SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigations of new members of signaling pathways during tooth development: the effects of Toll-like receptor 4 and Heat-shock protein 60.

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The Examination takes place in the Lecture Hall 210 of the Faculty of Dentistry, University

of Debrecen; April 19, 2016, at 11 o'clock.

Head of the **Defense Committee**: Prof. Ildikó Márton MD, PhD, DSc

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen; April 19, 2016. at 13 o'clock.

Introduction

Summary of tooth development

Tooth development forms by continuous reciprocal interactions between the epithelial cells of stomodeum and the neural crest derived ectomesenchymal tissue. The reciprocal interactions are coordinated by cross-talk between the neighbouring tissues of the tooth germ, similarly to the development of other ectodermal appendices (such as hair, nail). However, more than a dozen of signal transduction pathways take part in organogenesis, the following ubiquitous pathways play cardinal role during the tooth development: TGF (transforming growth factor), Notch, WNT (wingless-related integration site protein), FGF (fibroblast growth factor), TNF (tumour necrosis factor)and SHH (sonic hedgehog) pathways. The literature divides the tooth development to three principal stages. These stages are: initial, morphological, and histodifferentation parts.

- The initial part of the mouse incisor development starts at the 11.5 embryonic day (E11.5). During the initial part, the underlying ectomesencymal cells secretes FGF and BMP molecules, which causes the proliferation of cells of the oral epithelium at the territories of the dental lamina. Later the territory of the epithelial proliferation will be thicker and the cells will start to migrate into the mesenchyme and start to form the epithelial band.

- The second phase of the tooth development is the morphogenesis (from E12.5 to E18.5). The literature divides the morphogenetic phase into bud, cap and bell stages. Briefly, under the morphogenesis the epithelial cells of epithelial band form the enamel organ, which grows continuously into the mesenchyme. Later the enamel organ separate into several parts: inner and outer enamel epithelium, stratum intermedium, stellate reticulum. Enamel organ also forms the labial and lingual roots of the tooth germs, which parts contain pluripotent stem cells. These stem cells are responsible for the continuous growing of tooth of the murine incisors.

Enamel organ is encloses ectomesenchymal tissue, which originating from the neural crest and form the mesenchyme of the dental papilla. The enamel organ is cover by mesenchymal cells, which form the dental follicle.

- During the histodifferentation part of development (from E15.5) the enamel organ and dental papilla form special highly specialized cells, which can forms the hard tissues of the tooth. The morphological and histodifferentation parts of the tooth development overlap with each other in time under embryogenesis. The dental cells turn into special, which can support only one function during the formation of the tooth germ. The ameloblasts originates from the inner enamel epithelium, they produce enamel matrix proteins. During the histodifferentation of ameloblasts, the literature separate different stages of the maturing of ameloblasts. These stages are the followings: morphogenetic stage, histodifferentation, presecretory, secretory, postsecretory and protective stage. During the secretory stage the ameloblasts produce enamel matrix proteins. The inner enamel epithelium also forms the enamel knot. This is the key structure of the cuspal morphogenesis and act as a signaling centre. The outermost layer of dental papilla turns into odontoblasts, which cells are responsible for the formation of dentin matrix. After the birth of animals only the histodifferentation stage is active during the lifetime of murine.

The tooth germs are intact organs, however formed by different tissues. Based on this property it gives a great possibility to examine signal cascades during the morphogenesis under *in vitro* culturing.

Functions of Toll-like receptors related to tooth

Toll-like receptors (TLRs) were identified almost 40 years ago related to the development of Drosophila. Against this developmental aspect, nowadays the Toll-like receptors have a more

prominent function in medical practice, namely detection of pathogens. The specific molecular components of bacteria, viruses are identifying by different TLRs. Each TLRs have a specific ligand, however generally they can recognize a wide spectrum of agonists. Subsequently, numerous Toll-like receptors (TLR2; 4; 7; 8; 9) expressed in odontoblasts. Which repectors can identify different antigens since the tooth odontoblasts are the first vital structures, which can identify pathogens under caries. The modification of the activity of odontoblasts by TLRs can results the alteration of dentin matrix proteins synthesis. Another important function of TLRs in our focus is the modification of the mineralization processes of hard tissues such as bone, dentin.

Our experiments were designed around the function of TLR4. The widely accepted specific ligand of the TLR4 is the lipopolysaccharide (LPS), a component of the bacterial cell wall. The activation of TLR4 complex (MD-2, CD14 and TLR4) causes the degradation of the IκB (inhibitor of κappa B) molecule, which causes activation of the downstream signaling pathway of NF-κB (nuclear factor-κB). The cells of dental pulp also contain several TLRs, in order to control the inflammatory processes under infection. Screening the literature we can not find any available information about the possible role of the TLR4 during the tooth development. Concerning that mature odontoblasts contain TLR4 and the take a part the mineralization of the dentin, we were focusing to the developmental aspect of TLR4 signaling.

