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NEW OBSERVATIONS FOR THE MOLECULAR BIOLOGICAL AND GENETIC BACKGROUND OF THE BONE REMODELING OF OTOSCLEROSIS

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NEW OBSERVATIONS FOR THE MOLECULAR BIOLOGICAL AND GENETIC BACKGROUND OF THE BONE REMODELING OF OTOSCLEROSIS

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The examination takes place at the library of the Department of Ophthalmology, Faculty of Medicine, University of Debrecen, 16 June 2015, at 11 a.m.

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 16 June 2015, at 1 p.m.

CONTENT

1. INTRODUCTION

Otosclerosis is a unique inflammatory bone remodeling disorder of the human otic capsule with an unknown etiology. Inflammatory bone resorption foci means the main feature of the disease, which develops solely in the human otic capsule and in the stapes footplate. The foci can evolve anywhere in the otic capsule, however; they appear mainly at the fissula ante fenestram, a.k.a predilection site for otosclerosis. The rising of otosclerotic focus narrows the oval window niche, discourages the movements of the stapes footplate and blocks the outwardly coming vibration of sound towards to the inner ear. Accordingly, conductive hearing loss can be diagnosed more often in a patient suffering from otosclerosis. Nevertheless, clinical symptoms depend on the location of the foci. In case of endosteal involvement of the cochlea, progressive sensorineural hearing loss might occur, while in case of semicircular canal lesions, vertigo can come up.

In the Caucasian white population, the prevalence of manifest otosclerosis is about 0.3–0.38% of the general population, 5–7% of those with hearing loss and 18–25% of those with conductive hearing loss. It follows that the annual estimated incidence of otosclerosis is 6-8/10.000. Subclinical or histological otosclerosis is presumably more common: 8–11 % of the general white population develops otosclerotic foci, as confirmed by histologic autopsy studies. The onset of the disease is usually around at the third or fourth decades and is also characterised by female dominance. In Hungary, 200-250 stapes surgeries are performed annually and approximately in 150 cases, otosclerosis constitutes the background.

During the past decades, molecular mechanisms of bone metabolism have been intensively studied. Bone metabolism has double regulation. In addition to the well-known endocrine network, a local network of complex and refined interactions between osteoclasts, osteoblasts and numerous mediators has been identified. The balance between bone resorption and formation is closely regulated by cytokines and other mediators described below.

Osteoclasts are multinucleated cells formed by the fusion of the progenitors of monocyte/macrophage family. Their principal

function is bone resorption. In contrast, osteoblasts are bone-forming cells with equally important role in physiological bone turnover. Normally, the resorptive and bone-forming processes run simultaneously and maintain the physiological balance of bone metabolism.

Receptor activator of nuclear factor kappa B ligand (RANKL) is expressed and secreted by a variety of cells, including osteoblasts. RANKL facilitates the differentiation, activation and survival of osteoclasts by the activation of its specific receptor RANK, which is located on osteoclasts. Osteoprotegerin (OPG) is a powerful inhibitor of bone resorption and attenuates osteoclastogenesis. OPG is a soluble 'decoy' receptor that competes with RANK for RANKL. Due to the presence of RANK in osteoclasts and their precursors, OPG inhibits the differentiation, survival and fusion of osteoclastic precursor cells, and suppresses the activation of osteoclasts and promote their apoptosis. In otosclerosis, high expression levels of OPG mRNA were detected in the spiral ligament, supporting cells of the organ of Corti and interdental cells of the spiral limbus. In contrast, no OPG expression could be detected within the bone of the otic capsule using immunohistochemistry. Elevated concentrations of OPG were found in the perilymph. Thus, OPG may be produced in the soft tissues of the cochlea, secreted into the perilymph and the lacunocanalicular system of the otic capsule. There is intensive diffusion of OPG from the perilymph into the surrounding bone. Probably, this is one of the reasons why the otic capsule is unique in its morphology and development.

The normal otic capsule exerts very little bone turnover and is almost devoid of osteoclasts. The histologic activity of the otosclerotic foci may be categorized from grade I (most active) to grade IV (completely inactive) depending on the cellularity, degree of vascularization, amount of extracellular collagen matrix and the presence of osteoblasts and osteoclasts. There are numerous osteoclasts, fibroblasts, giant cells and proliferating endothelial cells in the active otosclerotic lesions, which can be responsible for the increased metabolic activity and the developing spongiotic structure. In response to this enhanced bone resorption, a regenerative process occurs in the foci leading to fibrous transformation by osteoblasts

and fibroblasts. The early stages of otosclerosis have been associated with measles virus infection and concurrent inflammation. In brief, the active phase of the disease has been characterized by incremental inflammation, detectable measles virus particles, abundant local expression of tumor necrosis factor alpha (TNF-α) and OPG negativity. The inactive phase was represented by measles virus and OPG positivity, TNF-α negativity and lack of inflammation. In the early stage of otosclerosis, TNF-α is detectable in the otosclerotic foci and correlates with the expression levels of measles virusderived RNA fragments.

