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Analysis of gene expression profiles in tympanic membrane following perforation using PCR Array in rats—Preliminary investigation

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ABSTRACT

Objectives: The goal of this work was to identify genes, known to be involved in the skin wound healing, that express differentially in the healthy and injured tympanic membrane (TM), and designate the molecules potentially beneficial for treatment of TM perforation. The molecular mechanisms controlling the course of TM regeneration are far from being elucidated.
Methods: Twenty rats had their tympanic membranes perforated, while four served as a control. Animals were sacrificed on either days 1, 2, 3, 5 and 10 post injury, and TMs were immediately dissected and frozen in liquid nitrogen. Total TM RNA was isolated and reversely transcribed. qPCR was performed using Rat Wound Healing RT^2 Profiler PCR Array (QIAGEN) containing primers for 84 genes.
Results: Statistically significant changes in the expression of 42 genes were found in various stages of TM healing. The increased expression of genes taking part in the inflammatory reaction (interleukin 6, granulocyte and macrophage chemotactic proteins) was observed from day 2. The expression of several genes of extracellular matrix components and their remodeling enzymes was also changed. Among growth factor genes: *Vegfa*, *Igf1* and *Hbegf* showed increased expression at the beginning of the healing process, while *Hgf* expression was highest on day 3.
Conclusions: Several changes in the expression of genes involved in remodeling of extracellular matrix point to important role of connective tissue in TM healing. The molecules accelerating this process, like HbEGF and HGF, seem to be good candidates for further evaluation of their possible use in clinical treatment.

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1. Introduction

Tympanic membrane (TM) perforations are commonly seen in clinical practice as a result of trauma or in the course of otitis media. Myringotomy is one of the most frequently performed surgical procedures in children. Although traumatic perforations and those resulting from myringotomy show remarkable ability to close spontaneously, in some cases closure of perforation fails to occur causing several problems as hearing loss, recurrent infections of the ear and necessity of water precautions.

The TM is a unique structure suspended in air between an external and middle ear. This feature makes healing processes different than in skin wounds. The main difference between the healing of TM perforations and skin wounds is a lack of provisional matrix formation. The regeneration of TM perforation starts with an inflammatory response followed by proliferation of the keratinizing squamous epithelium bridging the gap. The keratin spur guides migrating epithelial cells. Healing of the fibrous layer occurs secondarily. Finally, the inner epithelium regenerates [1–4]. There is support for the notion, that healing of TM involves several biological processes such as epithelial proliferation and migration, fibroblasts' proliferation, neoangiogenesis, extracellular matrix (ECM) formation and remodeling [5]. However, the mechanisms controlling this process are far from being elucidated.

Recently many experimental and clinical studies assess the possibility of using various biomolecules to improve the tympanic membrane healing [6–9]. An appropriate selection of these molecules requires in-depth knowledge of their role in TM healing which may differ from the skin healing. The aim of this study was identification which of the genes, known to be involved in skin wound healing, express differentially in healthy and injured TM.

The rat TM shares structural similarities with human TM and often serves as an animal model for studies of inflammatory and

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healing processes. However, several histological and biochemical techniques are difficult to perform because of the small size of rat TM. The techniques using DNA arrays allow to assess the expression of many genes in the small amount of tissue at the same time. In this study, the profiled array of genes involved in the skin wound healing was used to evaluate their expression in healthy and injured TM.

2. Materials and methods

The experimental procedures were carried out according to the European Council Directive of 24 November 1986 (6/609/EEC) and were approved by the Local Animal Ethics Committee in Białystok.

2.1. Animals, tympanic membrane perforation and sample collection

Twenty-four male Wistar rats weighing 250–300 g were used. Before surgery ears of all rats were examined otomicroscopically to verify a normal status of middle ear. Twenty animals were anaesthetized by intraperitoneal injection of 0.1 mL/100 g of equal parts of 20 mg/mL xylazine hydrochloride and 100 mg/mL ketamine hydrochloride and perforations about 1 mm in diameter in both ears were performed under an operating microscope. The perforations of uniform size were made in the upper anterior quadrant of pars tensa of tympanic membrane with a sterile, sharp point, microsurgical needle. Tympanic membranes in the control group of 4 rats (8 ears) were not perforated. The rats were randomly divided into five equal time point groups (4 rats in each group) and sacrificed on days 1, 2, 3, 5 and 10 after the perforation by aortic exsanguination under deep anesthesia with 100 mg/kg of pentobarbital. Before sacrifice the ears were examined otomicroscopically to assess the healing process. No signs of infection were found in any ear. After decapitation the temporal bones were removed immediately, tympanic bulla opened under operating microscope and TM was dissected out from the side of the bulla. Both eardrums of each individual were collected into one microtube, flash frozen in liquid nitrogen and stored at −80 °C for later processing.