Nevertheless, not only particles of pathogen agents (as exogenous ligand), but also different endogenous ligands such as heat shock proteins (Hsp), Gp96; proteoglycans can activate the TLRs. One widely accepted group of non-specific activator of the TLRs are the different heat shock proteins. Which were present in the developing tooth germs.

Current knowledge about the effects of heat shock proteins on tooth germs

The members of Hsp family are ubiquitous proteins in every cell, the Hsp-s act as housekeeping proteins, and the absence of Hsps cause lethal developmental failures. The Hsp expression seems to be cardinal at the case of the balance between proliferation and apoptosis under embryogenesis. We can make difference between these proteins according to the molecular weights and cellular localization. Numerous Hsp have been reported to be related to embryogenesis in recent years, however originally the literature describes, that its as stress inducible proteins.

During the tooth development several heat shock proteins were investigated. The expression pattern and possible functions of heat shock proteins 25, 27, 86, Hsc73 and Hsj2 were already described. The Hsp 25 has been studied the most in mice incisors which can increase the proliferation rate of dental cells and present basically in the enamel organ. The odontoblasts showed strong Hsp 25 expression, the cells of dental pulp, preodontoblasts and ameloblasts were transiently positive. The Hsp 27 was detected in the early stage of the tooth development which can modify the morphological development of the tooth. Screening online databases the mRNA expression pattern of Hsp 60 changes dramatically, which indicates the possible role it during tooth development.

The Hsp 60, also a member of heat shock proteins family, a highly conserved molecule is expressed in prokaryotic and eukaryotic cells. The majority of Hsp 60 is localized in the mitochondria, approximately 20% of the total amount presents in cytoplasm and under healthy condition only a few percentages can be secreted into the extracellular space. The mitochondrial Hsp 60 has ability to help in the refolding of proteins, which are translocates into the mitochondria. The cytosolic Hsp 60 able help the folding of the proteins and it can plays role in the modification of the NF-κB signal pathway through IKK. The IKK has 3 parts: IKKα, IKKβ

and IKKγ. According to the literature the free cytosolic Hsp 60 can attach to IKKα, which has an NF-κB independent role during the early stage of embryonic development. The IKKα influencing the invagination of the ectodermal derived structures (tooth germ, whiskers) into the underlying mesenchyme, and absence of it can cause abnormal tooth phenotype. Hypoxia, fever increases the amount of Hsp within minutes, the intracellular Hsp can act as pre- and pro-apoptotic protein. The high concentration of extracellular Hsp 60 acts as a danger signal during stress.

Aims of the studie

-Our first aim was to investigate the possible presence and role of TLR4 during the tooth development.

- Our second goal was to detect any possible endogenous ligand of TLR4 which can activate it during embryonic development.

Material and methods

Animal care

All experimental procedures followed the guidelines of the Animal Care and Use Committee of the University of Debrecen [DE FSZ/2010/10]. Pregnant females were euthanized according to these guidelines. Pregnant mice were sacrificed by cervical dislocation and embryos were killed by decapitation. Embryonic age was estimated using the appearance of the vaginal plug (E0.5), and from their exterior features.

Organoid culture from tooth germs

According to earlier articles, we performed Trowel-type tissue culture taken from E16.5-day old lower mice incisors. During isolation we prepared two of the incisors under Nikon SMZ 1000 stereomicroscope (Nikon Corporation, Tokyo, Japan). Only the unwounded and healthy tooth germs were selected for culturing. We placed the tissues on 0.1-μm pore-size nucleopore filters (Sigma, St. Louis, MO, USA) supported by metal grids in a humidified atmosphere of 5% CO_2 in air at 37 °C. One of the incisors was used as the treated explant and the pair of it was control. Both treated and control were excised from the same mouse, which ensuring that the alteration of the development of these tooth germs were minimal. The culture medium consisted of 15% foetal bovine serum (Gibco Brl, Gaithersburg, MD, USA) in DMEM (Gibco Brl, Gaithersburg, MD USA). 1 μg/mL LPS (InvivoGen, San Diego, USA) or 1 μg/mL Hsp 60 (Abcam, Cambridge, UK) was added to the medium. The culture medium was changed on the third day of culturing (after 48 hours), and the experiment was terminated on the fifth day. The data of *in vitro* culturing was based at least three different experiments.