Antigens of defective measles virus particles are expressed on the surface of the infected cells by the MHC-I molecules on osteoclasts, osteoblast, fibroblasts and endothelial cells. Therefore, CD8+ T cell-dependent immune responses lead to TNF-α release and consequent bone resorption. This is the most viable theory, but activated monocytes, macrophages, B cells, T cells and osteoclasts are also able to secrete TNF-α into the osteolytic foci; thus, they may further perpetuate the inflammatory events. TNF-α is a pro-inflammatory cytokine that plays an essential role in the differentiation of bone marrow-derived mononuclear cells to osteoclasts and stromal cells to osteoblasts. This cytokine is also an indispensable paracrine mediator during intercellular communication between osteoclasts and osteoblasts. High levels of TNF-α stimulate osteoclast activation, induce RANKL expression and decrease osteoclast apoptosis leading to osteolysis and spongiosis. TNF-α over expression in otosclerosis further stimulates osteoclast formation through the dual action of inhibiting secretion of OPG and stimulating that of RANKL. TNF-α may enter into the perilymph and cortilymph utilizing the same channels as OPG when migrating from the perilymph to the otic capsule. TNF-a may interfere with the electromotility of outer hair cells, leading to sensorineural hearing loss. As TNF-α is generated within the otosclerotic bone, one of the potential causes of sensorineural hearing loss in otosclerosis might be the abundant release of TNF-α,

Apart from the RANKL/OPG system and proinflammatory cytokines, such as TNF-α, proteolytic enzymes may also be involved in the bone resorption underlying otosclerosis. The role of cathepsins in otosclerosis has long been postulated. For example, cathepsin D

and B expression has been reported to be in association with histologic activity of otosclerosis. In case of active otosclerosis, early inflammation and bone resorption is followed by increased bone formation and sclerosis. Recently, the wingless protein (Wnt)-β-catenin system has been implicated in osteoblast activation and bone formation. Sclerostin and the Dickkopf-1 (DKK-1) proteins are inhibitors of Wnt and thus bone formation. Thus, apart from the RANKL/OPG system, the Wnt/DKK-1-sclerostin balance has also beenimplicated in bone remodeling. Interestingly, TNF-α—the key pro-inflammatory cytokine described above—not only stimulates bone loss by inducing RANKL but also induces DKK-1 and thus indirectly blocks Wnt-mediated bone formation. Although there is very little information available on the possible role of theWnt-β-catenin/sclerostin-DKK-1 system in otosclerosis, Wnt and its target genes have been identified in the cochlea and various other parts of the inner and middle ear.

An important cytokine, transforming growth factor β (TGF-β) has also been implicated in the pathogenesis of otosclerosis.TGF-β1 is themost abundant growth factor in the human bone. It plays a critical role in inducing mesenchymal cell differentiation to osteoblasts. TGF-β influences osteoblast differentiation, matrix formation, tissue fibrosis and mineralization. TGF-β interacts with many well-known pathways in osteoblasts biology, such as the Wnt-β-catenin pathway. As discussed above, otosclerosis is associated with fibrosis and bone formation as repair mechanism following bone resorption. Bone morphogenetic proteins (BMP) that also belong to the TGF-β gene family, have recently been implicated in the pathogenesis of otosclerosis.

At present, stapes surgery (stapedectomy and stapedotomy) still remained the main option for the treatment of otosclerosis. These interventions are able to almost abolish the conductive component of hearing loss, however; they are ineffective for the treatment of progressive sensorineural hearing loss. Therefore, one needs to understand the pathogenesis and the molecular mechanisms underlying otosclerosis in order to develop pharmacological compounds that may be able to interfere with the cellular and molecular mechanisms described above.

2. AIMS

2.1. Mapping of the expression levels and patterns of α-1 and α-2 alleles (COL1A1 and COL1A2) of type-I collagen with immunofluorescent staining in otosclerotic stapes footplates.

2.2. Demonstration of COL1A1 and COL1A2 allele-specific mRNA with RT-PCR from ankylotic stapes footplates and in case of pathologic transcription variants the determination of those.

2.3. Evaluation of protein expression of BMP2, 4, 5 and 7 with immunofluorescent staining in otosclerotic stapes footplates. Comparison of the results with the histologic staging and the clinical activity of otosclerosis.

2.4. Performation of renin-angiotensin-aldosterone (RAAS) specific immunofluorescent analysis in otosclerotic stapes footplates and cadaver human kidney tissues. Explanation of the resulted protein expression levels and patterns.

