temporal bones. In the majority of the material, every tenth section is stained with hematoxylin and eosin. The remaining 90% of the sections are stored unstained in ethanol. Medical records in Danish are available for most cases, including auditory and vestibular test data for many cases. The entire collection is registered in an Access® database.

The collection contains cases of bacterial infections, otosclerosis and osteogenesis imperfecta, vestibular schwannoma and carcinoma involving the temporal bone, and sensorineural disorders such as deafness from ototoxic drugs, Ménière's disease, dysplasia, thalidomide deafness and Pendred's syndrome. Based on this collection, N. H. Buch, M. B. Jørgensen and E. Hentzer studied middle- and inner-ear histology in the fetus and the newborn, otopathology in diabetes mellitus and in chronic otitis media. M. B. Jørgensen and P. Bretlau studied the light microscopic histology of otosclerosis in 80 specimens from the collection, and the ultrastructure of otosclerosis on separate material in collaboration with L. Chevance and J. Causse.

The histopathology of otosclerosis has remained a major field of interest of our laboratory. Since otosclerosis represents pathological remodeling of the bony otic capsule, it was decided to investigate the process of bone remodelling in non otosclerotic nor-

mal temporal bones to establish a contemporary basis for pathogenetic considerations. Beginning in 1988, M. S. Sorensen prepared undecalcified temporal bone specimens from rats, domestic pigs, dogs, rabbits and monkeys labelled *in vivo* with tissue time markers of a variety of osteofluorochromes as well as undecalcified human temporal bones labelled accidentally *in vivo* with tetracycline during antibacterial treatment. These studies revealed a morphology of otic capsular bone remodelling, modelling and repair which was essentially similar to that of any other compact bone tissue. Histomorphometric studies with CAST® software confirmed the general impression of many otopathologists of an unusually low rate of capsular bone turnover. By pooling the observations from a large number of sections, the exact rate of capsular bone remodelling was calculated by T. Frisch in 2000. Moreover, a unique pattern of perilyabyrinthine modelling and remodelling emerged, in which bone resorption was apparently highly restricted around the inner ear spaces, while gradually increasing to normal levels towards the capsular periphery. Following additional *in vitro* studies on the resorption of capsular- and other bony specimens by isolated osteoclasts, it was proposed that the unique restriction and spatial distribution observed in perilyabyrinthine bone dynamics might result from inhibition of resorption by a local inner ear mechanism. Recent studies by others on the RANK/RANKL/OPG system of the ear have identified a major candidate for such a signalling pathway, presenting the first truly local control factor in perilyabyrinthine bone dynamics, a pathogenetic "missing link" and a new avenue of research in otosclerosis.

Our collection currently includes more than 200 undecalcified animal temporal bones time labelled *in vivo*, and 340 undecalcified human temporal bones bulk



Prof. Poul Bretlau M.D., Prof. Harald K. Kristensen M.D., Klaus Qvortrup M.D., Thomas Frisch M.D., Mads Sølvsten Sørensen M.D. and Morten Friis M.D. in the Otopathological Laboratory (left to right).

stained with basic fuchsin for ongoing studies of intravital micro cracks, which are expected to accumulate when the remodeling rate is low. We use a KDG95 microslicer or an LKB base sledge cryomicrotome (now Leica CM3600) for undecalcified processing. Light microscopy is conducted with an Olympus BX50 microscope fitted with an automatic Mertzhäuser object table/ Colorview II digital camera/ CAST® software and a Zeiss PALM laser capture microscope. We are currently focusing on the genetics and biochemistry of inner ear signalling pathways as well as the histological and ultrastructural basis for signal transduction between the inner ear and the otic capsule, with additional studies of lining cells, fatigue microdamage and the viability and microstructure of resident capsular osteocytes and their canalicular network. Other areas of interest are the development of the air spaces monitored in animal temporal bones with sequential tissue time markers, histomorphometric analysis of the postnatal development of hair cells and 3-D representations of the temporal bone in the development of a high fidelity, low cost, interactive surgical simulator.

For the last 15 years, we have been engaged in an attempt to explain the underlying cause of Ménière's disease by ultrastructural (SEM and TEM) investigations headed by K. Qvortrup. Based at the Department of Anatomy, The Panum Institute, University of

Copenhagen (across the street from Rigshospitalet) we have benefited from a solid background in comparative anatomy in salt-water transporting epithelia in other organs, developed previously by Professor J. Rostgaard. A Philips CM100 equipped with a digital MegaView2 camera is used for TEM and a Philips FEG30 for SEM. With the development of a new improved fixation technique employing glutaraldehyde in combination with oxygenated fluorocarbon, we characterized new ultra structural details in Reissner's membrane, the vascular stria and the endolymphatic sac. Based on the latter investigations, we suggested a new nomenclature of the cells of the endolymphatic sac, since this fixation method preserved the mitochondria-rich cells, which due to fixation artifacts were previously named 'light-cells'. The other type of cell within the endolymphatic sac

was suggestively named 'chief' cells, since our investigations pointed to the endolymphatic sac as a putative endocrine gland. Extracts of this gland indeed revealed that it contained/produced a natriuretic factor, tentatively named 'saccin'. We have identified the amino acid sequence of this peptide, employing molecular and protein chemical techniques. Saccin could play a major role in the cause of Ménière's disease and possibly tinnitus. If the endolymphatic sac proves to have endocrine capacities, the microcirculation of the inner ear needs further investigation which is currently in progress employing vascular corrosion casts by M. Friis. A clinical study of patients with Ménière's disease is also underway, attempting to link the possible presence of elevated blood levels of saccin to this disease. Along with our in-house studies, P. Bretlau has been taking part in *in vivo* MR studies of experimental endolymphatic hydrops and in studies of the protective action of neurotrophins and antioxidants in experimental hearing loss as a visiting professor at the Karolinska Hospital, Stockholm, Sweden.

At the fifty year anniversary of the Otopathological Laboratory, we are joining efforts with the Panum Institute to establish a united "inner ear research institute" in Copenhagen in order to extend our capabilities on molecular biology in support of a fundamentally morphological approach to temporal bone biology and disease.



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News and Announcements

The Association for Research in Otolaryngology (ARO)

The ARO will be holding their 29th annual MidWinter Meeting on February 5-9, 2006 at the Marriott Waterfront in Baltimore, MD.
<http://www.aro.org>

American Academy of Audiology (AAA)

The American Academy of Audiology will be holding their annual meeting on April 5-8, 2006 in Minneapolis, Minnesota.
<http://www.audiology.org>

The Combined Otolaryngology Spring Meetings (COSM)

The next COSM meeting will be held on May 19-22, 2006 in Chicago, IL at the Hyatt Regency.
<http://www.entnet.org>

Self Help for Hard of Hearing People (SHHH)

The SHHH will be holding their annual meeting on June 29 - July 2, 2006. The meeting will be held at the Disney's Coronado Springs Resort in Disney World, FL.
<http://www.shhh.org>

Did you know.....

Did you know that the NIDCD produces a newsletter for clinicians, researchers and patients. *Inside* is produced by the Office of Health Communication and Public Liaison, NIDCD. For more information about this newsletter, please contact the editor, Mary Sullivan, at sullivml@od.nih.gov.

For general health information about communication disorders, contact the NIDCD Information Clearinghouse at: Voice: (800) 241-1044, TTY: (800) 241-1055 or E-mail: nidcdinfo@nidcd.nih.gov

DRF News

The Deafness Research Foundation (DRF) has moved. The new address is 2801 M Street NW, Washington, DC 20007 and new phone number is (202) 719-8088.