Morphological analysis

In order to detect the exact alteration of Hsp 60 treated tooth germ, we performed morphological analysis. We investigated the angle closed by the labial, lingual loops and the enamel knot. We used in vitro data from the tooth germ, in order to avoid the shrinkage of the histological section related to the histochemical process.

Alkaline phosphatase activity assay

Mineralization process of E16.5 day-old tooth germs were determined by alkaline phosphatase (ALP) activity assay and alizarin-red staining. Samples from the treated and control groups were stored at −70 °C in ALP assay buffer. Samples were sonicated by pulsing burst for 30 sec at 40 A (Cole-Parmer, Illinois, USA). After centrifugation at 10.000g for 10 min at 4 °C, supernatants with equal protein concentrations were used for enzyme activity measurements. ALP activity assay (Abcam, Cambridge, UK) was performed following the manufacturer's protocol

Western blot

The applied exogenous Hsp 60 protein (Abcam, Cambridge, UK) was tested by a monoclonal anti-Hsp 60 antibody (Thermo Scientific, Rockford, IL, USA) to qualify the specify. WB experiments were carried out on isolated mouse tooth germs from E13.5 to E18.5 stages. The tooth germs remained intact, and the surface of the tooth germs did not contain connective tissue. Isolated tooth germs were placed in 50 µL homogenisation buffer containing 50 mM Tris–HCl buffer (pH 7.0), 10 µg/mL Gordox, 10 µg/mL leupeptin, 1 mM phenylmethylsulphonyl-fluoride, 5 mM benzamidine, and 10 µg/mL trypsin inhibitor. Starting the procedure tooth germs were sonicated by pulsing burst (Cole-Parmer, East Bunker Court Vernon Hills, IL, USA). For WB, total cell lysates were used. Samples for SDS–PAGE were

prepared by the addition of two-fold concentrated electrophoresis sample buffer to cell lysates to equalise the protein concentration in samples, followed by boiling for 10 minutes. 10-20 μg of protein was separated by 7.5% SDS-PAGE gel for detection of Hsp 60 and actin. Proteins were electrophoreticaly transferred to nitrocellulose membranes. After blocking with 5% non-fat dry milk in PBS for 1 hour at room temperature, membranes were washed and exposed to the primary antibodies overnight at 4 °C. Monoclonal anti-Hsp 60 antibody (Thermo Scientific, Rockford, IL, USA) in 1:200 and monoclonal anti-actin antibody (Sigma, St. Louis, MO, USA) in 1:10.000 were used. After washing for 3x10 minutes in PBST, membranes were incubated with anti-mouse IgG secondary antibody (Bio-Rad Laboratories, Hercules, CA, USA) in 1:1500 dilution for 1 hour at room temperature. Signals were detected by enhanced chemiluminescence (Millipore, Temecula, CA, USA) according to the manufacturer's instructions. Signals were manually developed on X-ray films.

Immunohistochemical detection of TLR4

Tooth germs were fixed in Sainte-Marie fixative for 30 min at 4 °C immediately after termination of *in vitro* culturing. Samples were washed for 10 minutes in PBS 3 times. Whole mount tooth germs were incubated by Vectastain Elite ABC Kit (Vector Laboratories Ltd., Peterborough, UK) to label the attached LPS-EB biotin. Control samples were stained in the same way, but Vectastain Elite ABC Kit was replaced with PBS. During the visualization of the immunoreaction we used DAB (Vector Laboratories Ltd., Peterborough, UK) for conventional light microscopy. No specific signal was recorded from control sections. DAB immunprecipitations were evaluated independently by 3 researchers on immunostained samples.

Immunohistochemical detection of Hsp 60

Samples from E10.5 to E18.5 were fixed in Sainte Marie's fixation the earlier stages of tooth

development (from E16.5) we demineralized samples in 10% EDTA. During dehydration graded series of alcohol and xylol was used. Sections of µm were made, followed by deparaffination, and were preincubated in 1% normal horse serum in PBS for 30 min at 24 °C to prevent non-specific binding of primary antibody. Previously to block the endogen peroxidase activity samples were treated with 3% H_2O_2 for 5 minutes. We immunostained our samples with anti-Hsp 60 antibody at 1:200 in PBS (Thermo Scientific, Rockford, IL, USA) overnight, at 4 °C, as secondary antibody anti-mouse Ig-G (Vector Laboratories Ltd., Peterborough, UK) at 1:400 in PBS was applied for 2 hours at room temperature, and visualised by Vectastain Elite ABC Kit (Vector Laboratories Ltd., Peterborough, UK) according to the manufacturer's protocol. The control sections originate from the same incisor in each case, these sections were stained in the same way, but the primary antibody was omitted and replaced with PBS. No signal was recorded from control. Immunoreaction were visualised with DAB. The histological sections of tooth germs from *in vitro* culturing were stained with picrosirius F3B as described in the literature.

