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Identification of genes involved in gentamicin-induced nephrotoxicity in rats – A toxicogenomic investigation

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ABSTRACT

For the application of microarray technology as an additional endpoint in toxicological studies, there is a need to understand associations between pathological processes and gene expression alterations. In the current study, we investigated gentamicin as a nephrotoxic model compound. Gene expression changes of the kidney in response to a dose of 80 mg/kg gentamicin were analyzed by using DNA microarray technology and alterations in gene expression were associated with results from conventional histopathological investigations and with the described pathomechanisms of gentamicin. Under the conditions of our experiment, the mRNA level of 211 genes were found to be deregulated by gentamicin. The gentamicin-induced affection of proximal convoluted tubules was associated with a strong up-regulation of mRNAs encoding for proteins which are used as nephrotoxicity markers in urine and plasma such as Kim-1, Osteopontin and TIMP1. Candidate marker genes for nephrotoxicity such as GATM were deregulated. Gentamicin-induced lysosomal phospholipidosis was indicated by deregulation of lysosomal located gene products such as ATP6V1D, a subunit of the lysosomal H+ transporting ATPase. Effects on glucose transport and metabolism were indicated by the down-regulation on SGLT-2 and glucose-6-phoshatase. Renal cell apoptosis was indicated by up-regulated genes as TP53 and BAX. The role of oxidative stress in gentamicin toxicity was reflected by deregulation of transferrin receptor and heme oxygenase. The results of the study show the potential of microarray technology to study a complex mechanism of toxicity in a single study.

Introduction

Microarray technology makes it possible to study genome-wide expression profiles in response to the administration of pharmaceutical compounds and xenobiotics. The description of altered gene expression offers the possibility to understand the mode of action and to gain new insight into side effects at the molecular level. There is also the potential to study complex mechanisms of toxicity within a single animal study. Bridging between previous knowledge generated by classical biochemical or pathological work and gene expression analysis is needed to understand associations between gene expression alterations and described pathological processes. In the current study we have chosen gentamicin as nephrotoxic model compound. The pathomechanism of this compound was studied in detail in many studies but only a limited number of transcriptional gene expression changes after gentamicin administration has been published (Amin et al., 2004; Kramer et al., 2004; Nagy et al., 2004).

Gentamicin was chosen as a nephrotoxic model compound as this compound was the object of many mechanistic investigations. Its tissue-specific toxicity is considered to be caused by a selective accumulation of this drug in the tubular epithelial cells in renal cortex, where its concentration is several times higher than in the plasma (Nagai and Takano, 2004; Goodman et al., 2001; Mingeot-Leclercq and Tulkens, 1999). On the cellular level an intense binding of the drug to the brush border of the proximal tubule could be demonstrated (Moestrup et al., 1995). After binding, gentamicin is taken into the cell by endocytosis where it inhibits the activities of lysosomal phospholipases and sphingomyelinase (Laurent et al., 1982). Therefore, gentamicin also inhibits the degradation of phospholipid-rich cell membranes which leads to an accumulation of phospholipids. This phenomenon is reflected ultrastructurally by the appearance of myeloid or lamellar bodies implicating the accumulation of cell organelles like lysosomes and endosomes. The overload of phospholipid membranes may result in compromised lysosomal membrane integrity which might lead to occurrence of lysosomal enzyme leakage. This may explain the tubular necrosis seen after administration of gentamicin (Bennett et al., 1988). Within 1

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week a replicative rate increase in the proximal tubule is observed. In addition, apoptosis plays an important role in gentamicin toxicity which could also contribute to clear off damaged cells and to control the compensatory proliferative response (Servais et al., 2006). Finally, there is evidence for oxidative stress in gentamicin-induced nephrotoxicity.

In the current study a dose of 80 mg/kg gentamicin was given daily to male rats for 1 week. Selected parameters of clinical pathology were measured and the kidney were evaluated by histopathology. Affymetrix GeneChip® microarrays were used for gene expression analysis of the kidneys.

Materials and methods

Chemicals

The study performed at Boehringer Ingelheim used gentamicin obtained from Sigma (Steinheim, Germany).

