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Uloga angiotenzina II i 4-hidroksinonenala na stanice slične osteoblastima s posebnim osvrtom na metabolizam stapesa

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RESUME

I. Introduction

1. Physiopathologie d'otospongiose

L'otospongiose est une dystrophie osseuse limitée à la capsule otique et responsable de surdité. Cette surdité est d'abord une surdité de transmission (mécanique, liée au blocage de l'étrier empêchant ainsi la progression de l'onde sonore vers la cochlée et les cellules sensorielles). Elle évolue par la suite vers une surdité mixte avec une composante neurosensorielle liée à l'atteinte de l'oreille interne. Les foyers d'otospongiose sont composés de zones actives hypercellulaires et basophiles avec des signes d'inflammation et des foyers de résorption osseuse appelées otospongiose, et des zones inactives ou cicatricielles denses et peu cellulaires appelées également otosclérose. Les zones d'otospongiose et d'otosclérose sont juxtaposées au sein d'un même tissu avec une disposition en mosaïque. Dans les zones actives, il existe de nombreux ostéoclastes multi-nucléés, cellules ostéocytes et la prolifération des cellules endothéliales dans les espaces pseudo vasculaire des foyers actifs otospongiose. Généralement, CD8+, les lymphocytes T cytotoxiques et des ostéoclastes activés se trouvent dans la zone périphérique des foyers actifs. Des études antérieures ont suggéré différents types de cytokines pro inflammatoires jouent un rôle dans la physiopathologie de l'otospongiose. Plusieurs facteurs étiologiques ont été évoqués pour expliquer le déséquilibre de la résorption/formation osseuse: auto-immunité, micro-organismes (virus de la rougeole), facteurs génétiques et hormonaux (incidence plus élevée chez la femme et aggravation au décours des grossesses). Le traitement de l'otospongiose repose sur la correction de la surdité par la prothèse auditive ou par le remplacement chirurgical de l'étrier atteint, aucun traitement étiologique ne peut être proposé. L'utilisation de fluor ou, plus récemment des biphosphonates (inhibiteurs de l'activité ostéoclastique) a été proposée afin de ralentir l'évolution de la maladie. Aujourd'hui, le traitement chirurgical

(platinotomie calibrée au laser) reste la meilleure option de traitement pour otospongiose. L'otospongiose est essentiellement une maladie touchant les caucasiens et fortement liée à leur répartition géographique dans le monde. Sa prévalence est estimée à 0,3%. L'âge moyen au moment du diagnostic est de 33 ans. Des rapports récents montrent un passage de cet âge à 40-50 ans. Le sexe ratio (hommes/femmes) est de 1:2 et il a été signalé que la progression de l'otospongiose serait plus rapide chez les femmes que les hommes. Environ 60% des patients ont des antécédents familiaux d'otospongiose. Les 40% restants sont soit des cas liés à une transmission autosomique dominante avec absence pénétrance chez les autres membres de la famille, soit de nouvelles mutations, ou enfin des cas ou les cas familiaux existant n'ont pas été détectés.

2. Le système rénine-angiotensine-aldostérone

Le système rénine-angiotensine-aldostérone (SRAA) est un régulateur physiologique majeur de la pression artérielle et de l'équilibre hydro-électrolytique. Ce système est également considéré non seulement comme un système endocrinien, mais aussi comme un modulateur autocrine / paracrine des fonctions tissulaires de différents organes comme le cœur, les vaisseaux sanguins, le rein, le cerveau et les glandes endocrines. Il y a trois éléments importants de ce système: 1) la rénine, 2) l'angiotensine, et 3) l'aldostérone. Néanmoins, l'angiotensine II (Ang II) est à plusieurs titres l'agent central du SRAA.

Ang II qui est un octa-peptide bioactif, joue un rôle clé dans la régulation de l'homéostasie cardiovasculaire. Ang II peut également fonctionner comme une hormone paracrine/autocrine induisant la croissance et la prolifération cellulaire. Les études pharmacologiques ont montré plusieurs types de récepteurs de l'Ang II. Des récepteurs de type 1 (AT1) régulent deux voies de signalisations distinctes qui impliquent des protéines G. Une des voies implique l'activation de la phospholipase C et la production de l'inositol 1,4,5-triphosphate, et l'autre

voie concerne la régulation d'AMPc via l'activation ou l'inhibition de l'adénylate-cyclase. La voie de signalisation des récepteurs de type 2 (AT2) reste à être élucidée. Plus récemment, le type 4 (AT4) a été décrit dans le système nerveux central. Ce récepteur semble être impliqué dans l'acquisition et la mémoire.

La plupart des effets connus de l'Ang II sont médiés par le récepteur AT1. Des études *in vitro* et *in vivo* suggèrent que le récepteur AT2 a un effet antagoniste sur AT1. L'activation de l'AT2 entraîne une inactivation des MAPK, a un effet anti-prolifératif, favorise l'apoptose, la différenciation cellulaire, la régénération, l'ouverture de canaux K + lents et la fermeture des canaux de Ca2 + de type T. Ce récepteur AT2 est exprimé dans les tissus cibles dans diverses maladies et semble donc impliqué dans leur physiolpâthologie. Comme le récepteur AT2, le récepteur AT4 s'oppose également aux effets déclenchés par l'activation du récepteur AT1.

Il existe des antagonistes non peptidiques des récepteurs AT1 (Sartans) et AT2 (PD123317 et PD123319).

Ang II active plusieurs facteurs de transcription nucléaires, comprenant le récepteur et activateur des facteurs de transcription (STAT), protéine activatrice 1 (AP-1), NF-kB et l'adénosine monophosphate cyclique (AMPc) et CERB. Ang II est également connu pour activer la famille des protéine-kinases activées par les mitogènes (MAPK), comprenant ERK 1/2, c-jun N-terminal kinase (JNK, également connu sous le nom SAPKs), et MAPK (p38MAPKs). Les MAPK sont des protéines régulatrices qui contrôlent la réponse cellulaire aux signaux de croissance, l'apoptose et le stress.

L'angiotensine II est connue pour moduler le remodelage osseux. Nous avons récemment mis en évidence que plusieurs polymorphismes génétiques liés à une activation du SRAA (M235T, ACE I/D) étaient associés à l'otospongiose. Par ailleurs, l'angiotensine II stimule la production d'une cytokine pro-inflammatoire (interleukine 6) et diminue l'activité de la

phosphatase alcaline dans des cultures primaires d'os otospongieux, contrairement aux cultures d'étriers témoin.

3. Le stress oxydatif

Le stress oxydatif a lieu dans les tissus sous l'effet d'agents toxiques, l'inflammation, l'ischémie ou un traumatisme physique. Ces situations peuvent conduire à une formation excessive d'espèces réactives de l'oxygène (DRO) et un déséquilibre entre la formation et l'élimination des DRO. Ce stress peut être provoqué dans diverses pathologies aiguës comme les maladies cardiovasculaires, les maladies neuro-dégénératives et les tumeurs malignes. Avec une quantité excessive de DRO dans l'environnement cellulaire, les lipides membranaires sont peroxydés et les constituants lipidiques des cellules sont endommagés. Ce processus aboutit à la production d'aldéhydes très réactifs, qui sont appelés des seconds messagers du stress oxydatif. Contrairement aux DRO, les aldéhydes réactifs sont relativement stables dans le temps. Par conséquent, ils pourraient diffuser à partir du site de leur production et attaquer des cibles éloignées intracellulaire ou extracellulaire. Parmi de nombreux aldéhydes réactifs, les plus étudiés sont malondi-aldéhyde, l'acroléine et de la famille 4-hydroxyalkenal, en particulier le 4-hydroxynonénal (HNE).

HNE est également considéré comme le principal produit de la peroxydation lipidique des acides gras polyinsaturés ω-6.

Cet aldéhyde réactif est un composant cytotoxique et mutagène, mais aussi une molécule de signalisation, avec possibilité de modifier la prolifération cellulaire, la différenciation et l'apoptose. L'importance de la HNE a été démontrée dans la physiopathologie de plusieurs maladies telles que celles impliquant une inflammation chronique, les maladies neuro-dégénératives, les états d'ischémie-reperfusion, le syndrome de détresse respiratoire aiguë, l'athérogenèse et le diabète. HNE est également détectable dans des conditions

physiologiques dans de nombreux tissus humains et animaux. Son rôle physiologique reste encore à être clarifié, mais il est probable que HNE ait un rôle important dans la régulation de la croissance cellulaire.

Contrairement aux radicaux libres, HNE a la particularité de rester stable pendant plusieurs heures et se lie aux macromolécules avec une réactivité élevée, en particulier aux protéines, entraînant la modification de leur structure tertiaire et leur fonction. Les conjugués HNE-protéine peuvent être détectés par des anticorps monoclonaux. HNE réagit le plus souvent avec les peptides et les protéines par leurs groupes SH. Ce type de réaction entre HNE et le glutathion (GSH) représente le mécanisme le plus important de détoxification de cet aldéhyde réactif.

4. L'objectif de l'étude

La phase active de l'otospongiose est accompagnée d'inflammation. L'origine ou le facteur déclenchant de cette inflammation n'est pas élucidée. La participation de microorganismes tels que bactéries, virus, champignons ou même le traumatisme causé par la mastication a été évoquée. Une autre question dans la physiopathologie de l'otospongiose est l'inflammation persistante pendant plusieurs années. Dans un modèle physiopathologique proposé pour le diabète sucré de type 1, un virus coxsackie est le facteur déclenchant de la destruction des îlots de Langerhans. L'inflammation est maintenue par une réaction auto-immune qui apparaît secondairement et achève la destruction des tissus endocriniens. Ce modèle est une explication possible de l'inflammation chronique dans l'otospongiose et soutenu par plusieurs rapports sur la réaction auto-immune chez les patients présentant une otospongiose. Cependant, l'auto-immunité n'est pas constamment observée dans l'otospongiose.

Afin d'élucider les facteurs qui peuvent participer à l'entretien de l'inflammation au cours de l'otospongiose, Ang II a été étudiée. En induisant ou en entretenant l'inflammation, Ang II

peut potentiellement induire un stress oxydatif dans l'otospongiose. Potentiellement, ce stress a des effets importants sur le remodelage osseux. Les DRO qui diffusent dans l'oreille interne pourraient également expliquer les lésions de l'oreille interne au cours de l'otospongiose.

Le but de ce projet était d'étudier le rôle de l'Ang II et du stress oxydatif dans les os et en particulier dans l'otospongiose à travers 2 modèles expérimentaux :

1 Etude de l'effet de l'Ang II sur les voies de l'inflammation dans des cultures primaires de cellules osseuses dans l'otospongiose et étrier normal

2 Etude de la présence d'un messager du stress oxydatif dans les étriers otospongieux (HNE) et de son interaction avec Ang II dans une lignée cellulaire de type ostéoblaste humaine (HOS)

III. Etude de l'effet de l'Ang II sur les voies de l'inflammation dans des cultures primaires de cellules osseuses dans l'otospongiose et étrier normal

INTRODUCTION

Les effets de l'Ang II sur le remodelage tissulaire et de réparation ont été déjà étudiés chez l'homme et les animaux. Ang II est impliquée dans les événements de l'inflammation et modifie la composition de la matrice extracellulaire par divers facteurs de croissance et cytokines. Dans la présente étude, nous avons cherché à étudier l'effet de l'Ang II sur la production de cytokines dans les cultures cellulaires primaires d'étriers otospongieux et normaux obtenu de patients.

MATERIELS ET METHODES

Des fragments d'os ont été prélevés chez des patients atteints d'otospongiose lors de la chirurgie de réhabilitation auditive et chez des patients subissant une chirurgie pour une tumeur angle ponto-cérébelleux par une voie transpétreuse. Les cultures cellulaires primaires ont été obtenues de ces deux types de prélèvement. Ces cultures avaient été déjà caractérisées lors des études précédentes. Elles présentent des cellules ostéoblastiques avec plusieurs marqueurs phénotypiques (récepteurs de PTH, sécrétion d'ostéocalcine, production de phosphatase alcaline, nodules de calcification).

Concentration des cytokines dans le milieu de culture

Les surnageants des cultures cellulaires d'otospongiose (n=6) et d'étriers témoins (n=6) ont été recueillis après 24h de traitement par Ang II (10^{-7}M, 24H) ou le diluant pour l'analyse par des biopuces à anticorps (RayBio ® Human Inflammation G3 réseau d'anticorps, RayBiotech, Norcross, GA). Cette technique a permis d'analyser les surnageants pour 40 cytokines et de

chémiokines. La concentration de chaque molécule a été déterminée par une mesure semi-quantitative.

Expression de l'ARNm des cytokines pro inflammatoires

L'ARN total a été extrait de 3 cultures de cellules primaires parmi les 6 dans chaque groupe (otospongiose, témoin). Après synthèse et purification, l'ARNc marqué à la biotine a été hybridé sur des membranes de biopuce a ARN pour la détéction et la mesure semi-quantitative de l'expression ARNm de 126 gènes codant pour les cytokines et les molécules impliquées dans l'inflammation (Oligo GEArray ® cytokines inflammatoires de l'homme et des récepteurs de biopuces, EHS-011, SA Biosciences, Frederick, MD).

Quantification de l'expression ARNm de BCL-6 par la RT-PCR quantitative

L'ARN total de 4 cultures de cellules primaires parmi les 6 dans chaque groupe (otospongiose, contrôle) a été utilisé dans ces expériences. Tous les échantillons utilisés dans les tests de biopuce à ARN ont été inclus dans l'évaluation par une transcription inverse suivie d'une réaction de polymérase en chaîne quantitative (qRT-PCR). L'ADN complémentaire a été synthétisé par RT. Les paramètres de la qRT-PCR ont été déterminés dans des expériences préliminaires sur des lignées de cellules d'ostéosarcome (HOS, et SaOS, American Type Culture Collection, Molsheim, France). Seul l'ARNm de BCL-6 qui a montré une différence d'expression entre otospongiose et témoin et une modification sous l'effet d'Ang II lors des essais par la biopuce à ARN a été étudiée par la qRT-PCR.

Immunocytochimie

Quatre cultures cellulaires de chaque groupe ont été utilisées pour immunohistochimie afin de détecter des différences de population cellulaires entre les étriers témoins et les étriers otospongieux. Les anticorps primaires suivants ont été utilisés: pour des ostéoblastes, l'anticorps monoclonal de souris anti-humain CDH11, clone 4D10 (Sigma-Aldrich, France);

pour des ostéoclastes, l'anticorps monoclonal de souris anti-CD61 humain, clone Y2/51 ; et enfin pour des macrophages, l'anticorps monoclonal de souris anti-CD68 humain, clone KP1 (DakoCytomation, Glostrup, Danemark).

RÉSULTATS

La libération de cytokines et des protéines liées aux cytokines dans le milieu de culture

En condition basale, les concentrations d'IP-10 et IL-1b étaient plus élevées dans l'otospongiose que dans l'étrier témoin (88 ± 6.9 versus 36 ± 4.7 unités arbitraires pour IP-10 et 51 ± 6.9 versus 26 ± 5.1 unités arbitraires pour IL-1b respectivement, $p<0,05$, ANOVA, n=6). Cette observation était en accord avec une plus haute activité de leucocytes ou des ostéoclastes (IL-1b), et une augmentation de l'activité angiogénique (IP-10) dans les cultures issues de l'étrier otospongiose. En outre, une concentration plus faible de l'inhibiteur de métalloprotéases TIMP-2 a été détectée dans des conditions basales dans des cultures cellulaires otospongiose (11296 ± 1705.7 versus 21410 ± 3278.2 unités arbitraires, $p<0,05$, ANOVA, n=6). Cette observation était également compatible avec une remodelage osseux plus important détecté dans l'otospongiose et rapporté dans d'autres études. L'expression d'autres protéines ne différaient pas entre les échantillons d'otospongiose et témoins en condition basale.

Ang II a stimulé la libération d'IFN-γ dans le milieu de culture dans l'otospongiose et les témoins (99 ± 27 versus 136 ± 9.1 unités arbitraires pour l'otospongiose et 90 ± 25.3 versus 141 ± 27 unités arbitraires pour les témoins, $p<0,05$, ANOVA, n=6). Ang II a également augmenté la concentration d'IL-10 dans les milieux de culture otospongiose (70 ± 7.2 versus 116 ± 12.5 unités arbitraires, $p<0,05$, ANOVA, n=6). Il a inhibé la protéine inflammatoire des macrophages, MIP-1a, (22 ± 6.7 versus 9 ± 3.8 unités arbitraires, $p<0,05$, ANOVA, n=6) et l'interleukine 11, IL-11, (124 ± 7.3 versus 98 ± 8 unités arbitraires, $p<0,05$, ANOVA, n=6)

seulement dans les étriers témoins. Ang II a réduit la concentration de sTNFRII dans les milieux de culture otospongiose (18330 ± 2991.9 versus 11547 ± 2130.6 unités arbitraires, $p<0,05$, ANOVA, n=6) sans modifier le facteur de nécrose tumorale alpha (TNF-α).

Expression de l'ARNm des cytokines

Au total, 126 gènes impliqués dans la réponse inflammatoire ont été analysés par biopuces à ARN. Expression de l'ARNm de 15 cytokines ou des molécules liées aux cytokines a pu être détectée par cette technique. Une importante variation inter-individuelle a été observée. En condition basale, l'expression d'ARNm de BCL-6 était plus élevée dans l'otospongiose que chez les témoins (328 ± 39,7 unités arbitraires dans le groupe otospongiose contre 48 ± 21,7 dans les témoins, n = 3, $p <0,05$, test-t non pairé). L'Ang II a réduit l'expression de l'ARNm du BCL-6 dans l'otospongiose seulement (424 ± 63,3 en condition basale versus 105 ± 64,7 après Ang II, n=3, $p <0,05$, test-t pairé). La qRT-PCR après normalisation par l'expression du gène codant pour une protéine ribosomique S14 a confirmé des niveaux plus élevés de BCL-6 dans l'otospongiose par rapport aux témoins en condition basale. Toutefois, la réduction de l'expression d'ARNm du BCL-6 après Ang II dans l'otospongiose observée par puces à ARN n'a pu être confirmée par qRT-PCR. Des résultats similaires ont été obtenus après qRT-PCR après normalisation par le gène de référence GAPDH.

Immunocytochimie

Les cultures témoins et otospongiose ont montré un marquage positif avec des anticorps spécifiques pour des ostéoblastes, des ostéoclastes et des macrophages. Il n'y avait pas de différence de marquage entre le groupe otospongiose et des cultures témoins (n=3). Cette observation suggère que la différence de la production de cytokines pro-inflammatoires n'était pas liée à des différences quantitatives de sous-populations cellulaires entre les cultures otospongiose et témoin.

CONCLUSION

Ces données soutiennent l'hypothèse que l’Ang II est impliquée dans l'inflammation et la régulation du remodelage osseux dans l'otospongiose, via différents facteurs de croissance et cytokines. Ils indiquent plusieurs cibles potentielles pour leurs actions anti-inflammatoires ou anti-cataboliques telles que l'IL-11 et TIMP-2. Néanmoins d'autres investigations sont nécessaires pour clarifier le rôle de l 'Ang II dans la physiopathologie de l’otospongiose et le métabolisme osseux dans l'étrier.

III. L'intéraction entre angiotensine II et de 4-hydroxynonenal (HNE) en dans les lignées cellulaires humaines ostéoblastiques et dans l'otospongiose

INTRODUCTION

Le stress oxydatif et Ang II interagissent dans le remodelage des tissus par via les DRO. Ces dérivés interviennent dans de nombreuses voies de signalisation de l'Ang II. HNE qui représente un des DRO les plus étudiés se trouve dans de nombreux tissus humains en tant que second messager, mais ses rôles physiologiques ne sont pas encore entièrement compris. Des études récentes sur l'influence de la HNE sur les cellules osseuses ont démontré son effet inhibiteur sur la prolifération et la différenciation des cellules osseuses et son effet stimulateur sur l'apoptose.

L'objectif de la présente étude était d'étudier l'interaction entre l'Ang II et la HNE dans la physiopathologie de l'otospongiose en étudiant le modèle *in vitro* des lignées cellulaires humaines ostéoblastiques et dans les étriers humains otospongieux.

MATERIELS ET METHODES

La présence et la localisation de la HNE a été étudié dans les étriers humains otospongieux et témoins par immuno-histiochimie. Cette technique a été réalisée sur des échantillons de tissus prélevés chez 15 patients atteints d'otospongiose et 6 témoins (tumeurs de l'angle ponto-cérébelleux opérées par voie transpétreuse). Un anticorps anti-HNE a été utilisé. Une lignée de cellules ostéblastiques humaines (HOS, Human Osteosarcoma) a été utilisée pour analyser l'effet de l'Ang II et de la HNE sur la prolifération (incorporation de ^{3}H-thymidine), la différenciation (détection de l'activité phosphatase alcaline par un test de NBT / BCIP, Roche) et l'induction de l'apoptose par la cytometrie de flux (marquage des cellules apoptotiques par

un kit de coloration annexine-V, Roche, Allemagne). Une analyse par immunoblot a été effectuée pour déterminer s'il existe une interaction directe entre l'Ang II et HNE.

RESULTATS

La détection immuno-histochimique des conjugués HNE-protéine a été positive à la fois dans le groupe otospongiose et les témoins. Cependant la distribution du marquage était différente entre les deux groupes : diffuse dans l'otospongiose et limitée à l'espace sous-périostée dans les étriers témoins. L'association de l'Ang II à une concentration de 0,1nM à la HNE à 2,5 µM pendant 24 heures a stimulé la prolifération des cellules HOS ($p < 0,0002$). La diminution de l'activité de la phosphatase alcaline (ALP) a été observée dans des cultures traitées par une concentration de 0,1 nM Ang II associée à la HNE à une concentration de 1 ou 2,5 µM ($p < 0,01$). Au contraire, HNE seul à 10 µM ($p < 0,05$) ou en combinaison avec 0,1 ou 0,5 nM d'Ang II ($p < 0,01$) augmente l'activité ALP. La diminution de l'apoptose a été également observée dans les cellules traitées par Ang II seule à 0,1 ou 0,5 nM ($p < 0.05$). Au contraire, une augmentation de l'apoptose a été détectée dans les cultures traitées avec HNE à 10 µM seule ou en combinaison avec 0,1 ou 0,5 nM d'Ang II, ($p < 0,01$). Le traitement avec 0,5 nM d'Ang II et 1 µM de HNE réduit le niveau de cellules nécrotiques ($p < 0.05$). L'augmentation de la nécrose a été observée dans toutes les conditions : HNE seule à 10 µM ($p < 0.01$), en combinaison avec Ang II à 0,1 nM ($p < 0,02$), 0.5 et 1 nM ($p < 0,002$), ainsi que la combinaison de HNE à 5 µM avec Ang II à 0,1 0,5 ($p < 0.05$) ou 1 nM ($p < 0,003$). L'analyse immunoblot a montré une liaison significative entre l'Ang II et la HNE.

CONCLUSION

Nos résultats étayent l'hypothèse de l'implication des dérivés réactifs d'oxygène et notamment la HNE dans la physiopathologie de l'otospongiose. L'intéraction entre la HNE et l'Ang II peut expliquer la différence de réponse à l'Ang II observée entre les étriers otospongieux et

témoins. En outre, les résultats l'effet de l'Ang II et HNE sur la prolifération, la différenciation et l'apoptose des ostéoblastes, soutiennent leur rôle important dans le remodelage osseux au cours de l’otospongiose.

IV. Conclusion générale

Les résultats de ces 2 études fournissent un éclairage nouveau sur le rôle de l'Ang II et le stress oxydatif (DRO et ses seconds messagers) dans la physiopathologie du métabolisme étrier au cours de l'otospongiose. L'Ang II induit la sécrétion de différentes cytokines pro-inflammatoires dans les cultures primaires de cellules otospongieux. Notre investigation sur les effets de l'Ang II et les messagers secondaires du DRO (HNE), montrent l'interaction de ces deux facteurs ainsi que l'influence positive sur la prolifération cellulaire humaine d'osteosarcome (HOS). La distribution hétérogène et diffuse des HNE dans l'os otospongieuse appuie le rôle possible du stress oxydatif dans la physiopathologie de la maladie. Enfin, les produits du stress oxydatif interagissent avec l'Ang II pour modifier la réponse des cellules osseuses à cette hormone. Comme indiqué par immunoblot, au moins une partie de cette interaction a lieu dans le milieu extracellulaire et avant la liaison aux récepteurs de l'Ang II. Dans des études antérieures, nous avons observé une différence de réponse à l'Ang II entre étrier otospongieux et normal. La présence des dérivés réactifs d'oxygène dans les foyers actifs d'otospongiose suite à l'inflammation est au moins l'une des explications de cette différence de réponse à l'Ang II.

DISCIPLINE : PHYSIOLOGIE ET PHYSIOPATHOLOGIE DES EPITHELIUMS

MOTS-CLES: dystrophie osseuse, surdité, angiotensine II, stress oxydatif, inflammation

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SAŽETAK

I. Uvod

1. Patofiziologija otoskleroze (bolesti stapesa)

Otoskleroza je bolest labirintne kapsule koja postupno zahvaća i osikularni lanac srednjega uha, točnije strukture stapesa. Osikularni lanac srednjega uha sastoji se od triju slušnih košćica (maleusa, inkusa i stapesa). Stapes (stremen) sastoji se od prednjeg i stražnjeg krusa (suprastruktura) i pločice kojim prijanja na ovalni prozorčić (fenestra ovalis). Patološke promjene koje ga zahvaćaju širenjem iz područja labirintne kapsule, obilježene su stalnim procesom koštane razgradnje i predgradnje. Rezultat toga je fiksacija stapesa i posljedično konduktivna nagluhost, što je jedna od prvih i glavnih karakteristika otoskleroze. Incidencija bolesti kreće se od 0,3 do 1% u različitim populacijama i obično se manifestira između drugoga i petoga desetljeća života. Bilateralni oblik bolesti prisutan je u 80% slučajeva. Histološki razlikujemo tri faze bolesti. Koštana resorpcija i pojačana vaskularizacija karakteriziraju prvu fazu. Kako sazreli kolagen nestaje, kost poprima spongiozni oblik (u francuskoj literaturi drugi naziv za bolest je otospongioza). Zadnju fazu bolesti obilježava čvrsta sklerotična kost, odakle i konačno ime otoskleroza. Otosklerotična kost pokazuje jaku koštanu pregradnju s dva jasno ograničena područja aktivnosti: inaktivno fibrozno područje i aktivno područje bogato osteoklastima, osteoblastima i krvnim žilama formiranima u zajednički mozaik. Etiologija bolesti još uvijek nije do kraja razjašnjena. U različitim istraživanjima predloženo je više potencijalnih etioloških faktora (inflamatorni, infektivni, genetski, autoimuni, hormonalni). Kliničku sliku karakterizira progresivan konduktivan gubitak sluha i šum. Neki pacijenti javljaju bolju razumljivost u bučnoj okolini, što je poznato kao fenomen Wilisi. Vestibularni simptomi su rijetki. Kliničkim pregledom ustanovi se normalan vanjski zvukovod i bubnjić. U određenoga broja bolesnika vidljivo je crvenilo iznad promontorija (Schwartzov znak), a rezultat je pojačane vaskularizacije kosti odmah ispod

periosta. Audiološka testiranja pokazuju umjerenu do jaku provodnu nagluhost, sa zračno-koštanim gapom više izraženim u nižim frekvencijama. Senzoneuralni gubitak od 20dB na frekvenciji od 2000Hz (Carhartov znak) tipičan je audiološki nalaz. Medikamentozna terapija bolesti usmjerena je ka koštanom sazrijevanju i smanjenju otosklerotične aktivnosti. Upotreba natrij florida predložena je kao moguća terapija vezano za uspjeh iste kod osteoporoze. Upotreba bifosfonata koji inhibiraju aktivnost osteoklasta i antagoniste citokina koji inhibiraju koštanu resorpciju, drugi je izbor terapije. Ostali mogući oblici terapije uključuju upotrebu antioksidansa (koji blokiraju stimulatorni efekt slobodnih radikala na osteoklaste), inhibitora neuralne apoptoze, upotrebu kortikoida i konačno antagonista angiotenzina. Za sada ipak jedni je učinkovit oblik terapije kirurški postupak koji uključuje izvođenje stapedotomije (uklanjanje otosklerotično izmijenjene kosti i njezina zamjena posebno proizvedenom protezom).

2. Angiotenzin II

Vaskularni sustav igra važnu ulogu u remodeliranju koštanoga tkiva bilo u fiziološkim bilo u patofiziološkim uvjetima. Zbog bliskoga anatomskoga odnosa, endotelijalne i koštane stanice izlučuju slične parakrine medijatore. Jedan od njih je i angiotenzin II koji se lokalno sintetizira upravo u endotelijalnim stanicama. Angiotenzin II glavni je izvršni peptid sustava angiotenzin-renin i kao takav poznati je regulator kardiovaskularnog sustava i sustava tjelesne homeostaze. Angiotenzin II nastaje konverzijom iz angiotenzina I pomoću angiotenzin konvertirajućeg enzima (ACE). Povezuje ga se s više različitih procesa koji uključuju kontrolu neuralne aktivnosti, staničnoga rasta i razvoja. Dokazani su njegovi stimulatorni utjecaji na sintezu DNA i sintezu kolagena na staničnim linijama osteoblasta i to preko receptora tipa 1, kao i stimulatorni učinak na ekspresiju receptora aktivatora NF-kB liganda

(RANKL) u osteoblastima. Poznata su dva subtipa angiotenzin II receptora. Tip 1 (AT_1) i tip 2 (AT_2) receptori. AT_1 receptori reguliraju dva signalna puta koji uključuju G proteine. Jedan put uključuje aktivaciju fosfolipaze C, uz posljedičnu stimulaciju produkcije inozitol 1,4,5-trifosfata, dok drugi uključuje regulaciju stupnja aktivnosti cAMP, bilo aktivacijom ili inhibicijom adenilat-ciklaze. Signalni put angiotenzin II receptora (AT_2) još valja do kraja razjasniti. Specifični antagonisti peptida kao losartan i ostali sartani blokiraju AT_1 receptore, dok ne-peptidne komponente kao PD123317 i PD123319 antagoniziraju AT_2 receptore. Angiotenzin II upleten je i u patofiziološke procese povezane s vaskularnim ozljedama i oporavkom od njih, kao i s mehanizmima koji promovirajući oksidacijski stres u vaskularnom zidu vode do endotelijalne disfunkcije, stanične apoptoze i lipoproteinske peroksidacije. Kao takav povezuje se s medijatorima oksidacijskog signalnoga puta.