In situ hybridization, RT-qPCR

During the *in situ* hybridization pregnant NMRI animals were anaesthetised with sodium-pentobarbital (applied dose 50 mg/kg), embryos were dissected in PBS on ice. Probes were designed and used according to the manufacturer's protocol (Roche, Mannheim, Germany) as described earlier. PCR primers sequences were chosen for regions containing exons from 7 to 12 and the 3'UTR regions using the mouse Hsp 60 mRNA (HSPD1, GenBank accession No: NM_010477.4) and ameloblastin mRNA (AMBN, GenBank accession No: NM_016519.5) as a template. The sense primer was flanked by the T3 sequence, and the antisense primer contained the T7 primer sequence at the 5' ends. Heat shock preotein 60 T3 flanked sense: 5'-ATTAACCCTCACTAAAGGTCCCTGCTCTTGAAATTGCT, T7 flanked antisense: 5'-AATACGACTCACTATAGGCTCCACAGAAAGGCTGCTTC Ameloblastin T3 flanked

sense: 5'-AATTAACCCTCACTAAAGGCAGAAGGCTCTCCACT GCAA, T7 flanked antisense: 5'-TAATACGACTCACTATAGGAGCAGTCAGGGTTTTCCACC Probes were manufactured by Integrated DNA Technologies Inc., (Coralville, IO, USA). Dissected 16.5 day-old tooth germs were fixed in RNA-ase free 4 % paraformaldehyde in PBS for overnight at 4°C. After fixation, samples were washed for 10 minutes in PBS 3 times, incubated for 15 minutes in 0.1 m TPBS, finally we washed in PBS. Proteinase K (4 μg/mL) was used for digestion in TE buffer for 20 minutes at 37 °C. Samples were prehybridized for 30 minutes at 60 °C, then hybridized with the respective probes for over night at 70 °C. The hybridization buffer contained 50 % formamide, 5× SSC, 10 % Dextran sulfate, Denhardt's solution and 2.5 mg/mL probes. After incubation, sections were washed in 2× SSC, SSC and 0.2× SSC, 20 min each. Then we used 20 μg RNAse A in 1 mL NTE for 30 min in 37 °C to break down the unhybridized probes. Finally samples were washed 3 times in TBS containing 0.1% Triton-X 100. During the detection blocking reagent contained 1 % Boehringer Blocking Reagent, 10 % goat serum in TBS. Alkaline-phosphatase-conjugated anti-digoxygenin (Roche, Mannheim, Germany) was applied for 8 h at 4 °C. Samples were washed in TBS 3 times for 10-10 minutes. Detection was performed with 20 μL NBT/BCIP (Roche, Mannheim, Germany) in 1 mL TRIS. After the colour development samples were washed in TRIS and stored in Scale A2 solution till documentation.

RT-qPCR was performed as described earlier with minor modifications. Briefly, total RNA was prepared using Trizol reagent (Invitrogen Waltham, USA) according to the manufacturer's instructions from 7-7 LPS treated and control samples. The samples formed 2 pools, which were used during the RT-qPCR. cDNA was synthesized from 200 ng RNA sample using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR reactions contained 10 ng cDNA from each samples. The quantitative PCR reactions were performed using the LightCycler 480 system (Roche, Basel, Switzerland) and a

qPCR supermix (PCRBiosystems, London, UK) with the following primers: ameloblastin (fwd: 5'- CATGCAGGACTTCTTGCTTTC; rev: 5'-GGTGCACTTTGTTTCCAGGTA) and cyclophilin as control (fwd: 5'-TGGAGAGCACCAAGACAGACA; rev: 5'-TGCCGGAGTCGACAATGAT). Ameloblastin expression was normalized for cyclophilin expression. In all cases qPCR reactions were performed on untranscribed RNA to verify the absence of genomic DNA contamination.

BrdU Assay

At the case of the *in vitro* culturing of Hsp 60 treated samples 10 µL BrdU labelling reagent (Life Technologies, Carlsbad, CA, USA) was administrated into the culture medium for 2 hours prior to fixation of the samples. The samples were fixed in Sainte-Marie's fixative at 4°C for 30 minutes then dehydrated in graded series of ethanol and embedded in paraffin. Sections were made in the sagittal plane at 5 µm and processed for further immunohistological analysis. The BrdU was immunodetected by BrdU Detection Kit according to the manufacturer's protocol (Zymed, Carlsbad, CA, USA).

