Characterization of hepatocyte-like cells differentiated from human embryonic stem cell lines

Short PhD thesis

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Introduction

Liver transplantation is the only option for those with irreversible liver failure. The lack of whole organ donors, cell-based therapies have been considered an alternative strategy for hepatic injuries. Therefore, different type of stem cells (such as human embryonic stem cells, induced pluripotent stem cells and bone marrow-derived stem cells) has been used in various experiments to evaluate their regenerative potential.

In our experiments we studied two human embryonic stem (ES) cell lines the HUES1 and HUES9, which were established from the inner cell mass cells of the human blastocyst stage embryos. Human ES cells are pluripotent; they can develop in all three germ layers when different growth factors and morphogens are added. The differentiation process of the human ES cells can be well characterised by different cellular markers.

The parenchymal cells of the liver, i.e., hepatocytes and cholangiocytes play distinct roles in diverse hepatic functions, thus, they differ from each other in several aspects including expression pattern of ABC transporters, and tight junction (TJ) components. The matured hepatocytes are highly polarised cells, where intercellular junctions, including TJs, determine cell polarity. In the polarised hepatocytes, the ABC transporters are expressed selectively at the basolateral (sinusoidal) and at the apical (canalicular) membrane, which proper localisation in hepatocytes is essential for liver function (metabolism, glycogen storage, bile excretion, cholesterol synthesis, detoxification, etc.).

To date during the hepatic differentiation of human ES cells has not been studied the continuous gene expression level changes of various ABC transporters and also many TJ components; including claudins, the recently identified tricellulin and an extracellular matrix component agrin.

The expression level changes of different TJ components is in connection with cancer formation, therefore we also aimed to evaluate the changes in gene expression and protein composition of TJ components in different type (liver and pancreas) of cancers.

Aims

The primary objective of our work was to differentiate human ES cells into hepatic direction, we set out to

- select an appropriate human ES cell line to differentiate into hepatocyte-like cells,
- select the most efficient and reproducible hepatic differentiation protocol,
- follow the progress of the differentiation by studying the changes of different cellular differentiation markers in the various stages of the differentiation process,
- apply various functional assays to functionally characterize the hepatocyte-like cells and demonstrate the effectiveness of the differentiation protocol.

In the well characterized stages of the human ES cell differentiation into hepatic direction, we aimed to

- map the continuous gene expression level changes of the hepatocyte basolateral (sinusoidal) membrane expressed *ABCA1, ABCC1 (MRP1), ABCC3 (MRP3), ABCC6 (MRP6),* at the hepatocyte apical (canalicular) membrane expressed *ABCB1(MDR1), ABCB4 (MDR3), ABCB11 (BSEP), ABCC2 (MRP2), ABCC4 (MRP4), ABCG2 (BCRP), ABCG5/G8, the* cholangiocyte specific *ABCC7* and in the liver macrophage membrane expressed ABCG1 and also
- map the expression level changes of various TJ components such as *claudin-1, -4, -5, -7, tricellulin* and an extracellular matrix component, *agrin* genes.

Most tumor cells originate from cancer stem cells with stem cell-like properties, including self-renewal and multilineage differentiation capacity, we set out to

- compare the gene expression level and protein localization of different TJs and ABC transporters in some type of cancers with these healthy tissues,
- draw a parallel between the formation and severity of cancers and our results in the differentiation process.

Materials and methods

We differentiated two human embryonic stem cell lines, HUES1 and HUES9 into hepatic direction using two directed differentiation protocols. By way of various methods – morphology studies, glycogen storage, oragnic anion uptake/release, albumin and urea secretion and CDF-DA hydrolysis measurement – we demonstrated the capability of human ES cells to differentiate to the hepatic lineage. We followed the gene expression level changes of some stem cell and hepatocyte markers during the differentiation process at the well-defined differentiation stages.

We followed by quantitative real time (RT) PCR the gene expression level changes of some ABC transporters and TJ components at the above mentioned differentiation stages.

We compared some type of liver cancers with healthy liver tissue samples on the basis of their TJ gene expression level by quantitative RT-PCR and on the basis of the localization of different TJ components by immunohistochemistry. We draw a parallel between the different grade of the tumors and the healthy tissues on the basis of their TJ gene expression level by quantitative RT-PCR.