Angiotenzin II isto tako može aktivirati više nuklearnih transkripcijskih faktora, signalnih transdusera i aktivirajućih faktora kao STAT, AP-1, NF-kB i proteina ovisnih o sustavu ciklične adenozin monofosfataze (cAMP). Među njima osobito je zanimljiv NF-kB sustav, budući da je upravo on odgovoran u kontroli imunološki odgovornih gena, uključujući citokine i adhezijske molekule. Inhibicija NF-kB sustava može prevenirati upalni odgovor i promijeniti ekspresiju proinflamatornih citokina. Angiotenzin II može aktivirati NF-kB sustav, inducirajući fosforilaciju i degradaciju inhibitora kB faktora (IkB). Ipak, mehanizam toga djelovanja nije do kraja razjašnjen. Poznato je, osim toga, da angiotenzin II može aktivirati obitelj mitogen-aktivirajućih protein kinaza (MAPK) koji su uključeni u NF-kB indukciju putem različitih citokina. Imajući u vidu sve navedeno, moguće je pretpostaviti da bi angiotenzin II mogao imati regulatorni učinak na koštani metabolizam.

U prethodnim istraživanjima dokazano je da angiotenzin II na staničnim linijama osteoblasta porijekla stapesa stimulira produkcija interleukina 6 te smanjuje aktivnost alkalne fosfataze.

Genetskim analizama ustanovljena je povezanost polimorfizma genotipa sustava renin-angiotenzin–aldosteron kod osoba oboljelih od otoskleroze (M235T, ACE I/D).

3. Oksidacijski stres

Oksidacijski stres u osnovi je patološki proces. Reaktivni kisikovi spojevi koji nastaju njegovom aktivacijom izuzetno su štetni i mogu dovesti do lipidne peroksidacije. Vrlo su kratkotrajnoga života ali sudjeluju u stvaranju takozvanih sekundarnih glasnika, kao što je 4-hidroksinonenal (HNE) čiji je vijek života dulji, može se vezati za stanične proteine i tako djelovati na sustavnoj, a ne samo na lokalnoj razini. Mjerenjem ekspresije alkalne fosfataze uočeno je da medijatori oksidacijskog stresa, kao vodikovi radikali H_2O_2 iz ksantin/ksanin oskidaza sustava, mogu uzrokovati staničnu diferencijaciju u staničnim kulturama osteoblasta. HNE je aldehid koji se smatra jakim induktorom fibrinogeneze (mezenhemijalnih stanica) i glavnim markerom lipidne peroksidacije. Ovaj reaktivni aldehid je citotoksični i mutageni spoj, ali i signalna molekula s utjecajem na staničnu proliferaciju, diferencijaciju i apoptozu. Upletenost HNE istraživana je u patofiziologiji različitih bolesti kao što su: kronična upala, neurodegenerativne bolesti, respiratorni distres sindrom odraslih, ateroskleroze i šećerne bolesti. U studijama staničnih kultura osteosarkoma zamijećeno je da ovaj aldehid uzrokuje inhibiciju stanične proliferacije u istim koncentracijama kojima uzrokuje apoptozu i inhibira staničnu diferencijaciju. Kao takav, moguće je da je upravo HNE jedna od važnih signalnih molekula koje reguliraju metabolizam humanih koštanih staničnih kultura

4. Cilj istraživanja

Aktivnu fazu otoskleroze karakterizirju znakovi upale uz prisustvo brojnih multinuklearnih osteoklasta, osteocita i proliferativnih endotelijanih stanica u pseudovaskularnim prostorima i aktivnim otosklerotičnim žarištima. Prethodna istraživanja potvrdila su ulogu različitih proinflamatornih citokina u patofiziologiji otoskleroze. Unatoć dosadašnjim istraživanjima (virusi, bakterije, mehanička trauma) potencijalni tzv. trigerring faktor inflamacije u patofiziologiji otoskleroze još uvijek nije otkriven. Angiotenzin II istraživan je kao mogući faktor koji pokreće inflamatorne putove u patofiziologiji otoskleroze. Aktivirajući inflamaciju Ang II može aktivirati i oksidacijski stres koji može biti odgovoran za različite oblike kohlearnoga oštećenja (difuzija reaktivnih kisikovih spojeva u unutarnje uho). Osim transformirajućega faktora rasta B, faktora rasta fibroblasta, endotelina i inzulin faktora rasta, i produkti lipooksigenacije važni su medijatori djelovanja angiotenzina. Jedan od glavnih produkata procesa oksidacijskog stresa i lipidne peroksidacije s dokazanim učincima na stanične kulture osteoblasta je 4-hidroksinonenal (HNE). Povezujući učinke angiotenzina II i produkta oksidacijskog stresa (HNE) na stanične kulture osteoblasta, za pretpostaviti je da bi upravo angiotenzin II mogao biti novi lokalni regulator koštanoga metabolizma.

Cilj ovoga istaživanja bio je ustanoviti ulogu angiotenzina II i oksidacijskoga stresa na koštani metabolizam općenito odnosno u patofiziologiji otoskleroze, provedenim istraživanjem na dva eksperimentalna stanična modela:

1 Istražiti utjecaj angiotenzina II na upalni odgovor primarnim staničnih kultura stapesa (zahvaćenog otosklerozom i zdravoga stapesa)

2 Istražiti interakciju angiotenzina II i HNE-a (kao produkta oksidacijskoga stresa) na koštani metabolizam na stanicama humanoga osteosarkoma (HOS)

II. Utjecaj angiotenzina II na sekreciju proinflamatornih citokina iz primarnih staničnih kultura otoskleroze

UVOD

Uloga angiotenzina II (Ang II) na koštani metabolizam prethodno je već istraživana. Ang II važan je regulator sustava tjelesne homeostaze, a upleten je i u različite stanične inflamatorne putove i kao takav važan je modulator staničnoga rasta. U ovom dijelu istraživanju ispitali smo utjecaj Ang II na sekreciju proinflamatornih citokina iz primarnih humanih staničnih kultura otoskleroze.

MATERIJALI I METODE

Primarne stanične kulture stapesa

Uzorci zdravoga stapesa i stapesa zahvaćenog otosklerozom iskorišteni su za dobivanje primarnih staničnih kultura. Ove stanične kulture karakterizirane su u prethodnim istraživanjima. Sastoje se od osteoblasta različitih fenotipova (s izraženim receptorima za PTH, sposobnošću sekrecije osteokalcina, produkcije alkalne fosfataze i stvaranju mineralnih nodula).

Sekrecija citokina iz staničnoga medija

Stanični medij uzoraka otoskleroze (n=6) i kontrolne grupe (n=6), sakupljeni su nakon 24h tretmana (Ang II 10^{-7}M, ili bazalni uvjeti) i analizirani na sekreciju proinflamatornih citokina. Uzorci su analizirani na 40 proinflamatornih citokina koristeći RayBio® Human Inflammation antibody array G3, RayBiotech, Norcross, GA.

mRNA ekspresija proinflamatornih citokina

Ukupna RNA izolirana je iz staničnih kultura otoskleroze i kontrolne grupe (RNA 2Plus, MP Biomedicals, France). Sinteza, purifikacija i hibridizacija biotin označenih komplementarnih (cRNA) uzoraka učinjena je korištenjem Oligo GEArray® Human inflammatory cytokines

and receptors MicroArray, EHS-011, SA Biosciences, Frederick, MD. Ukupno je testirano 126 gena.

Kvantifikacija mRNA ekspresije pro-inflamatornih citokina putem RT-PCR

Ukupna RNA izolirana je iz uzoraka otoskleroze i kontrolne grupe. Komplementana DNA sintetizirana je RT-PCR metodom uz prethodnu standardizaciju na uzorcima humanih stanica osteosarkoma (HOS i SaOS, American Type Culture Collection, Molsheim, Francuska). Amplifikacija putem PCR metode učinjena je za BCL-6 i CCL25.

Imunocitokemija

U cilju diferenciranja staničnih kultura korištena su sljedeća monoklonalna antitijela: za humane osteoblaste i osteoklaste (CDH11 klon 4D10 i CD61 klon Y2/51, Sigma-Aldrich, Francuska i za humane makrofage (CD68 klon KP1 DakoCytomation, Glostrup, Danska).

REZULTATI

Sekrecija citokina iz staničnog medija

U bazalnim uvjetima uočena je veća koncentracija IP-10 i IL-1b u uzorcima otoskleroze nego u kontrolnoj grupi (88 ± 6.9 prema 36 ± 4.7 srednje vrijednosti za IP-10 et 51 ± 6.9 prema 26 ± 5.1 srednje vrijednosti za IL-1b, $p<0,05$, ANOVA, n=6). Ovaj rezultat u skladu je s prethodno poznatim o jačoj aktivnosti leukocita i osteoklasta (IL-1) i jačom angiogenetskom aktivnosti (IP-10) u kulturama otoskleroze. Također u bazalnim uvjetima u uzorcima otoskleroze uoćena je i smanjena koncentracija inhibitora metaloproteinaze 2, TIMP-2 (11296 ± 1705.7 prema 21410 ± 3278.2 srednje vrijednosti, $p<0,05$, ANOVA, n=6). Ekspresija ostalih citokina nije se bitno razlikovala u bazalnim uvjetima između otoskleroze i kontrolne grupe.

U uvjetima stimulacije s Ang II uočena je povećana koncentracija interferon gamma (IFN-γ) u obje grupe uzoraka (99 ± 27 prema 136 ± 9.1 srednje vrijednosti za otosklerozu i 90 ± 25.3

prema 141 ± 27 srednje vrijednosti za kontrolu, p<0,05, ANOVA, n=6). Također u uvjetima stimulacije s Ang II uočena je povećana koncentracija interleukina 10 (IL-10) u uzorcima otoskleroze (70 ± 7.2 prema 116 ± 12.5 srednje vrijednosti, p<0,05, ANOVA, n=6). U kontrolnim uzorcima u uvjetima stimulacije s Ang II uočena je manja koncentracija inflamatornoga proteina makrofaga, MIP-1a, (22 ± 6.7 prema 9 ± 3.8 srednje vrijednosti, p<0,05, ANOVA, n=6), i interkleukina 11, IL-11 (124 ± 7.3 prema 98 ± 8 srednje vrijednosti, p<0,05, ANOVA, n=6). U uzorcima otoskleroze u uvjetima stimulacije s Ang II uočena je smanjena koncentracija sTNFRII citokina (18330 ± 2991.9 prema 11547 ± 2130.6 srednja vrijednost, p<0,05, ANOVA, n=6), ali bez utjecaja na sekreciju TNF-α.

mRNA ekspresija proinflamatornih citokina

Ukupno je testirano 126 proinflamatornih citokina. Detektirana je ekspresija 15 citokina i citokinima sličnih molekula. Uočene su siginifikantne međusobne razlike. U bazalnim uvjetima, ekspresija BCL-6 bila je veća u otosklerozi nego u kontrolnom uzorku (48 ± 21.7 srednje vrijednosti kontrolnih uzoraka u odnosu na otosklerozu 328 ± 39.7 p<0.05, unpaired t-test). Stimulacija s Ang II smanjila je ekspresiju BCL-6 u uzorcima otoskleroze (424 ± 63.3 u bazalnim uvjetima prema 105 ± 64.7 nakon stimulacije p<0.05, paired t-test). Rezultati qRT-PCR analize nakon normalizacije sa S14-genom potvrdili su više vrijednosti BCL-6 u uzorcima otoskleroze. Ipak, inhibicija BCL-6 nakon stimulacije angiotenzinom II nije potvrđena. Slični rezultati dobijeni su i nakon normalizacije s GAPDH genom.

Imunocitokemija

U obje grupe uzoraka diferencirane su stanice prema korištenim antitijelima (osteoblasti, osteoklasti, makrofazi). Nije uočena razlika između ispitivanih uzoraka što ukazuje da prethodno uočena razlika u produkciji proinflamatornih citokina nije uvjetovana kvantitativnom razlikom staničnih populacija među ispitivanim uzorcima.

ZAKLJUČAK

Dobiveni rezultati podržavaju hipotezu o upletenosti angiotenzina II u patofiziologiji otoskleroze aktiviranjem različitih proinflamatornih citokina i faktora rasta. Ipak, neophodna su dodatna istraživanja u cilju daljnjeg razumijevanja uloge angiotenzina II u patofiziologiji ove bolesti, ali i u metabolizmu stapesa općenito.

III. Interakcija angiotenzina II i 4-hidroksinonenala na koštani metabolizam i u patofiziologiji otoskleroze

UVOD

Oksidacijski stres i angiotenzin II (Ang II) imaju zajednički utjecaj na stanični rast i razvoj putem reaktivnih kisikovih tvari koji su upleteni u različite signalne puteve angiotenzina II. 4-hidroksinonenal (HNE) kao produkt oksidacijskoga stresa je pronađen kao sastavni dio različitih stanica i tkiva, ali njegova fiziološka uloga još uvijek nije u potpunosti razjašnjena. Prethodna istraživanja o ulozi HNE-a na koštani metabolizam pokazala su njegov inhibitorni utjecaj na staničnu proliferaciju i diferencijaciju te stimulaciju apoptoze. Cilj ovoga dijela istraživanja bio je utvrditi mogući zajednički utjecaj Ang II i HNE-a na koštani metabolizam općenito i u patofiziologiji otoskleroze.

MATERIJALI I METODE

Imunohistokemija je provedena na histološkim uzorcima otoskleroze (n=15) i kontrole (n=6). Korištena su monoklonska antitijela na HNE. Humane stanice osteosarkoma (HOS) korištene su za analizu utjecaja angiotenzina II i HNE na proliferaciju (inkorporacija ^{3}H-timidina), diferencijaciju (detekcija aktivnosti alkalne fosfataze putem NBT/BCIP eseja) i indukcija apoptoze putem protočne citometrije (Annexin-V-Fluos Staining Kit). Imunoblot metodom procijenjena je moguća interakcija između angiotenzina II i HNE-a.

REZULTATI

Imunohistokemijom utvrđena su protutijela na HNE u svim ispitivanim uzorcima. Uočena je jasna razlika u distribuciji navedenih protutijela u dvije grupe uzoraka. U kontrolnim uzorcima uočeno je jednoliko nakupljanje protutijela i to u području periosta, dok je u uzorcima otoskleroze uočeno povećano nakupljanje protutijela u područjima novog stvaranja

kosti i područjima pojačane koštane pregradnje. Rezultati proliferacije pokazali su da u određenim koncentracijama Ang II (0.1 nM) i HNE (2.5 μM) stimuliraju proliferaciju HOS ($p<0.0002$). Smanjene aktivnosti alkalne fosfataze uočeno je kod koncentracije Ang II od 0.1 nM i HNE-a od 1 μM i 2.5 μM ($p<0.01$). Suprotne tomu koncentracije HNE-a od 10 μM samostalno povećavaju aktivnost alkalne fosfataze ($p<0.05$) i u kombinaciji s Ang II u koncentracijama od 0.1 ili 0.5 nM ($p<0.01$). Inhibicija apoptoze zabilježena je kod stanica tretiranima samostalno s Ang II i to u koncentracijama od 0.1 i 0.5 nM ($p<0.05$). Suprotno tomu stimulacija apoptoze ($p<0.01$) zabilježena je kod stanica tretiranima samostalno s HNE-om od 10 μM ili u kombinaciji s Ang II za sve koncentracije osim za 1 nM. Rezultati ispitavanja nekroze stanica pokazali su da tretiranje stanica s Ang II u koncentracijama od 0.5 nM i HNE-a od 1 μM smanjuje postotak nekrotičnih stanica ($p<0.05$). Povećanje postotka nekrotičnih stanica zabilježeno je kod koncentracija HNE od 10 μM samostalno ($p<0.01$) ili u kombinaciji s Ang II u koncentracijama od 0,1 nM ($p<0.02$) i 0,5 nM i 1 nM ($p<0.002$) kao i u kombinaciji HNE od 5 μM s 0,1 nM i 0.5 nM Ang II ($p<0.05$) i koncentraciji Ang II od 1 nM ($p<0.003$). Imunoblotom je dokazano postojanje interakcije između HNE-a i Ang II.

ZAKLJUČAK

Dobiveni rezultati potvrđuju hipotezu o upletenosti HNE (oksidacijskoga stresa) u patofiziologiji otoskleroze. Rezultati istraživanja interakcije Ang II i HNE-a pokazuju njihov značajan zajednički utjecaj na proliferaciji, diferencijaciju i apoptozu osteoblasta odnosno na koštani metabolizam općenito.

IV. Konačan zaključak

Rezultati ova dva istraživanja pružaju novi uvid u ulogu angiotenzina II i oksidacijskoga stresa i njegovih sekundarnih glasnika (4-hidroksinonenala, HNE) u patofiziologiji otoskleroze i općenito metabolizmu stapesa. Angiotenzin II inducira sekreciju različitih proinflamatornih citokina u primarnim staničnim kulturama otoskleroze. Također uočena je interakcija angiotenzina II i oksidacijskoga stresa (HNE) na stimulaciju proliferacije HOS stanica. Heterogena distribucija HNE u uzorcima otoskleroze i kontrolnim uzorcima potvrđuje ulogu oksidacijskoga stresa u patofiziologiji bolesti. Konačno, rezultati upućuju na moguću modifikaciju uloge angiotenzina II u patofiziologiji otoskleroze putem produkata oksidacijskoga stresa. Imunoblot metoda ukazala je na moguću direktnu interakciju HNE i Ang II. U prethodnoj studiji uočen različit odgovor staničnih kultura otoskleroze i kontrolnih uzoraka na stimulaciju angiotenzinom II mogao bi dijelom biti moduliran i pod utjecajem oksidacijskoga stresa.

DISCIPLINA : FIZIOLOGIJA I PATOFIZIOLOGIJA EPITELA

KJUČNE RIJEČI : koštani metabolizam, gubitak sluha, angiotenzin II, oksidacijski stres, upala

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I. INTRODUCTION

1. The anatomy and physiology of the human ear

1.1. Anatomy

Based on anatomical and physiological considerations, the ear can be divided into 3 parts: outer (external), middle and inner ear (Figure 1). The external ear consists of the pinna (auricle) and the external auditory canal. The middle ear consists of the tympanic membrane, ossicular chain with supporting ligaments and middle ear space. The sound is conducted from the tympanic membrane to the inner ear by three bones, the malleus (hammer), incus (anvil) and stapes (stirrup), Figure 2. The malleus is shaped like a club, its handle is embedded in tympanic membrane, running from its centre upwards. The head of the club lies in a cavity of the middle ear above the tympanic membrane (the attic) where is suspended by a ligament. Here the head articulates with the cone shaped incus. The incus runs backwards from the malleus and has sticking down from it, a very little thin projection known as its long process, which hangs freely in the middle ear. It has right angle bend at its tip which is attached to the stapes (stirrup), the third bone, shaped with an arch and a foot plate. The stapes is the smallest bone in the body. It is about 3 millimeters long and weights about 3 milligrams. It consists of two legs, the anterior and posterior crura and a footplate. The two crura are joined superiorly at the head, which articulates with the lenticular process of the incus. Each leg resembles a trough, with the concave surfaces facing each other. Of the two legs, the anterior crus is very delicate and less curved. On the posterior crus is a roughened area in the region of the head for the attachment of the tendon of the stapedial muscle. Cartilage covers the articulating surface of the head. The stapes lies almost horizontally, at right angles to the process of the incus. Its base, or footplate, is surrounded by the elastic annular ligament that attaches it to the margins of the vestibular (oval) window. However the stapes remains free to vibrate in

transmitting the sound to the labyrinth. The foot plate covers the oval window (an opening into the vestibule of the inner ear or cochlea), with which it articulates by the stapedio-vestibular joint (Duckert *et al*., 1993). The inner ear is the inner most portion of the ear. It is filled with fluid (perylimph and endolymph) and comprises both hearing and balancing organs (cochlea and labyrinth).

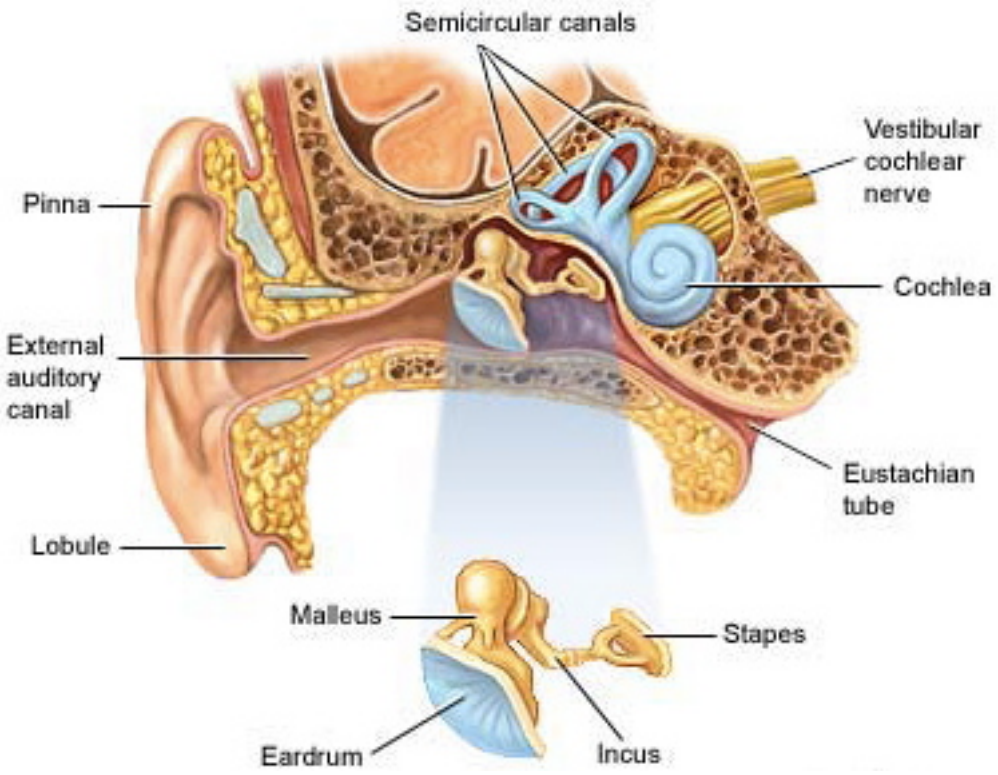

Figure 1- *Schematic view of the anatomy of the human ear*

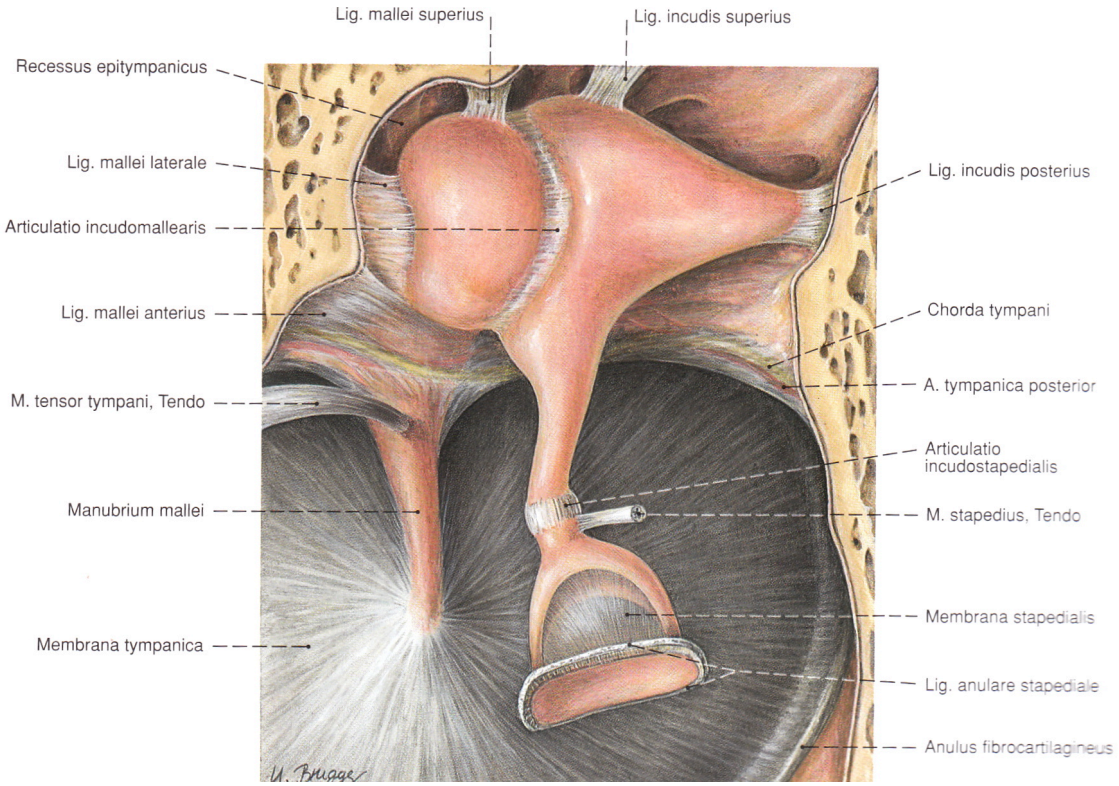

Figure 2*- Auditory ossicles connections, Atlas of Human Anatomy (Sobotta 2001)*

1.2. Physiology

Given the immobility of the human external ear, it is assumed that it serves only as a passive tunnel that collects and filters the sound. However, experimental studies have shown that there are two main functions of the human external ear. Firstly it aids in sound localization and secondly it increases the acoustic pressure at the tympanic membrane in the 1.5 to 5kHz range, which is the frequency range most important for speech perception. The middle ear ossicles form a transmission pathway that conducts sound energy from the tympanic membrane, at the interface of the external and middle ear, to the oval window of the cochlea. The tympanic membrane moves the manubrium or handle of the malleus. In turn, the long

process of the incus and manubrium move together because the malleoincudal joint is essentially fixed. In contrast, the joint between the incus and the stapes is flexible. Therefore, because the stapes is fixed at its posteroinferior border, movement of the tympanic membrane causes it to rock in and out of the oval window. The changes in acoustic pressure caused by the stapes moving in and out of the oval window are transmitted instantaneously by the perilymph through the cochlear partition and then to the round window. This pressure transmission through the cochlear partition causes it to move either upward or downward, depending on the direction of the pressure change. Change initiates a mechanical traveling wave that reaches a maximum at some point on the basilar membrane depending on the frequency of the stimulating sound. The mechanical traveling wave moves from the base to the apex of the cochlea largely owing to a reduced stiffness of the basilar membrane in the apical direction. This traveling wave disturbance causes the hair cells in the organ of Corti to stimulate the dendritic endings of the cochlear nerve, thus signaling to the central auditory system that a sound stimulus has occurred (Lonsbury-Martin *et al*., 2003).

1.3. The development of the human ear

The development of the ear structures is a complex process. The critical period of ear development begins in the early embryologic period (three weeks of gestation), the inner ear appearing first. The inner, middle and outer portions of the ear have different embryological origins and development can be arrested at any stage. The result is a range of abnormalities from mild to severe and a disorder of one part does not necessarily signify in disorder in another, but proximity in terms of time development, originating tissue, anatomic

characteristics, and function does mean that multiple malformations are possible (Wareing *et al*., 2006).

The middle ear cavity and lining of the middle ear and Eustachian tube develop from the expanding terminal end of the first pharyngeal pouch with a small contribution from the second pharyngeal pouch. This is apparent in the fourth week of gestation. In the fifth and sixth week of gestation the mesenchyme between the branchial cleft and the developing inner ear has condensations destined to become the ossicles. The head of the malleus, the body and short process of the incus develop from the neural crest mesenchyme of the first branchial arch (Meckel cartilage). The handle of the maleus, the long process of the incus, and tympanic surface of the stapes footplate develop from the second branchial arch (Reichert cartilage). The vestibular surface of the footplate and annular ligament arise from the mesoderm of the otic capsule. The stapes appears slightly before maleus and incus. It is initially ring shaped and penetrated by the stapedial artery. By 10 weeks, the stapes has already started to assume the familiar stirrup shape. By the time ossification begins from the solitary centre at 16 weeks, the structure is a model of the future stapes. It is reduced in bulk throughout fetal life to develop its slender architectural form.

The maturation of the bony labyrinth plays a role in the pathogenesis of the otosclerosis. The otic capsule arises from mesenchyme surrounding the otic vesicle at 4 weeks of embryologic development. At 8 weeks, the cartilaginous framework is begun. At 16 weeks, endochondral bone replacement of this framework begins in 14 identifiable centers (Donaldson *et al*., 1992; Nager 1993). In some people complete bony replacement does not occur and leaves cartilage in certain locations. One of these regions, the *fissula ante fenestram*, is anterior to the oval window and is usually the last area of endochondral bone formation in the labyrinth. According to the temporal bone studies this region is affected in 80-90% of patient with

otosclerosis (Bastian *et al*., 1996). The otic capsule reaches its adult size by week 22 of gestation (Wareing *et al*., 2006).

2. Overview of the stapes (otosclerosis) pathophysiology

Otosclerosis (localized bone dysplasia) is a primary disease of the human otic (labyrinthine) capsule and stapes footplate, causing hearing and balance disorders, depending on the site, size, and histologic features of the pathologically involved area. When describing otosclerosis it is important to distinguish between the histological and clinical form of the disease (Declau *et al*., 2007). Histological otosclerosis refers to a disease process without clinical symptoms and is discovered only on routine sectioning of the temporal bone. Clinical otosclerosis refers to the presence of otosclerosis foci at the site where it causes conductive hearing loss by interfering with the motion of the stapes or of the round window membrane (Guild 1944; Shambaugh 1949; Arnold *et al*., 1987). Otosclerotic plaques are mainly localized anterior to the oval window (fissula ante fenestram region) and on the stapes footplate (80%), round window (30%), pericochlear region (21%) and the anterior part of the internal auditory canal (19%). Other localizations although very rare have also been described: malleus, incus, facial canal, semi-circular canals and endolymphatic canal (Guild 1944). So far, otosclerotic foci have not been described in the external auditory canal (Hueb *et al*., 1991).

There are three forms of histologic lesions of the otosclerosis: otospongiosis (early phase), transitional phase and otosclerosis (final phase). The early, active phase lesions consist of histiocytes, osteoblasts and the most active cell group osteocytes (Fraysse *et al*., 1994). They absorb the bone around pre-existing blood vessels that causes higher and better

microcirculation. Otoscopic finding can reveal the reddish hue caused by this higher vascularization, known as Schwartze sign (Roland *et al*., 2006).

As osteoblast becomes more involved, these areas grow rich in amorphous ground substance and deficient in mature collagen, resulting in formation of new spongy bone. At hematoxylin-eosin staining, this new bone appears densely blue known as mantles of Manasse (1912). The late phase is characterized with the formation of sclerotic, dense bone in areas of previous bony resorption. The vascular spaces that were previously dilated are narrowed due to bony deposits. Otosclerosis begins in endochondral bone, as the spongious and sclerosis continue the endosteal and periosteal layers also become involved (Roland *et al*., 2006). Its clinical presentation is mainly conductive hearing loss (CHL), although sensorineural hearing loss (SHL) and mixed hearing loss (MHL) may also occur. Symptom onset usually occurs by the early third decade of life, but onset is not unusual later in life. Bilateral form of disease is present in almost 80% of cases (Roland *et al*., 2006).