ELISA

Tooth germs from the organoid culture were put and stored in RIPA buffer at -70 °C immediately after the culturing. During the processing of samples tooth germs were mechanically homogenized in TRIS-glycine buffer containing 1% SDS and protease inhibitors. Protein concentration of the tooth germs was measured with BCA assay. NUNC Maxisorp plates (Nunc Intermed, Copenhagen, Denmark) were coated with 10 µg protein/well in coating buffer (15mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% NaN_3, pH=9,6). Free binding surfaces of the polystyrene well were blocked with 1% BSA, followed by anti-collagen X primary antibody (Abcam, Cambridge, UK). During the detection of the primary antibody we used horse radish peroxidase-conjugated goat-anti-rabbit IgG as secondary antibody (DAKO, Glostrup,

Denmark). The color reaction was developed with o-phenylene-diamine substrate and absorbency was measured at 492 nm with a microplate reader. The positive control of the reaction was determined by the same procedure, on epiphyseal cartilage

Alizarin-red staining

The tooth explants were fixed in Saint-Marie's fixative for 2 hours at room temperature, and after washing in deionized water the tooth germs were stained with Alizarin-red (Sigma Aldrich, St. Louis MO, USA) for 10 min at room temperature. Excess dye was removed and washed with 20 % glycerol for 5 minutes at room temperature. Samples were stored in Scale A2 solution for 10 minutes before documentation.

Data analysis and image capturing

Images were captured by conventional light microscopy (Nikon Eclipse E800, Tokyo, Japan) The images acquired were representative of all the tissue sections examined. For documentation, images were processed by Adobe PhotoShop Software CS4 (Adobe Systems Inc., San Jose, CA, USA). Data of morphological analysis was measured with ImageJ 1.46 (National Institutes of Health, Maryland, USA). For statistical analysis, 7-7 control and treated samples from at least 3 independent experiments were compared with statistical analysis at the case of BrdU incorporation test. Data are expressed as mean ± SEM. Statistical analysis was performed by Mann-Whitney test. For statistical analysis of ELISA and IκB degradation (WB) at least 3 individual samples from 3 different culturing groups were used. Where applicable, data were expressed as mean ± SEM. Statistical analysis was performed by Student's T test, where statistical method reported significant differences among the groups ($p<0.05$).

Results

Functionally active TLR4 presented from the bell stage of tooth development

Protein expression of TLR4 was detected from the bell stages (from E14.5 to E18.5 stages) of the developing tooth. LPS addition in the applied dose to the culturing medium resulted significantly ($p<0.05$) reduce of the amount of IκB in case of the E16.5 day-old tooth germs. Our result was normalized to the amount of tubulin.

LPS accumulation was detected in enamel organ and in preodontoblasts

The whole mount samples showed strong immunoreaction against biotin conjugated LPS in the enamel organ of the treated tooth germs. After histological processing of samples, the following layers contained LPS: presecretory, secretory ameloblasts, stratum intermedium and preodontoblasts**.** Interestingly the postsecretory ameloblasts not contained LPS. Concerning, that the LPS a specific ligand of TLR4, this structures contain active TLR4 at the case of 16.5 day-old tooth germs.

LPS inhibited the mineralization of hard tissues of tooth germ, without any morphological changes

No morphological alternation was detected in the tooth germs after LPS administration during the 5 day of in vitro culturing (E16.5). Histochemical staining was used to detect the mineralization process. In control samples strong extracellular Ca^{2+} deposits were demonstrated with alizarin-red in the dentin and enamel. LPS treated tooth germs showed obviously weaker signal in the same structures. LPS treated tooth germ showed no obvious mineralization either lingual or labial side. ELISA was performed in order to investigate the amount of type X

collagen. The amount of it decreased significantly related to LPS treatment, this result coincides with the result of alizarin-red staining.

LPS treatment can elevate the amount of ameloblastin mRNA

For investigation of possible effect of LPS on ameloblasts, we performed *in situ* hybridization against ameloblastin RNA. The ameloblastin is a specific marker of the secretory ameloblasts and indicates the maturation of these cells. Higher ameloblastin mRNA expression was detected at the case of LPS treated samples. It is noteworthy that presence of ameloblastin was visible only in the enamel matrix with this technique in both control and LPS treated cultures. Data of RT-qPCR indicates the signifcantly enhanced synthesis of ameloblastin mRNA, which coindicate the result of in situ hybridization.