2.2. RNA isolation

Total RNA from each rat (2 TM) was isolated using RNeasy® Fibrous Tissue Mini Kit (QIAGEN) according to the manufacturer instruction. Slight modifications in DNase I treatment were used. For better penetration of silicone bead volume of DNase incubation mix was increased from 80 to 160 μL and incubation time was prolonged to 30 min. Elution was performed with 30 μL of RNase-free water. Immediately, after elution RNA concentration was measured with NanoDrop. In order to obtain minimal amount of material for genes' expression assessment by Rat Wound Healing PCR Array (Qiagen Company) four tympanic membranes from two rats needed to be pooled (250 + 250 = 500 ng) and reversely transcribed using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo-Fermentas). Reaction mixtures of 50 μL containing oligo$(dT)_{18}$ and random hexamer primers were incubated for 10 min at 25 °C followed by 30 min at 50 °C. Reaction was terminated by heating at 85 °C for 5 min. cDNA stored in −20 °C overnight was used on the following day in real-time PCR.

2.3. Quantification assay

qPCR was performed using ready to use Rat Wound Healing RT^2 Profiler PCR Array (QIAGEN) containing primers for 84 tested and 5 housekeeping genes, and controls for RT and PCR reactions. The

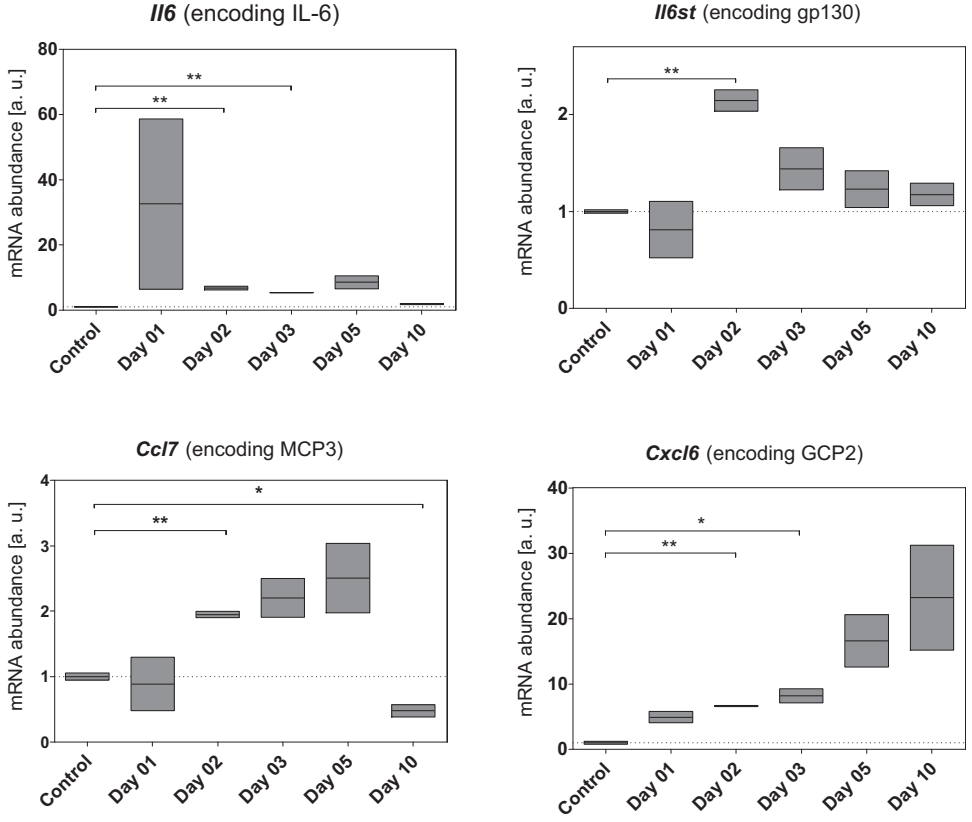

Fig. 1. Abundance of mRNA for IL-6, IL-6 transducer (gp130) and chemokine ligands (C–C ligand 7, C–X–C ligand 6) at different time points of tympanic membrane healing. mRNA level for all investigated groups is presented in relation to control group with mRNA abundance set up arbitrarily as 1. $^*p < 0.05$, $^{**}p < 0.01$; Student's *t*-test.

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