Otosclerosis is also often associated with osteogenesis imperfecta (van der Hoeve syndrome) in a classic triad of hearing loss (conductive, mixed, or sensorineural), spontaneous bone fractures, and blue sclera (McKenna *et al*., 1998).

Otoscopic examination findings are usually normal, although 10% of patients demonstrate previously mentioned Schwartze sign. Tuning-fork examination reveals signs of conductive hearing loss. Early in the disease course, pure-tone audiometry usually demonstrates low-frequency conductive hearing loss. High-frequency losses begin to manifest with gradual air-bone gap widening. If cochlear involvement is not present, otosclerosis is limited to maximal conductive loss of 50-65 dB across all frequencies. If cochlear involvement is present, a mixed hearing loss appears, with high frequencies more affected. Stapes fixation produces an audiometric artifact known as the Carhart notch, which is characterized by elevation of bone

conduction thresholds of 5 dB at 500 Hz, 10 dB at 1000 Hz, 15 dB at 2000 Hz, and 5 dB at 4000 Hz (Roland *et al*., 2006). Cochlear otosclerosis is characterized by the presence of mixed or primary sensorineural hearing loss where air-bone gaps are minimal, and the speech discrimination scores are better than what would be anticipated with the degree of hearing loss (Donaldson *et al*., 1993). Tympanometry usually reveals a type As or A tympanogram. Acoustic reflexes are often abnormal and may provide the earliest evidence of otosclerosis. Advancing fixation affects both ipsilateral and contralateral acoustic reflexes, even in unilateral disease. The course of the otosclerosis is very variable and so far there are no known factors that could suggest the progression of the disease (Glasscock *et al*., 1990). However, there are evidence showing that during puberty, pregnancy and the menopause hormonal factors could influence the disease progression (Glasscock *et al*., 1990; Donaldson *et al*., 1993).

Today surgical treatment (modern stapedotomy) remains the best possible treatment option for otosclerosis (Gjurić *et al*. 2007).

3. Epidemiology of otosclerosis

The otosclerosis is predominantly a Caucasian disease correlating well with their geographic distribution throughout the world and with the mean prevalence estimated at 3/1000 or 0.3% (Karosi *et al*., 2009). Clinical otosclerosis is very rare among black, oriental and American Indian populations. The Japanese and South American populations have the 50% of the incidence of that of Caucasians (Altman *et al*., 1967; Tato *et al*., 1967; Thy *et al*., 2009). In distinguishing the clinical of histological form of otosclerosis several authors have studied the prevalence of the later. The mean prevalence was estimated to be 8.3% in Caucasian

population (Altman *et al*., 1967; Donaldson *et al*., 1993). Interestingly the study of Declau *et al*. (2007) reported the histologic prevalence of otosclerosis to be much lower, 3.4%. These results are in agreement with the histologic prevalence among Japanese people that is reported to be 2.56% (Ohtani *et al*., 2003) suggesting a possible common biochemical disease pathway. The average age at presentation is 33 years. Recent reports show shift to the age between 40 and 50. The incidence ration between female and male is 2:1 and it has been reported that otosclerosis advances more rapidly in females than males. About 60% of the patients with clinical otosclerosis report a family history of the disease. The remaining 40% of cases are either autosomal dominant inherited cases with failure of penetrance in other family members, phenocopies, new mutations or rare cases transmitted with alternate model of inheritance (Gordon 1989; Sabitha *et al*., 1997; Thys *et al.,* 2009).

4. Etiology of otosclerosis

The etiology of otosclerosis despite the variety of theories proposed in the past is still not clear. In 1977 Wright suggested a vascular thrombosis and Glasscock in 1990 a repetitive micro fractures in the fissula ante fenestram region as a possible causal factors. These theories could not be confirmed by histological findings done by Guild in1994. Donaldson in 1993 based on a similar histological finding between the osteogenesis imperfecta and otosclerosis proposed a possible causal-link. In half of the otosclerosis cases there is a hereditary trait and multiple genetic and environmental factors contribute to its development.

4.1. The genetics of otosclerosis

In 1861 the first report on a familiar pattern of hearing loss due to otosclerosis was presented by Toynbee. Later, another family case of hearing loss was documented by Magnus (1876). In 1922, Albrecht was first to conclude that otosclerosis could be inherited as an autosomal dominant disease in certain families. In 1960 Larson supported this hypothesis and found that in most autosomal dominant families, the penetrance is incomplete and lies between 25-40%. Fowler in 1966 conducted a twin study and found concordance for clinical otosclerosis in nearly all 40 pairs of monozygotic twins, which supported the initial theory that otosclerosis has a genetic basis. But this author concluded that due to the lack of evidence on the genetic transmission of histologic otosclerosis, it is not known whether the genetic basis of inheritance is related to the formation of the otosclerotic focus within the temporal bone or the tendency for a lesion to progress once it has already started, or both. Further studies showed that the genetic background facilitates the progression of the disease, but a triggering factor is probably needed. Morrison and Bundy (1970) in a detailed genetic study in larger otosclerotic families concluded that otosclerosis is autosomal dominant disease with 40% of penetrance. The results of that study were confirmed in 1975 (Gapany-Gapanavicus *et al*.) and 1984 (Causse *et al*.). Although a strong familial background exists, 40-50% of all clinical cases have been reported to be sporadic, by reduced penetrance and characterized by other model of inheritance besides autosomal dominant. Today several known loci for otosclerosis have been localized (OTSC1, OTSC2, OTSC3, OTSC4, OTSC5, OTSC6, OTSC7, OTSC8 and OTSC10) on chromosomes 15q, 7q, 6p, 16q, 3q, 6q, 9p and 1q (Tomek *et al*., 1998; Van Den Bogaert *et al*., 2001, 2002, 2004; Chen *et al*., 2002; Brownstein *et al*., 2006; Thys *et al*., 2007; Bel Hadj *et al*., 2008; Schrauwen *et al*., 2011). However, to date none of the otosclerosis-

causing genes have been identified despite the known chromosomal localization. Recently a new location on chromosome 7q22.1, located in the gene RELN (reelin protein that has a crucial role in the regulation of neuronal migration and positioning in the brain development) has been associated with otosclerosis. What is known today of RELN function has not an evident link with otosclerosis pathogenesis, but this finding could offer a new insight in the molecular mechanism related to the development of the disease (Schrauwen *et al*., 2009, 2010). Still, many otosclerosis patients do not have a family history of otosclerosis or do not follow a clear Mendelian segregation of disease and they represent the so called complex form of otosclerosis, caused by environmental and genetic factors.

Autoimmune reaction against the otic capsule has been suggested as a possible etiologic factor in otosclerosis, and autoantibodies against type II collagen have been detected in the sera of patients with otosclerosis. The first genetic case-control association study of collagen types I and II genes with otosclerosis was done by McKenna in 1998. The COL2A1 gene was first analyzed due to its connection with the globuli interossei and the hypothesis that an auto-immune reaction to type II collagen could be involved in otosclerosis (Yoo 1984; Sorensen *et al*., 1988). Studies on association of COL1A1 and otosclerosis have demonstrated an even more significant association between clinical otosclerosis, both familial and sporadic, and a Sp1 binding site polymorphism in the first intron of the COLIA1 gene (McKenna *et al*., 2004). In 2007 Chen *et al*. further confirmed the correlation between the COL1A1 expression and otosclerosis.

Like in many other diseases, the associations with the human leukocyte antigen (HLA) have been investigated for otosclerosis. HLA is an important factor associated with many diseases, especially those with an immunological component. The HLA system represents the major histocompatibility complex in humans and is encoded by a complex DNA segment on

chromosome 6. Several studies showed an association between HLA system and otosclerosis (Miyazawa et al., 1996) while in others no association could be determined (Nibu *et al*., 1990).

The transforming growth factor-β1 (TGF- β1) is a member of the TGF-β superfamily and plays an important role in the embryonic development and maintenance of both cartilage and bone. It is also important for the development of the otic capsule. In human otosclerotic bone cell cultures TGF- β1 can modify the phenotypic expression of glycosamynoglycans (GAG), fibronectin and collagen of the extracellular matrix (Bodo *et al*., 1995). In 2 large independent population studies (Janssens *et al*., 2005; Thys *et al*., 2009) TGF-β1 has been associated with otosclerosis. Based on the association with the TGF-β1 family the 13 new candidate genes were selected and further analyzed related to the metabolism of the otic capsule, involvement in the syndromic or non-syndromic forms of stapes fixation and other hypothesis related to the development of the otosclerosis. Two genes, bone morphogenetic proteins 2 and 4 (BMP2 and BMP4) showed a significant association. Results showed that the individuals who possess the T allele of a single nucleotide polymorphism (SNP) in the 3' untranslated region of BMP2 have a higher risk of developing otosclerosis. The other single nucleotide polymorphism (SNP) association was an amino acid-changing SNP lying on exon 4 of BMP4 (Conne *et al*., 2000; Schrauwen *et al*., 2008).

4.2. Measles virus infection

Over the past 20 years many authors have studied the potential role of measles virus infection in the pathophysiology of otosclerosis. The hypothesis was assumed on a similarity between otosclerosis and Paget's disease where evidence suggested a possible viral etiology (Singer,

1999). In 1986 McKenna *et al*. for the first time reported the presence of paramyxoviral neucleocapsids resembling structures in the osteoblast–like cells of the otosclerosis origin. Later, several studies supported this hypothesis based on electron microscopy observations, immunohistologic studies, and RNA detection in the otosclerotic tissue (Arnold *et al*., 1987; McKenna *et al*., 1990; Niedermayer *et al*., 1994; Arnold *et al*., 1996; McKenna *et al*., 1996). On the contrary, a study reported by Bozorg Grayeli *et al*. in 2000 could not confirm these results. The study was carried out on a large number of otosclerotic samples (35 patients) and measles virus could not be detected in none of the analyzed samples by multiple highly sensitive detection methods. They concluded that high incidence of acute infections by viruses such as herpes simplex virus and measles virus in the general population, and the presence of these viruses in different organs including the inner ear in normal individuals challenges the possible causal relationship between the measles virus and the chronic disease. In 2007 Arnold *et al*. suggested the decrease in otosclerosis incidence due to a measles vaccination in the early 1970s. But since the otosclerosis develops mostly between the 3rd and 5th decade of life it is too early to draw a definitive conclusion. The measles virus shows certain organotropism to the otic capsule and only human and primates are host of the measles virus due to their complementary cell surface structures CD46 and CD150 (Dorig *et al*., 1993; Tatsuo *et al*., 2000). Latest study done by Karosi et al. in 2008 showed the existence of 4 new novel splice variants of the measles virus receptor CD46 only present in the otosclerotic stapes footplate. Histologic study of Sizklai *et al*. in 2009 confirmed the presence of measles virus in the stapes footplate of patients with histologically diagnosed otosclerosis. Measles virus may be a triggering factor in the inflammatory events which occur during the active phase of otosclerosis in a subpopulation of patients. However, the exact role of measles virus in the pathogenesis of otosclerosis remains to be further analyzed.

4.3. Hormonal influences

Estrogen deficiency is considered to be a cause of osteoporosis in women, and estrogen substitute therapy has shown beneficial effect in those cases. Although it is well established that estrogen inhibits bone resorption (Pacifici *et al*., 1996), its effect on osteoblasts is still unclear. In otosclerosis, hearing deterioration has been associated with pregnancy, and thus sex hormones were believed to be involved in the progression of the disease (Menger *et al.*, 2003). A more recent study compared female patients with and without children in a relatively large population (n=154, Lippy *et al.*, 2005). This retrospective comparison did not show a difference of hearing loss between the 2 groups. However, considering the methodological limitations of such a study, definitive conclusions cannot be drawn. Estrogen has an inhibitory effect on bone resorption by directly inhibiting osteoclast activity as well as decreasing auto- and paracrine production of cytokines such as IL-1 and IL-6 and tumor necrosis factor (TNF, Pacifici *et al*. 1996). Contradictory observations have been published concerning the estrogenic effect in osteoblasts, depending on the model and experimental protocol. Both decrease and increase of alkaline phosphatase activity and osteoblastic proliferation have been reported. This variability might be explained by the predominant type of estrogen receptor present in the target tissue (Bord *et al*., 2000). Imauchi *et al*., (2004) investigated the effect of 17β-Estradiol on bone remodeling via diastrophic dysplasia sulphate transporter (DTDST) in otosclerosis and in a human osteoblast-like cell line (SaOS-2). 17β-Estradiol inhibited DTDST in SaOS-2 which expressed type alpha and beta receptors mRNAs. In otosclerotic cell cultures which expressed only type alpha receptors, no effect could be observed. Authors concluded that the response to estrogens in terms of DTDST activity might be related to the expressed receptor type. It is possible that exacerbating effects of estrogens in patients with

otosclerosis may be mediated by peculiar profiles of estrogen receptor in otosclerotic cells and therefore regulatory mechanisms related to the estrogen receptor profile in the otosclerotic cells need to be further analyzed.

4.3.1. Parathyroid hormone and parathyroid hormone-related peptides receptor expression in otosclerosis

Considering the major role of parathyroid hormone in the physiology of bone metabolism mediated via osteoblasts (Aurbach *et al*., 1992) and the histological aspect of otosclerosis, the role of this hormone in the etiology of the disease has been investigated. Fano *et al*., (1993) showed that higher PTH concentration was required to stimulate the adenylate cyclase activity (AC) in stapes cells. The study conducted by Bozorg Grayeli *et al*., (1999) showed a lower PTH-PTHrP receptor mRNA expression in the otosclerotic samples associated with a lower cAMP response. This difference supports the hypothesis that abnormal cellular response to PTH may contribute to the abnormal turnover in otosclerosis.

4.4. Inflammation factors

Previous studies have demonstrated the possible role of inflammatory and regulatory cytokines in the etiopathogenesis of otosclerosis (Karosi *et al*., 2005). Tumor necrosis factor-alpha (TNF-α) is an inflammatory cytokine produced by monocyte-macrophage system which regulates the differentiation and activation of bone-derived mesenchymal cells. Its increased expression in otosclerotic bone could result in an extensive osteoclast activation cascade and pathologic bone-turnover (Karosi *et al*., 2005, 2006; Stankovic *et al*., 2006). Cytokines such

as osteoprotegerin (OPG), receptor activator of nuclear factor-kappa B (RANK) and RANK ligand (RANK-L) play a major role in the system that directly controls bone turnover (Zehnder *et al*., 2005). RANK-L is expressed by many cells including osteoblasts which are directly involved in bone turnover. It also regulates the differentiation, activation, and survival of osteoclasts by its specific RANK receptor. Osteoprotegerin or tumor necrosis factor–alpha super family member 11b (TNFalphaSF11b) is the main inhibitor of the tumor necrosis factor alpha and an antagonist of the RANK-L. It blocks the osteolysis and osteoclast formation and induces the osteoclast apoptosis. Its main function is to maintain and secure the normal bone turnover (Boyce *et al*., 2007).

Cytotoxic enzymes (elastase, collagenase, cathepsin-D/B), inflammatory cytokine mediators such as TNF-alpha, interleukin-1 (IL-1), interleukin-6 (IL-6) and complement fragments (C3a, C3b, C5a) are released from otosclerotic foci in the early stages of the disease. It is possible that these molecules flow into the perilymph and interfere with the electromotility of outer hair cells, causing the sensorineural hearing loss (Karosi *et al*., 2005, 2006). In a recent study, Sziklai *et al*. (2009) showed that short-term effects of the TNF-α will not cause hearing loss, but its long-term effects might lead to sensorineural hearing loss associated with changes in the gene expression of the sensory epithelium. The increased TNF-α production could trigger the focal bone resorption, thus the administration of monoclonal anti TNF-α antibody could be considered as a potential medical treatment option in types of cochlear otosclerosis with sensorineural hearing loss (Fricker *et al*., 2007).

5. Medical treatment of otosclerosis

So far there have been several medical treatment options suggested for otosclerosis (NaF, biphosphonates and bioflavonoids).

The hypothesis of a potential preventive role of sodium fluoride (NaF) in otosclerosis was supported by epidemiological studies showing that disease was associated with areas of low-fluoride content in the drinking water (Daniel, 1969). In 1980s Causse *et al.* thought that moderate doses of NaF could inhibit proteolytic enzymes and therefore decrease disease progression. In 1989 Bretlau *et al*. evaluated the effect of sodium fluoride treatment in patients with otosclerosis in a prospective clinical double-blind, placebo-controlled study of 95 patients. Their results showed a statistically significant greater deterioration of hearing loss in the placebo group than in the group actively treated with 40 mg of sodium fluoride daily. These results supported the view that sodium fluoride can change otosclerotic active lesions to more dense, inactive otosclerotic lesions. More recently Bozorg Grayeli *et al.* in *2003* studied the possible molecular effect of sodium fluoride (NaF) in otosclerosis. They showed that sodium fluoride affects the otosclerotic cells by inhibiting the diastrophic dysplasia sulphate transporter (DTDST) activity via intracellular calcium and cAMP. This transporter is implicated in the sulfation of bone matrix glycosaminoglycans (GAG). Previous studies (Bodo et al., 1997) had demonstrated that otosclerotic cell cultures produce bone matrix GAG with a higher level of sulfation. The inhibitory effect of the sodium fluoride on DTDST activity may reduce GAG sulfation, participate in the reduction of bone remodeling and eventually lead to the preservation of the sensorineural hearing function in otosclerotic patients (Bozorg Grayeli *et al*., 2003). In 2006, Imauchi *et al*. further showed that the observed increased activity of DTDST in otosclerotic cells can be inhibited by Dexamethasone. The effect could be mediated via inhibition of autocrine/paracrine

interleukin 6 (IL-6) secretion that has stimulatory effect on DTDST. This observation opened new pharmacological insights in the treatment of hearing loss in otosclerotic patients.

The use of biphosphonates have also been studied as a potential therapy option in otosclerosis. In 1993 Keneddy *et al.* performed a study to evaluate the role of etidronate disodium for the treatment of progressive hearing loss in patients with otosclerosis. The results showed a trend (not statistically significant) toward stabilization or improvement in air conduction thresholds in some frequencies (1000 and 4000 Hz) and in bone conduction thresholds at other frequencies (500, 1000, and 2000 Hz). Other studies on the use of biphosphonates in otosclerosis showed no biphosphonates effect on hearing preservation and severe side effects were observed (Yesil *et al*., 1998). In 2008 Kanzaki *et al*. performed a study on an animal model (osteoprotegerin knock out mice) and showed that the use of nitrogen-containing biphosphonates, risedronate, could prevent both degenerative changes of auditory ossicles and progressive hearing loss.

Flavonoids or biflavonoids (flavonols and flavanols) have also been studied as potential therapeutical agents in otosclerosis. They are most commonly known for the antioxidant activity. Additionally, at high experimental concentrations that would not exist *in vivo*, the antioxidant abilities of flavonoids *in vitro* are stronger than those of vitamin C and E. Sziklai *et al*. (1995) investigated the possible beneficial role of bioflavonoids in an otosclerosis-like lesions. On an animal model it was observed that flavonoids interference in bone remodeling process is induced by prostaglandin E2 (PGE2). The fact that PGE2 effects are mediated through cyclic AMP in bone turnover and flavonoids act synergistically with PGE2 in collagen synthesis confirm a cyclic AMP phosphodiesterase inhibitory role of flavonoids. Previously the beneficial role of Ipriflavone (7-isopropoxy-isoflavone) was observed while in

the 1995 the same effect was observed but for another flavonoid (Quercetine or 7-pentahydroxyflavone), Sziklai *et al*.

6. Otosclerosis-like lesions

So far there are several otosclerosis-like lesions of the stapes footplate and otic capsule observed: *Osteogenesis imperfecta*, *Paget's disease, osteopetrosis, osteoporosis, rare bone diseases*.

Osteogenesis imperfecta is characterized by multiple bone fractures, often as a consequence of only minimal trauma. In about 50% of cases, hearing loss of conductive or mixed type is present. There is a small similarity of the histological findings between osteogenesis imperfecta and otosclerosis. However there are some obvious differences. Most important, in otosclerosis the disease affects manly endochondral layer of the otic capsule as opposed to the *osteogenesis imperfecta* where all three layers of the otic capsule are always involved (Alkadhi *et al*., 2004).

Paget's disease is a metabolic bone disease characterized by excessive bone resorption and formation due to activated osteoclasts. An overall prevalence is about 3% (Klein *et al*., 1995). Like otosclerosis it has a later onset in life and a possible viral pathogenesis has been reported. Histological images are far different from those seen in otosclerosis and hearing loss in affected patient is usually not due to ossicular lesion (Thys *et al*., 2009).

Osteopetrosis is another bone disease characterized by lack of resorption of cartilage in primitive bone, resulting in an increased bone density of different bones in the body, including the temporal bone. Hearing loss is most likely due to a compression of acoustic nerves (Balemans *et al*., 2005).

Osteoporosis is a disease characterized by a reduced bone mineral density and changes in bone structure. Studies of the disease have shown a higher prevalence of the disease in otosclerosis patients suggesting a possible common genetic pathogenesis (Clayton *et al*., 2004).

Otosclerosis and otosclerosis-like lesions have also been reported in different rare bone diseases like: Camurati-Engelmann disease (Huygen *et al*., 1996). A very rare sclerosing bone dysplasia characterized by a constant bone turn-over. Sensorineural hearing loss observed in the course of the disease is most likely caused by narrowing of the internal auditory canals. In families with certain genetic diseases the congenital stapes fixation has been noted: X-linked stapes fixation with perylimphatic gusher (de Kok *et al*., 1995), stapes ankylosis, broad thumbs and toes syndrome (Teunissen *et al*., 1990), proximal syphalangism (Gong *et al*., 1999), and facio-audio-symphalangism (van den Ende *et al*., 2005). The last three syndromes are associated with the Noggin gene mutation. The Noggin protein binds and inactivates BMPs (bone morphogenetic proteins) and has a role in the bone metabolism (Zimmerman *et al*., 1996; Liu *et al*., 2003).

7. Renin-Angiotensin-Aldosteron System

7.1. Overview

The renin-angiotensin-aldosterone system (RAAS) is a major physiological regulator of arterial pressure and hydro-electrolyte balance (Haber *et al*., 1983; Santos *et al*., 2000). This system is also considered not only as an endocrine system but also as an autocrine/paracrine modulator of tissue functions of different organs like heart, blood vessels, kidney, brain and

endocrine glands (Gasparo *et al*., 2000). There are three important components of this system: 1) renin, 2) angiotensin, and 3) aldosterone. Renin, a proteolytic enzyme that is primarily released in the circulation by the kidneys, stimulates the formation of angiotensin in blood and tissues, which in turn stimulates the release of aldosterone from the adrenal cortex. When renin is released into the blood, it acts upon a circulating substrate, angiotensinogen, that undergoes proteolytic cleavage to form the decapeptide angiotensin I. Vascular endothelium, particular in the lungs, has an enzyme, angiotensin converting enzyme (ACE), that cleaves off two amino acids to form the octapeptide, angiotensin II (Ang II) although many other tissues in the body (heart, brain, vascular) can also form angiotensin II. Once formed angiotensin I can be processed by several proteases originating, in addition to angiotensin II, in to several biologically active angiotensin peptides (Figure 2). Thus angiotensin (Ang 2-10), and angiotensin (Ang 3-10) are formed via amino peptidases (Page *et al*., 1974). These amino-derivates of angiotensin I can then be further processed into the biologically active peptides Ang-2-8 (Ang III), Ang-3-8 (Ang IV) via angiotensin-converting enzyme and to Ang-3-7 via post-proline cleaving enzymes (Green *et al*., 1982; Neves *et al*., 1995). Nevertheless, angiotensin II remains to be the central product of the renin-angiotensin system.

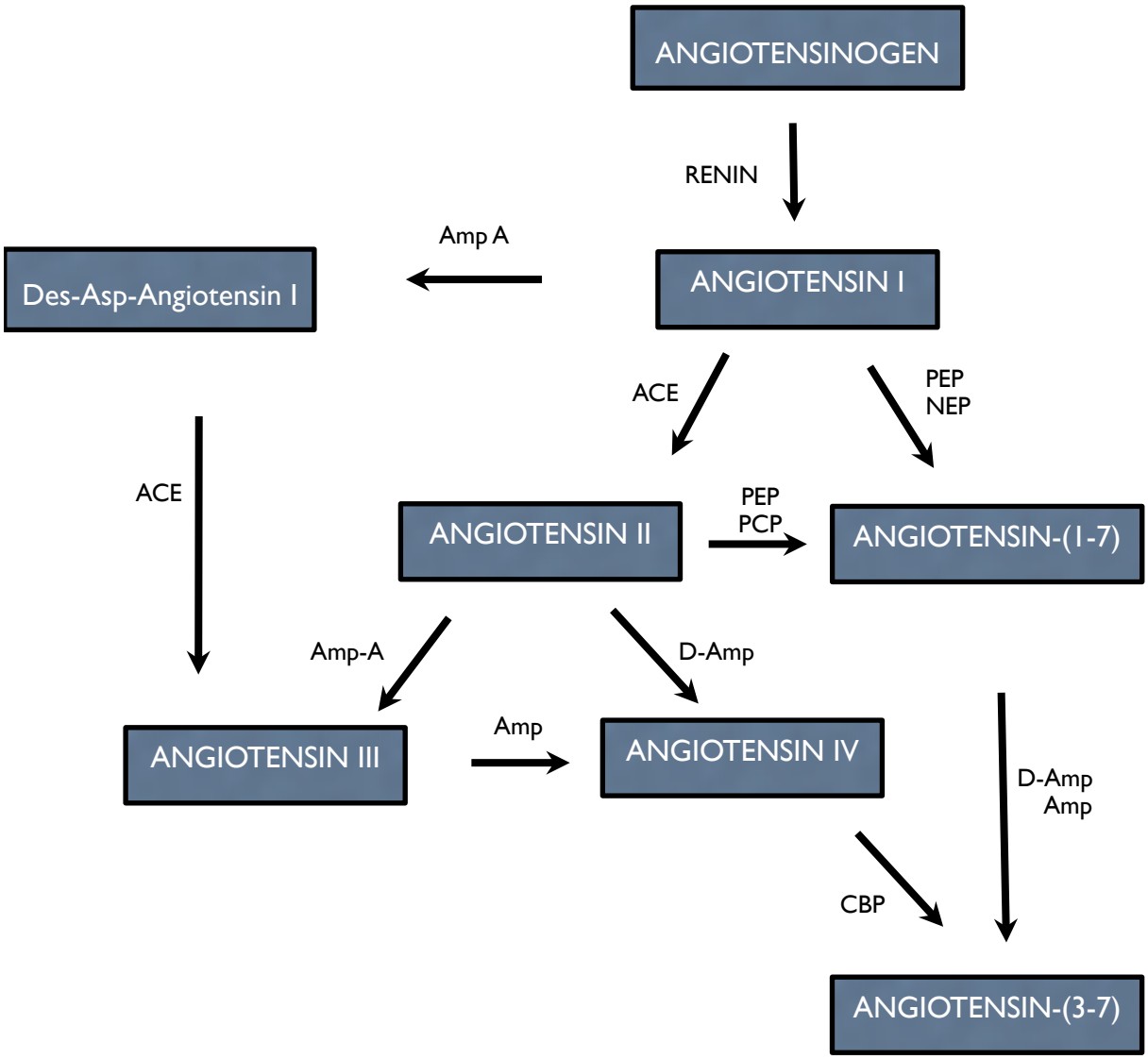

Figure 3- *Simplified illustration of the protheolytic pathway for the formation of biologically active angiotensin peptides. ACE: Angiotensin converting enzyme; Amp A: Aminopeptidase A; Amp: Aminopeptidases; Cbp: Carboxypeptidases; D-Amp: Dipeptidyl-aminopeptidase I-III; NEP: Neural-endopeptidase 24.11; PCP: Prolyl-carboxypeptidase; PEP: Prolyl-endopeptidases (Santos RAS et al., 2000)*

7.2. Angiotensin II

The first insight into the regulation of blood pressure came from the discovery of a vasopressor agent by Tigersted and Bergman in 1898. They called this factor "renin" because it was extracted from the kidney. This pioneering work led to the description of reno-vascular hypertension in animals and humans (Goldblatt *et al*. 1934). In 1940, Braun Menendez *et al*. isolated a vasoconstrictor substance from the renal venous blood. A similar finding was made simultaneously by Page and Helmer (1940) after the injection of renin to an animal. The group also isolated a so-called "renin activator" later know as angiotensinogen. The pressor substance was named hypertensin and angiotonin and later it was isolated and shown to be an octapeptide (Skeggs *et al*., 1956; Bumpus *et al*., 1957; Elliot and Peart, 1957). In 1958, Braun-Memendez and Page agreed on the term angiotensin for this highly potent pressor octapeptide.

Afterwards various components of the cascade leading to the formation of Ang II were characterized, including angiotensinogen, angiotensin converting enzyme (ACE), and Ang I, and Ang III. The synthesis of the peptide Ang II by Bumpus *et al*. (1957) and by *Rittel et al*. (1957) was followed by a continuing series of investigations on the structure-activity relationship of angiotensin analogs, mainly in the hope of finding a peptide antagonist. The sequence of angiotensin II was described as Asp-Arg-Val-Tyr-Ile-His-Pro-Phe in the human and various animals.

In 1987, a committee of the International Society for Hypertension, The American Heart Association, and the World Health Organization proposed abbreviating angiotensin to Ang using decapeptide angiotensin I as the reference for numbering the amino acids of all angiotensin peptides (Dzau *et al*., 1987).

Ang II, a bioactive octapeptide, plays a key role in the regulation of cardiovascular homeostasis (Gasparo *et al*., 2000). The wide spectrum of Ang II target tissues includes the adrenals and kidney (aldosterone release, which in turn acts on kidneys to increase sodium and fluid retention), brain (stimulates thirst centers within the brain), pituitary gland (stimulates the release of vasopressin, antidiuretic hormone), vascular smooth cells (constricts resistance vessels, thereby increasing systemic vascular resistance and arterial pressure), and the sympathetic nervous system (stimulates norepinephrine release from sympathetic nerve endings and inhibits norepinephrine re-uptake). Ang II may also function as a paracrine and autocrine hormone inducing cellular growth and proliferation and controlling extracellular matrix formation (Dzau *et al*. 1987; Griffin *et al*., 1991; Weber *et al*., 1995). Other angiotensin-derived metabolites such as angiotensin 2-8 (Ang III), angiotensin 1-7, or angiotensin 3-8 (Ang IV) have all been shown to have biological activities (Table 1), (Peach, 1977; Schiavonne *et al*., 1990; Ferrario *et al*., 1991; Ferrario et Iyer, 1998; Wright *et al*., 1995).