Results

The selected differentiation protocol was relatively simple and had well-defined differentiation stages. The selected HUES9 cell line was kept for three days at undifferentiated state, the cells following an Activin A treatment was differentiated into endoderm-like cells. The cells were further differentiated into hepatocyte-like cells in the presence of hepatocyte growth factor (HGF).

The efficiency of the HUES9 cell differentiation was investigated by various methods (morphology, functional assays and RT-PCR of stage-specific gene expression) which showed that we could differentiate the human ES cells into hepatic direction. The differentiation process was monitored at different stages on the basis of their morphology. By the end of the process, the hepatocyte-like cells exhibited polygonal shape and we observed a lot of granules in the cytoplasm. The human ES cell-derived hepatocyte-like cells, showed hepatic functional activities. The PAS (periodic acid-schiff) assay is routinely used to detect the characteristic cytoplasmic glycogen accumulation in hepatocytes. At the end of the differentiation process the cells were PAS positive. In the present study, we examined cellular

uptake and release of ICG (indocyanin green) organic anion, to evaluate liver function since it is eliminated exclusively by hepatocytes. The hepatocyte-like cells differentiated from human ES cells were taken up the ICG, but the undifferentiated stem cells did not. The functional hepatocyte-like cells eliminated the ICG six hour later. These hepatocyte-like cells produced significantly high level of urea at the end of the differentiation. Interestingly the albumin secretion significantly increased and reached maximal values at the end of the endodermal stage, but during the hepatic stage the albumin secretion declined, however we can say that the albumin expression increased overall during the differentiation. Using the CDF-DA hydrolysis study we could detect the proper apical (canalicular) membrane protein localisation of the ABCC2 (MRP2), which is responsible for the hepatobiliary disposition of the green fluorescent CDF. During the CDF-DA hydrolysis study we observed no bile canaliculi-like structures, although these cells initiated from HUES9 cells accumulated the green fluorescent CDF inside the cells and in the intercellular spaces, because the hepatocyte-like cells at the end of the differentiation were not fully polarised, and the targeting of the membrane proteins were still in process.

We monitored the mRNA expression level of stem cell marker genes such as *Oct3/4* and *Nanog*, which showed significant decrease, suggesting that during the differentiation process the stem cells lost their stem character. By the end of the differentiation process the gene expression level of the hepatocyte marker genes, such as *α-fetoprotein* and *albumin* was significantly increased.

The further studies have shown that the ABC transporter and TJ component set of the hepatocyte-like cells differentiated from human ES cells had more or less characteristic to hepatocytes. From the total of fourteen examined ABC transporters there were eight apical, four basolateral and two of them were expressed in liver macrophages and cholangiocytes. At the basolateral (sinusoidal) membrane of the hepatocytes *ABCA1* (15-fold), *ABCC3 (MRP3)* (15-fold) and *ABCC6 (MRP6)* (30-fold) were significantly induced during the HUES9 cells differentiation. At the apical (canalicular) membrane of the hepatocytes *ABCB11 (BSEP)* (110-fold), *ABCC2 (MRP2)* (30-fold), *ABCG2 (BCRP)* (20-fold) and *ABCG5* (8-fold) were significantly induced during the hepatic differentiation. *ABCG1* which is expressed in the membrane of the liver macrophages (Kupffer cells) was also significantly induced (85-fold). Similarly, the expression of *ABCC7 (CFTR)*, which is expressed in the membrane of cholangiocytes, was significantly induced (80-fold) too.

The endoderm-like cells expressed significantly higher level of *claudin-1* (30-fold) than the hepatocyte-like cells at end of the differentiation process. During the differentiation

the expression of *claudin-4* (20-fold) and the *agrin* (10-fold) genes increased continuously, and they reached the significantly highest level at the end of the hepatic differentiation. However, the gene expression of *tricellulin* was not changed significantly compared to the parent cells.