2.5. Establish the first genetic association study, which is based on the whole DNA of patients with histologically confirmed otosclerosis and non-otosclerosis, and investigate the role of the previously described gene specific SNPs.

3. MATERIALS AND METHODS

This scientific study is based on four series of experiments, thus the methodology chapter is discussed accordingly separately.

3.1. *Investigation of type-I collagen A1 and A2 alleles:* 55 ankylotic stapes (*n=55*, female=34, male=21) was removed and 30 cortical bone fragments were harvested by lateral atticotomy. The samples were divided unequally two portion, 40 stapes and 20 control samples was examined by conventional hematoxilin-eosin and COL1A1/A2 specific immunofluorescent staining. 15 stapes and 10 control samples were investigated by COL1A1/A2 specific RT-PCR.

3.2. *BMP2, 4, 5 and 7 specific immunofluorescent investigation:* 67 ankylotic stapes (*n=67*, male=26, female=41) were collected and 35 bone specific control cortical bone fragments were removed. Conventional hematoxilin-eosin and BMP2, 4, 5 and 7 specific histologic analysis were also performed.

3.3. *RAAS specific immunofluorescent analysis:* 20 ankylotic stapes (*n=20*, female=16, 4=male), 10 cortical bone fragment and 10 human cadaver kidney tissue sample were removed. Conventional hematoxilin-eosin and RAAS (renin, angiotensin II, angiotensin II receptor, angiotensin converting enzym) specific histologic analysis were performed.

3.4. *Genetic association study:* 153 histologically confirmed otosclerotic stapes (*n=153*, male=65, female=88) and postoperative blood samples were harvested from patients. 300 blood samples were also collected by healthy volunteers, whom had no hearing loss and during otoscopic examination could find no illness. The diagnosis was based on conventional hematoxilin-eosin staining. According to previously described scientific datas SNPs of otosclerosis susceptible genes (COL1A1, TGF-β1, BMP2, BMP4, AGT, RELN) were examined by genotyping and association testing.

4. RESULTS

Results were summarized according to the subdivision of the ’Materials and methods’ chapter.

4.1. *Investigation of type-I collagen A1 and A2 alleles:* Normal and consistent COL1A1 (α-1 chain) and COL1A2 (α-2 chain) expression was detected in both otosclerotic and non-otosclerotic stapes footplates. The intensity of α-1 and α-2 chain-specific immunoreaction was independent from the histological diagnosis of stapes fixation. The immunoreactivity did not vary between different parts of the stapes samples. The superstructures, the footplates and the hyaline cartilage layer of the vestibular surface displayed very similar expression pattern. Cortical bones, incus and malleus specimens applied as negative controls for stapes fixation represented normal type-I collagen expression. Expression patterns of COL1A1/A2 alleles did not show significant correlation with the histological diagnosis of otosclerosis.

Human cellular RNA was detected in 15 ankylotic stapes footplates and in all negative controls (n=12) confirming that mRNA extraction process was. RT-PCR detection of A1 and A2 allele specific mRNA was successful in all ankylotic stapes footplate specimens. Cortical bones and hearing ossicles applied as negative controls also displayed normal COL1A1/A2 allele expression by RT-PCR. Pathologic transcription variant was not detectable.

4.2. *BMP2, 4, 5 and 7 specific immunofluorescent investigation:* Active otosclerosis cases were characterized by simultaneously increased expression of BMP2, 4, 5, and 7, that resulted in intense granular immunoreaction. Both osteoclasts and osteoblasts showed considerable positivity for different types of BMPs, indicating active bone remodeling and new bone formation within the lesion. The BMP immunoreactivity varied between different parts of the stapes samples. The superstructures were negative for BMPs, while the footplates containing the lesion and the hyaline cartilage layer of the vestibular surface displayed very similar expression patterns. In contrast, in inactive otosclerotic foci, no significant BMP expression could be demonstrated on the surface of cellular remnants. In these

cases, expression levels and patterns of BMPs were strongly associated with the histopathological activity of otosclerosis. Non-otosclerotic stapes fixations displayed negative immunoreactions for BMPs. Cortical bones and incus specimens applied as negative controls for stapes fixation displayed negative BMP-specific immunoreactions. Finally, expression of different types of BMP was strongly associated with the histologic diagnosis of otosclerosis.

4.3. *RAAS specific immunofluorescent analysis:* Stapes footplates with active or inactive otosclerosis were characterized by negative immunoreactions against renin, angiotensin II, angoitensin converting enzyme and angiotensin II receptor. Missing protein expression was independent from the histologic diagnosis and histologic activity of otosclerosis.

