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The role of aryl hydrocarbon receptor (AhR) in the pathology of pleomorphic adenoma in parotid gland

Agnieszka Drozdzik[a,*], Robert Kowalczyk[b], Mariusz Lipski[c], Joanna Łapczuk[d], Elzbieta Urasinska[e], Mateusz Kurzawski[d]

[a] Department of General Dentistry, Pomeranian Medical University, Powstancow Wlkp 72, 70-111 Szczecin, Poland
[b] Department of Maxillofacial Surgery, Pomeranian Medical University, Powstancow Wlk 72, 70-111 Szczecin, Poland
[c] Department of Preclinical Conservative and Endodontic Dentistry, Powstancow Wlkp 72, 70-111 Szczecin, Poland
[d] Department of Pharmacology, Pomeranian Medical University, Powstancow Wlkp 72, 70-111 Szczecin, Poland
[e] Department of Pathomorphology, Pomeranian Medical University, Unii Lubelskiej 1, 71-252 Szczecin, Poland

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ABSTRACT

Objectives: Pleomorphic adenoma (benign mixed tumor) is one of the most common salivary gland tumors. However, molecular mechanisms implicated in its development are not entirely defined. Therefore, the study aimed at definition of aryl hydrocarbon receptor (AhR) involvement in pleomorphic adenoma pathology, as the AhR controlled gene system was documented to play a role in development of various human tumors.
Design: The study was carried out in pleomorphic adenoma and control parotid gland tissues where gene expression of *AHR*, AhR nuclear translocator (*ARNT*), AhR repressor (*AHRR*), as well as AhR controlled genes: *CYP1A1* and *CYP1B1*, at mRNA and protein (immunohistochemistry) levels were studied. Functional evaluation of AhR system was evaluated in HSY cells (human parotid gland adenocarcinoma cells) using 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as AhR specific inducer.
Results: Pleomorphic adenoma specimens showed cytoplasmic and nuclear AhR expression in epithelial cells as well as in mesenchymal cells. In parotid gland AhR was expressed in cytoplasm of duct cells. Quantitative expression at mRNA level showed significantly higher expression of *AHR*, *ARNT* and *CYP1B1*, and comparable levels of *CYP1A1* in pleomorphic adenoma tissue in comparison to healthy parotid gland. The HSY cell study revealed significantly higher expression level of *AHRR* in HSY as compared with MCF-7 cells (human breast adenocarcinoma cell line used as reference). Upon TCDD stimulation a drop in *AHRR* level in HSY cells and an increase in MCF-7 cells were observed. The HSY and MCF-7 cell proliferation rate (measured by WST-1 test) was not affected by TCDD.
Conclusions: Summarizing both *in vitro* and in *vivo* observations it can be stated that AhR system may play a role in the pathology of pleomorphic adenoma.

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1. Introduction

Pleomorphic adenoma (benign mixed tumor) is one of the most frequent salivary gland tumors. Histologically, within pleomorphic adenoma tumor mass both epithelial and mesenchymal elements with a marked morphological diversity are found. It is generally accepted that the adenomas arise from intercalated duct cells with myoepithelial cell differentiation into epithelial and connective-tissue structures (Batsakis, Sneige, & El-Naggar, 1992).

Despite numerous studies, pathophysiology of pleomorphic adenomas is still not well defined. Therefore, there is a need for studies on mechanisms associated with tumorigenesis in order to better characterize factors contributing to the development and progression of these tumors.

AhR is a transcription factor, which coordinates expression of genes involved mainly in xenobiotics' metabolism (including carcinogens), in response to endo- and exogenous compounds. Ligand binding to AhR triggers its translocation into the nucleus, combined with subsequent heterodimerisation to AhR nuclear translocator (ARNT). AhR/ARNT heterodimer recognizes response elements in regulatory sequences (promoter or enhancer regions) of target genes, and modulates transcription. AhR signaling is regulated by AhR repressor (AHRR) by its competition with AhR for

* Corresponding author at: Department of General Dentistry, Pomeranian Medical University, Powstancow Wlkp 72, 70-111 Szczecin, Poland. Fax: +48 91 4661600.
E-mail address: agdro@pum.edu.pl (A. Drozdzik).

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ARNT dimerization and binding to AhR-responsive elements (AHREs) (Beischlag, Luis Morales, Hollingshead, & Perdew, 2008; Harper, Riddick, & Okey, 2006; Hahn, Allan, & Sherr, 2009; Murray, Patterson, & Perdew, 2014).

The available data, both experimental and clinical, supports an important role of AhR in carcinogenesis. Ahr-D (defective) mouse Hepa1c1c7 cells are not well differentiated and functionally defective. Loss of AhR results also in decreased rate of cell proliferation and an increased number of cells in G0/G1 phase of the cell cycle (Ma & Whitlock, 1996). Characteristics of 967 cancer cell lines for AHR mRNA expression revealed that esophageal, upper respiratory and digestive, pancreatic, and liver cancer cell lines were characterized by relatively high *AHR* levels, whereas many subtypes of leukemia cells expressed low mRNA levels (Safe, Lee, & Jin, 2013). Therefore, contribution of AhR to carcinogenesis may involve various mechanisms, which are cell type specific. Likewise, clinical studies provide observations that AhR protein expression in pancreatic, prostate, urinary tract, lung, esophageal tumors and papillary thyroid carcinoma (especially with BRAF mutations) is relatively high but not in pituitary tumors. The cellular location of the receptor, which defines its functional state, i.e., cytosolic and/or nuclear, was variable (Safe et al., 2013; Mian et al., 2014).