During the examination of the different tumor diseases, we observed that the *tricellulin* expression significantly decreased while the grade of the pancreatic ductal adenocarcinomas (PDACs) increased, although the overexpression of *tricellulin* correlated with unfavourable prognosis in a subset of hepatocellular carcinomas (HCCs). The tricellulin was successfully detected by immunohistochemistry in both normal and cancerous liver tissues; in HCC, focal nodular hyperplasia (FNH) and in cirrhosis (CIR)**.** We found that, well-differentiated tumours showed strong spotty immunoreactivity at tricellular contacts of the tumour cells. In CIR the tricellulin protein expression was slightly stronger, while in case of FNH the tricellulin protein expression was much stronger than in normal liver tissue samples. Among the investigated liver cancers we found the highest tricellulin production in HCCs. Expression of *claudin-1* and *tricellulin* was lower, while that of *claudin-5* was higher in fibrolamellar hepatocellular carcinomas (FLC) than in HCCs. We detected for the first time the presence of tricellulin in human hepatic tumors.

Discussion

Human embryonic stem (ES) cells can be differentiated *in vitro* into all three germ layers by adding different morphogens and growth factors. Our aim was to monitor the expression pattern of some tight junction (TJ) components and ABC (ATP-binding cassette) transporters during differentiation of human ES cell lines (HUES1, HUES9) toward the hepatic lineage. Human ES cells were differentiated first into endoderm-like cells, and further into hepatocyte-like cells.

We performed various functional studies to confirm the success of the differentiation. The polygonal morphology and the functional assessments, such as glycogen storage, organic anion uptake/release, albumin and urea secretion, demonstrated the hepatocyte characteristics of the differentiated cells. The expression level of stem cell marker genes *(Oct3/4* and *Nanog)* significantly and gradually decreased, while liver-associated genes *(α-fetoprotein, albumin)* culminated by the end of the differentiation. The endoderm-like cells expressed *claudin-1*, which declined eventually. The expression levels of cholangiocyte markers including *claudin-*

4, *CK-7*, *CK-19*, and *agrin* gradually increased and reached their highest level at the final stage of differentiation. In contrast, these cells did not express notable level of *claudin-7*, *CK-8*, and *tricellulin*. The hepatocyte-like cells at the end of the differentiation process expressed significantly high level of some hepatocyte-specific apical (canalicular) ABC transporters *(ABCC2/MRP2*, *ABCG5*, and *ABCB11/BSEP)* as well as some hepatocyte-specific basolateral (sinusoidal) ABC transporters *(ABCA1*, *ABCC3/MRP3*, and *ABCC6/MRP6).* By the marker set used for monitoring the differentiation process we could conclude, that the cells at the end of the differentiation revealed both hepatocyte and cholangiocyte characteristics. The hepatic differentiation process provide a model that may reveal molecular mechanisms of the pathogenesis of different type of liver cancers, which have both hepatocellular and cholangiocellular character. Our results support that monitoring the gene expression level changes of the TJ components and ABC transporters during hepatic differentiation, leads us to a better understanding of *in vivo* liver organogenesis.

We compared the gene expression level and protein localization of different TJs and ABC transporters in some type of liver and pancreas cancers (FLC, HCC, FNH, CIR, PDAC) with these healthy tissues. We described that high *tricellulin* expression in HCCs, while decreased expression in PDACs was correlated with poor prognosis. Our results predict the possibility that, the TJs, tricellulin and ABC transporters could be potential targets in cancer therapy.

Publications related to the thesis

B. Erdélyi-Belle, Á. Apáti, Gy. Török, B. Sarkadi, Zs. Schaff, A. Kiss, L. Homolya
Expression profiling of hepatocyte-like cells initiated from human embryonic stem cells
PATHOLOGY AND ONCOLOGY RESEARCH (2014) accepted
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Expression profiling of ABC transporters during hepatic differentiation initiated from human embryonic stem cells

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Erdélyi-Belle Boglárka

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Korompay Anna, Borka Katalin, Lotz Gábor, Kovalszky Ilona, Somorácz Áron, Patonai Attila, Erdélyi-Belle Boglárka, Kiss András, Schaff Zsuzsa

A tricellulin jelenlétének, lokalizációjának és mennyiségi változásainak vizsgálata humán pancreasban és egyes daganataiban

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