Table 1. *Amino acid sequences of Angiotensin II precursors and metabolites (De Gasparo et al., 2000)*

Designation	**Peptide Sequence**
Angiotensinogen	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His Leu-LeuVal-Tyr-Ser
Ang I	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
Ang II	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
Ang III	Arg-Val-Tyr-Ile-His-Pro-Phe
Ang IV	Val-Tyr-Ile-His-Pro-Phe
Angiotensin 1-7	Asp-Arg-Val-Tyr-Ile-His-Pro

Pharmacological studies have shown several types of Ang II receptors (Hagiwara *et al*., 1998). Ang II receptor type 1 (AT1) regulates two distinct signaling pathways that involve G proteins. One pathway involves activation of phospholipase C, with subsequent stimulation of the production of inositol 1,4,5-triphosphate, and the other involves the regulation of the level of cAMP via the activation or inhibition of adenylate cyclase. The signaling pathway of Ang II receptor type II (AT2) remains to be explained. More recently the Ang II receptor type 4 (AT4) has been described and it appears to be involved in memory acquisition and recall (Gasparo *et al*., 2000).

Most of the known effects of Ang II are mediated through the AT1 receptor. *In vitro* as well as *in vivo* studies suggest that the AT2 receptor has antagonistic effect on AT1 (Gasparo *et al*., 2000). There is an inactivation of MAPK, anti-proliferative effect, promotion of apoptosis, differentiation and regeneration, opening of delayed K^+ channels and closing of T-type Ca^{2+} channels. The re-expression of AT2 receptor in various diseases suggests a role of this receptor in its pathophysiology. Like the AT2 receptor, the AT4 receptor also opposes the effect of AT1 receptor (Gasparo *et al*., 2000).

Specific non-peptide antagonist, such as losartan and other sartans block AT1 receptors, while non-peptide compounds, such as PD123317 and PD123319, antagonize AT2 receptors.

7.3. Angiotensin II signaling pathways

Ang II activates several nuclear transcription factors, including signal transducer and activator of transcription factors (STAT), activator protein 1 (AP-1), NF-κB and cyclic adenosine monophosphate (cAMP) response element-binding (CREB) protein (Zahradka *et al*., 2002; He *et al*., 2006). Among these, the NF-κB is particularly interesting because it plays a pivotal

role in control of multiple immune response genes, including cytokines and adhesion molecules. Its inhibition is shown to prevent inflammatory responses and attenuate the expression of pro inflammatory cytokines. NF-κB is retained in the cytoplasm by an inhibiting subunit, inhibitor of κB (IκB). In response to pro inflammatory cytokines the IκB is phosphorylated leading to the NF-κB activation. A previous study has clearly shown that Ang II activates NF-κB by inducing the phosphorylation and degradation of IκB (Kawano *et al*., 2006).

Ang II is also known to activate the family of mitogen-activated protein kinases (MAPK), including extracellular signal-regulated kinases (ERK 1 /2), c-jun N-terminal kinase (JNKs, also known as SAPKs), p38MAPKs. The MAPKs are regulatory proteins that control cellular response to growth, apoptosis and stress signals (Martin *et al*., 2005). Ang II activates all these MAPKs except big MAPK-1 (Daniels *et al*., 2005; Znao *et al*., 2005; Wang *et al*., 2006). The activation of the other three MAPK signaling pathways can result in the NF-κB activation by phosphorylating and degrading the IκB. A study in human endothelial cells by Guo *et al*., (2006) demonstrated that stimulation of NF-κB DNA binding by Ang II is dependent in MAPK activation. Results showed that p38MAPK, but not JNK or ERK, is involved in Ang II-induced NF-κB activation.

7.4. Angiotensin II and pathophysiology of otosclerosis

The progression of hearing loss in otosclerosis occurs in 25 to 76% of females during pregnancy and may explain the female predominance reported in this disease (Schuknecht *et al*., 1974). No consistent biological explanation has been advanced for this phenomenon (Donaldson *et al*., 1993). During pregnancy, the plasma angiotensinogen

concentration is increased and the RAAS is stimulated resulting in elevated plasmatic Ang II concentrations (Schrier *et al*., 1997). The tissue Ang II production may also increase during pregnancy as suggested by observations in the smooth muscles of decidual spiral arteries (Moran *et al*., 1997). Considering the progression of otosclerosis during pregnancy and the increase of angiotensinogen production during this period, the implication of Ang II in otosclerosis seemed to be possible. In a study by Imauchi *et al*. in 2008, this hypothesis was investigated through two types of experiments. Authors tested whether functional polymorphisms of RAAS could be associated with otosclerosis in a large case-control study. The results of the study demonstrated that T235 variant allele related to higher angiotensinogen plasmatic concentrations was associated with otosclerosis. Furthermore, ACE I/D polymorphism seemed also to be associated with the occurrence of the disease. The proportion of DD genotype associated to an increased ACE activity was also higher in patients with otosclerosis than in control subjects. Patients with both TT and DD genotypes had an increased risk of otosclerosis compared with patients with DD or TT genotypes only.

The results of the same study also demonstrated the presence of angiotensinogen and Ang II receptor types 1 and 2 in otosclerotic cultures. This study also showed in increased IL-6 release in the culture media and a reduced activity of alkaline phosphatase under the effect of Ang II (10^{-7} M, 24h) only in cultures derived from otosclerosis and not in control stapes. These results suggested that RAAS is implicated in the progression of otosclerosis, and that Ang II does not have the same effects in otosclerosis as in normal stapes. It has been previously described that Ang II increases the autocrine-paracrine production of IL-6 in vascular smooth muscle cells, and this interleukin is a well-known mediator of bone resorption and a pro inflammatory cytokine. The difference between otosclerotic and normal

stapes could be related to a different Ang II receptor profile or differences in the intracellular signaling pathways. This issue is crucial and has to be investigated further.

8. Oxidative stress

8.1. Overview

Oxidative stress takes place in tissues under the effect of toxic agents, inflammation, ischemia, or physical trauma. These situations may lead to an excessive formation of reactive oxygen species (ROS), and disequilibrium in the steady–state between formation and elimination of ROS (Halliwell *et al*., 1989). This stress can cause various acute, as well as chronic diseases, such as cardiovascular (Ignaro *et al*., 2007), neurodegenerative diseases (Emerit *et al*., 2004) and malignant tumors (Dreher et al., 1996). Nowadays, there is growing evidence about the physiological generation of ROS that can occur as a byproduct of other biological reactions. NADPH oxidase is known to be the major source of ROS, but ROS generation also occurs with mitochondria, peroxisomes, cytochrome P-450, and other cellular elements (Bedard *et al*., 2007). Several growth factors and cytokines (e.g. TGF-β and PDGF) through binding to different types of cell membrane receptors can elicit a rise in intracellular ROS. Furthermore, it has been reported that ROS activate MAPK pathways in several systems, including extracellular signal regulated kinase (ERK) 1/2, Jun-NH2-terminal kinase and P38 MAPK cascades (Henrotin *et al*., 2003). With an excessive amount of ROS in the cellular membrane environment, lipid peroxidation occurs and lipid cell constituents are damaged. Lipids are essential in cell membranes and among the first molecules susceptible to the damage caused by reactive oxygen species. Free radical reactions in tissues are

accompanied by oxidative degradation of polyunsaturated fatty acids of membrane lipids (Esterbauer et al., 1990). This process results in the production of highly reactive aldehydes which are named “second messengers” as opposed to the primary free radicals that initiate lipid peroxidation (Esterbauer *et al*., 1967, Schauenstein *et al*, 1977). Unlike reactive oxygen species (ROS), reactive aldehydes are relatively long-lived. Therefore, they might diffuse from the site of their production and attack distant intracellular or extracellular targets. Among many different aldehydes, most thoroughly studied are malondialdehyde, acrolein and the 4-hydroxyalkenal family, in particular 4-hydroxynonenal (HNE).

8.2. 4-hydroxynonenal (HNE)

4-hydroxyalkenals are endogenous end-products of polyunsaturated membrane fatty acid, lipoprotein, and phospholipid peroxidation. HNE is the major lipid peroxidation product of ω-6 polyunsaturated fatty acids (linoleic and arachidonic acid) (Esterbauer *et al*., 1991; Zarkovic 2003; Schaur 2003) Figure 4.

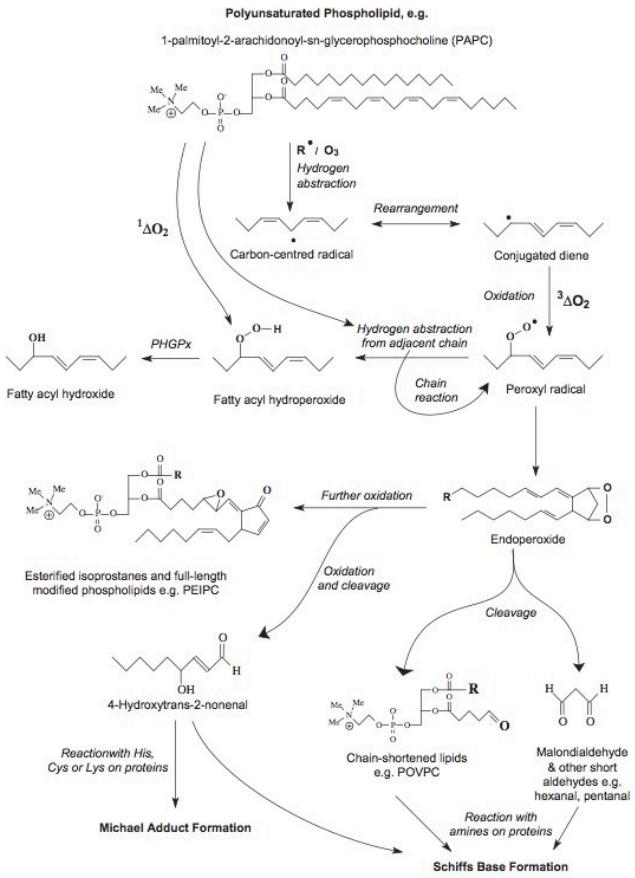

Figure 4- *Pathway of lipid peroxidation (Spicket et al., 2010)*

HNE is a second messenger of free radicals and a major bioactive marker of lipid peroxidation (Zarkovic, 2007). This reactive aldehyde has been generally considered to be merely a toxic end-product of lipid peroxidation contributing to the deleterious effects of oxidative stress. Thus, the importance of HNE has been considered in the pathophysiology of several diseases such as those involving chronic inflammation, neurodegenerative diseases, ischemia/ reperfusion, adult respiratory distress syndrome, atherogenesis and diabetes (Borovic *et al*., 2007). On the other hand, HNE has attracted a great deal of interest in recent years because of its role as a signaling molecule. Numerous studies in the last decade have clearly demonstrated that HNE can cause apoptosis, differentiation, modulate cell growth, and

affect various kinases involved in signal transduction (Esterbauer *et al*., 1991; Zarkovic *et al*., 2002; Dianzani *et al*., 2003). The role of HNE in signaling processes is intriguing because its pleomorphic effects are concentration dependent; at low levels, HNE promotes cell proliferation, whereas at higher concentrations, it induces cell cycle arrest, differentiation, and finally apoptosis (Awasthi *et al*., 2005). For this reason, the regulation of intracellular concentrations of HNE might be crucial for the nature of cell cycle signaling. It was found that HNE can induce cell growth, accompanied by the activation of MAPKs (ERK, JNK and p38) and induction of c-fos, c-jun gene expression, and AP-1 DNA binding activity (Kakishita *et al*., 2001). Studies have also shown that HNE induces various enzymes including phospholipase C, adenylate cyclase, caspase 3, protein kinase C and other kinases involved in signal transduction cascades, strongly comprising a role of HNE in signaling cascades. It is also able to influence cellular functions by regulating the genes encoding for other molecules such as heat-shock protein genes (Allevi *et al*., 1995), c-myc (Barrera *et al*., 2005), c-fos , c-jun (Ruef *et al*., 1998), c-myb (Barrera 1996), cyclins (Barrera *et al*., 2005), p53 gene family (Laurora *et al*., 2005), procollagen type I, and TGF-β1 (Poli *et al*., 1995).

Moreover, HNE is found to be a physiological constituent of many human and animal tissues (Esterbauer *et al*., 1967, 1990; Poli *et al*., 2000; Zarkovic *et al*., 2009) however its physiological role has yet to be clarified, but it is likely that HNE could have an important role in the regulation of cellular growth (Zarkovic *et al*., 1993; Barrera *et al*., 1991; Kreuzer *et al*., 1998).

Relatively high steady state concentrations of HNE in cell membranes indicated the primary site of its origin, from where it can diffuse acting as a second messenger of free radicals. Unlike free radicals or the other ROS, HNE has the unique feature to remain stable for some time and binds to macromolecules with high reactivity, especially to proteins, causing

alteration of their tertiary structure and their function. These alterations are mainly conducted through formation of covalent bond with cysteine, lysine and histidine amino acid residues (Zarkovic et al., 2003; Uchida et al., 2003). HNE mostly reacts with SH groups of proteins and peptides generating HNE-protein conjugates that can be determined by the use of monoclonal antibodies. On the other hand, such reaction with glutathione (GSH) represents its most important detoxification mechanism (Borovic *et al*., 2007).

In addition, detoxification mechanisms of HNE are important to define the amount of cellular protein modified by HNE. GSH is the most important intracellular antioxidant that protects cells from HNE. Consequently, after administration of HNE, the amount of HNE-modified proteins can be deduced by intracellular level of GSH. This level is crucial for cellular sensitivity to HNE toxicity (Borovic *et al*., 2007). Although GSH could act alone, detoxification reaction is accelerated by GSH metabolizing enzymes such as glutathione S-transferases (GSTs). Noteworthy, Ramana et al., (2006) reported that conjugates of GSH with HNE activate PKC and stimulate NF-κB and AP-1-dependent gene transcription leading to an increase in cell growth.

In conclusion, the unique pathways and the primary molecular targets of intracellular signal transduction mediated by HNE are still unknown. However, it seems that a unique receptor is not necessary for HNE to achieve growth regulating effect. On the other hand complexity of possible interactions with signal-transduction pathways increased enormously. Possible signaling pathways of HNE involved in oxidative stress influenced bone cell growth are summarized in Figure 5

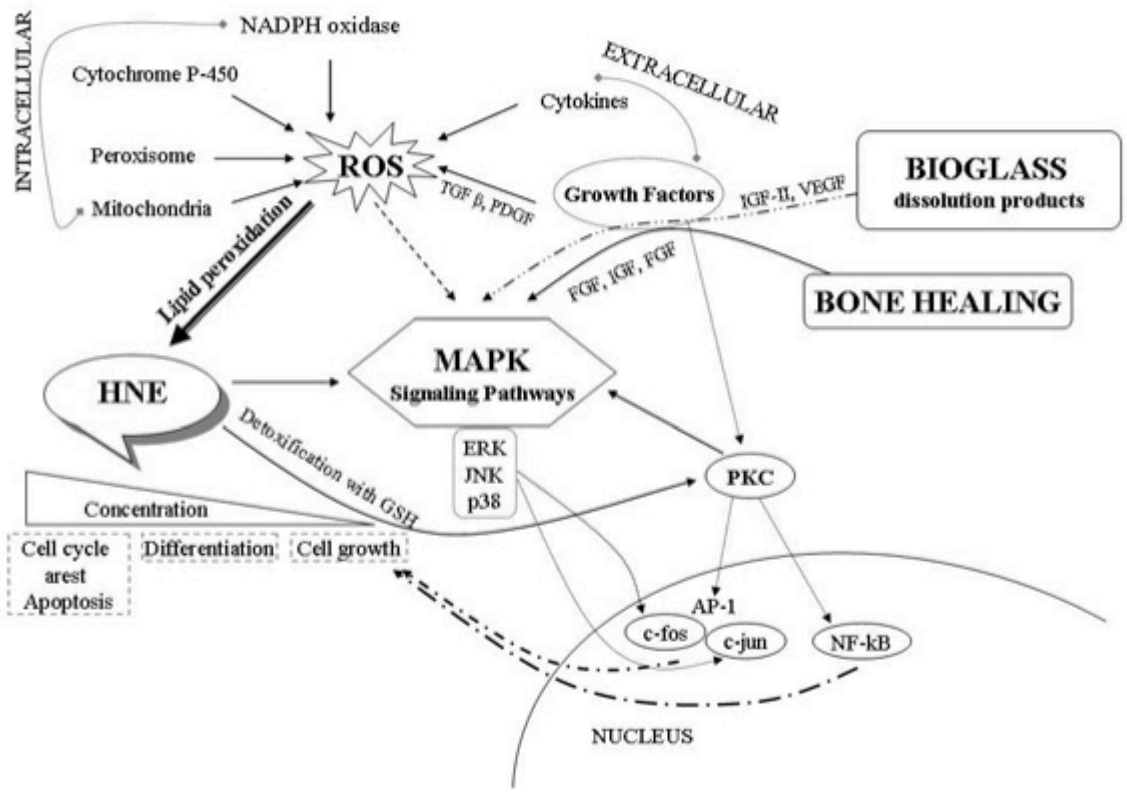

Figure 5- *4-hydroxynonenal (HNE) potential signaling pathways in pathophysiology of bone cell growth as defined using bioactive glass in vitro (Mrakovcic et al., 2010)*

9. The goal of the study

Active phase of otosclerosis is accompanied by signs of inflammation. There are numerous multi nucleated osteoclasts, osteocyte-like cells and proliferating endothelial cells in the pseudo-vascular spaces of the active otosclerotic foci. Mostly, CD8 positive, cytotoxic T lymphocytes and activated osteoclasts with possible cytokine secretion are found in the peripheral zone of active foci. Previous studies have suggested different types of pro-inflammatory and regulatory cytokines playing a role in the etiopathogenesis of otosclerosis (Sziklai *et al*., 2009, Karosi *et al*., 2006). The origin or the triggering factor of this inflammation is not elucidated. Participation of microorganisms such as bacteria, virus, fungi or even mastication trauma has been advocated (Schrauwen *et al*., 2010; Niedermayer *et al.*, 1996). Another issue in the pathophysiology of otosclerosis is the persistent inflammation during several years. In a pathophysiological model proposed for diabetes mellitus type 1, a coxsackie virus is the triggering factor in the destruction of the islets of Langerhans. The inflammation is maintained by an auto-immune reaction which appears secondarily and completes the endocrine tissue destruction (Tirabassi *et al*., 2010). This model is a possible explanation for the chronic inflammation in otosclerosis considering several reports on auto-immune activity in patients with otosclerosis (Schrauwen *et al*., 2010). However, auto-immunity is not constantly observed in otosclerosis (Karosi *et al*., 2010).

In order to elucidate other factors which may participate in the maintenance of the inflammation, Ang II was investigated (Imauchi *et al*. 2008). By inducing or promoting inflammation, Ang II can potentially induce oxidative stress in otosclerosis. Potentially, this stress has dramatic effects on bone remodeling. Reactive oxygen species (ROS) diffusion into the inner ear might also explain inner ear lesions during otosclerosis.

The goal of this research was to study the role of Ang II and oxidative stress in bone and especially in otosclerosis through 2 experimental models:

1) Investigation on the effect of Ang II on inflammation pathways in human primary bone cell cultures in otosclerosis and normal human stapes

2) Study of Ang II and HNE interaction in a human osteoblast-like cell line (HOS)

II. ANGIOTENSIN II AND CYTOKINE PRODUCTION IN HUMAN OTOSCLEROTIC PRIMARY BONE CELL CULTURES

1. Introduction

Ang II effects on tissue remodeling and repair have been previously studied in humans and animals. Ang II is implicated in the key events of inflammation and alters the composition of the extracellular matrix through various growth factors and cytokines. In the present study, we aimed at further investigation of Ang II effect on cytokine production in human primary otosclerotic bone cell cultures.

1.1. Angiotensin II effect on bone cells

Ang II tissular effects

In addition to its well-known effect on cardiovascular system and body fluid homeostasis, Ang II can act as a local growth factor with effect on both proliferation and cellular hypertrophy (Lamparter *et al.,* 1998). Ang II can stimulate the proliferation of heart myocytes, fibroblasts, fetal mesangial cells, and adrenal cells. It also plays an important role in various models of tissue repair in various models of tissue injury (Lamparter *et al.,* 1998). Bone is one of the most prominent tissue in which constant remodeling requires a continuous regulation of growth activities. A variety of systemic and local growth factors have been implicated in this process (Mundy *et al.,* 1993). Vasculature plays an important role in bone remodeling under normal and pathological conditions. Ang II binding sites have been located in almost every organ system and it has been shown that Ang II is generated locally by endothelial cells (Millan *et al.,* 1989). Endothelial and bone cells are likely to interact through paracrine mediators because of their close anatomical relationship. One of these mediators could be Ang II.

Ang II significantly contributes to bone demineralization in hypertensive patients (Shimizu et al., 2008). Previously, it was observed that the use of ACE inhibitors reduced the risk of fractures in a large retrospective case-control study on osteoporosis (Rejnmark *et al*., 2006).

Ang II cellular effects

Ang II has potent effects on the proliferation and function of osteoblasts (Lamparter *et al.* 1998). *In vitro* Ang II not only stimulates bone cell proliferation but has also proved to be a stimulator of collagen synthesis, suggesting that it might play an anabolic role in the remodeling process in rodents and in human. Moreover, Ang II appears to be a potent suppressor of osteoblastic differentiation in the new-born rat calvaria model (Hagiwara *et al*., 1998). Ang II was found to decrease the mRNA expression of osteocalcin, a well-known marker of osteoblastic differentiation. Similarly, the alkaline phosphatase activity and the formation of mineralization nodules were also inhibited.

These effects seem to be mediated not only by Ang II receptors (AT1 and 2) but also by paracrine mediators (Shimiza *et al*., 2008, Inagami *et al.*, 1999). Indeed, incubation of bone cells responsive to Ang II with the hormone followed by co-cultures of responsive cells with those lacking Ang II binding sites also elicited effects on co-cultured cells. Several mediators of Ang II have been previously described such as transforming growth factor-beta (TGF-β, Gibbons *et al.*, 1992), basic fibroblast growth factor (bFGF, Stirling *et al*., 1990), platelet derived growth factor (PDGF, Naftilan *et al*., 1989), insulin-like growth factor I, (IGF-I, Delafontaine *et al*., 1993) parathyroid hormone-related protein (PTHrP, Pirola *et al.*, 1992), and prostaglandins (Trachte *et al*., 1990), which could be responsible for these paracrine effects of Ang II on bone cells. These mediators are produced by different bone cell populations and act on osteoclasts (Hatton *et al.,* 1997).

In osteoporosis, Ang II treatment in osteoblasts up-regulate RANKL expression through ERK pathway, leading to osteoclast activation and differentiation (Kim *et al*., 2003). RANKL up-regulation appears to be a key step in osteoclast differentiation, activation and bone resorption by Ang II. Indeed, osteoblast / stromal cells express RANKL in response to several bone-resorbing factors including vitamin D3 to support osteoclast differentiation from their precursors. Osteoclast precursors, which express RANK, recognize RANKL through cell-to-cell interactions with osteoblasts / stromal cells and differentiate into mature osteoclasts in the presence of M-CSF. Targeted disruption of either RANKL or RANK in mice causes a lack of osteoclasts and an osteopetrotic phenotype. Moreover, Ang II activates NF-κB in osteoclasts leading to an anti apoptotic effect and the immune response activation in these cells (Kong *et al*., 1999). As osteoclast differentiation is also regulated by a variety of hormones, local factors, and inflammatory cytokines such as IL-1 and tumor necrosis factor alpha (TNF-α), RAAS seems to be a significant modulator of the osteoclast differentiation via several pathways.

The important role of Ang II in bone metabolism is integrated in a larger regulation system where this hormone and others implicated in bone metabolism (e.g. estrogens) interact. In an animal osteoporosis model, the continuous administration of Ang II accelerates osteoclast activation induced by estrogen deficiency. Indeed, estrogen antagonizes the bioactive effect of Ang II through signaling cross-talk in vascular smooth muscle cells (Takeda Masubara *et al*., 2002, Liu *et al*., 2002). This interaction opens therapeutic insights for Ang II receptor blockers. These agents could be used to treat hypertensive elderly patients, especially the female population to prevent or reduce osteoporosis. Further clinical trials with Ang II receptor blockers are necessary to confirm this hypothesis.

The goal of this part of the project was to further investigate the role that Ang II might have on the osteoblast-like cells (primary otosclerotic stapes cell cultures) and to assess the pro-inflammatory cytokine secretion in response to the Ang II stimulation.

2. Article: “Effect of Angiotensin II on Inflammation Pathways in Human Primary Bone Cell Cultures in Otosclerosis “

Milan Rudić, Christine Nguyen, Yann Nguyen, Lidija Milković, Neven Žarković, Olivier Sterkers, Evelyne Ferrary, Alexis Bozorg Grayeli

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Effect of Angiotensin II on Inflammation Pathways in Human Primary Bone Cell Cultures in Otosclerosis

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ABSTRACT

Introduction: The aim of this study was to assess the expression and production of inflammation mediators in basal condition and after AngII in otosclerosis. *Materials and methods*: Human stapedial cell cultures (6 otosclerosis and 6 controls) were incubated with AngII (10^{-7} M, 24 H) or vehicle. Cytokines and their mRNA expression were assessed by antibody and cDNA arrays. *Results*: In basal conditions, otosclerosis cultures produced higher amounts IL1b and IP-10, and smaller amounts TIMP-2. AngII promoted inflammation by increasing IFN-gamma, and IL-10, and by decreasing MIP-1 alpha and sTNF-RII. *Conclusions*: Otosclerotic cultures produced higher pro inflammatory cytokines in basal condition. AngII appeared to promote inflammation via these mediators in otosclerosis.

Key words: Bone dystrophy, angiotensin II, hearing loss, inflammation, bone remodeling

INTRODUCTION

Otosclerosis is a bone remodeling disorder of the human otic capsule and one of the most frequent causes of acquired hearing loss in adult Caucasians [Roland & Samy, 2006]. Its prevalence is estimated as 0.3% of population in Western countries [Roland & Samy, 2006]. This disease affects both ears in 80% of the cases [Roland & Samy, 2006]. The disease is usually discovered between the third and fifth decades of life with a significant female predominance (2:1 sex ratio) [Roland & Samy, 2006]. Pregnancy has been reported to accelerate the progression of hearing loss due to otosclerosis [Roland & Samy, 2006]. This relation suggests the implication of hormonal changes during the pregnancy in the disease progression.

Initially, the bone remodeling occurs on the anterior part of the oval window and stapes *(*i.e. *fissula ante fenestram*), reducing the stapedial mobility, and leading to a conductive hearing loss. As the disease progresses, the bone surrounding the cochlea is involved causing a sensorineural hearing loss [Menger & Tange, 2003]. Cytotoxic enzymes (elastase, collagenase, cathepsin-D/B), inflammatory cytokine mediators (TNF-alpha, interleukins, IL-1 and 6) and complement fragments (C3a, C3b, C5a) released from otosclerotic foci can diffuse into the perylimph, interfere with the electromotility of the cochlear sensory cells, lead to tissue damage and cause sensorineural hearing loss [Sziklai et al., 2009].

The etiology of otosclerosis remains still unclear. Even if several monogenic forms of this disease have been reported, otosclerosis frequently appears as a complex disease in which multiple environmental and genetic factors are involved [Stankovic et al., 2010].

The hypothesis of angiotensin II (AngII) implication in the progression of otosclerosis is based on the observation that otosclerosis progresses during pregnancy, and that plasmatic AngII concentrations increase during this period [Schrier & Durr, 1987] .

AngII effects on tissue remodeling and repair have been largely studied in human and animals [Guo et al., 2006]. This hormone is implicated in the key events of inflammation, and alters the composition of the extracellular matrix through multiple growth factors and cytokines (e.g. prostaglandins, transforming growth factor-beta, basic fibroblast growth factor, platelet derived growth factor, insulin-like growth factor, parathyroid hormone related peptide, and IL-6) [Lamperter et al., 1998]. AngII stimulates bone cells directly (stimulation of DNA and collagen synthesis, cell proliferation), and modulates their activity indirectly via other bone cell populations such as osteoclasts [Lamperter et al., 1998; Asaba et al., 2009]. In a previous study, we reported a genetic association between otosclerosis and 2 genetic polymorphisms increasing the plasmatic AngII concentration (AGT M235T and ACE I/D) [Imauchi et al., 2008]. AngII receptors and angiotensinogen mRNA could be detected in primary bone cell cultures derived from human stapedial samples. Furthermore, AngII stimulated the production of IL-6, and reduced the alkaline phosphatase activity in otosclerotic cell cultures but not in control cultures [Imauchi et al., 2008]. This observation suggested that the AngII affects otosclerotic bone differently from normal stapes, and may elicit local inflammation during pregnancy.

The objective of this study was to further investigate the expression and production on inflammation mediators in human otosclerotic primary bone cell cultures and the effect of AngII on this activity.

MATERIALS AND METHODS

Human primary bone cell cultures

Stapedial bone fragments were harvested from patients undergoing surgery for otosclerosis and a group of control patients undergoing transpetrous surgery for a cerebellopontine angle tumor. All patients were adults (mean age: 52 years, range: 31-62). In the otosclerosis group (2 males and 4 females), the diagnosis was based on clinical data, audiometry, and CT-scan. In the control group (3 males and 3 females), the diagnosis was based on preoperative imaging, the intraoperative observations and the pathology. In this group, no past history of middle ear disease, no conductive hearing loss, and no otosclerosis foci on CT-scan was noted. Ethics committee approval and patients' consent were previously obtained for the samplings. The diagnosis of otosclerosis was based on clinical, audiometric, and computed tomography scan findings and confirmed by the preoperative aspect of the stapes.

The primary culture of bone cells from human stapes has been previously described and characterized [Bozorg Grayeli et al., 1999]. Briefly, stapedial explants were placed in a culture medium composed of DMEM (Dulbecco Modified Eagle Medium) with 4.5% glucose (Gibco BRL Life Technologies, Cergy-Pontoise, France), 12.5 mg/L vancomycin (Lilly, Saint Cloude, France), and 30% fetal calf serum (FCS, Gibco BRL Life technologies, Cergy-Pontoise, France) in 10 cm^2 culture dishes, in a humidified atmosphere with 5% carbon dioxide at 37°C. All experiments were performed on confluent cells between first and third passages. After 3 washes with a serum-free media, cells were incubated culture media without FCS containing AngII (Sigma-Aldrich, St; Louis, MO, final concentration, 10^{-7} M) or vehicle (basal condition) during 24 hours.