Hsp 60 is present in high levels in the structures of the enamel organ and in odontoblasts

We used Western blot and immunohistochemistry methods to detect Hsp 60 protein in tooth germs from E13.5 to E18.5. The results showed continuous and strong expression of Hsp 60 from the cap stage of enamel organ development. The first appearance of Hsp 60 was detected by only a weak DAB signal in the epithelial band (EB) during the initial stage of the tooth development (E11.5). During the bud and cap stage (E13.5-E15.5), the inner enamel epithelium and outer enamel epithelium and enamel knot of the enamel organ showed strong immunoreaction against Hsp 60. The dental papilla and dental follicle contain weak signal. This weak signal is considered to be the baseline expression of the Hsp 60 protein which is expressed by every mammalian cell.

During the bell stage, the Hsp 60 signal was strong in the inner enamel epithelium, outer enamel epithelium, presecretory ameloblasts, secretory ameloblasts, and stratum intermedium (E16.5-E18.5). Extracellular Hsp 60 signals were also found in the stratum

intermedium. The immunoreactivity was increased in the cytoplasm of the preodontoblasts (PreO) and odontoblasts (O) at E16.5.

The mRNA of Hsp 60 confirms the result of immunohistochemistry

Our goal was to reinforce the result of immunohistochemistry and detect the cellular origin of extracellular Hsp 60 which was present in stratum intermedium at E16.5. To reach this goal we detected the expression pattern of Hspd1gene (mRNA of Hsp 60) by *in situ* hybridization. The whole mount sample labelled strong Hspd1 expression in the labial part of enamel organ. The territory of the presecretory ameloblasts and preodontoblasts contain high concentration of Hspd1. The weak background in the dental papilla could be a basic expression of Hspd1. However strong signal was observed in the enamel organ, according to the histological sections the distribution pattern is uneven in the samples. Especially the outer enamel epithelium, inner enamel epithelium, presecretory ameloblasts, secretory ameloblasts, stratum intermedium, preodontoblasts and odontoblasts showed strong positive reaction. The majority of Hspd1 situated in the cytosol of the apical part of dental cells. The lingual side of the tooth germ showed weak Hsp 60 mRNA expression. Interestingly the enamel knot was not labelled by the *in situ* hybridization. We can consclused that, the result of *in situ* hybridization correlates with the result of immunohistochemistry and the extracellular Hsp 60 originated from the cells of stratum intermedium.

Exogenous Hsp 60 alters the morphology of the tooth germs

To investigate the possible effects of extracellular Hsp 60 on tooth germs, we used tooth germs in *ex vivo* organotypic culture in the presence of Hsp 60. The bell stage was chosen because this time point contains the morphological stage of the tooth development and this is the starting point of the histodifferentation part of tooth development. Another argument to choose this

stage is, that the mineralization of the enamel may inhibit physically the uptake of exogenous Hsp 60. During the first day of culturing we did not detect any morphological difference between the Hsp 60 treated and non-treated tooth germs. Altered morphology in treated cultures was observed at the third day of culturing. The apical part of the treated tooth germs became blunted in shape whereas the proximal part of the tooth germ did not show any visual difference. More profound morphological changes between the treated and control cultures were observed at the fifth day of culturing. The distal parts were sharp in the control explants and blunted in the treated explants. The proximal part of the tooth germ showed no visual differences. Significant morphological alteration was detected between the treated and control groups (Mann-Whitney test, $p>0.05$). Between the lingual and labial loops significantly higher degree was closed by in treated samples (21,01°; SD:3,77; SEM:1,68), than control samples (11,88°; SD:2,94; SEM:1,31). This result underlining the macroscopic observations (Figures 5, G-H). We performed picrosirius staining to identify the morphology of the explants. Sections were oriented parallel to the longitudinal axis of the tooth germs. Distal parts of the treated tooth germs were blunt in comparison to the control samples, confirming the observed macroscopic morphology.

Cell cycle analysis:

The apical parts of the samples did not contain any dividing cells in the treated and control groups. Several BrdU positive cells were observed in the labial roots and in the proximal part of enamel organ in both groups. Although, distribution of the BrdU positive cells seemed slightly different in the two experimental groups; we did not find any significant differences between the numbers of proliferating cells (Mann-Whitney test, $p>0.84$). According to our results, Hsp 60 does not influence the cell cycle in the labial root of the 16.5 day-old tooth germs.