Our previous study revealed expression of AhR in human parotid gland in cytoplasm of striated duct cells (Drozdzik, Kowalczyk, Urasińska, & Kurzawski, 2013). In a further study we observed regulation of AhR expression and function by its specific inducer, i.e. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in rat parotid gland (Drozdzik, Wajda, Łapczuk, & Laszczynska, 2014). The AhR expression in human parotid gland as well as its response to ligands suggest its role in the physiology and pathology of the gland. However, there is no information on AhR role (expression, regulation) in pleomorphic adenoma in parotid gland. The aforementioned findings suggesting AhR involvement in tumorigenesis focused our present study on evaluation of AhR involvement in pleomorphic adenoma pathology.

2. Materials and methods

2.1. Tissue specimens

Tissue specimens were from 14 patients, aged 49–66 years (8 females, 6 males) diagnosed with pleomorphic adenoma. From each patient a neoplastic tissue as well as tissue from healthy part of the parotid gland were dissected. A part of each specimen sampled was immediately preserved in RNAlater (Applied Biosystems, USA) for RNA expression analysis and the adjacent healthy and neoplastic tissues were embedded in formalin for immunohistochemistry. The study protocol was approved by local ethics committee, and all patients gave informed consent.

2.2. Cell culture

A human parotid gland adenocarcinoma cells (HSY) (provided by Dr. M. Sato, Tokushima University, Japan) and human breast adenocarcinoma cell line (MCF-7, reference cell line) cells were seeded in 24-well tissue culture plates, 5×10^4 per well into DMEM medium (Sigma, Germany), supplemented with 10% FBS (Invitrogen, USA) and 0.4% streptomycin/penicillin (Sigma, Germany), and incubated at 37 °C in a humidified incubator supplied with 5% CO_2. After 24 h, the medium was replaced with DMEM medium without FBS, containing 0.5% BSA and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)—final concentration 10 nmol/l, as well as in control cells a respective medium with DMSO (without TCDD). After the subsequent 24 or 48 h of incubation, the medium was decanted and RNA immediately extracted from cells for subsequent qRT-PCR analysis, using RNAqueous Micro Kit (Ambion, USA). The experiments were performed in triplicate.

2.3. Cell proliferation/viability assay

Cell proliferation was evaluated using water-soluble tetrazolium salt WST-1 assay (Roche Applied Science, Mannheim, Germany). The cell proliferation WST-1 test is based on the reduction of the tetrazolium salt WST-1 to a soluble red-colored formazan by mitochondrial dehydrogenase of metabolically active cells. The amount of formazan dye formed directly correlates with the number of metabolically active cells. For the present study HSY and MCF-7 cells were seeded into a 96-well plate (at a density of 8×10^3/well and 4×10^3/well respectively) in DMEM medium (Sigma, Germany) supplemented with 10% FBS (Sigma, Germany), 0.4% penicillin-streptomycin (Sigma, Germany) and L-glutamine (2 mM, Sigma, Germany). Following 24 h of incubation period, cells were treated with TCDD (10 nmol/l) or vehiculum (0.1% DMSO) in DMEM medium for 24 and 48 h. After the respective incubation time, WST-1 reagent was added to each well for 0.5 h. Subsequently, the plate was shaken gently for 1 min, and the absorbance was measured at 450 nm and 620 nm wavelength (reference wavelength, background correction) using a multifunctional microplate reader (Infinite 200 PRO, Tecan, Switzerland). Cell proliferation/viability was calculated using the following equation: % of cell number = $[(A_{test} - A_{blank})/(A_{control} - A_{blank})] \times 100\%$.

2.4. Quantitative real-time PCR analysis

Total RNA was extracted from 20 mg tissue specimen by means of Direct-zol RNA MiniPrep Kit (Zymo Research Corporation, USA). Subsequently cDNA was prepared from 500 ng of total RNA in 20 μl of reaction volume, using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Lithuania) with oligo-dT primers, according to the manufacturer's instructions. Quantitative expression of the following genes was measured using two-step reverse transcription PCR, using pre-validated Taqman Gene Expression Assays (Life Technologies, USA): *AHR* (assay ID: Hs00169233_m1), *ARNT* (Hs00231048_m1), *AHRR* (Hs00324967_m1) *CYP1A1* (Hs00153120_m1) and *CYP1B1* (Hs00164383_m1), together with house-keeping endogenous control genes: *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase, Hs99999905_m1), *PPIA* (cyclophilin A, Hs99999904_m1) and *GUSB* (beta-glucuronidase, Hs99999908_m1). qRT-PCR was performed in ViiA7 Real Time PCR System (Life Technologies, USA), with TaqMan Fast Advanced Master Mix (Life Technologies, USA) and 1.5 μl of cDNA for each reaction mix of 15 μl. Each sample was analyzed simultaneously in two technical replicates, and mean C_T values were used for further analysis. Calculations were performed using the ΔΔCt relative quantification method, using integrated instrument software (Life Technologies, USA). The thresholds were set manually to compare data between runs, and C_T values were extracted. All C_T values for each sample were normalized to the geometric mean value obtained for three control genes, processed in the same run. In the present study, each tumor sample was compared to the adjacent normal tissue. The results of relative gene expression for each patient (tumor and normal tissue) were treated as repeated measurements. Fold change between groups was calculated from the means of the logarithmic expression values.

2.5. Immunohistochemical staining

Formalin-fixed, paraffin-embedded 5 μm sections from the specimens from healthy parotid gland tissue as well as from pleomorphic adenoma were deparaffinized, rehydrated and immersed in pH 9.0 buffer. Heat-induced antigen retrieval was

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