Cytokine protein expression in the culture medium

Cell-free culture supernatants in otosclerosis (n=6) and control groups (n=6), were collected after 24h of treatment (AngII 10^{-7}M, or vehicle) for protein microarray assay. Supernatants were assayed for the following 40 cytokines and chemokines by a human cytokine antibody array (RayBio® Human Inflammation antibody array G3, RayBiotech, Norcross, GA), according to the manufacturer's instructions: Eotaxin, Eotaxin-2, granulocyte, granulocyte macrophage, and macrophage colony stimulating factors (G-CSF, GM-CSF, and M-CSF respectively), intercellular adhesion molecule 1 (ICAM-1), interferon gamma (IFN-g), I-309, Interleukins (IL) 1a,1b, 2-4, 6, 6sR, 7, 8, 10, 11, 12 p40, 12p70, 13, 15-17, interferon gamma induced protein 10 (IP-10), monocyte chemotactic proteins (MCP-1 and 2), monokine induced by IFN-g (MIG), macrophage inflammatory proteins (MIP-1a, b, and d), RANTES (regulated upon activation, normal T cell expressed and secreted), transforming growth factor (TGF)-b1, tumor necrosis factors (TNF-a and b), soluble TNF receptors (s TNF RI and II), platelet derived growth factor (PDGF BB), and metallopeptidase inhibitor-2 (TIMP-2). In addition, the array contained positive (biotinylated protein), negative (bovine serum albumin), and internal control (spiking-in protein with no cross reactivity with proteins in the array). The following molecules were also assessed for control and normalization: glyceraldehyde 3-phosphodehydrogenase (GAPDH), beta-2 miocroglobulin (B2M), heat shock protein 1 beta (HSPCB), and actin beta (ACTB). In our experiments, values obtained for the internal control were highly reproducible between different slides (data not shown). Hence, the internal control signal was not used for normalization between slides.

Briefly, after hybridization at room temperature, slides were incubated with biotynilated antibodies and labeled with conjugated streptavidin. The images were

captured by a laser scanner. Signal for each protein was normalized according to the positive control intensity (ACTB in this study) after subtraction background. The positive control of the first sample was considered as 1 and the signal intensities of all other samples were calculated according to the formula: Normalized signal intensity of particular spot = signal intensity of particular spot * (positive signal intensity sample 1 /positive signal intensity sample in particular spot).

Pro-inflammatory cytokines mRNA expression

Total RNA was extracted from 3 primary cell cultures among the 6 in each group (otosclerosis, control) using RNA 2Plus extraction solution (MP Biomedicals, France), and by a previously described method [Chomczynski & Sacchi, 1987]. Synthesis, purification and hybridization of biotin-labeled cRNA to custom array membranes were performed according to the manufacturer's recommendations (Oligo GEArray® Human inflammatory cytokines and receptors MicroArray, EHS-011, SA Biosciences, Frederick, MD). Briefly, cRNA was labelled by TruLabeling AMP 2.0 (0.1-3 µg of total RNA was used). Target cRNA was added to the labeled cRNA on GEArray slides for hybridization and washing. After local background subtraction, average signal intensity from duplicate spots was normalized by the expression of several housekeeping genes (ribosomal protein S27, glyceraldehyde 3 phosphate dehydrogenase, beta-2 microglobulin, heat shock protein 90 alpha B1, and ACTB) using Alpha Imager HP automatic image capture software (AlphaInnoTec, France). Similarly to antibody arrays, the presented results are relative concentrations normalized by ACTB. Normalization by other house-keeping proteins yielded similar results (data not shown).

The mRNA expression of the following cytokines and cytokine-related molecules was assessed in all samples : ATP-binding cassette, sub-family F, member 1 (ABCF1), B-cell CLL/lymphoma 6 (BCL6), Burkitt lymphoma receptor 1 (BLR1), Complement components 3, 4A, 5, Chemokine (C-C motif) ligands (CCL) 1-5, 7, 8, 11, 13, 15-21, 23-26, Chemokine (C-C motif) receptor (CCR) 1-9, CCAAT/enhancer binding protein, beta (CEBPB), C-reactive protein, pentraxin-related (CRP), Chemokine (C-X3-C motif) ligand 1 (CX3CL1), Chemokine (C-X3-C motif) receptor 1 (CX3CR1), Chemokine (C-X-C motif) ligands (CXCL)1-3,5, 6, 9-14, CXCR4 Chemokine (C-X-C motif) receptor 4, Endothelial differentiation, sphingolipid G-protein-coupled receptor 3 (EDG3), ICEBERG caspase-1 inhibitor, interferon alpha 2 (IFNA2) and gamma (IFNG), Interleukin 10 (IL10), and its receptors, alpha and beta (IL10RA, IL10RB), IL11 and its receptor alpha (IL11RA),IL12A and B, and their receptors beta 1 and 2 (IL12RB1, IL12RB2), IL13, IL13RA1, IL15, IL15RA, IL16, IL17A, IL17C, IL17RA, IL18, IL18R1, IL1A, IL1B, IL1R1, IL1R2, Interleukin 1 receptor antagonist (IL1RN), IL2, IL20, IL21, IL22, IL2RA, IL2RB, IL2RG, IL3, IL4, IL5, IL5RA, IL6, IL6R, Interleukin 6 signal transducer (IL6ST), IL8, IL8RA and B, IL9, IL9R, Lymphotoxin alpha and beta (TNF superfamily, members 1 and 3, LTA and LTB) Leukotriene B4 receptor (LTB4R), Macrophage migration inhibitory factor (MIF) Platelet factor 4 (PF4 or CXCL4), Small inducible cytokine subfamily E, member 1 (endothelial monocyte-activating, SCYE1), Secreted phosphoprotein 1 (SPP1, osteopontin, bone sialoprotein I, early T-lymphocyte activation 1), Tumor necrosis factor (TNF, TNF superfamily, member 2), Tumor necrosis factor receptor superfamily members 1A, and B (TNFRSF1A, TNFRSF1B), CD40 ligand (CD40LG), Toll interacting protein (TOLLIP), Chemokine (C motif) ligand, and receptor 1 (XCL1, XCR1).

Quantification of cytokine mRNA expression by qRT-PCR

Total RNA from 4 primary cell cultures among the 6 in each group (otosclerosis, control) were used in these experiments. All the samples in the gene array assays were included in qPCR evaluation. The RNA pellet was suspended in 20 µL of water and stored at –20°C. Complementary DNA was synthesized by a reverse transcription (RT-PCR). The reaction mix (final volume 20 µL) contained 1 µg of the total RNA, SuperScript II RT (Invitrogen, France), 5X first-strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) and 0.1 M DTT. The mix underwent: 95°C for 30 sec, 70°C for 7 min, 42°C for 65 min, 95°C for 6 min and 30°C for 1 min. qRT-PCR parameters were determined in preliminary experiments on human osteosarcoma cell lines (HOS, and SaOS, American Type Culture Collection, Molsheim, France). BCL-6, CCL25 cDNA were amplified by PCR using 5 µL of reverse transcription product and the following primers: BCL-6 sense 5'-ACCTGGTGGAGAACAACCTG-3' and antisense 5'-TTCTGGGATTGTTTCCTTGG-3'; CCL25 sense 5'-GATAAAACCGTCGCCCTACA-3' and antisense 5'-ATCAGGCCAACTCCCTCTTT-3'. The amount of targeted mRNA was corrected by those of S14 and GAPDH mRNA using the following primers: S14 sense 5'-TCACTCAGGAAGAATACCATTTTG-3' and antisense 5'-CCGATTTCTGATTCTAACAGGAC-3'; GAPDH sense 5'-AAGGCTGGGGCTCATTTG-3'and antisense 5'-GTGTGGTGGGGGACTGAG-3' (Gibco BRL Life technologies, Cergy-Pontoise, France). Quantitative PCR was performed in total volume of 20 µL with the use of SYBER Green JumpStart Taq ReadyMix (Sigma-Aldrich, France) containing Tris-HCl 20 mM pH 8.3, KCl 100 mM, $MgCl_2$ 7 mM, 0.4 mM each dNTP (dATP, dCTP, dGTP, dTTP) stabilizers, Taq DNA Polymerase 0.05 unit/µL, JumpStart Taq antibody and

SYBER Green I. DNA was amplified for 40 cycles (Chromo 4, Biorad, Marnes-la-Coquette, France). Reactions were prepared in duplicate and heated to 94°C for 2 min, 58°C for 30 sec, 72°C for 30 sec, melting curve 55°C to 95°C every 0.5°C. To detect the amplification log phase, the fluorescence quantification of product was determined at each cycle. The cycle at which the fluorescence reached the threshold (CT) was recorded, averaged between duplicates and normalized to the averaged CT value. The ratio between samples with and without treatment was calculated by relative quantification. Melting curves were also measured to ensure that only a single product was amplified. A 10 µL sample of the PCR product was run on a 2% agarose gel with Sybersafe (Invitrogen, France) and 100-bp molecular weight ladder. Control and otosclerosis samples were paired during experiments to allow comparison of otosclerosis versus control as well as basal versus AngII conditions.

Immunocytochemistry

Cell cultures from patients with otosclerosis (n=4) and control patients (n=4) were grown on permanox slides (Lab-Tek Chamber Slide, Nalge Nunc International, Rochester, NY). At confluence, cells were fixed in 4% paraformaldehyde for 20 minutes. Upon fixation they were rinsed in phosphate buffered saline (PBS, Gibco, Invitrogen, France) and incubated with NH_4Cl for 5 minutes. Cells were permeabilized by triton-100-X 0.1% (Sigma-Aldrich, France). They were further saturated in blocking solution composed of PBS, 1% albumin bovine powder (Sigma Aldrich, France), 0.5% of TWEEN 20 (Sigma-Aldrich, France) and 5% of goat serum (Invitrogen, France) for 10 minutes. Cells were further incubated with primary antibody diluted 1/100 for 1 hour at room temperature. They were subsequently rinsed in PBS and incubated with the secondary antibody diluted at 1/20 for 30 minutes at room temperature. Again cells were rinsed with PBS and incubated with

ExrAvidine Peroxydase (Sigma-Aldrich, France) diluted 1/20 for 30 min at room temperature. They were finally stained with the AEC (3-Amino-9-ethylcarbazole) Staining Kit (Sigma-Aldrich, France) for 10 minutes at room temperature.

The following primary antibodies have been used: for osteoblasts, monoclonal mouse anti-human CDH11, clone 4D10 (Sigma-Aldrich, France); for osteoclasts, monoclonal mouse anti-human CD61, clone Y2/51 and for macrophages, monoclonal mouse anti-human CD68, KP1 clone (DakoCytomation, Glostrup, Denmark).

Statistical Analysis

For antibody and gene array analysis a repeated measures 2-way ANOVA was performed followed by a Bonferroni post-test when ANOVA showed a significant effect ($p<0.05$). For gene array analysis, basal versus AngII conditions in the same samples were compared by a paired t-test. Controls were compared to otosclerotic samples by an unpaired t-test. For qPCR analysis, the difference fold was tested by a one-sample t-test for a difference from a hypothetical value of 1. Data were expressed as mean ± standard error of the mean. Difference or effect was considered significant at $p<0.05$.

RESULTS

Release of cytokines and cytokine-related proteins in culture medium

In basal condition, IP-10 and IL-1b concentrations were higher in otosclerosis than in control stapes (Table 1). This observation was in accordance with a higher leukocyte or osteoclast activity (IL), and an increased angiogenetic activity (IP-10) in cultures derived from otosclerotic stapes. Furthermore, a lower activity of metalloprotease inhibitor 2 (TIMP-2) was detected in basal conditions in otosclerotic cell cultures. This observation was also compatible with a higher bone turn-over in this disease (Table 1). The expression of other proteins did not differ between otosclerosis and control samples in basal condition.

AngII stimulated the IFN-γ release in culture media in both otosclerosis and control cell cultures. Ang II also increased the IL-10 concentration in otosclerosis culture media. It inhibited macrophage inflammatory protein (MIP-1a) and Interleukin 11 (IL-11) release only in control stapes and not in otosclerosis. AngII appeared also to reduce the soluble tumour necrosis factor receptor II (sTNFRII) concentration in otosclerosis culture media without altering the tumour necrosis factor-alpha (TNF-α) levels.

mRNA expression of cytokines and cytokin-related proteins

In total, 126 key genes involved in the inflammatory response were analyzed by gene array. mRNA expression of 15 cytokines or cytokine-related molecules could be detected by this technique (Fig 1). A significant inter individual variation was observed. In basal condition, BCL-6 mRNA expression was higher in otosclerosis than in control (48 ± 21.7 arbitrary units in control versus 328 ± 39.7 in otosclerosis for BCL-6, n=3, $p<0.05$, unpaired t-test). Angiotensin II reduced BCL-6 mRNA

expression only in otosclerosis (424 ± 63.3 in basal condition versus 105 ± 64.7 after AngII, $p<0.05$, paired t-test). Real-time quantitative RT-PCR (qPCR) after normalization by S14 mRNA expression confirmed higher levels of BCL-6 mRNA in otosclerosis in comparison to control stapes in basal condition (Table 2). However, the reduction of BCL-6 mRNA expression after Ang II in otosclerosis observed by gene array could not be confirmed by qPCR. Similar qPCR results were obtained after normalization by GAPDH (data not shown).

Immunocytochemistry

Both control and otosclerotic cells showed positive labeling with osteoblast, osteoclast and macrophage specific antibodies. There was no difference of labeling between otosclerosis and control samples (Fig 2). This observation suggested that difference in pro-inflammatory cytokine production was not related to quantitative differences of cell types between otosclerosis and control cultures.

DISCUSSION

AngII is implicated in the key events of inflammation and bone turnover via various growth factors and cytokines [Lamperter et al., 1998; Asaba et al., 2009]. In previous studies, we showed that two genetic polymorphisms related to higher plasmatic AngII concentrations (AGT M235T and ACE I/D) were associated to a higher relative risk of otosclerosis occurrence in a French Caucasian population. We also observed that AngII receptors, and angiotensinogen mRNA were expressed in bone cell cultures obtained from otosclerotic and control stapes. Moreover, *in vitro* AngII stimulated the production of a pro inflammatory cytokine (interleukin-6, IL-6) as measured by ELISA,

and reduced the alkaline phosphatase activity in otosclerotic osteoblast-like cell cultures. These effects appeared to be specific to otosclerosis since they were not observed in control cell cultures [Imauchi et al., 2008]. In order to elucidate the mechanism of the increased bone turnover and to identify targets for therapeutic action, this study focused on the effect of AngII on inflammation mediators in otosclerosis.

In basal condition, 2 pro inflammatory cytokines (IL-1b, IP-10) were released by otosclerotic cells in larger quantities than in control stapes. Interleukin 1-beta is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by caspase 1 (CASP1/ICE). This cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular activities including cell proliferation, differentiation and apoptosis [Kapoor M, et al. 2011]. Its higher production in otosclerosis is in accordance with the abnormally high tissue turnover in the disease foci. Interferon (IFN) inducible protein (IP-10) is expressed in various cells including lymphocytes, monocytes, and fibroblasts. Its production is mainly induced by IFN-gamma and tumor necrosis factor (TNF)-alpha among others. IP-10 is mainly known for its hemotactic activity but it is also produced in response to acute or chronic inflammation by attracting CD8 and NKT lymphocytes to tissues involved in the pathogenesis of many disorders such as skin inflammation, multiple sclerosis, pulmonary/liver fibrosis, type 1 diabetes, autoimmune myasthenia gravis, acute cardiac allograft rejection and Celiac disease [Dufour et al., 2002]. In this study we did not observe the increased IL-6 production after AngII which we reported previously [Imauchi et al., 2008]. This could be explained by the difference of assessment technique (antibody array versus ELISA), or the lower number of samples in the present study.

In basal condition, tissue inhibitor of metalloproteinases TIMP-2 had a lower concentration in otosclerosis than in control cultures. This cytokine has a unique role among TIMP family members to directly suppress the proliferation of endothelial cells. Its role is critical in the maintenance of tissue homeostasis by suppressing the tissue proliferation in response to angiogenic factors, and in inhibiting the protease activity in tissues undergoing extracellular matrix remodeling (e.g. otosclerotic foci). Hence, lower concentrations of TIMP-2 might be related to higher bone turnover. Abnormalities of TIMP expression in the connective tissue could be related to the incidence of otosclerosis [McPhee JR et al., 1991].

In our study, AngII stimulation led to an increased release of IFN-gamma which is a pro-inflammatory cytokine. IFN-gamma is secreted by Th1, Tc, dendritic, and NK cells. It is known for its antiviral, immunoregulatory and anti-tumor properties [Shroder K et al., 2004]. In bone, IFN-gamma suppresses osteoclast formation and promotes apoptosis by rapidly degrading the RANK (receptor activator of nuclear factor-kappa B) adaptor protein TRAF6 in the RANK-RANKL (ligand) signaling system. RANK stimulates the production of NF-kappa B which induces the transcription of anti apoptotic genes and plays a major role in remodeling. IFN-gamma is currently employed in the treatment of chronic granulomatous diseases and osteopetrosis [Shroder K et al., 2004].

AngII also stimulated IL-10 or human cytokine synthesis inhibitor factor (CSIF). IL-10 is primarily produced by monocytes and to a lesser extent by lymphocytes. It has pleiotropic effects, inhibiting synthesis of pro inflammatory cytokines such as IFN-γ, IL-2, IL-3, TNFα and GM-CSF produced by macrophages and regulatory T-cells. IL-10 also displays potent abilities to suppress the antigen presentation capacity of antigen presenting cells. However, it is also stimulatory towards certain T cells, mast

cells and stimulates B cell maturation and antibody production. Similarly to IFN-gamma, it can block NF-kappa B activity [Moore et al., 2001].

AngII inhibited the production of macrophage inflammatory protein (MIP)-1a, and IL-11 only in control cells. MIP-1a is a chemokine, produced by macrophages and activates human granulocyte leading to an acute neutrophilic inflammation. It is also capable of inducing the synthesis of other pro inflammatory cytokines such as TNF-alpha, IL-1 and 6 from fibroblasts and macrophages [Sherry et al., 1988]. IL-11 participates in the regulation of bone cell proliferation and differentiation and could be used as a therapeutic agent for osteoporosis. It increases the cortical thickness and strength of long bones in mice [Yang & Yin, 1993].

Finally, tumor necrosis factors are involved in systemic inflammation at its acute phase. Inhibition of sTNF RII by AngII in otosclerosis cultures would promote inflammation by increasing the amount of free TNF [Locksley et al., 2001].

Gene array and qPCR results indicated a higher BCL-6 mRNA expression in otosclerosis and in basal conditions in comparison to control stapes. BCL-6 gene encodes for the B-cell lymphoma-6 protein which acts as a sequence-specific repressor of transcription, and has been shown to modulate the transcription of STAT-dependent IL-4 B-cell response. BCL-6 has also been shown to regulate the expression of several chemokine genes, to negatively regulate the proliferation of the monocytic / macrophage lineage via IL-6/STAT signaling, and to modulate chemokine gene expression [Woo et al., 2002]. Chemokine expression is repressed by BCL-6 both in B-cell lines and macrophages. MIP-1alpha and IP-10 appear to be repressed by BCL-6 in B-cells. This effect probably passes through a functional binding site in the MIP-1alpha promoter but not in the IP-10 gene [Schaffer et al., 2000]. In our study, BCL-6 mRNA expression was higher in otosclerosis than in controls, in basal

condition supporting its role in the cellular proliferation. Its inhibition by AngII may explain the activation of different pro-inflammatory cytokines.

In conclusion, these data support the hypothesis that AngII is implicated in inflammation and bone turnover regulation in otosclerosis, via different growth factors and cytokines. They indicate several potential targets for their anti-inflammatory or anti-catabolic actions such as IL-11 and TIMP-2. Nevertheless further investigations are necessary to clarify the role of AngII in the pathophysiology of otosclerosis and stapes metabolism.

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Figure 1- Cytokine and cytokine-related mRNA expression levels in control and otosclerotic human primary bone-cell cultures and the effect of Angiotensin II. Relative levels of mRNA expression were assessed by gene array in primary bone cell cultures (n=3) after total RNA extraction. Circles indicate individual values, horizontal bars the mean value and vertical bars the SEM. Significant value dispersion was noted. Only the 15 cytokines or cytokine-related proteins out of 126 had a detectable mRNA expression by this method. *: $p<0.05$, paired t-test for basal versus angiotensin II, and unpaired t-test for control versus otosclerosis. Cont: control, Otos: Otosclerosis, -: Basal condition, +: Incubation with angiotensin II at 10^{-7} M, 24H.

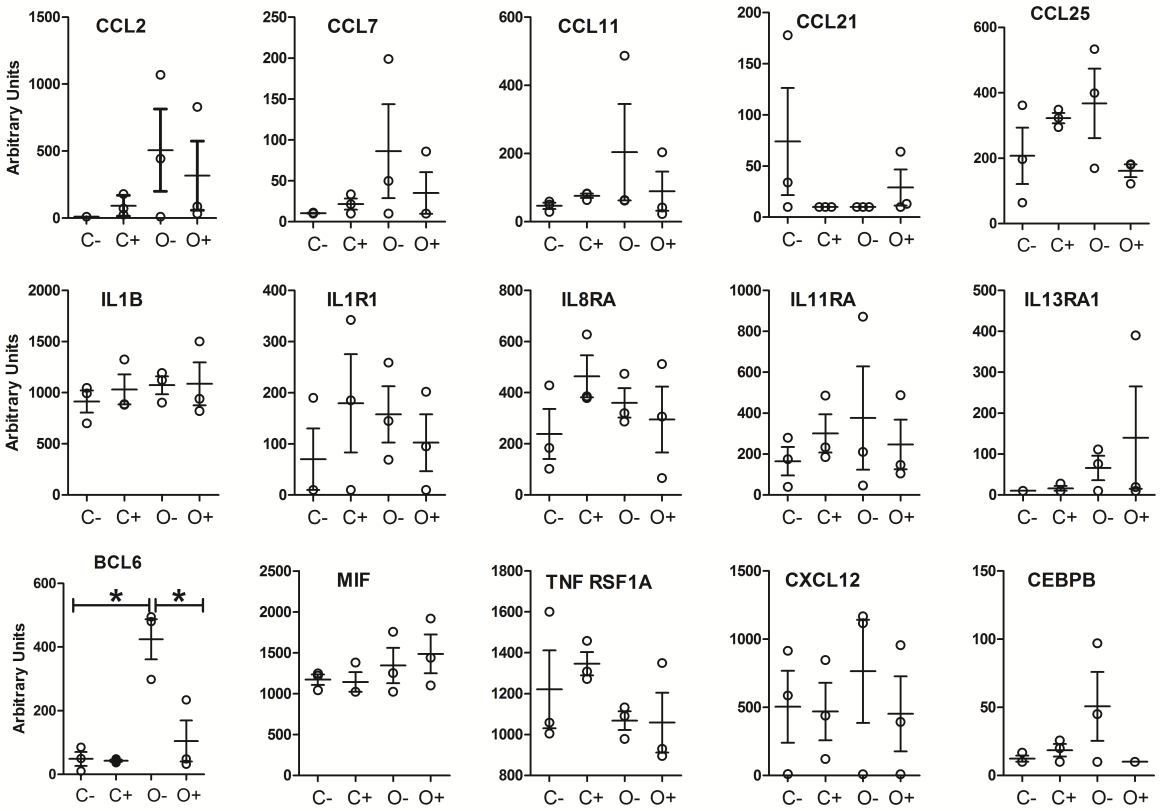

Figure 2- Immunocytochemistry in primary bone cell cultures of control and otosclerotic stapes: Both control (A, C, E) and otosclerotic (B, D, F) stapedial bone-cell cultures showed positive reactivity for macrophage (CD68, A and B), osteoclast (CD61, C and D) and osteoblast (cadherin 11, E, and F). There was no difference of reactivity between otosclerosis and control by microscopic examination.

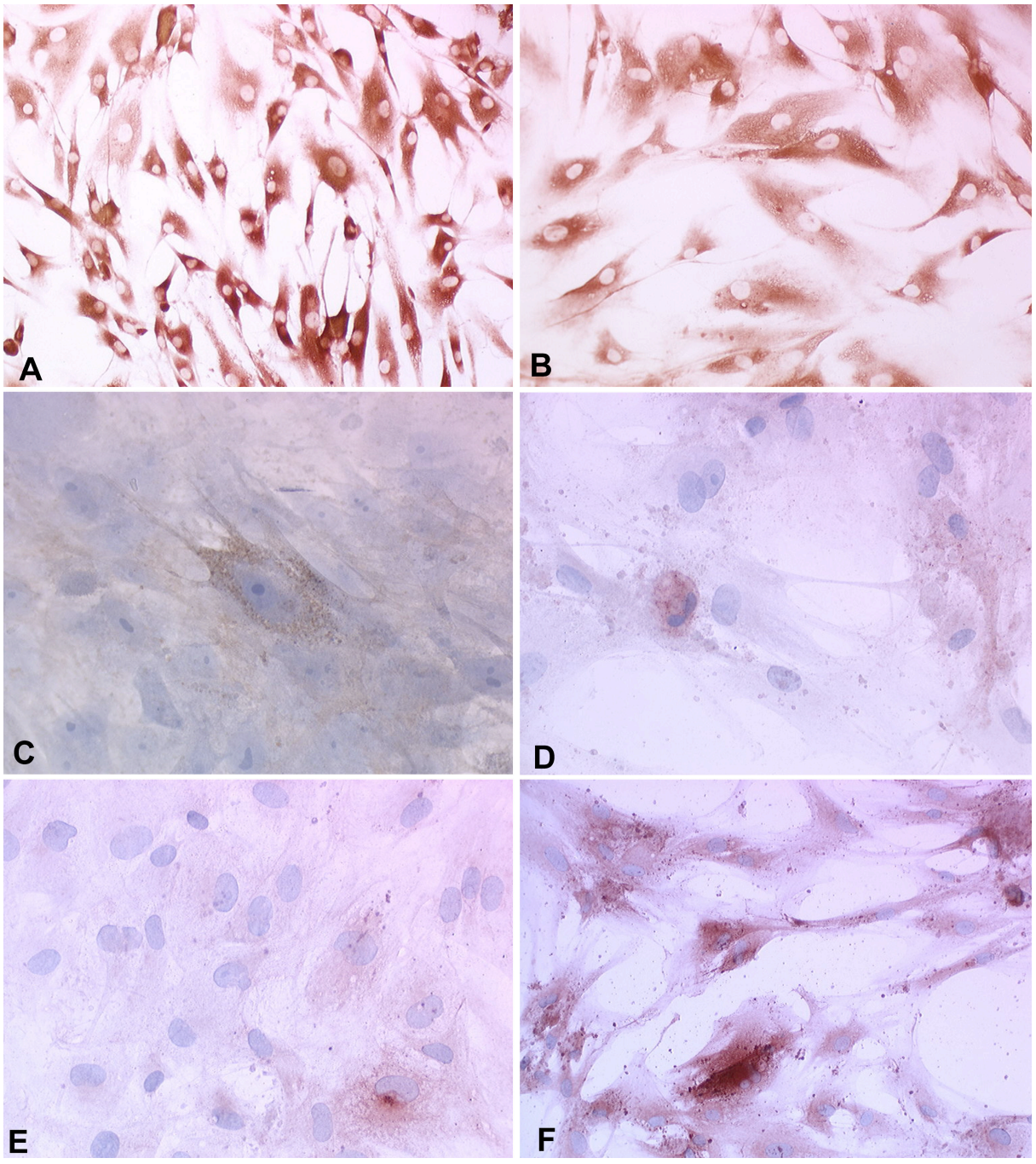

Table 1- Comparison of cytokine relative concentrations between control and otosclerotic human primary bone-cell cultures

	Control basal	Control +Ang2	Otosclerosis basal	Otosclerosis +Ang2
EOTAXIN	87 ± 8.9	88 ± 8.6	108 ± 10.3	102 ± 12.9
EOTAXIN-2	77 ± 12.7	70 ± 14.8	109 ± 15.8	108 ± 29.1
GCSF	191 ± 20.5	217 ± 19.2	203 ± 22.9	212 ± 7.2
GM-CSF	83 ±14.2	79 ± 4.64	89 ± 6.8	85 ± 4.5
ICAM-1	81 ± 56.3	106 ± 86.6	21 ± 3.4	23 ± 4.1
IFN-g	90 ± 25.3	141 ± 27.0§	99 ± 27.0	136 ± 9.1§
I-309	4 ± 1.39	10 ± 4.8	16 ± 5.0	11 ± 2.6
IL-1a	172 ± 20.5	197 ± 13.0	189 ± 25.4	208 ± 16.4
IL-1b	26 ± 5.1	33 ± 11.3	51 ± 6.9£	65 ± 7.4£
IL-2	184 ± 14.9	207 ± 12.5	182 ± 6.1	199 ± 8.9
IL-3	1406 ± 105.6	1631 ± 145.7	1384 ± 111.5	1421 ± 143.4
IL-4	49 ± 12.0	48 ± 5.5	61± 5.3	67 ± 7.6
IL-6	432 ± 93.3	448 ± 67.3	566 ± 219.4	519 ± 192.5
IL-6sR	42 ± 4.2	38 ± 6.5	45 ± 2.8	42 ± 5.1
IL-7	210 ± 32.6	270 ± 20.5	211 ± 30.7	252 ± 23.2
IL-8	999 ± 216.0	1212 ± 431.0	818 ± 263.7	554 ± 125.0
IL-10	82 ± 9.1	111 ± 13.5	70 ± 7.2	116 ± 12.5*
IL-11	124 ± 7.3	98 ± 8.0*	117 ± 14.8	104 ± 6.8
IL-12 p40	29 ± 4.4	23 ± 3.1	39 ± 7.6	34 ± 10.4
IL-12 p70	191 ± 10.7	225 ± 28.7	262 ± 24.2	212 ± 15.1
IL-13	122 ± 15.6	141 ± 7.7	125 ± 19.2	155 ± 12.9
IL-15	166 ± 8.7	180 ± 9.7	162 ± 7.8	175 ± 6.2
IL-16	58 ± 14.9	77 ± 24.4	91 ± 13.4	114 ± 7.5
IL-17	113 ± 9.7	126 ± 10.1	119 ± 9.5	116 ± 8.7
IP-10	36 ± 4.7	55 ± 12.1	88 ± 6.9£	70 ± 13.1£
MCP-1	589 ± 153.1	630 ± 192.6	379 ±75.9	359 ± 46.7
MCP-2	29 ± 6.0	25 ± 5.5	45 ± 6.6	46 ± 10.4
M-CSF	54 ± 7.6	41 ± 7.9	47 ± 5.9	46 ± 5.2
MIG	229 ± 26.6	242 ± 13.5	228 ± 25.7	218 ± 15.6
MIP-1a	22 ± 6.7	9 ± 3.8*	22 ± 4.3	17 ± 1.6
MIP-1b	240 ± 16.7	225 ±10.8	287 ± 27.5	309 ± 23.5
MIP-1d	24 ± 5.8	30 ± 2.6	34 ± 6.6	33 ± 3.6
RANTES	23 ± 3.8	29 ± 6.2	40 ± 10.7	35 ± 7.9
TGF-b1	276 ±15.8	281 ± 22.6	275 ± 9.3	274 ± 4.0
TNF-a	184 ±10.9	212 ± 11.4	185 ± 12.3	197 ± 9.24
TNF-b	222 ±16.1	224 ± 7.0	189 ± 11.7	200 ± 5.2
s TNF RI	592 ± 297.1	764 ± 472.0	194 ± 33.3	192 ± 35.6

s TNF RII	113 ± 6.0	121 ± 6.7	129 ± 5.6	106 ± 7.4*
PDGF-BB	9 ± 1.9	11 ± 4.5	19 ± 3.1	14 ± 2.9
TIMP-2	21410 ± 3278.2	18330 ± 2991.9	11296 ± 1705.7£	11547 ± 2130.6£

Relative concentrations (in arbitrary units) were assessed by antibody array in primary cell culture media (n=6) for 40 cytokines and normalized by the expression of beta actin in the same culture in basal condition and after stimulation by angiotensine 2 (AngII, 10^{-7} M, 24h). Results are expressed as means ± SEM in arbitrary unites. A 2-way repeated-measures ANOVA compared otosclerosis to control stapes, and basal to AngII conditions. It was followed by a Bonferroni post-test only when ANOVA showed a significant effect.