Discussion

Toll-like receptors are widely investigated in immunological aspect, but only a few articles available about their roles during embryonic development. In our study we focused to the functions of these receptors during tooth development, which area does not contain any available information about the function of Toll-like receptor 4. We described that TLR4 expressed from the cap stage (from E 14.5) and the expression later is continuous during the murine tooth development, which can indicate the developmental aspect of TLR4 during odontogenesis. Lipopolysaccharide, a potent agonist might cause the degradation of IκB during the *in vitro* culturing at the case of E16.5 tooth germs. The significantly decreased amount of IκB can indicate the functional activity of TLR4 complex during the early stages of the tooth development, since the IκB has major function through masking the nuclear translocation sequence of NF-κB molecule which is the effector molecule of the downstream signaling of TLR4. One of the most prominent effects of TLR4 is the modification of mineralization of hard tissues according to earlier findings. Comparing the mineralization status of our samples by alizarin red staining, we can declare that LPS treatment decreases dramatically the amount of Ca^{++} in the enamel at E16.5 day-old tooth germs in the 5 days of culturing. The LPS can inhibit the mineralization of hard tissues, through the modification of non-tissue specific alkaline phosphatase. Enhances this, we measured low alkaline phosphatase activity in the tooth germ. Underlining the result by alizarin-red staining related to mineralization, significantly decreased amount of collagen type X was measured by ELISA. During tooth development type X collagen presents exclusively in the enamel matrix. The lower level of type X collagen also indicates the weaker mineralization of enamel matrix.

During the *in vitro* culturing of tooth germs we used biotin conjugated LPS. Concerning that LPS is specific agonists of TLR4, the structures which contain LPS have to express functionally active Toll-like receptor 4. According to immunohistochemistry the LPS presents in

presecretory, secretory ameloblasts, stratum intermedium and in preodontoblasts. The stratum intermedium and secretory ameloblasts contain the highest amount of biotin labeled LPS. These structures normally contain the major part of ALP, which control the enzymatic steps of the mineralization of the tooth germ.

Ameloblastin is a member of the enamel matrix protein and synthetized by secretory ameloblasts. Ameloblastin is an indicator of ameloblast maturing, however transiently it is expressed in the dentin matrix. *In situ* hybridization was performed to detect the expression pattern of AMBN at E16.5. Since AMBN was expressed only in the enamel organ, the whole amount of AMBN originated from ameloblasts. The treated tooth germs show accelerate ameloblastin mRNA synthesis by RT-qPCR, which indicates the role of TLR4 during differentiation of ameloblasts. Earlier findings describe the same effect of TLR4 on odontoblasts, namely that TLR4 activation increases the synthesis of matrix proteins of the dentin (decorin), and promote differentiation of odontoblasts. The accelerated ameloblast maturing can be the direct effect of the TLR4 signaling as well as on odontoblasts, or it can be compensatory mechanism of the inhibition of ALP activity. To find an answer to this question we have investigated the possible role of MAPK signaling pathway, being an open question during the tooth development, which is also activated by TLR4.

The second goal was to detect any possible endogenous ligand of TLR4, which can activate TLR4 under developmental processes. According to the literature several endogenous ligands can activate the toll-like receptors, for example: Hsp-s, Gp96; HMGP; proteoglycans from these candidates we choose the Hsp 60, because the Hsp 60 can activate or taken up by TLR4 receptor and it can modify the NF-κB signaling pathway through the modification of IKK complex. Earlier findings describe the expression patterns and possible roles of several heat

shock proteins (Hsp 25, 27, Hsp 86, Hsc73, Hsj2) during tooth development, which can take part of the morphological development and the differentiation of dental cells

The expression pattern of Hsp 60 shows unequal concentration in the tooth germs, the enamel organ shows very strong expression against the rest of the tooth germ where we can find basal expression of HSP 60. The *in situ* hybridization against mRNA of Hsp 60 is a more sensitive method to enhance the result of IHC. The whole mount sample shows also uneven concentration of Hsp 60 between the different parts of the tooth germ. The histological section from the whole mount sample labels the Hsp 60 mRNA in the enamel organ, which overlapping to the results of immunohistochemical reactions. Important to note, that we collect the samples under nearly physiological conditions, which indicate that the high amount of Hsp 60 protein is not the result of any harm to tooth germs.

However pathological conditions such as hypoxia and low pH can cause elevated level of Hsp 60. Related to this, we investigate the effect of high concentration of Hsp 60 in *ex vivo* cultures from 16.5 day–old tooth germs. Since Hsp 60 KO animal is not viable, we mimic the effect of elevated level of Hsp 60. The exogenous Hsp 60 cause blunt apical part of the treated tooth germs, while the proximal part shows no alteration, when compared the control and treated samples. This alteration can result the activation of NF-κB signaling pathway. Recent experimental data describe that the free cytosolic Hsp 60 can attach to the IKK complex. IKK complex activates the IκB which break down and the free NF-κB can activate gene expression. IKK consists of three subunits: IKKα, IKKβ and IKKγ. Absence of IKKα cause similar blunted apical part of lower incisors of IKKα KO tooth phenotypes in mice. Hsp 60 can attach directly to IKKα which can modify the effects of this protein. The IκB KO mouse has normal phenotype related to lower incisors; it also indicates the principal role of the IKK complex. Another possible answer to the abnormal morphology can be the modification of the ectodysplasin/TNF signaling pathway, which has main role in morphological development of

ectodermal appendices. The effector of ectodysplasin/TNF pathway is the NF-κB. The ectodysplasin/TNF mutant mice have similar tooth phenotype what we found in the case of the Hsp 60 treated samples. Interestingly the downstream signaling under the IKK complex did not altered the morphological development of tooth. During the BrdU incorporation test, the mitogenic effect of Hsp 60 could not be confirmed in dental cells. However the number of proliferating cells is slightly different in the territory of the labial root and the distal part of the enamel organ, this changing is not significant.