In agreement with previous observations, human kidney specimens used as positive controls showed robust cytoplasmic RAAS expression by IFA. Epithelial cells of glomeruli, proximal and distal canaliculi, and the modified neuroendocrine cells of juxtaglomerular apparatus showed rather intense immunoreaction standing out delineating the complex structure of individual nephron units.

Cortical bone fragments applied as bone-specific controls displayed considerable RAAS specific immunoreactions. In the perivascular region, bone marrow progenitor cells showed weak annular and weak homogeneous immunoreactivity against the 4 studied members of RAAS.

4.4. *Genetic association study:* Thirteen SNPs that were significantly associated with otosclerosis in previous studies were genotyped. Patients with active and inactive otosclerosis were compared with the controls The group with active otosclerosis and inactive otosclerosis were not compared separately to the control group because of the lack of power. The estimated power of the samples set to detect statistically significant SNPs association with the disease varied from 16% to 80.5%. None of the SNPs showed a deviation from Hardy-Weinberg equilibrium. Association by logistic regression analysis revealed one SNP (rs1800472, TGFB1) significantly associated with otosclerosis.

5. NEW OBSERVATIONS

5.1. No association can be found in the expression of type-I collagen α-1 and α-2 chan between histologically confirmed otosclerotic stapes footplates and control bone, nevertheless no pathologic transcription variants can be detected in COL1A1 and COL1A2 allele with RT-PCR. In our opinion type-I collagen is not liable for the evaluation of otosclerosis.

5.2. Coincidently with literature data, BMP2, 4 and 7 can be observed with immunofluorescent staining in histologically confirmed otosclerotic stapes footplates, furthermore increased expression of BMP5 in otosclerotic stapes footplates were first reported. In our imagination BMPs play essential role in the regeneration phase after lytic process.

5.3. In contrast to previous suggestions, no detectable RAAS expression can be detected in histologically confirmed otosclerosis, thus in our judgement RAAS has no responsibility in the developement of otosclerosis

5.4. Within the confines of an international cooperation, we first investigated histologically confirmed otosclerotic stapes footplates in an association study. Based on our results, presumably the mutation of TGF-β1 plays a crucial role in the pathogenesis of otosclerosis.

6. DISCUSSION

We suppose the model of the etiology of otosclerosis, that antigens of defective measles virus particles are expressed on the surface of the infected cells by the MHC-I molecules on osteoclasts, osteoblast, fibroblasts and endothelial cells. Therefore, CD8+ T cell-dependent immune responses lead to TNF-α release and consequent bone resorption. Meantime the embryonic chondroblasts and osteoblasts of the otic capsule and the migrated osteoclasts reactivated by the effect of measles virus infection. The osteoclasts and osteoblasts of the otosclerotic foci express CD51/61 (osteoclast functional antigen) molecule, morover increased BMP, TGF-β and TNF-α expression can be detected. Activated monocytes, macrophages, B cells, T cells and osteoclasts are also able to secrete TNF-a into the osteolytic foci; thus, they may further perpetuate the inflammatory events

The current series of experiment certify, that the rs1800472 SNP of the TGF-β1 (T263I) plays role in the patogenesis of otosclerosis. Besides this, the high tissue expression of BMP2, 4, 5 and 7 is quite important, however these molecules participate exclusively in the regenerative mechanism of the osteolytic inflammation. Furthermore, we suggest, based on our experimental work, that COL1A1, COL1A2 and RAAS do not take part in the pathogenesis of otosclerosis, in contrast to previous literature proposals.

7. LIST OF PUBLICATIONS

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Subject: Ph.D. List of Publications

Candidate: Balázs Liktor
Neptun ID: YI9JCN
Doctoral School: Doctoral School of Clinical Medicine

List of publications related to the dissertation

1. Sommen, M., van Camp, G., **Liktor, B.**, Csomor, P., Fransen, E., Sziklai, I., Schrauwen, I., Karosi, T.: Genetic Association Analysis in a Clinically and Histologically Confirmed Otosclerosis Population Confirms Association With the tgfb1 Gene but Suggests an Association of the RELN Gene With a Clinically Indistinguishable Otosclerosis-Like Phenotype.
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3. Csomor, P., **Liktor, B.**, Liktor, B., Szekanecz, Z., Sziklai, I., Karosi, T.: Expression of bone morphogenetic protein 2, 4, 5, and 7 correlates with histological activity of otosclerotic foci.
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5. Takács I., **Liktor B.**, Förster G., Karosi T.: Ismeretlen eredetű, orbitába törő orrüregi tumor műtéti megoldása.
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8. **Liktor, B.**, Révész, P., Csomor, P., Gerlinger, I., Sziklai, I., Karosi, T.: Diagnostic value of cone-beam CT in histologically confirmed otosclerosis.
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Fül-Orr-Gégegyógyászat. 58 (3), 101-106, 2012.

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