*: $p<0.05$ Bonferroni post-test, versus basal condition.

£: $p<0.05$ ANOVA for otosclerosis effect, Bonferroni post- test: not significant

§: $p<0.05$ ANOVA for AngII effect, Bonferroni post- test: not significant

Table 2- mRNA expression levels of BCL-6 in primary human osteoblast-like cell cultures of control and otosclerotic stapes evaluated by real-time qRT-PCR

BCL-6	$\Delta C_{T\ Basal}$	$\Delta C_{T\ Ang2}$	$\Delta\Delta C_T$ (Basal-Ang2)	Stimulated-Basal Difference fold ($2^{-\Delta\Delta C_T}$)
Control (n=4)	-3.5±0.27	-3.2±0.75	0.3±0.97	1.8±0.59 (ns)
Otosclerosis (n=4)	-2.7±0.34	-2.8±0.76	-1.3±0.72	1.4±0.62 (ns)
$\Delta\Delta C_T$ (Otosc-Cont)	0.3±0.49	0.4±0.82		
Otosc-Cont difference fold ($2^{-\Delta\Delta C_T}$)	1.7 ±0.20*	1.2±0.74 (ns)		

Cell cultures were obtained from 4 patients with otosclerosis and 4 control patients. Total RNA was extracted from cultures in basal condition and after angiotensin II (AngII, 10^{-7} M, 24H). Real-time qRT-PCR was performed in duplicates. Ribosomal S14 was used as the reference gene. Control and otosclerosis samples were paired during experiments to allow comparison of otosclerosis versus control as well as basal versus AngII conditions. Values are expressed as mean ± SEM. Difference fold refers to basal versus angiotensin II condition in rows and to control versus otosclerosis in columns.

ΔC_T = Avg.Bcl-6 or CCL25 C_T- Avg. S14 C_T, $\Delta\Delta C_T$ = Avg. $\Delta C_{T,\ AngII}$ - Avg. $\Delta C_{T,\ basal}$ for the rows and $\Delta\Delta C_T$ = Avg. $\Delta C_{T,Otosclerosis}$ - Avg. $\Delta C_{T,\ control}$ for the columns.

* $P<0.05$, one sample t test for a difference from a hypothetical value of 1. ns: not significant

III. ANGIOTENSIN II AND OXIDATIVE STRESS IN PATHOPHYSIOLOGY OF OTOSCLEROSIS

1. Introduction

Oxidative stress and Ang II interact for tissue remodeling via reactive oxygen species (ROS) which intervene in numerous signaling pathways of Angiotensin II (Ang II). HNE or 4-hydroxynonenal as a second messenger of ROS is found in many different human tissues and its physiological roles are still not entirely understood. Recent studies of the HNE influence on bone cells have demonstrated its inhibitory effect on the bone cell proliferation and differentiation and its stimulatory effect on apoptosis.

The goal of the present study was to further investigate and understand the interaction between Ang II and HNE in the pathophysiology of otosclerosis by studying the *in vitro* model of osteoblast-like cells.

1.1. Angiotensin II and reactive oxygen species (ROS)

ROS are involved in transducing many of the Ang II effects and are produced in response to agonist-receptor binding. ROS are molecules ultimately derived from oxygen but have undergone univalent reduction, so that they readily react with other biological products (Griendling *et al*., 2000). ROS include superoxide anion (O_2°-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH°), nitric oxide (NO°) and peroxynitrite (OON°), (Griendling *et al*., 1994; 2000, Zafari *et al*., 1998). Among these, the superoxide anion and the hydrogen peroxide have been shown to be produced under Ang II effect and to alter the activity of specific signaling proteins and enzymes (Griendling *et al*., 2000). The signaling mechanism by which Ang II activates the oxidase is not entirely understood. Zafari *et al*. (1998) provided evidence that arachidonic acid metabolites are involved in the oxidase activation. Arachidonic

acid is produced by Ang II in several different pathways. This molecule can be directly generated by phospholipase A_2 action on membrane phospholipids or indirectly via phospholipase D-mediated degradation of phohphatidylcholin to phosphatidic acid and its subsequent dephosphorylation to diacylglicerol and monoacylglycerol (Lassegue *et al*. 1993). Touyz *et al*. (1999) showed that inhibition of phospholipase D attenuated the oxidase activation under Ang II effect.

In vascular smooth muscle cells superoxide anion production induced by Ang II is mediated by NAD(P)H oxidase (Ushio-Fukai *et al*., 1996). The superoxide anion produced upon Ang II stimulation is converted to hydrogen peroxide. In endothelial cells, Ang II also increases the production of superoxide anion via activation of membrane-associated NAD(P)H oxidase (Munzel *et al*., 1999, Zhang *et al*., 1999, Lang *et al*., 2000). In addition, it activates nitric oxide synthase to release nitric oxide (Saito *et al*., 1996, Schena *et al*., 1999). Arachidonic acid metabolites are not the only lipid activators of NAD(P)H oxidase, and phospholipase A inhibition has very little effect on NAD(P)H oxidase activity (Griendling *et al*., 2000).

ROS are involved in multiple physiologic responses to Ang II, including vascular smooth cell growth, induction of vascular inflammatory response, impairment of endothelium-dependant relaxation and cardiac hypertrophy (Griendling *et al*., 2000). Ang II also induces the inflammatory response in the vessel walls. Such response is observed in otospongiotic foci during the active phase of otosclerosis (Schrader *et al*. 1990). It is present in monocytes and macrophages and induces such pro-inflammatory molecules such as VCAM-1, MCP-1 and the thrombin receptor. These molecules are redox sensitive, and especially in the case of MCP-1 and the thrombin receptor, interact with ROS in Ang II mediated gene expression has been demonstrated (Griendling *et al*., 2000).

Determination of which Ang II signaling pathways are redox sensitive has been subject of several recent studies. Study of the early signaling events found that neither phospholipase C nor Ca^{2+} influx are mediated by ROS after Ang II stimulation (Griendling *et al*., 2000). On the contrary, phosphorylation of the epidermal growth factor receptor (EGF-R) appears to be an important step in the activation of downstream kinases by Ang II and is mediated through NAD(P)H oxidase derived ROS (Murasawa *et al*., 1998, Eguchi *et al*., 1998). Moreover, Ang II induces the tyrosine phosphorylation of the platelet derived growth factor (PDGF) receptor. This phenomenon leads to the conclusion that ROS participate in the formation of the most proximal tyrosine kinase-related signaling complexes in response to Ang II (Linseman *et al*., 1995). It has been already mentioned that Ang II can activate the MAPKs signaling pathway including ERKs, JNK and p38MAPKs (Abe *et al*., 1996, Ushio-Fukai *et al*., 1998). Research on which of these MAPKs is redox sensitive has shown that Ang II activation of p38MAPK and JNK are indeed redox sensitive (NAD(P)H oxidase) while some studies showed that activation of ERKs is insensitive to redox (Griendling *et al*., 2000). As for other enzymes activated via Ang II (i.e. phospholipase D, phospholipase A2, FAK, Fyn, janus kinase 2 (JAK2), STAT1 and ras), it still remains unclear whether they are redox sensitive or not (Griendling *et al*., 1997, Berk et al., 1999).

1.2. Reactive oxygen species in human ear diseases

It is known that inner ear hair cell damage and death may occur as a result of local ROS production. The main sources of these molecules within the cochlea appear to be the hair cells' mitochondria, or enzymes such as xanthine oxidase and NADPH oxidase. The electron transport chain in the mitochondria is thought to be a major production means of intracellular

superoxide, since hair cells are known to be highly energy-demanding and oxygen-consuming cells (Henderson *et al*., 1999; Ciorba *et al.*, 2010).

Once generated, ROS are responsible for direct cellular damage to lipids, proteins, and DNA, triggering apoptosis or necrosis and may also diffuse among the inner ear scalae (Henderson *et al*., 2006; Clerici *et al*., 1996). In hair cells, death may occur either through necrosis or apoptosis (Henderson *et al*., 1999). In animal models, several conditions of increased ROS production related to cochlear damage have been described, including exposure to noise and to drugs (i.e. aminoglycosides and cisplatin, Ciorba *et al*., 2010). In humans, ROS appeare to be involved in hair cell damage in some cases of genetic hearing loss (i.e. Pendred syndrome), presbycusis, and Menière's syndrome (Ciorba *et al*., 2010). Several animal or in vitro models have been proposed for treatment against ROS-mediated damage (Henderson *et al*, 2006; Goldvin *et al*., 1998; Wang *et al*., 2002). In rats the increase of antioxidant supplies in the cochlea may effectively prevent hair cell damage and hearing loss (Lorito *et al*., 2006). Pharmacological interventions have been directed at: interrupting the lipid peroxidation process, thus preserving the integrity of cell membranes (i.e. by lazaroids), preventing noise-mediated inner ear damage by cochlear ischemia/reperfusion (i.e. by pentoxifylline and sarthran) and preventing cell apoptosis, by inhibitors of cell death pathways such as JNK inhibitors, D-JNK-1, riluzole or minocyclin (Henderson *et al*., 2006; Goldvin *et al*., 1998; Corbacella *et al*., 2004).

Recently Ciorba *et al*., (2010) provided the first evidence of the presence and production of a biologically relevant ROS, superoxide in the perilymph of patients affected by profound hearing loss and treated with cochlear implantation. This molecule is produced by xanthine dehydrogenase/xanthine oxidase (XA/XO) enzyme system. High levels of ROS and related

enzymes in human inner ear perilymph (above 25 mmoles per mg of protein) may be an expression of previous damage to the organ of Corti and to the inner ear.

In otosclerosis, ROS may be produced in otosclerosis foci. Their first target is bone cell populations. In bone, ROS might participate in the acceleration of bone turnover (Borovic *et al*., 2007). After diffusion into the inner compartment through the cochlear endosteum, they can participate in the cochlear damages and the sensorineural hearing loss (Henderson *et al*., 2006).

The aim of this part of the project was to investigate the presence of HNE in otosclerotic and normal human stapes, and to evaluate the interaction between Ang II and HNE in human osteoblast-like cells in terms of cellular proliferation, differentiation, and apoptosis.

2. Article: “Angiotensin II and 4-hydroxynonenal (HNE) interaction in otosclerosis and in osteoblast-like cells”

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(submitted for publication)

Angiotensin II and 4-hydroxynonenal (HNE) interaction in otosclerosis and in osteoblast-like cells

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ABSTRACT

Introduction: Otosclerosis is a complex disease characterized by an abnormal bone turnover of the human otic capsule resulting in conductive hearing loss. Angiotensin (Ang) II, a major effector peptide of the renin-angiotensin system is an important regulator of the cardiovascular system and body fluid homeostasis, and an important local cellular growth factor. Oxidative stress and reactive oxygen species (ROS) have a major role in different signaling pathways of Ang II. The aim of the present study was to investigate the role of Ang II and 4-hydroxynonenal (HNE) a second messenger of ROS, interaction in otosclerosis, on *in vitro model* of osteoblast-like cells in order to further understand the pathophysiology of the disease.

*Materials and Methods***:** Immunohistochemistry was performed on tissue samples taken from 15 otosclerotic patients and 6 controls. Antibodies against HNE have been used. Human Osteosarcoma cell lines (HOS) were used to analyze the Ang II and HNE effect on proliferation (^{3}H-thymidine incorporation assay), differentiation (detection of alkaline phosphatase activity by NBT/BCIP assay, Roche) and induction of apoptosis by flow citometry (presence of apoptotic cells was determined by Annexin-V-Fluos Staining Kit, Roche, Germany). Immunoblot analysis was performed to determine whether there is an existing binding between the Ang II and HNE.

*Results***:** Immunohistochemical detection of HNE-protein adducts showed positivity on both control and otosclerotic samples but with different distribution areas. Association of Ang II in concentration of 0,1 nM and HNE of 2,5 μM stimulated the proliferation of the HOS cells ($p<0.0002$). Decrease in alkaline phosphatase activity (ALP) was observed in cell cultures treated with 0,1 nM Ang II and HNE concentrations of 1 μM or 2,5 μM ($p<0,01$). On the contrary, 10 μM HNE alone ($p<0,05$) or in combination with 0,1 or 0,5 nM Ang II ($p<0,01$) increased ALP activity

with the exception of 1 nM Ang II. Decrease in apoptosis ($p<0,05$) was observed in cells treated just with Ang II (0,1 and 0,5 nM). On the contrary increase in apoptosis ($p<0,01$) was detected in almost all cell cultures treated with 10 μM HNE, alone or in combination with Ang II, with the exception of 1 nM Ang II. Results of necrosis have shown that treatment with 0,5 nM Ang II and 1 μM HNE reduced the level of necrotic cells ($p<0,05$). Increase in necrosis was observed in all treatments with 10 μM HNE, alone ($p<0,01$) and in combination with 0,1 nM Ang II ($p<0,02$) and 0,5 nM Ang II and 1 nM Ang II ($p<0,002$) as well as in all treatments with combination of 5 μM HNE with 0,1 nM and 0,5 nM Ang II ($p<0,05$) and 1 nM Ang II ($p<0,003$). Immunoblot analysis showed clear binding between the Ang II and HNE.

Conclusion: Our results support the hypothesis of the involvement of the HNE and thus ROS in the pathophysiology of otosclerosis. Furthermore the results of the Ang II and HNE effect on the osteoblasts proliferation, differentiation and apoptosis, support their observed role as an important signaling molecules in bone remodeling.

INTRODUCTION

Otosclerosis is a complex disease characterized by an abnormal bone turnover localized to the human otic capsule resulting in conductive hearing loss[1]. Its incidence in Western populations is 0.3%. In 80% of cases the disease has a bilateral form. It is more frequent in women than in men[2,3]. Several hypotheses have been proposed concerning the etiology of otosclerosis including viral infection, and auto immunity, but the etiology appears to be complex multifactorial and still unclear. Potential implication of various environmental and genetic factors have been reported (measles virus, auto immunity, HLA system) [4-8].

Angiotensin (Ang) II, a major effector peptide of the renin-angiotensin system is an important regulator of the cardiovascular system and body fluid homeostasis, and an important local cellular growth factor[9-11]. Research of the Ang II effects on bone cells have demonstrated its stimulatory effects on osteoblasts (stimulation of DNA synthesis, cell number and collagen synthesis in osteoblastic precursor cells)[12-17]. Previously we have shown an existing association between otosclerosis and two genetic polymorphisms related to plasmatic Ang II concentrations (AGT M235T and ACE I/D)[18]. We also demonstrated the *in vitro* effects of Ang II in otosclerotic stapes (pro-inflammatory cytokine secretion, reduction in alkaline phosphatase activity)[18].

Oxidative stress and reactive oxygen species (ROS) have a major role in different signaling pathways of Ang II[19-22]. Ang II stimulates the ROS formation via activation of NADPH oxidase[9,23]. Furthermore, several important physiological functions of Ang II (e.g. cell growth, hypertrophy, proliferation, apoptosis and cell migration) are directly mediated by ROS[22]. As highly reactive and unstable free radical molecules, ROS, can react and damage cell macromolecules such as proteins, DNA and lipids. Primary targets of ROS are polyunsaturated fatty acids in membrane phospholipids

and in other lipid containing structures thus producing more stabile reactive radicals in a process called lipid peroxidation. Recent studies have shown that lipid peroxidation products can cause lipid hydroperoxide-derived modifications of Ang II which can modulate its biological activity[19]. A major bioactive marker of lipid peroxidation, derived from ω-6 polyunsaturated fatty acids like arachidonic acid and linoleic acid, is 4-hydroxynonenal (HNE), an reactive aldehyde, that acts as a second messenger of free radicals and a signaling molecule[24,25]. It is found in different human tissues and its physiological roles are not entirely understood[26-30]. Our previous research has revealed the HNE role in differentiation, apoptosis and cell proliferation in human osteoblast-like cells [26,29,30] and a positive impact on cell growth when it is present in physiological concentration[31]. We have also found that HNE is generated by osteoblasts while grown *in vitro* on the surfaces of bioactive glasses (e.g. material used in medicine for enhancement of osteogenesis) [32].

The aim of the present study was to understand the role of Ang II and HNE, in otosclerosis, on *in vitro model* of osteoblast-like cells in order to further understand the pathophysiology of the disease.

MATERIALS AND METHODS

Tissue samples

In this series, the stapedial bones were harvested from patients undergoing surgery for otosclerosis (n=15) and control group of patients undergoing translabyrinthine surgery for a vestibular schwanoma (n=6). Ethics committee approval and patients' consent were previously obtained for the bone samplings. The diagnosis of otosclerosis was based on clinical, audiometric, and computed tomography scan

findings and confirmed by the preoperative aspect of the stapes. All samples were fixed in 10% buffered formalin and decalcified in 0.5 mol/L Na-EDTA for 72 hours. Samples were then dehydrated in a series of alcohol solution (70%, 96%, absolute) and xylene and then embedded in paraffin blocks.

Immunohistochemistry

Immunohistochemistry for HNE-modified proteins was carried out on formalin-fixed paraffin embedded stapes samples using monoclonal antibodies obtained from culture medium of the clone 'HNE 1g4', produced by a fusion of Sp2-Ag8 myeloma cells with B-cells of a BALB-c mouse immunized with HNE-modified keyhole limpet hemocyanine. The antibody is specific for the HNE-histidine epitope in HNE-protein (peptide) conjugates and gives only 5% cross-reactivity with HNE-lysine and 4% with HNE-cysteine.

Immunohistochemistry was performed in a two-step procedure using an EnVision kit (DAKO, Denmark), where the first step was incubation with anti-HNE monoclonal antibodies (dilution 1:10) during 2 h in humid chambers at room temperature. The second step was incubation with peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulins during 30 min. Finally, the reaction was visualized by a DAB (3,3-diaminobenzidine tetrahydrochloride in organic solvent) giving a brown colour and using haematoxylin contrast staining (blue). Negative control was done on one histological slice of the same tissue, without application of HNE-histidine-specific monoclonal antibodies. All immunohistochemical analyses were done by a pathologist experienced in the HNE-immunohistochemistry without prior knowledge of the study group design.

Preparation of fresh HNE

(E)-4-hydroxynonenal-dimethylacetal (HNE-DA, Alexis Biochemicals) was obtained as 1mg/ml liquid, preserved in chloroform and kept at –20°C. Before use, it is activated according to the manufacturer's protocol. Briefly, HNE-DA is evaporated by nitrogen blow and 1mM HCl is added to hydrolyze HNE for 1h. HNE concentration is determined by spectrophotometer measuring the spectra from 200 to 350nm and calculated value upon the absorption maximum at 223nm. HNE concentration is calculated upon the formulae c(mol/l)= Axd/13750, where is c-concentration of HNE in mol/l; A-measured absorbance; d-sample dilution; 13750-molar extinction coefficient of HNE in water.

Human osteosarcoma (HOS) cell culture

The human osteosarcoma cell line (HOS) was obtained from the American Type Culture Collection (ATCC). Cells were maintained in DMEM (Dulbeco's Modified Eagle's Medium, Sigma USA) with 10% fetal calf serum (FCS, Sigma, USA) in an incubator (Heraeus, Germany) at 37°C, with a humid air atmosphere containing 5% CO_2. The cells were detached from confluent cultures with a 0.25% trypsine solution for 5 minutes. Viable cells upon (trypan blue exclusion assay) were counted on a Burker-Turk hemocytometer and used for experiments.

Cell Proliferation

Cells have been seeded at $1x10^4$ per well in 96-well microwell plate in 100 µl of DMEM medium with 0.25% of FCS and left for 48 hours. Upon 48 hours medium was removed and replaced with a fresh medium free of FCS. Cells were further treated with different concentrations of HNE (1µM, 2.5µM, 5µM and 10µM) and Ang II

(Sigma-Aldrich, France, 0.1nM, 0.5nM, 1nM) in two different sets of experiments. In the first type of experiment the HNE was added first followed by Ang II after one hour, while in the second experiment first the Ang II was added followed by HNE after one hour. Previously we used higher concentrations of Ang II (10 nM and 100 nM). The obtained results have shown that these concentrations have deleterious effect on HOS cell proliferation both alone and even more if cells were treated with HNE in concentration-dependent manner. For this reason, and since normal human plasma Ang II levels are from 12-35 pg/ml when measured at rest and increasing on standing, exercise, dehydration, or sodium depletion, we have focused on lower Ang II concentrations.

The rate of radioactive ^{3}H-thymidine incorporation into DNA is used to estimate the replicate activity of HOS cells. After 12 hours of incubation with compounds, radioactive ^{3}H-thymidine ([6-^{3}H] thymidine, 1mCi/ml, Amersham Biosciences, USA) was added 0.1 µCi to each well and incubation is continued for the following 6 hours. The cells were then harvested on glass filter in a cell harvester (Skatron, Norway) and ^{3}H-thymidine incorporation was measured using a liquid scintillation β-counter (Beckman 7400, USA). Results are expressed as percentage of positive control.

Cell Differentiation

Cells were seeded 2x10^{4} per well in 96-well plate in DMEM with 1% of FCS and left for 24 hours. Afterwards the medium was replaced by fresh medium containing different concentrations of HNE (1µM, 2.5µM, 5µM and 10µM) and Ang II (0.1nM, 0.5nM and 1nM). Given that the proliferation results where more interesting where HNE was added first and upon 1 hour Ang II, we decided to carry out all our treatments in this way. The treatment was repeated every second day. Control cells

were stimulated to differentiate with 10^{-7} M dexamethasone (Sigma, USA), $5x10^{-6}$ M L-ascorbic acid (Sigma, USA) and 10^{-3} M β-glycerophosphate (Sigma, USA). After 10 days of treatment, cells were stained for alkaline phosphatase (ALP) by NBT/BCIP assay (Roche, Switzerland). This assay is based on a chromogenic reaction initiated by the cleavage of the phosphate group of BCIP by alkaline phosphatase present in the cells. Cell cultures were washed three times with tris-buffered saline (TBS), pH 7.0 and fixed with 4% buffered paraformaldehyde for 30 minutes at 4°C. Staining was performed by adding 200 µl/well of stain and incubated at 37°C for 60 minutes. Insoluble purple precipitates were solubilised with 200 µl of SDS 10% HCl and incubated for 18 h. The optical density measurement was done at 595 nm.

Induction of Apoptosis

Flow Cytometry

Cells were seeded in 6-well plates (Sarstedt, Germany) at the density of 250 000 cells/well in DMEM with 0.25% FCS for 48 hours. After 2 day cultivation, medium was replaced and cells were treated with HNE and Ang II according to previously described treatment and left for 24 hours. The presence of apoptotic cells was determined by Annexin-V-Fluos Staining Kit (Roche, Germany) according to manufacturer protocol. Briefly, cells were collected and washed in phosphate buffer saline (PBS), centrifuged and resuspended in Anexin-V-Fluos labeling solution (Anexin-V-Fluoresce/Propidium iodide (PI) negative). After 15 minutes incubation at room temperature cells were analyzed on FACSCalibur flow cytometer (Becton Dickinson, USA). The percentage of cells nonapoptotic live cells (Annexin V-FITC negative/PI negative), apoptotic cells (Annexin V-FITC positive/PI negative) and

necrotic cells (Annexin V-FITC positive/PI positive), was calculated by application of FlowJo software (Tree Star Inc., USA).

Immunoblot Analysis

Ang II and bovine serum albumin (BSA, fatty acid free, Sigma, USA) were prepared in a concentration of 0.1 mg/ml, mixed with $5x10^{-5}$ and $5x10^{-4}$ M HNE (final concentrations) and left for one hour at room temperature. Prepared samples (100 µl) were spotted onto nitrocellulose membrane (Amersham). The membrane was incubated in blocking solution (2 % nonfat milk powder in PBS) at room temperature for 60 min and subsequently incubated overnight with mouse monoclonal antibody directed against HNE-histidine epitope. The blot was incubated with 3 % H_2O_2 to block endogenous peroxidases and then washed and incubated with EnVision (Dako, Denmark) for 30 min. Immune complexes were visualized using the 3,3'-diaminobenzidine tetrahydrochloride (Dako, Denmark) staining and scanned for quantification of signals. Negative controls were included in all experiments in which the antibody was omitted and replaced by control diluent (1% BSA in PBS).

Statistical Analysis

All assays were performed in triplicates. Values were expressed as means ± SD. Comparisons were assessed a by two-tailed Student's test considering values of $p<0.05$ as significant.

RESULTS

Immunohistochemistry

Both control and otosclerotic samples showed positivity for HNE antibodies. Control samples showed clearly marked HNE positivity in periosteal bone regions while otosclerotic samples showed localized regions of HNE positivity with no regular distribution (Figure 1).

Proliferation of HOS cells

The influence of Ang II and HNE on the cellular growth was analyzed on human osteosarcoma cell line (HOS). The effect of Ang II and HNE on the cell proliferation depended on the concentration used. Results showed that association of Ang II in concentration of 0.1nM and HNE of 2.5 μM stimulated the proliferation of the HOS cells ($p<0.0002$, Figure 2). On the other hand association of 10 μM HNE with all Ang II concentration or alone showed significant decrease in cell proliferation (alone $p<0.00005$; 0.1 and 0.5 nM Ang II $p<0.001$; 1 nM Ang II $p<0.0004$). All values were calculated as percentage of the respective control.

Differentiation of HOS cells by Ang II and HNE

Alkaline phosphatase (ALP) activity

Results are expressed as a percentage of the positive control (treatment with dexamethasone, L-ascorbic acid and β-glycerophosphate as inducers of osteoblastic differentiation). We observed decrease in ALP activity in cell cultures treated with 0.1 nM Ang II and HNE concentrations of 1 μM or 2.5 μM ($p < 0.01$). On the contrary, 10

μM HNE alone ($p < 0.05$) or in combination with 0.1 or 0.5 nM Ang II ($p < 0.01$) increased ALP activity with the exception of 1 nM Ang II (Figure 3).

Apoptosis induction

The used Annexin-V-Fluos Staining Kit can distinguish apoptotic cells, FITC+PI-, from necrotic cells, FITC+PI+, so we presented both results. Results are expressed as a percentage of the control value (FITC+PI- cells without any treatment for apoptotic cells and FITC+PI+ cells without any treatment for necrotic cells).

We have observed decrease in apoptosis ($p<0.05$) in cells treated just with Ang II (0.1 and 0.5 nM). On the contrary increase in apoptosis ($p<0.01$) was detected in almost all cell cultures treated with 10 μM HNE, alone or in combination with Ang II, with the exception of 1 nM Ang II (Figure 4).

Results of necrosis have shown that treatment with 0.5 nM Ang II, 1 μM HNE or combination of 1 μM HNE with 0.5 nM Ang II reduce the level of cells in necrosis ($p<0.05$). Increase in necrosis was observed in all treatments with 10 μM HNE, alone ($p<0.01$) and in combination with 0.1 nM Ang II ($p <0.02$); 0.5 nM Ang II and 1 nM Ang II ($p<0.002$) as well as in all treatments with 5 μM HNE with Ang II of 0.1 and 0.5 nM ($p<0.05$) and 1 nM Ang II ($p<0.003$, Figure 5).

Immunoblot analysis

Using the HNE antibodies immunoblot analysis showed clear binding between the Ang II and HNE (Figure 6).

DISCUSSION

In the present study we wanted to determine the interaction of Ang II and HNE in the otosclerosis by studying the *in vitro* model of osteoblast-like cells. In order to investigate whether HNE can be found in the otosclerotic bone we have performed immunohistochemical analysis of formalin fixed paraffin embedded control and otosclerotic stapes samples, with the monoclonal HNE antibody that detects HNE-protein conjugates, specifically the HNE-histidine epitope. Results showed that HNE-protein conjugates are present in both samples, control and otosclerotic ones. However, difference between these samples was observed in a way that the HNE positivity in control samples was present only in periosteal region while otosclerotic samples showed no regular HNE positivity but rather multifocal areas mainly in the new bone or pathologic bone formation regions. The HNE presence in periosteal region of control samples suggests its physiological role associated to cell proliferation and differentiation. On the other hand, otosclerotic samples, where abnormal and undesirable bone turnover is present, express HNE positivity mostly in multifocal areas and it diminishes in periosteal regions. This clear difference in distribution patterns of control and otosclerotic samples indicates HNE bifunctional role. Therefore, in normal conditions HNE regulates the cell growth and differentiation, however in pathological conditions, like otosclerosis, seems that it loses its role in cell growth regulation but rather has a part in the pathology of the otosclerosis. Although, this immunohistochemical analyses is mostly qualitative and not quantitative, yet when comparing the brown color intensity we can observe that it does not differ greatly. This implies that HNE is associated with the cell growth but the difference of its role (physiological-pathological) is mainly associated with the

origin of the cells. If the cell originates in periosteal region HNE is involved in normal physiological process of cell growth but if cell are forming new bone in multifocal regions of otosclerotic bone, HNE is involved in pathophysiology of this disorder.

As a second messenger of ROS, HNE has high reactivity to bind to macromolecules, especially proteins, and can also be carried to the distant places and released from protein in certain conditions such as pH change affecting biomolecules distant from the place of its origin[31]. This could be one of the ROS diffusion mechanisms into the inner ear. Previously ROS have been determined in the human inner ear and perylimph by the activity of other ROS such as xantine dehydrogenase/xantine oxidase (XA/XO) enzyme system[33]. In animal models, several conditions of increased ROS production related to cochlear damage have been described, including exposure to noise and to drugs (i.e. aminoglycosides and cisplatin). In humans, ROS appeared to be involved in hair cell damage in some cases of genetic hearing loss (i.e. Pendred syndrome), presbycusis, and Menière's syndrome[33].Once generated, ROS are responsible for direct cellular damage to lipids, proteins, and DNA, triggering apoptosis or necrosis and may also diffuse among the inner ear scalae[33].

