A combined biochemical and bioinformatic approach to investigate the induction of glutamine synthetase in hepatocytes

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Introduction The liver is the central organ of lipid, amino acid, protein and carbohydrate metabolism. In addition, drugs are metabolized and bile is produced in liver tissue. Although morphology of liver tissue is rather uniform it shows a highly structured organization. Each lobulus, which represents the smallest physiological unit in liver, consists of an efferent and several afferent vessels. The afferent vessels form portal fields at the periportal site. Blood flows from a portal field into the lobulus and drains through the central vein at the pericentral site. On average 3 to 6 portal fields surround a pericentral area. The main cell type in liver tissue is the hepatocyte. In order to perform the different metabolic functions hepatocytes show a heterogeneous gene expression, depending on the location relative to the periportal-pericentral axis. In particular, glutamine synthetase (GS) is located exclusively in the pericentral area in a single cell layer surrounding the central vein. The mechanisms, which determine this pericentral expression of GS have not been elucidated, yet. However, some factors involved in the signaling pathway have recently been resolved in HepG2 cells as well as in primary hepatocytes [1, 2, 3]. Affymetrix chip analysis in combination with a bioinformatics top-to-bottom approach was used to calculate a network for the induction of GS in HepG2 by LiCl. Enzymatic assays and western blot data were included to improve and validate the data. Here we present evidence that gene expression of GS is mediated by the Wnt/beta-catenin pathway in hepatoma cells as well as in primary mouse hepatocytes.

Material and Methods HepG2 cells were cultivated in DMEM supplemented with 10 % FCS, 2 mM glutamine and penicillin/streptomycin. Primary mouse hepatocytes were isolated from C57BL/6N mice by the collagenase perfusion technique and cultivated in Williams E supplemented with 100 nM dexamethasone and penicillin and streptomycin [4]. Hepatocyte subpopulations (periportal as well as pericentral) were isolated by the digitonin/collagenase perfusion technique. GS was induced in HepG2 cells by the addition of 50 mM LiCl and harvested before and 2, 4, 8, 12 and 24 h after the addition of LiCl. In primary nouse hepatocytes GS was induced by cocultivation with an epithelial cell line (RL-ET-14). The mRNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany). For the Northern blot hybridization a digoxigenin labelled probe from the rat GS gene was used. Cell viability was tested using the MTT assay. Determination of GS activity was performed by a modified method of Levintow [5]. Protein concentration was determined using the Bradford assay [6] and the specific GS activity of each sample was expressed in mU/mg. For the expression analysis in HepG2 cells oligonucleotide arrays HG-U133A and U95 and for the primary hepatocytes MOE4302 oligonucleotide arrays were used, respectively. The gene expression data were pre-processed using 'affyPLM' packages of the Bioconductor Software [7, 8]. The log-ratios of expression values from samples with and without LiCl stimulation were calculated after normalization by Probe-level Linear Models. Probesets representing differentially expressed genes were selected by the following criteria for GS: i) the fold change exceeds the threshold 1.62 for 2 h, 4 h of 8 h after stimulation and ii) there is a direct or indirect link to the known Wnt/beta-catenin pathway or to the GS induction described in literature. Processing and visualization of data were performed using MATLAB tools (The MathWorks Inc., Natick, MA). The reverse engineering algorithm [9] was used to identify the structure and parameters of a linear differential equation system and to visualize this by a network model. The most robust model was identified using different combination of models with varying the reverse engineering algorithm's configuration and by disturbing the input data. A gene-to-gene interaction was accepted in the HepG2 model if it occurred at least 40 times (80%). In the second step of search for robust structures the stimulus-gene interactions from LiCl to the three genes with the highest interaction were pre-set and the reverse engineering algorithm was applied 1000 times using normally distributed noise with a mean of zero and standard deviations of 0.05 added to the log ratios. A gene-gene interaction was accepted in the recalculated network, if it occurred in at least 80 % of the reconstructed models.

Results and Discussion Glutamine synthetase (GS) is heterogeneously expressed in the liver. The pericentral expression is shown by immunhistochemistry and mRNA expression analysis using quantitative RT-PCT (Light Cycler). In isolated primary mouse hepatocytes as well as in HepG2 cells LiCl induces the expression of glutamine synthetase. Since LiCl is an inhibitor of GSK3-beta a key regulator of the Wnt/beta-catenin pathway these experiments support recent results showing that the Wnt/beta-catenin pathway is involved in the induction of GS [1, 2, 3]. Based on a time series on the effect of LiCl on HepG2 a network of interacting components was calculated [10]. Preliminary data proved that the expression of Dickkopf is related to the expression of GS, since secreted Dickkopf reduced the expression of GS. In addition, the network showed that the insulin-like growth factor binding protein (IGFBP1) seems to be involved in the induction. The effect of IGFBP was investigated further using primary rat and mouse hepatocytes. Schrode and co-workers showed that secreted factor(s) of the RL-ET-14 cell line induce GS in primary rat hepatocytes [11]. Here we demonstrate for the first time that RL-ET-14 induce GS also in primary periportal mouse hepatocytes. These cells have a GS activity of 17.6 mU/mg 24 h after isolation. The weak expression of GS in periportal hepatocytes was shown by RT-PCR and Affymetrix oligonucleotide arrays. Co-cultivation of periportal mouse hepatocytes with RL-ET-14 cells increased the GS activity in the co-culture to 110.9 mU/mg within 96 h. In order to elucidate factors that induce GS in the co-culture Affymetrix oligonucleotide arrays were used and preliminary results will be discussed. There is no doubt that an intact Wnt/beta-catenin pathway is a prerequisite for the induction of GS. However, the expression is influenced by several other factors and/or pathways [12]. Own experimental data show that the induction in primary mouse hepatocyte cultures is much more complex compared to the HepG2 cell line. Increased biological variance of individual animals might effect the extend of induction as demonstrated by repeated experiments with primary hepatocytes.

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