Our data suggest that TLR4 may take a part during histodifferentation part of the tooth development. The Toll-like receptor 4 is expressed from the cap stage of the tooth development continuously. According to our findings the TLR4 is present in the structures of the enamel organ and in preodontoblasts. The activation of TLR4 at E16.5 inhibits the mineralization process of hard tissues of the tooth, which correlates with earlier data. Another important effect of LPS treatment is, that it increases the maturing of ameloblast according to the *in situ* hybridization and RT-qPCR. During our experiments we used an exogenous ligand of TLR4, however a cardinal question that which endogenous ligand(s) can activate this receptor. One of the candidates was the heat shock protein 60. The immunohistochemistry method detected the Hsp 60 protein (as a potent agonist of TLR4) in the enamel organ and in odontoblasts in higher concentration than in neighboring structures. The *in situ* hybridization against HSPD1 enhances the result of immunohistochemistry, namely the structures of enamel organ contains elevated amount of Hsp 60 mRNA.

If we compare the result of our experiments we find physical similarities between the localization of these peptides, enhancing the connection between the TLR4 and Hsp 60. Basically both TLR4 and Hsp 60 can modify the NF-κB pathway; however other signaling pathways also can activate the NF-κB pathway.

On the other hand, one part of our experiments does not support the role of Hsp 60 related to TLR signaling. Concerning that LPS (specific exogenous ligand) and Hsp 60 (non specific endogenous ligand) caused different morphology of the tooth germs, they may not use the same downstream signaling pathway or effector molecule. The activation of TLR4 break down the IκB level as we detect in our experiments and others authors described earlier. Experimental data indicate that IκB KO mice do not show any tooth abnormality in phenotype, however we found that Hsp 60 cause blunted distal part. Based on this, we can discuss that the Hsp 60 may not activate Toll-like receptor 4, but it can take up Hsp 60 and the cytosolic protein can attach to the IKK complex. According to these remarks the Hsp 60 does not acts as a typical ligand of the TLR4. Also important to note, that Hsp 60 can activate another TLRs, which can cause abnormal development of the tooth. Screening the literature we can not find any experimental data about the possible role of TLRs.

Summary

Our goal was to identify the presence and possible role of Toll-like receptor 4 signaling cascade under tooth development and find any endogenous ligand (Hsp 60) of it, which may take a part under the developmental processes. According to our finding we declare the followings:

- The TLR4 is expressed from the cap stage of the tooth development and is present in high concentration in the structures of enamel organ.
- The activation of it with LPS did not cause any morphological alternation, but LPS treatment decreased the mineralization of the tooth germ and accelerated the differentiation of the ameloblasts.
- A possible ligand (Hsp 60) is expressed from the first day of tooth development. However the enamel organ contained it in high concentrat in later stages.
- The Hsp 60 caused abnormal blunt distal part of the tooth germ, but did not affect proliferation rate of the dental cells.
- As a conclusion we described the expression pattern of the Hsp 60 and TLR4, but according to the different effects of these molecules the exact signaling pathway is not clear in details.

Publications

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Registry number: DEENK/2/2016.PL
Subject: Ph.D. List of Publications

Candidate: Tamás Papp
Neptun ID: FY4L4R
Doctoral School: Doctoral School of Dental Sciences
MTMT ID: 10037441

List of publications related to the dissertation

1. **Papp, T.**, Polyák, A., Papp, K., Mészár, Z., Zákány, R., Mészár-Katona, É., Terdik, T., Chang, H.H., Felszeghy, S.: Modification of tooth development by heat shock protein 60.
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DOI: http://dx.doi.org/10.3109/00016357.2015.1130853
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List of other publications

3. Albert, R., Vásárhelyi, G., Bodó, G., Kenyeres, A., Wolf, E., **Papp, T.**, Terdik, T., Módis, L., Felszeghy, S.: Computer-assisted microscopic analysis of bone tissue developed inside a polyactive polymer implanted into an equine articular surface.
Histol. Histopath. 27 (9), 1203-1209, 2012.
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