Our previous results have shown that HNE acts as a signaling molecule affecting cell proliferation, differentiation and apoptosis in dose-dependent manner[26,32]. This is in compliance with our current results clearly distinguishing physiological and supra-physiological role of HNE. Consequently, we have also observed interesting dose-dependent manner of combined Ang II effect. Our results with supra-physiological concentrations of HNE (5 and 10 μM) have shown that higher Ang II concentrations (0.5 and 1 nM) multiply the negative effect of HNE on cell proliferation, differentiation and stimulates cell apoptosis and necrosis. Moreover, 10 μM HNE have shown to

decrease cell proliferation and induce differentiation, apoptosis and necrosis while addition of 1 nM Ang II completely directs towards necrosis. Combined effects of 5 μM HNE with 1 nM Ang II and 10 μM HNE with 0.5 nM Ang II show attenuated effects compared to the highest concentration treatment. Thus 5 μM HNE reduces negative effect of HNE on cell proliferation, but on the other hand, with pronounced necrosis. Furthermore, lower Ang II concentration (0.5 nM) increases differentiation. The physiological concentrations of HNE (1 μM and 2.5 μM) are more likely interacting with lower concentration of Ang II (0.1 nM) indicating completely opposite effect mostly directed to cell proliferation, and reduction of cell differentiation, apoptosis and necrosis.

These results suggest that Ang II interacts with already observed bifunctional growth-regulating HNE effect in a dose-dependent manner. However, previous studies confirmed the involvement of ROS in signaling pathways of Ang II. NADPH oxidase is known to be the major source of vascular ROS. Moreover, Ang II stimulates ROS generation via activation of NADPH oxidase, mainly through its G protein-coupled Ang II type 1 (AT1) receptor[19]. Although our protocol minimized the possible interactions between Ang II and HNE and since is known that various cells metabolize 90-95% of 100 μM HNE within first 3 minutes[34], because Ang II consist of one histidine we also investigated whether there is an existing binding between Ang II and HNE. For that reason we performed the immunoblot analysis that showed clear positivity. The difference in color intensity of Ang II and bovine serum albumin (BSA) dots, for the same treatment, could be explained with the difference in their structure. BSA is known to have 16 histidines comparing to 1 histidine of Ang II. Since the used monoclonal antibody specifically recognizes HNE-histidine epitope in HNE-protein

conjugates this is most likely the explanation for intensity difference. Consequently, we can assume that HNE could modulate the effect of Ang II *per se*.

In conclusion our results support the hypothesis of the involvement of the ROS in the pathophysiology of otosclerosis. Furthermore the results of the Ang II and HNE effect on the osteoblast proliferation, differentiation and apoptosis, support their observed role as an important signaling molecules in bone remodeling.

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Figure 1- Immunohistochemistry for HNE of the otosclerotic and control samples: Both control (A,B) and otosclerotic (C,D) samples showed positivity for HNE antibodies. Control samples showed clearly marked HNE positivity in periosteal bone regions while otosclerotic samples showed localized regions of HNE positivity with no regular distribution

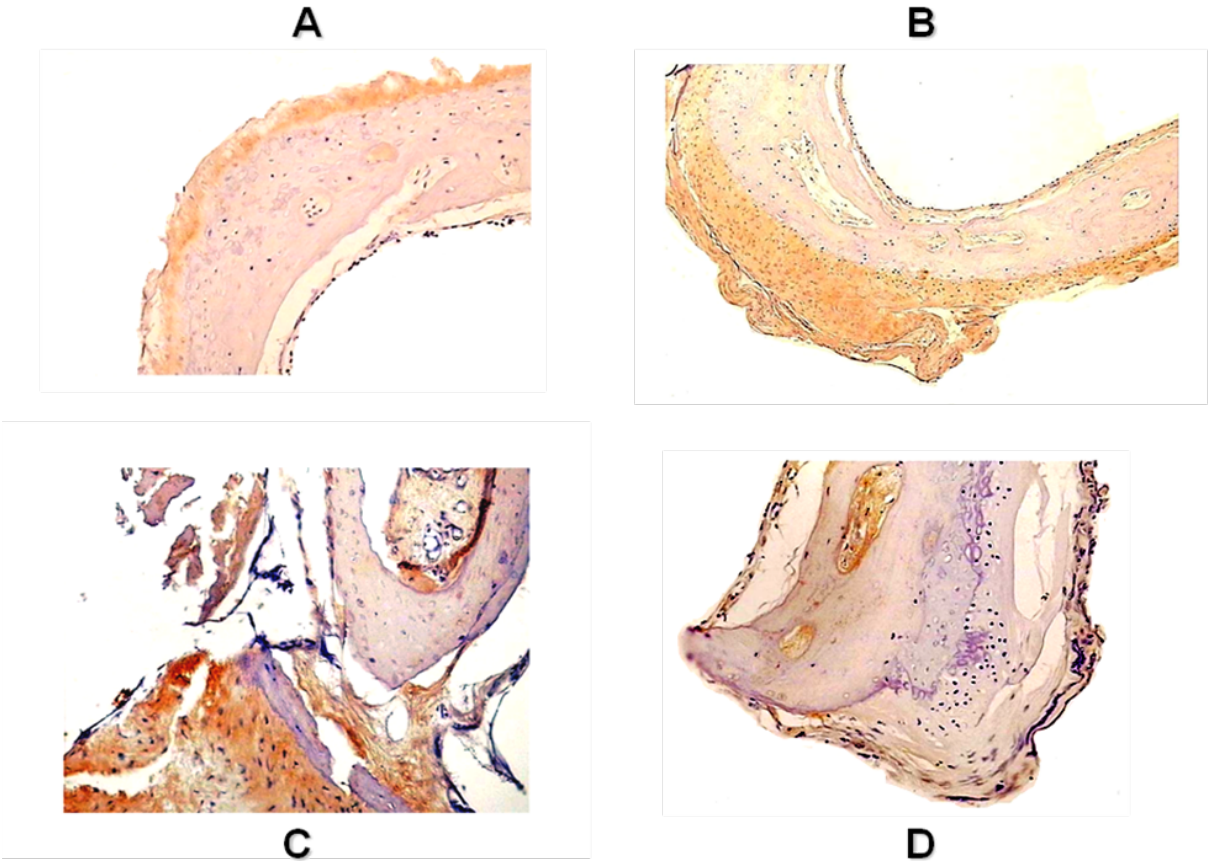
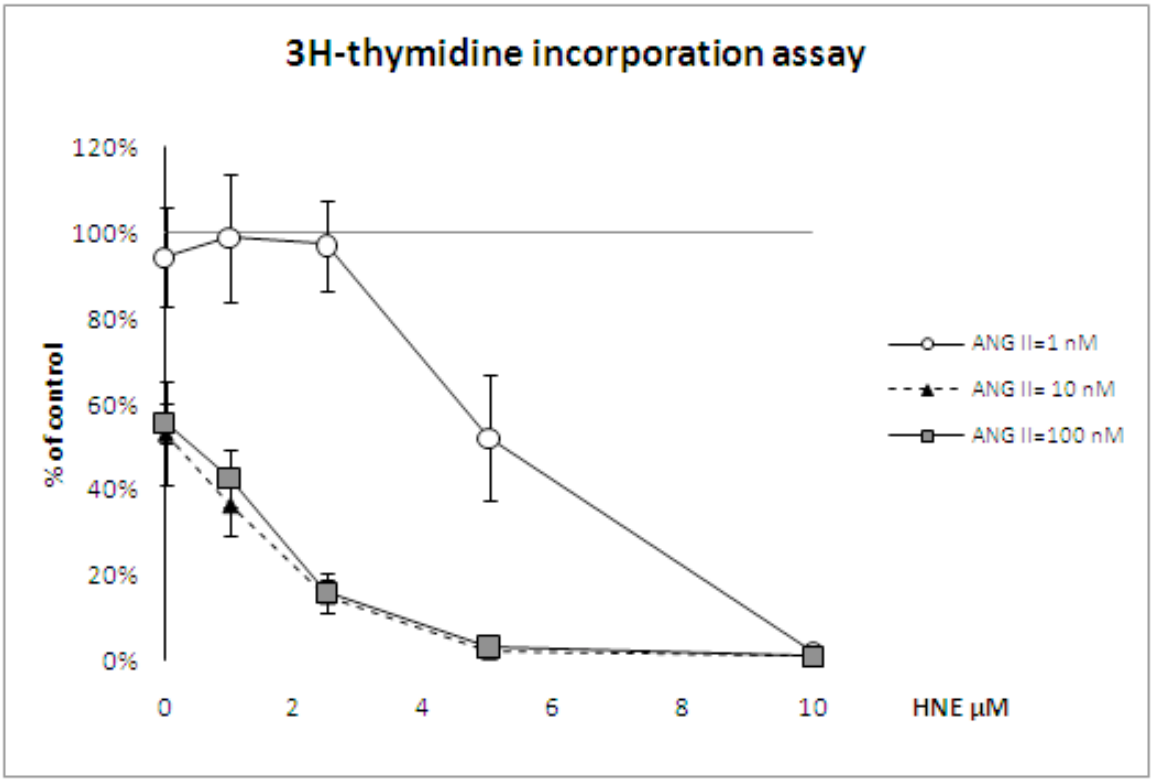
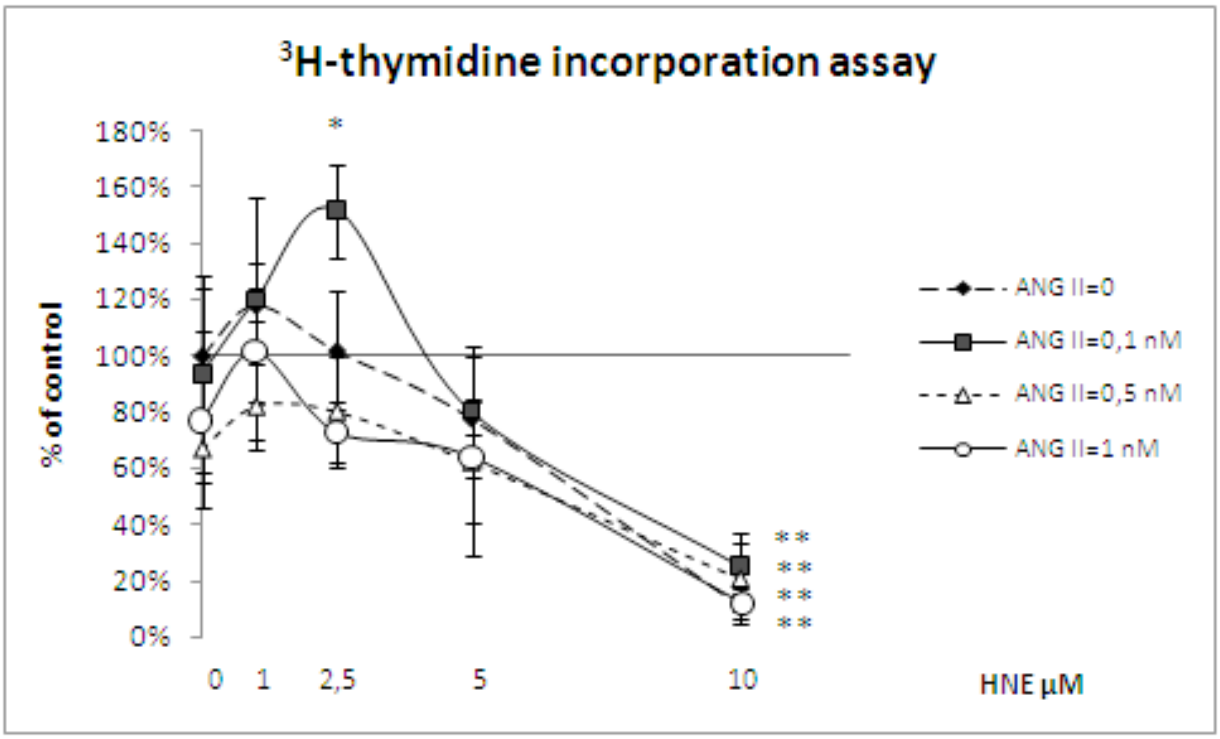


Figure 2-Proliferation of HOS cells:

a) The effect of Ang II and HNE on the cell proliferation depends on the concentration used. Higher concentrations of Ang II (10 nM and 100 nM) have deleterious effect on HOS cell proliferation both alone and even more if cells were treated with HNE in concentration-dependent manner.



b) Results showed that association of Ang II in concentration of 0,1nM and HNE of 2.5 µM stimulated the proliferation of the HOS cells ($p<0.0002$). Association of 10 µM HNE with all Ang II concentration or alone showed significant decrease in cell proliferation (alone $p<0.00005$; 0,1 and 0.5 nM Ang II $p<0.001$; 1 nM Ang II $p<0.0004$).



Results are expressed as percentage of respective control. Ang II: Angiotensin II, HNE: 4-hydroxynonenal.

Figure 3- Differentiation of HOS cells by Ang II and HNE: Results are expressed as a percentage of the positive control. Alkaline phosphatase activity (ALP) decreased in cell cultures treated with the combination of Ang II (0.1 nM) and HNE at or 2.5 µM ($p<0.01$, paired t-test). In contrast, 10 µM HNE alone ($p<0.05$) or in combination with 0.1 or 0.5 nM Ang II ($p<0.01$) increased ALP activity. ALP: Alkaline phosphatase, Ang II: Angiotensin II, HNE: 4-hydroxynonenal.

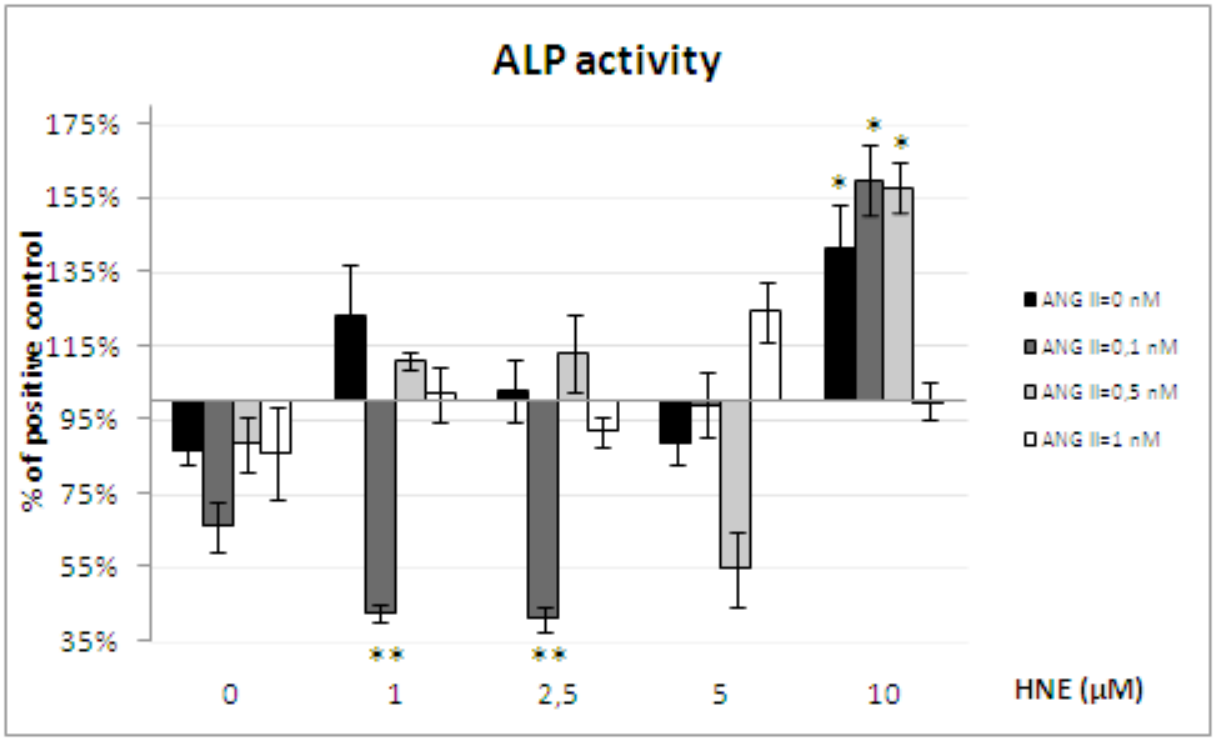


Figure 4 - Induction of Apoptosis (FITC+PI-): Decreased **apoptosis** in cells treated with Ang II (0.1 and 0.5 nM) alone ($p<0.05$, paired t-test). In contrast, increase in apoptosis ($p<0.01$) was detected in almost all cell cultures treated with 10 μM HNE, alone or in combination with Ang II, with the exception of 1 nM Ang II. Ang II: Angiotensin II, HNE: 4-hydroxynonenal.

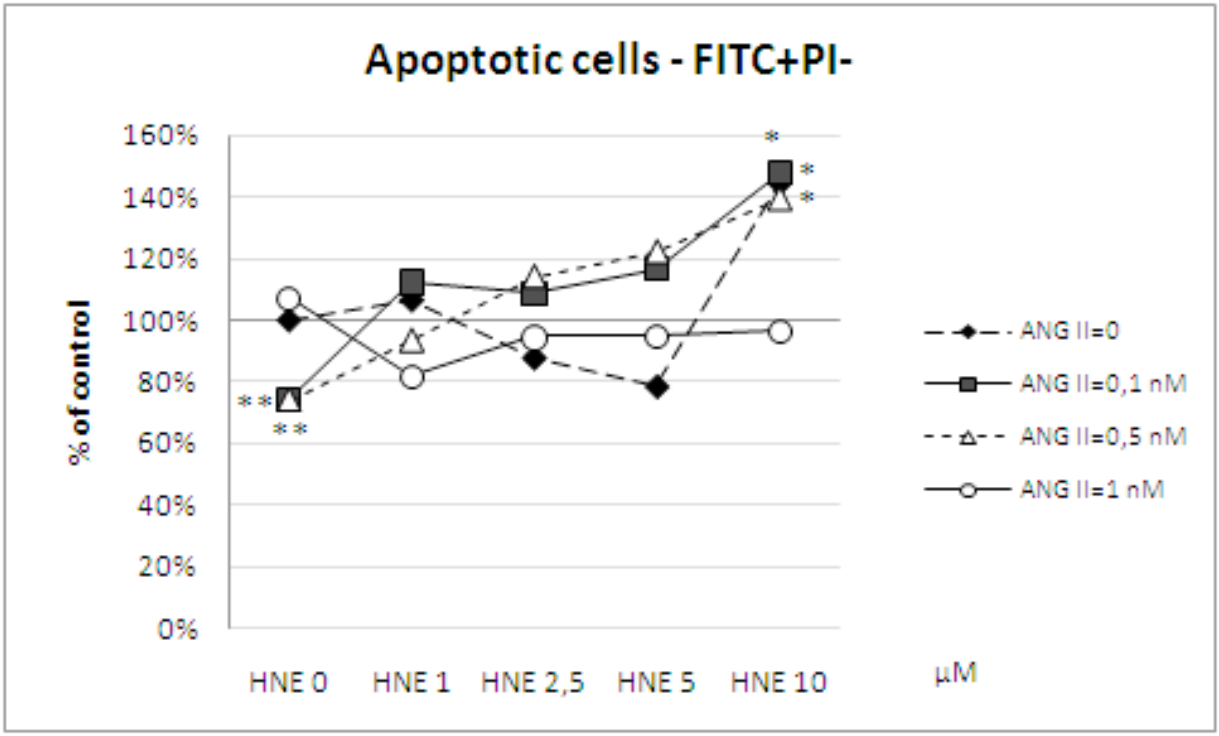


Figure 5- Induction of Apoptosis (FITC+PI+): Results of necrosis have shown that treatment with 0.5 nM Ang II, 1 μM HNE or combination of 1 μM HNE with 0.5 nM Ang II reduced the level of cells in necrosis ($p < 0.05$). Increase in necrosis was observed in all treatments with 10 μM HNE, alone ($p < 0,01$) and in combination with 0.1 nM Ang II ($p < 0.02$); 0,5 nM Ang II and 1 nM Ang II ($p < 0.002$) as well as in all treatments with 5 μM HNE with Ang II of 0.1 and 0.5 nM ($p < 0.05$) and 1 nM Ang II ($p < 0.003$). Ang II-Angiotensin II, HNE: 4-hydroxynonenal.

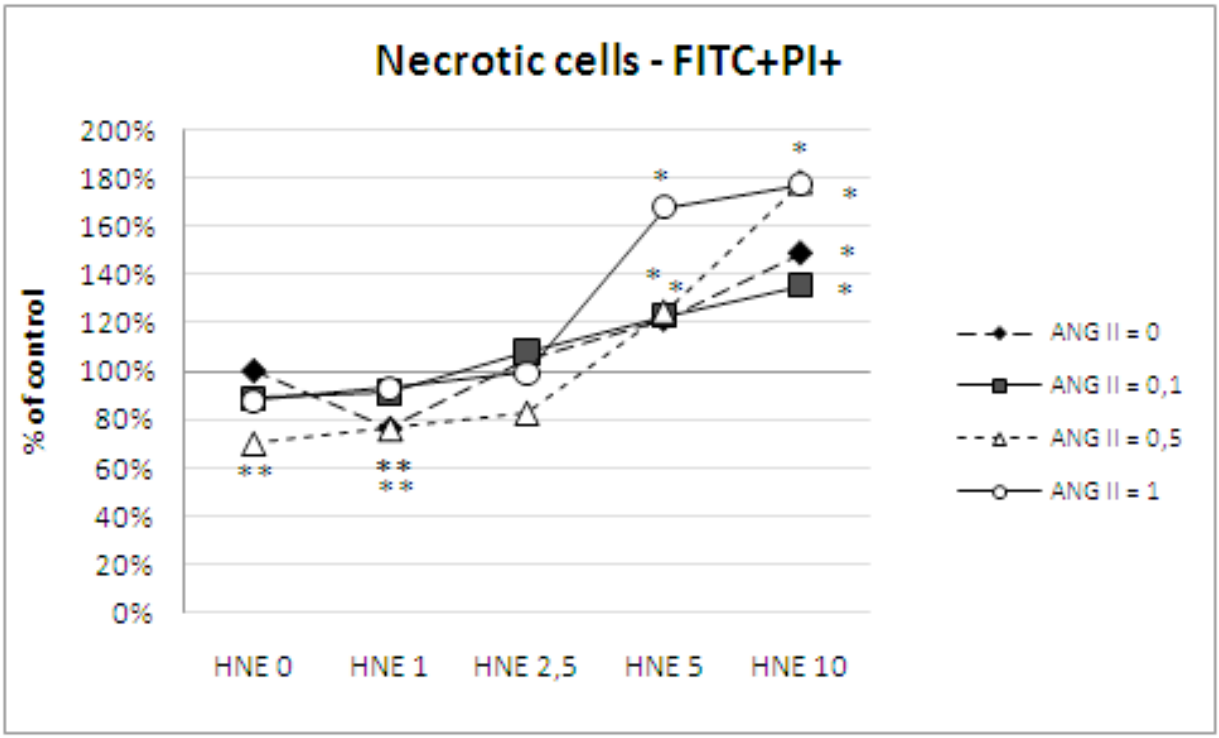
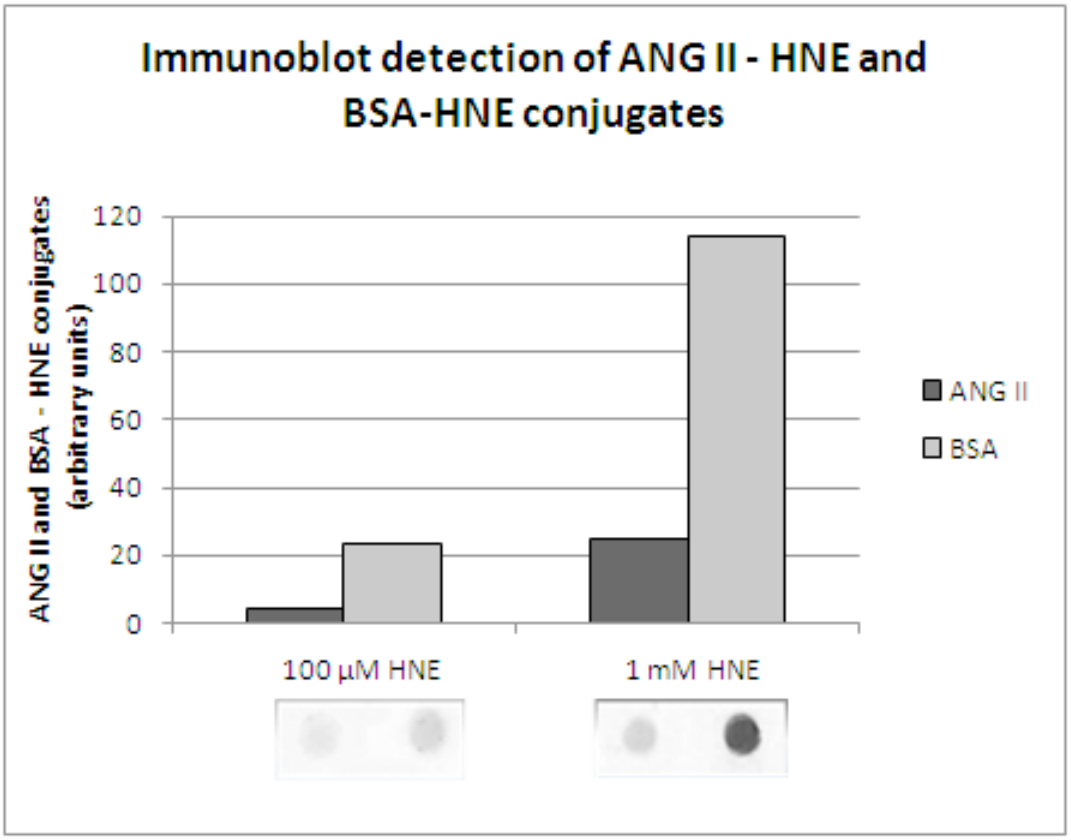


Figure 6- Immunoblot analysis with HNE antibodies showed clear binding between the Ang II and HNE. Ang II: Angiotensin II, HNE: 4-hydroxynonenal, BSA: bovine serum albumin.



IV. GENERAL DISCUSSION

The results of these 2 studies provide a new insight on the role of Ang II and oxidative stress (ROS and its second messengers) in the pathophysiology of stapes metabolism namely otosclerosis. Ang II induces the secretion of different pro-inflammatory cytokines in the primary otoslerotic cell cultures. Studies on Ang II effects and secondary messenger of the ROS, (HNE) show interaction of these two factors and show positive influence on proliferation of human osteosarcoma cell-lines. The heterogeneous and diffuse distribution of HNE in otosclerotic bone supports the possible role of oxidative stress in the pathophysiology of the disease. Finally, oxidative stress products interact with Ang II to alter bone cell response to this hormone. As indicated by immunoblot, at least a part of this interaction takes place at a level before Ang II receptors. In previous studies, we observed a difference of response to Ang II stimulation between otosclerotic and normal stapes. HNE is at least one of the explanations to this difference.

1. Bone (stapes) metabolism

Bones are constantly remodeled through the synthesis of bone matrix by osteoblasts and the resorption of bone by osteoclasts. Perturbations in inflammatory cytokines, growth factors and hormones cause an imbalance between osteoclasts and osteoblast activities and can result in different bone diseases such as osteoporosis, osteopetrosis and otosclerosis (Boyle *et al*., 2003; Theill *et al*., 2002; Wada *et al*., 2006). The discovery of the receptor activator of nuclear factor-kB ligand (RANKL)/RANK/osteoprotegerin (OPG) signaling system provided a major breakthrough in understanding of the bone metabolism (Martin *et al*., 2005; Boyce *et al.*, 2007). Under physiological condition the RANKL produced by osteoblasts binds to the RANK on the surface of the osteoclast precursors, leading to the NF-kB activation (via

adaptor protein TRAF6) and translocation to the nucleus causing transcription of the osteoclastogenic genes. Osteoprotegerin acts as a soluble, neutralizing antagonist which competes with RANK on pre osteoclasts and osteoclasts for RANKL produced by osteoblasts. Osteoprotegerin inhibits the differentiation, survival and fusion of osteoclastic precursor cells, supresses activation and promotes osteoclast apoptosis (Simonet *et al*., 1997; Udagawa *et al*., 2000; Hofbauer *et al*., 2001; Zehnder *et al*., 2008). Osteoprotegerin is expressed in high levels within the inner ear, and is secreted into the perilymph and the surrounding bone through a lacunocanalicular system. It inhibits the active bone remodeling within the otic capsule (Zehnder *et al*., 2005, 2008). Inhibition of otic capsule remodeling is most present close to the cochlea and decreases with the distance from cochlear endosteum (Frisch *et al*., 2000; Zehnder *et al*., 2008). The active remodeling process seen in the osteoprotegerin knockout mice shows many similarities to otosclerosis seen in human temporal bones (Zehnder *et al*., 2008).

Otosclerosis is a primary disorder of the bone metabolism unique to the human temporal bone and is characterized by a disturbed resorption and deposition in bone (Niedermeyer *et al*., 2002; Schrauwen *et al*., 2008). It is characterized by spongiosis (active phase) and sclerosis (inactive phase) of the bony labyrinth which normally undergoes very little bone remodeling after birth (Frisch *et al*., 2000; Ealy *et al*., 2008). The otic capsule is unique in its composition and pattern of bone remodeling. It remodels at a very low level compared with other bones in the body. Within the otic capsule, bone turnover varies from particularly full inhibition (0,13%) in the innermost perilymphatic zone to gradually increasing, yet still reduced, level at the periphery (Frisch *et al*., 2000; Zehnder *et al*., 2006). The reason for this low level of bone turnover remains unclear. It has been suggested that this pattern of bone remodeling is a fundamental prerequisite for the normal function of the inner ear and has led to the

speculation that bone turnover in the otic capsule is controlled by intrinsic factors produced by the inner ear (Frisch *et al*., 2000, Adams *et al*., 2002; Zehnder *et al*., 2008).

The causes of otosclerosis still remain unknown. Today otosclerosis is considered a complex disease caused by environmental and genetic factors. In addition to linkage analysis, association of several genes with otosclerosis has been reported. Previously, a TGF-ß1 gene was identified to be associated with the complex form of otosclerosis (Thys *et al*., 2007; Ealy *et al*., 2008). TGF-ß1 is an important factor in bone remodeling and in the induction of embryogenesis in the otic capsule. It can either stimulate chondrogenesis to promote otic capsule growth or selectively inhibit this process to permit perilymphatic space formation and capsular modeling (Bonewald *et al*., 1990; Frenz *et al*., 1991). The presence of TGF-ß1 at low levels stimulates osteoclast precursors to osteoclast differentiation, and at higher levels inhibits osteoclastogenesis (Karst *et al*., 2004). Recent studies showed that polymorphisms in BMP2 and BMP4, two members of the TGF-ß superfamily, play an important role in otic capsule chondrogenesis, and could contribute to the otosclerosis susceptibility (Lehnerdt *et al*., 2007, Schrauwen *et al*., 2007). Another gene TNF-α has been noted to be expressed in otosclerotic stapes. Its expression was associated with the detection of measles virus RNA (Karosi *et al*., 2005). Osteoprotegerin expression appears to be decreased in otosclerotic stapes footplates which are positive for measles viruses and TNF-α expression (Karosi *et al*., 2006).