Animal treatment and sample collection

The gentamicin study was performed by Boehringer Ingelheim Pharma GmbH & Co. KG. Male Han Wistar rats (CrlGlxBrlHan:WI) were purchased from Charles River Laboratories (Sulzfeld, Germany). At administration start the rats were about 12-week old and weighed approximately 200 g. The animals were dosed intramuscularly in the right hind limb once daily with 80 mg/kg gentamicin (solved in 0.9% saline) for 7 days. Corresponding control rats were dosed with equivalent volumes of vehicle (0.9% saline). The animals were assigned to groups (3 rats/group) by weight using weight stratification-based computer software. The animals received a food ration in pellet form (Kliba no. 3438.0.25, Provimi Kliba SA (Kaiseraugst, Switzerland)) ad libitum but it was withdrawn in the afternoon before necropsy. Municipal tap water (Stadtwerke Biberach; Germany) was available ad libitum via drinking bottles. The animals were kept under controlled temperature (20 ± 2 °C), humidity (45–75%), and lighting (12 h light/dark cycle) and were acclimatized for a minimum of 11 days. The animals were kept in groups of 3 per cage (Noryl, type V). The study has been performed in accordance with OECD Principles of Good Laboratory Practice (GLP; as revised in 1997, ENV/MC/CHEM(98)17) and the "Chemikaliengesetz" (Chemicals Act) of the Federal Republic of Germany. Standard operating procedures (SOPs) covering techniques, conditions and items involved in this study were available. The animal facilities of Boehringer Ingelheim are accredited by Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International).

At necropsy the kidneys were removed and hemi-dissected. One half of the kidney was preserved in 10% buffered formaldehyde for histological examinations, the other half were frozen in liquid nitrogen for RNA isolation.

Clinical chemistry

For clinical chemistry 550 µl blood per animal were collected at necropsy. Plasma creatinine and blood urea nitrogen (BUN) were determined with a Hitachi 917 (405657) and Roche Diagnostics Kits (Roche Diagnostics GmbH, Mannheim).

Histopathology

Representative sections of the kidneys were processed and embedded in paraffin, sectioned at a thickness of approximately 4 µm and stained with hematoxylin–eosin (H&E), Periodic Acid Schiff reagent and immunohistochemically for the Proliferating Cell Nuclear Antigen (PCNA) by application of the streptavidin–biotin method and diaminobenzidine as substrate.

RNA isolation

Total RNA was isolated individually from harvested snap frozen hemi-dissected left and right kidneys using QIAGEN RNeasy kits (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The RNA concentration was determined spectrophotometrically (BioPhotometer, Eppendorf, Wesseling-Berzdorf). The integrity of the RNA was checked by gel analysis using RNA 6000 Nano chips on an Agilent 2100 Bioanalyzer from Agilent Technologies (Waldbronn, Germany).

Hybridization of cRNA to oligonucleotide arrays

Biotin-labeled cRNA samples for hybridization on Affymetrix GeneChip® RG_U34A arrays were prepared according to the GeneChip Expression Analysis Technical Manual from Affymetrix Inc. (Santa Clara, CA, USA). Briefly, 5 µg total RNA was used for cDNA synthesis with the SuperScript Choice System from Invitrogen Life Technologies (Invitrogen GmbH, Karlsruhe, Germany) employing a modified T7 primer with 24 thymidines at the 5′ end. After spin column purification, biotin-labeled cRNA was synthesized from the cDNA using the ENZO RNA Transcript Labeling Kit (Affymetrix Inc., Santa Clara, CA, USA). Spin column-purified cRNA was quality controlled using an Agilent 2100 Bioanalyzer and spectrophotometrically quantified. The cRNA (15 µg) was then fragmented in buffer supplied with the GeneChip Sample Cleanup Module and hybridized for 16 h at 45 °C. The microarrays were washed and stained with streptavidin–phycoerythrin (Dianova, Hamburg, Germany) on the Affymetrix Fluidics Station 400. Fluorescent images were read using the Affymetrix GeneChip Scanner. The raw data image files (DAT) were converted into CEL files using the Affymetrix Microarray Suite 5.0.

Gene expression profiles

Each animal was analyzed individually on a Affymetrix RG_U34A Gene Chip. This chip contains 8740 probe sets corresponding to about 7000 annotated rat genes and 1740 expressed sequence tags (ESTs). For comparison between the gentamcin-treated group and the vehicle-treated group, the Wilcoxon test was applied using the software Expressionist Analyst (Version 5.1.2, Genedata AG, 4016 Basel, Switzerland). To identify the genes that had a significant increase or decrease in intensity values a significance level of $\alpha = 0.05$ was chosen. Due to the fact that a two-sided Wilcoxon rank test was used, the p-value cut-off was set at $p = 0.099$ which correspond to a p-value cut-off at 0.05 for a one-sided Wilcoxon rank test. Median fold changes in intensity values across the statistically significant genes were required to be ≥ 1.7-fold or ≤ -1.7-fold. This approach using a combination of a p-value cut-off with a fold change cut-off was chosen based on the results of the Micro Array Quality Control Consortium (Shi et al., 2006). The probe sets were annotated through the use of Ingenuity Pathways Analysis (Ingenuity Systems, Redwood City, CA, USA).