In our study of stapes metabolism and oxidative stress we used two different models: HOS (human osteosarcoma cell lines) which serves as a standard model for studying the bone metabolism, and primary otosclerotic cell cultures. The human otosclerotic stapes primary cell cultures were first described in 1968 (Petrovic and Shambough). The cultures were

described as highly heterogenic with the presence of osteoblast-like cells, fibroblasts and intermediary cells. Later Bozorg Gayeli *et al*. (1999) studied the primary otosclerotic cells and for the first time compared them to the normal stapedial cell cultures (2000). These cell cultures show several indicators of osteoblastic differentiation: alkaline phosphatase activity, mineralization nodules, presence of PTH receptors, osteocalcin production, and change of shape after PTH stimulation. Since the etiology of this disease is unknown, there is no valid animal model for the study of otosclerosis pathophysiology.

2. Angiotensin II and bone metabolism

In the local milieu of bone, two major types of cells, osteoblasts and osteoclasts, are located in close proximity, exchange their signals, and coordinately resorb and form bone matrix (Zhao *et al*., 2006; Izu *et al*., 2008). Such events are controlled by molecules present in the local micro environment. These include cytokines, their modulators, and matrix proteins secreted by osteoblasts and osteoclasts (Myazono *et al*., 2004; Usui *et al*., 2004; Morinobu *et al*., 2005; Izu *et al*., 2008). The bone environment is quite heterogeneous, and there are also cells other than osteoclasts and osteoblasts. Bone microvasculatures serve as root to supply osteoclast progenitors, which are derived from hematopoietic lineage cells. These bone vasculatures are also considered to give rise to progenitors for osteoblastic cells from their perivascular regions (Izu *et al*., 2008). In addition to the anatomical relationship between the vascular cells and bone cells, these cells may be functionally involved to coordinate regulation of bone mass (Izu *et al*., 2008).

High blood pressure has been associated with bone loss in various clinical studies in humans (Lynn *et al*., 2006; Rejnmark *et al*., 2006). The epithelia of distal renal tubule, amiloride-

sensitive epithelial sodium channels (ENaCs), which are controlled by the RAAS, represent the rate limiting step for sodium reabsorption. Recent clinical studies indicate that beta blockers and anti-hypertension drugs would reduce risk of bone fractures in the elderly populations (Sanada *et al*., 2004; Lynn *et al*., 2006). This suggests a possible link between vascular and skeletal systems. RAAS appears to act not only systematically but also locally in several tissues, and bone micro-environments have been studied in this regard (Lavoie *et al*., 1997; Sakai *et al*., 2007). Ang II expression has been reported in cartilage and bone (Kizer *et al*, 1997; Trujillo *et al*., 1999). Osteoblasts and osteoclasts both express AT1 receptor in cell cultures, suggesting the existence of local RAAS in bone (Hatton *et al*., 2997; Hiruma *et al*., 1997; Shimizu *et al*., 2008). However, whether bone expresses RAAS components *in vivo* is not known.

Ang II Receptors in bone

Ang II acts via AT1 and AT2 receptors, which are members of the 7-trans membrane-spanning G protein-coupled receptors. Many of Ang II actions are mediated through AT1 receptors (Senbonmatsu *et al*., 2003). In bony tissue, Ang II was reported to promote bone resorption via the AT1 receptor in cell culture system (Shimizu *et al*., 2008). Expression of AT1 receptor was observed in cultured osteoblasts and Ang II inhibits differentiation and bone formation via the AT1 receptor in rat calvarial osteoblastic cells (Bandow *et al*., 2007). Ang II significantly induces the expression of RANKL (receptor activator of NF-kB ligand) via AT1 in osteoblast, leading to the activation of osteoclasts.

Since osteoclast differentiation is regulated by a variety of hormones, local factors and inflammatory cytokines, such as IL-1 and TNF-α, RAAS might also be involved in this auto/ paracrine system. Previously published work provides several potential mechanistic

connections for the control of bone resorption by the AT1 receptor. For example, AT1 receptor signaling has been shown to inhibit the development of osteoclast and to block macrophage infiltration in inflammatory situations (Shimizu *et al*., 2008; Nagai *et al*., 2006). AT1 receptor antagonists also interfere with lipopolysaccharide activation of RAW264.7 osteoclast-like cells (Nagai *et al*., 2006). Ultrasound (mechanical strain) has been shown to increase RANKL, MP-1, and MIP in osteoblasts by signaling via AT1 receptor (Bandow *et al*., 2007).

In contrast to AT1 receptor, AT2 receptor blockade did not yield a significant effect in rat calvarial cells or in human osteoblast and osteoclast precursor cell co-cultures (Hagiwara *et al*., 1998; Shimizu *et al*., 2008).

However, a recent study showed that AT2 receptor blockade increased bone mass in adult mice (Izu *et al*., 2008). Indeed, AT2 receptor appears to inhibit osteoblastic basal activity *in vivo* as shown by the blockade-induced increase in bone formation rate. At the same time, AT2 receptor blockade suppresses *in vitro* bone resorption based on the analysis of osteoclast number and osteoclast surface and in organ culture. Thus, the dual role of AT2 receptor leads to a significant catabolic effect in bone. Latest studies demonstrating the presence of AT2 receptor, ACE, and renin in bone microenvironment suggest that bone is also regulated by local RAAS and Ang II functions at least in part via AT2 receptor (Izu *et al*., 2008).

Several publications raise the possibility that AT1 and AT2 receptors carry out negative cross-talk within fibroblasts and vascular endothelial cells with respect to each other's signaling pathways and responses. Similar antagonism may also exist in bone (Izu *et al*., 2008).

3. Angiotensin II and stapes metabolism

The role of Ang II was recently suggested in the human bone and other connective tissue remodeling. Considering the progression of otosclerosis during pregnancy and the increase of angiotensinogen (AGT) production during this period, the implication of Ang II in otosclerosis seemed an interesting hypothesis (Imauchi *et al*., 2008).

The angiotensinogen plasma concentration is associated with an ATG gene polymorphism represented by the substitution of a methionine by threonine at codon 235 (M235T) and yielding M235 and T235 alleles (Inoue *et al*., 1997). Individuals with T235 allele have a higher plasma AGT concentration and may have higher tissular Ang II concentrations (Jeunemaitre *et al*., 1992; Ward *et al*., 1993; Inoue *et al*., 1997). Two other polymorphisms are related to Ang II activity. The I/D polymorphism of the ACE, characterized by the insertion (I) or deletion (D) of a 287-base pair (bp) sequence in the intron 16 of this gene, strongly influences serum ACE levels and may be associated with an increased cardiovascular risk (Niu *et al*., 2002). Whether this polymorphism may influence the local tissular generation of Ang II is difficult to determine (Niu *et al*., 2002). Another polymorphism located in the 3' part of the Ang II receptor Type 1 gene (A1166C) may also modulate the tissular response to Ang II and has been associated with primary hypertension (Baudin *et al*., 2005).

Imauchi *et al*. (2008) investigated the hypothesis of a possible implication of Ang II in otosclerosis through two types of experiments. They tested whether the functional polymorphism of the RAAS could be associated with otosclerosis in a large case-control study. Furthermore, they tested whether the Ang II receptors (AT1R and AT2R) and AGT were expressed in these cells and whether Ang II could influence the production of interleukin-6 (IL-6) in bone derived cells. Previously, Ang II has been shown to increase the

autocrine-paracrine production of IL-6 in vascular smooth cells and IL-6 was shown to be a mediator of bone resorption and a pro inflammatory cytokine.

Results of Imauchi *et al*. genetic study (2008) in a large series of patients and controls showed that the T235 variant allele of the AGT gene, that has shown to be related to high plasma AGT concentrations, was associated with otosclerosis. The ACE I/D polymorphism seemed to be also associated with the occurrence of otosclerosis. The proportion of DD genotype that is related to an increased enzymatic activity was higher in patients with otosclerosis than in control subjects. Patients both with TT and DD genotypes had an increased risk of otosclerosis with those with DD and TT genotypes only. The relation between AGT plasma concentrations and the M235T polymorphism of the AGT gene has been demonstrated by several studies. AGT plasma concentrations are 14-20% higher in TT than in MM genotype. Several observations are in favor of increased tissue expression of AGT and Ang II in individuals with TT and MT genotypes in comparison with MM. The T235 allele is associated with hypertension, preeclampsia and diabetic nephropathy. The present study revealed higher blood pressure in the otosclerosis group, and this may be partly explained by the fact that inclusions in the otosclerosis group were only based on the middle ear disease and not the blood pressure.

The T235 variant of the AGT gene allele prevails in black and Asian populations, in which the prevalence of symptomatic otosclerosis has been reported to be lower than that in Caucasians (Chole *et al*., 2001; Menger *et al*., 2003). Furthermore, a case-control study in a Belgian-Dutch population did not find this association (Schrauwen *et al*., 2009). This observation may be due to the fact that otosclerosis has probably several and variable predisposing factors, depending on the studied population, and that RAAS polymorphisms strongly interact with the environment and other genes to induce their effects.

Imauchi *et al*. (2008) also showed the presence of AGT, AT1, and AT2 in otosclerotic cultures and increased Ang II induced secretion of IL-6 that inhibited the alkaline phosphatase activity specifically in the otosclerotic tissue. This observation is consistent with the implication of this hormone in the stapedial bone metabolism and supports the involvement of Ang II in otosclerosis. The observed difference between otosclerotic and control cultures in terms of response to Ang II may be attributed to the participation of inflammation in the otosclerotic process and thus a higher predisposition of otosclerotic cells to produce pro-inflammatory agents in response to hormonal stimulations.

4. Angiotensin II and activation of pro-inflammatory cytokines in primary otosclerotic bone cell cultures

In the present study we focused on the Ang II effect on the secretion of a large panel of pro-inflammatory cytokines in primary otosclerotic cell cultures. We showed differences in the secretion of inflammation mediators between the otosclerotic and control stapes both in basal and Ang II-stimulated conditions.

In otosclerotic stapes, in basal conditions two pro-inflammatory cytokines (IL-1b, IP-10) were released in higher amounts in comparison to the control stapes. Interleukin 1-beta is an important mediator of the inflammatory response, and is involved in a variety of cellular activities; including cell proliferation, differentiation and apoptosis. Its higher production in the otosclerotic stapes is in accordance with a higher bone turnover in the disease foci. The expression of interferon inducible protein IP-10 has been noted in various cells including lymphocytes, monocytes, and fibroblasts. Although IP-10 is mainly known for its hemotactic activity, it has a significant role in inflammation.

Control stapes showed higher production of TIMP2, a tissue inhibitor of metalloproteinases, in basal conditions and in comparison to otosclerosis. This cytokine plays an important part in tissue homeostasis by directly suppressing the endothelial cell proliferation, inhibiting the proliferation of various tissues in response to angiogenic factors, and reducing the protease activity in tissues undergoing extracellular matrix remodeling such as active otosclerotic foci (Stetler-Stevenson *et al*., 2005).

Upon stimulation with the Ang II, otosclerotic cultures showed higher expression of several pro-inflammatory cytokines (IFN-gamma, IL-10). IFN-gamma is known as an antiviral, immunoregulatory and anti-tumor factor (Shroder *et al*., 2004). Today, IFN-gamma is used to treat chronic granulomatous diseases and osteopetrosis, an otosclerosis like lesion characterized by an increased density of various bones, including the temporal bone (Todd *et al*., 1992; Key *et al*., 1992). Interleukin-10 or human cytokine synthesis inhibitor factor (CSIF) is an anti-inflammatory cytokine. It is primarily produced by monocytes and to a lesser extent by lymphocytes. This cytokine has pleiotropic effects on immunoregulation and inflammation. It can block NF-kappa B activity, and is involved in the AK-STAT signaling pathway (Moore *et al*., 2001).

When stimulated by Ang II, control cells showed inhibition of several other cytokines (MIP-1a, and IL-11). Macrophage inflammatory protein (MIP) belongs to the family of chemotactic cytokines, known as chemokines. It is a highly potent pro-inflammatory cytokine capable of inducing the synthesis of other pro-inflammatory cytokines like IL-1, IL-6 and TNF-α from fibroblasts and macrophages (Sherry *et al*., 1988). Interleukin 11 is a multifunctional cytokine and a key regulator of multiple events in hematopoiesis (Yang *et al*., 1993). It has been demonstrated that this interleukin participates in the regulation of bone-cell proliferation and differentiation and could be used as a therapeutic agent for osteoporosis.

Higher prevalence of osteoporosis has been observed in otosclerosis patients, suggesting a common genetic pathogenesis (Clayton *et al*., 2004). This common pathogenesis could be RAAS. In mice, IL-11 stimulates and increases the cortical thickness and strength of long bones (Locksley *et al*., 2001).

Tumor necrosis factor alpha (TNF-α) is a cytokine involved in systemic inflammation and is a member of a group of cytokines involved in the acute phase reaction (Locksley *et al*., 2001). Dysregulation, and in particular the overproduction of TNF have been implicated in a variety of human diseases. Among other it is produced by monocytes-macrophage system that regulates the differentiation and activation courses of bone-derived mesenchymal cells. Its increased expression in otosclerotic bone could result in an extensive osteoclast activation cascade and pathologic bone turnover. So far it has been observed that TNF-alpha along with other cytokines is released from the otosclerotic foci in the early stage of the disease. Thus these molecules could flow into the perilymph and could interfere with the electromotility of the outer hairy cells, causing the sensorineural hearing loss (Karosi *et al*., 2009; Sziklai *et al*., 2009). The increased TNF-alpha production could trigger the focal bone resorption, thus administration of monoclonal anti TNF-alpha antibody could be considered as a potential medical treatment option in types of cochlear otosclerosis with sensorineural hearing loss (Sziklai *et al*., 2009).

In our study, Ang II-stimulated otosclerotic cultures showed inhibition of soluble TNF RII. This agent acts as a buffer for TNF. Its reduction can promote inflammation by increasing the amount of free TNF.

We also tested the mRNA expression of 126 genes involved in the inflammatory response. Although several genes were differently expressed only one, BCL-6, showed statistically significant difference in otosclerotic versus control cultures. BCL-6 gene encodes the BCL-

lymphoma 6 protein that acts as a sequence-specific repressor of transcription, and has been shown to modulate the transcription of STAT-dependent Interleukin 4 responses of B cell. BCL-6 has also been shown to regulate the expression of several chemokine genes, while its function in other aspects of macrophage biology has not been studied. Also the precise role of BCL-6 in cell proliferation is poorly understood. Recent results show that BCL-6 negatively regulates proliferation of the monocytic/macrophage lineage by suppressing an autocrine IL6/ STAT3-mediated gene expression program (Woo *et al*., 2002). Namely, BCL-6 negatively regulates the IL-6 transcription and inactivation caused by BCL-6 leads to spontaneous IL-6 secretion and STAT3 activation, which in turn results in cellular hyper-proliferation phenotype characterized by an accelerated G1/S phase transition. Besides the IL-6/STAT signaling it is very likely that BCL-6 controls other aspects of cell cycle as well (Yick-Loi *et al*., 2005). An important class of genes regulated by BCL-6 is chemokines. Chemokines were repressed by BCL-6 both in the micro-array study in B cell lines, but also in specific study of chemokine expression by macrophages (Schaffer *et al*., 2000; Dent *et al*., 2002). It was already observed that chemokines MIP-1alpha (inhibited secretion in otosclerotic stapes when stimulated with angiotensin II) and IP-10 (higher expression in otosclerotic stapes in basal condition) were repressed by BCL-6 in B cells. It has been demonstrated as a functional binding site in the MIP-1alpha promoter but not the IP-10 gene (Schaffer *et al*., 2000). In our study the gene array analysis showed a significant over expression of BCL-6 gene in the otosclerotic stapes when compare to the control ones, in basal condition. The results were confirmed by the qPCR that showed significant higher BCL-6 expression in otosclerotic foci. When stimulated with Ang II the otosclerotic foci gene array analysis showed a significant inhibition of BCL-6 in otosclerotic foci. The results were not confirmed by the real time qPCR analysis. Nevertheless higher expression of BCL-6 in otosclerotic foci supports its role

in the cellular proliferation. And its inhibition caused by Ang II in otosclerotic foci suggests BCL-6 regulation of inflammation and activation of different pro-inflammatory cytokines.

From the obtained data, we concluded that Ang II could have an important role in the key events of inflammation and in the bone turnover regulation and metabolism of the human primary otosclerotic cell cultures, through different pro-inflammatory pathways. These data support are previous hypothesis of Ang II role in the pathophysiology of otosclerosis. However further investigations are necessary to additionally clarify the role of the Ang II effect in the pathophysiology of otosclerosis and stapes metabolism.

5. Angiotensin II and oxidative stress in otosclerosis

In the present study we focused on the possible Ang II and HNE (oxidative stress) interaction in pathophysiology of otosclerosis. By analyzing the histologic samples of normal and otosclerotic stapes we found that both samples expressed different positivity for HNE antibodies. While control samples showed clearly marked positivity in periosteal region, otosclerotic samples showed multifocal areas of HNE positivity mainly in the new bone or abnormal bone formation regions. These observations suggest that HNE (secondary messenger of ROS) is indeed involved in the pathophysiological processes of the otosclerosis. Moreover we could conclude that if the cells originate in periosteal region HNE is involved in normal physiological process of cell growth but if the cells are forming new bone like in multifocal regions of otosclerotic bone, HNE is involved in pathophysiology of this disorder. So far it has been observed that several important physiological functions of Ang II are mediated through ROS (i.e. cellular growth, and inflammatory responses, Griendling *et al*., 2000). Activating the NADPH oxidase enzyme system Ang II stimulates ROS generation

mainly through its G protein-coupled Ang II type 1 (AT1) receptor (Lee *et al*., 2008). In order to test the possible direct binding between the Ang II and HNE we have performed the Immunoblot analysis. The results showed that there could exist a possible interaction between the HNE and Ang II (histidine, serine, lysine and cysteine amino-acids could be possible binding sites). In the performed experiment due to higher number of histidine residues present in BSA higher binding was observed for BSA and HNE then for HNE and Ang II. However due to the experiment protocol (addition of HNE prior to Ang II) we cannot confirm direct binding between them. But we could conclude that interaction is likely to exist in a tissue and intracellular level but mainly on the transmembrane proteins and receptors. HNE is mostly formed close to the cellular membrane and Ang II acts as a cytokine-like hormone and could react with the HNE.

Indeed one of the signaling pathways of Ang II includes activation of mitogen-activated protein kinases (MAPK), including extracellular signal-regulated kinases (ERK 1 /2), c-jun N-terminal kinase (JNKs, also known as SAPKs), p38MAPKs and big MAPK-1. Previously it was observed that cells in respond to oxidative stress or HNE can also activate different mitogen activated proteins kinases (namely JNKs and ERK family MAP kinases, Uschida *et al*. 1999).

In studying the effect of Ang II and HNE on the cellular proliferation (HOS) we observed stimulation of cellular growth (DNA synthesis) in dose dependent concentrations. This might be due to the fact that HNE modulates the effect of Ang II or that Ang II adds to the already noted bifunctional-growth regulating effect of HNE on osteoblast-like cells.

To investigate the influence of Ang II and HNE on the cellular differentiation we analyzed the alkaline phosphatase activity (ALP). In certain concentrations HNE (10μM) and Ang II (0.1

and 0.5nM) increased the activity of ALP while in concentrations of HNE (1μM or 2.5μM) and Ang II (0.1nM) no such effect was noted.

The results of apoptosis induction showed that both HNE and AngII expressed the concentration dependent results. While in certain concentrations the decrease of apoptosis cells was noted in others no such effect was observed. Results of the effect of the HNE on the apoptosis in concentrations of 10μM are in concordance with the previous observed HNE effect on osteoblast like cells (increased percentage of apoptotic cells).

The observed bimodal effect of HNE on cellular growth is in concordance with previous reports suggesting that in cells exists a very narrow window of physiological levels of HNE. Alterations of those levels could strongly change the HNE effect on cell cycle. When intracellular HNE levels exceed its physiological range, programmed cell death (apoptosis) is triggered. On the contrary cellular proliferation is stimulated when HNE levels drop bellow the normal physiological range (Awasthi *et al.* 2004). The mechanisms through HNE affects signaling processes in dose-dependent manner still need to be investigated. HNE is a strong electrophile and as such it reacts with nucleophilic groups of proteins, nucleic acid and lipids. It is possible that at low concentrations HNE selectively affects pathways in favor for cellular proliferation by interacting with nucleophilic groups having high affinity for HNE. At higher concentrations HNE could react with low affinity groups of cellular nucleophils thus triggering the cellular apoptosis (Awasthi *et al*. 2004).

Gluthatione S-transferases (GSTs) can modulate the intracellular concentrations of HNE by affecting its generation during lipid peroxidation by reducing hydroperoxides and also by converting it into gluthatione (GSH) conjugate (GS-HNE). Previously it was observed that otosclerotic cells produce bone matrix glyocosaminoglycans (GAG) with higher level of sulfatation (Bodo *et al.* 1997). Moreover otosclerotic cells express higher activity of the

diastrophic dysplasia sulphate transporter (DTDST). Study of Imauchi *et al.* (2006) showed that Ang II in otosclerotic cells stimulate the over expression of IL6 that has stimulatory effect on DTDST. This activity could be inhibited with the NaF (Bozorg Grayeli et al., 2003) and Dexamethason (Imauchi *et al*., 2006).

Regarding our results of the observed interaction between Ang II and HNE on the cellular proliferation, differentiation and apoptosis and with respect to the influence of both on the sulfatation of matrix proteins further studies are needed on possible interactions of HNE and Ang II and the role of GST in the regulation of these processes. Possible interaction of HNE and ANG II on cellular proliferation, differentiation and apoptosis is explained in Figure 6.

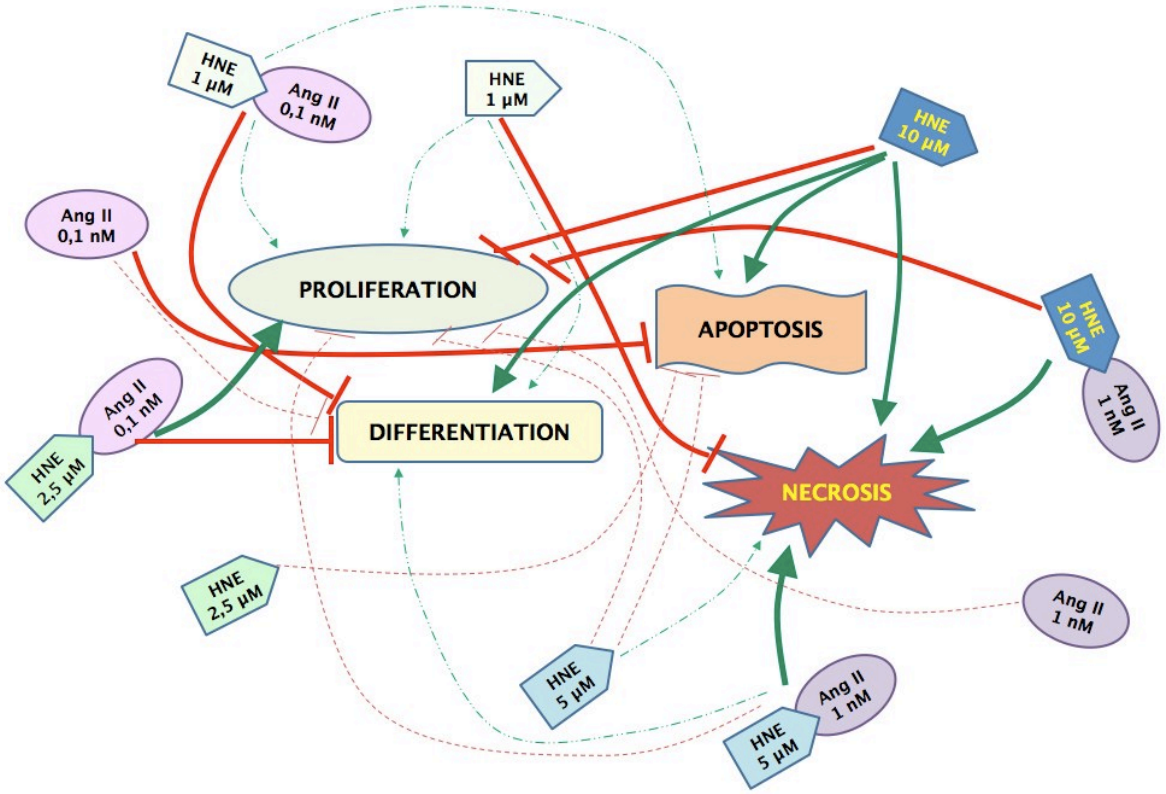


Figure 6- *Possible interaction of HNE and Ang II on cellular proliferation, differentiation and apoptosis and necrosis. Ang II interacts with already observed bifunctional growth regulating HNE effect in a dose-dependent manner. Higher Ang II concentration (1 nM) multiplies the negative effect of supra-physiological concentrations of HNE (5 and 10 µM) on cell proliferation, differentiation and stimulates cell apoptosis and necrosis. The best example is 10 µM HNE that, as itself, decreases cell proliferation and induces differentiation, apoptosis and necrosis whereas in combination with 1 nM Ang II completely directs towards necrosis. On the other hand, the physiological concentrations of HNE (1 µM and 2.5 µM) are more likely interacting with lower concentration of Ang II (0.1 nM) indicating completely opposite effect mostly directed to cell proliferation, and reduction of cell differentiation, apoptosis and necrosis.*

In conclusion we showed that there is an interaction between Ang II and oxidative stress on the cellular proliferation, differentiation and apoptosis of osteoblast-like cells. The diffuse immunostaining of HNE in the otosclerotic stapes indicates the possible role of oxidative stress in the otosclerosis. This phenomenon can explain the differences between normal and otosclerotic stapes in terms of response to Ang II. However, further studies on the interaction of Ang II and oxidative stress are needed in order to clarify their role in the pathophysiology of the disease.

V. PERSPECTIVES

Ang II and oxidative stress interaction has to be further studied in otosclerosis to clarify their role in the pathophysiology of the disease and eventually offer new therapeutic targets.

1. Angiotensin II and otosclerosis

Because the vasculature plays an important role in bone remodeling, the effect of renin-angiotensin system has been largely studied in this regard. Ang II has been postulated to be able to act on the cells involved in bone metabolism through receptors located in osteoblasts and osteoclasts. Ang II inhibited differentiation and bone formation via AT1 receptor in an animal model. Moreover AT2 receptor blockade increased bone mass in animal model and in addition AT2 receptor knock-out mice revealed also an increase in bone mass. The essential signaling pathway for normal osteoclastogenesis is that of RANK-RANKL-OPG system. Disruption of this system leads to numerous bone diseases. Osteoblasts express RANKL while osteoclasts express RANK that recognize RANKL through cell-to cell interaction and differentiate into mature osteoclasts. Osteoprotegerin (OPG) binds to the RANKL and prevents it from binding to RANK. Thus RANKL/OPG ratio is an important regulatory factor of bone mass and skeletal integrity.

Ang II increases RANKL expression in osteoblasts, while the angiotensin receptor blockade promotes osteoclastogenesis through down-regulation of RANKL expression.

The histopathological and pathophysiological findings in osteoprotegerin (OPG) knockout mice support the hypothesis that OPG is important in the inhibition of bone remodeling within the otic capsule and the maintenance of the normal auditory function. These mice demonstrate

abnormal remodeling of bone within the otic capsule and might be considered in the future as a valuable animal model of human otosclerosis.

In an animal model of osteoporosis (an otosclerosis like lesion) it has been shown that blocking of the synthesis of Ang II resulted in exacerbation of the low bone mass phenotype. Thus, blocking the synthesis of Ang II could be an effective treatment of osteoporosis. Similar studies could be done in otosclerosis in order to offer new therapeutical pathways of the disease.

As observed in the present study, Ang II is implicated in inflammation and bone-turnover regulation in otosclerosis via different growth factors and cytokines (i.e. IFN-gamma, IL-10, TIMP-2). Moreover, gene array analysis showed that Ang II modulated the secretion of different pro-inflammatory genes in otosclerotic stapes (i.e. down-regulation of BCL-6 and CCL25). The results support the hypothesis of Ang II implication in inflammation during the active phase of otosclerosis, but the exact signaling pathway still remains to be analyzed.

Future studies need to be more focused on investigation of the exact signaling pathway on how Ang II activates different pro-inflammatory genes particularly the BCL-6. Moreover the effect of specific Ang II receptor 1 and 2 antagonists on cytokine production in basal condition and upon Ang II stimulation in primary otosclerotic cultures should be evaluated.

Finally, whether the use of different anti-inflammatory (corticosteroids) and antioxidants (gluthation) agents could reduce the oxidative stress and thus the Ang II effect remains to be further analyzed.

2. Oxidative stress and Angiotensin II

As previously discussed, ROS are involved in multiple physiological responses to Ang II. Long-term response to Ang II require some level of oxidative stress, and many proteins and enzymes that serve as a signal transducers of Ang II are modified by ROS. Furthermore, many responses to Ang II share common signaling pathways, and future work should be focused on identifying which of these biochemical cascades are redox sensitive.

So far, studies on the redox-sensitivity of Ang II signaling have shown that Ang II activates many enzymes including phospholipase D, phospholipase A2, FAK, Fyn, janus kinase 2 (JAK2), STAT1 and ras, whose modulation by ROS is unknown,. For some of these proteins (JAK2, STAT) potential redox-sensitivity has been demonstrated using exogenous ROS. Others (such as Akt and MAP kinase) have still not been studied but their clear relationship with ROS suggests that they are potentially among the protein that mediate redox-sensitive physiological response to Ang II.

Recently, it has been shown that an isomer (4-ONO) of the so called second messenger of the ROS (4-hydroxynonenal, HNE) was formed in the endothelial cell subject to oxidative stress. This aldehyde can cause different modifications to the Ang II. Studies are now focused on the development of methodology of quantification of 4-ONO derived pyruvamide Ang II in biological fluids in order to better understand the influence that lipid peroxidation product could have on the role of Ang II in the pathophysiology of different diseases.

Although the role of ROS in mediating physiological responses to Ang II is currently under intense study, much remains to be learned concerning the signaling pathways and genes that are regulated by ROS. Animal studies have confirmed that this signal transduction system is important *in vivo*, but the dysregulation of ROS during disease like otosclerosis requires

further study. Furthermore, ROS have also been involved in cochlear damage in different inner ear pathologies. It is important to identify the possible source of cochlear ROS production and the pathways of ROS-mediated cochlear damage.

Further *in vitro* studies should be focused on investigating the presence of ROS in otosclerotic and control culture media. Furthermore the effect of antioxidants on proliferation, differentiation and apoptosis of otosclerotic cell cultures should be determined.

Clinical studies should be focused on evaluating the presence of ROS in stapedial samples (control and otosclerotic ones) as a possible prognostic factor for disease activity. Moreover measurement of the ROS presence in perilymph samples could be compared with the degree of hearing loss in otosclerosis.

Finally future studies should be focused on developing new approaches for prevention and treatment of oxidative stress caused inner ear pathologies (i.e. antioxidant therapy, interruption of the lipid peroxidation process, prevention of cochlear ischemia).

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VII. FIGURES AND TABLES INDEX

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