INVESTIGATION OF PROLIFERATIVE REACTIONS IN HUMAN AND MOUSE LIVER

PhD thesis

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Background

The liver is a vital organ with highly diversified functions. While it has an enormous reserve capacity, it is also capable of very effective regeneration.

Under experimental conditions, the most widely employed method for causing liver injury in rodents is partial hepatectomy. This surgical intervention removes a portion of the liver lobules. The procedure can be easily carried out in rodents without causing any further necrosis in the remaining tissues. The rodent liver fully regenerates within 5-7 days following partial hepatectomy. This process is somewhat slower in humans, requiring about a month to regenerate after the removal of one half of the liver parenchyma.

Strictly speaking liver regeneration is no regeneration, but compensatory hyperplasia, since lobes removed will not be replaced, and the original mass of the liver is generated by the growth of the remaining lobes.

A number of cytokines (IL-6, TNF), growth factors (HGF, TGFα), transcription factors (STAT3, NF-κB) and immediate early genes (c-fos, c-jun. c-myc) become activated and orchestrate the compensatory hyperplasia following partial hepatectomy.

Primary hepatocyte mitogens are able to induce hepatocyte proliferation in vivo without prior liver damage. Although the family of primary hepatocyte mitogens is quite heterogeneous, they share some common features: they commonly target nuclear transcription factor receptors, a single dose of them is able to induce significant DNA synthesis in the liver, and following their withdrawal, the liver regains its original weight and DNA content by the strictly regulated apoptosis of the hepatocytes. Their significance arises, among other things, from the fact that they activate signal transduction pathways that are different from the ones responsible for compensatory

hyperplasia following partial hepatectomy. Certain factors that play a central role in compensatory hyperplasia are absent during primary mitogen induced hepatocyte proliferation. The outcomes of these two different types of proliferations also differ from each other.
For our experiments we used 1,4-bis[2-(3,5 dichloropyridyloxy)]benzene (TCPOBOP), a halogenated carbohydrate acting through the constitutively active androgen receptors (CAR) as primary mitogens. Its target genes include the Cyp2b10, which plays an important role in the detoxication function of the liver.

Although TGFβ1, a member of the TGFβ family, is one of the most widely investigated cytokine involved in liver regeneration following partial hepatectomy, its exact role is still unclear. It acts as a very potent mitogen inhibitor in primary cell cultures, and most likely down regulates the regeneration processes during compensatory hyperplasia the exact mechanism of action is not yet understood.
We used a transgenic mouse model expressing active TGFβ1 in the hepatocytes for our experiments. Previous reports described a decreased and prolonged regeneration, on this mouse model, indicating that the product of this transgene inhibits the regenerative response following partial hepatectomy.
The effect of TGFβ1 on primary hepatocyte mitogen induced liver proliferation has not yet been described in the literature.

The decreased regenerative capacity of a cirrhotic liver compared to a healthy liver is at least partly attributed to the increased TGFβ1 concentration found in the cirrhotic liver. The decreased regenerative capacity of a cirrhotic liver represents a significant problem in everyday clinical routine; as the majority of hepatocellular carcinoma develops in cirrhotic

livers and the decreased regenerative capability of the remaining liver tissue negatively affect the outcome of patients undergoing tumor surgery.
The livers of old animals generally show decreased regeneration when compared to the young ones, however with the help of the primary hepatocyte mitogen TCPOBOP old livers regenerate similar to young ones.
Taking all this into consideration, it would be worthy to investigate weather the primary hepatocyte mitogens have a beneficial effect on the regenerative capacity of cirrhotic livers.

The appearance of ductular structures resembling bile ducts can be seen both in humans and in rodents following severe liver damage. This tissue response is known as the ductular reaction. Although ductular reactions have very diverse etiology and functions, attempts at a consensus classification have repeatedly failed, so they do not have a standardized nomenclature. This makes it difficult to compare and utilize the results of various reports and experiments. Regarding ductular reactions, even the most widely used typical and atypical terms are not recommended due to their poor interobserver reproducibility.

Therefore, as an example we can currently describe as “ductular reaction” the reactions seen in rats after hepatocyte injury (so called oval cells) and reactions following bile duct ligation (biliary proliferation), whereas in the former case the cells of the ductular reaction, similarly to stem cells, are able to show signs of both hepatocyte and bile duct differentiation, while in the latter case the cells are committed toward the bile duct lineage. Overlooking these important differences might lead us to false conclusions.

There is an increasing demand for a reproducible and easily adoptable classification system reflecting the etiology, morphology and function of the ductular reactions, but a widely accepted consensus is yet to be reached.
Thus far no attempts of an immunophenotype based classification have been described in the literature.

Goals

1. How does TGFβ1 influence the TCPOBO- induced hepatocyte proliferation in mice?

2. Can TCPOBOP induce hyperplasia in fibrotic livers?

3. Can we achieve a classification of ductular reactions of human livers based on their immunophenotype?

Materials and methods

We used TGFβ1 transgenic mice for our in vivo experiments. In these mice, the TGFβ1 gene is under the transcriptional control of the albumin promoter; therefore TGFβ1 is exclusively produced in the liver, resulting in stable and high serum concentrations. The replacement of two serine codons by cysteine residues has lead to a constitutively active form of TGFβ1, without the need for further activation. Age and sex matched C57Bl mice were used as controls. The C57Bl mice were also used for the hepatic fibrosis experiments.
The ductular reactions we evaluated on paraffin embedded tissue sections were selected from the archive of our department.
The genotype of the transgene animals was confirmed by PCR reactions. The expression analysis was performed by real-time quantitative RT-PCR studies, using GAPDH as reference.

The analysis of ductular reactions and cell proliferation studies was performed by immunohistochemistry.

Results

In our studies we compared the TCPOBOP induced proliferation between the TGFβ1 over expressing transgenic mice and the control (wild type) animals. Liver weights were measured and liver weight/bodyweight ratios were calculated. The DNA synthesis was evaluated by two approaches. Using the so called pulse method we assessed the ratio of cells entering the S phase, based on their BrdU incorporation at several different time points following the administration of a single dose of TCPOBOP. Furthermore, BrdU was added to the drinking water of the animals for 5 days following the TCPOBOP treatment for assessment of the ratio of all cells entering the S phase during this period, representing the so called proliferative pool. According to our observations neither the pulse method, nor the evaluation of the proliferative pool showed any significant difference between the two groups of our experimental design, indicating that TGFβ1 was unable to inhibit the TCPOBOP induced proliferation.
We examined the gene expression of Cyp2b10, a target gene of CAR, and found a significant increase in both groups, although the extent of increase was significantly lower in the transgenic mice. We studied the expression of two proliferation markers cyclin A and cyclin D1, and found that the peak of expression of both cyclins occurred at 34 hours, and levels of cyclin A was somewhat higher in the transgenic animals. The expression levels of TGFβ1 increased from 30 hours, and this change was more pronounced in the wild type mice.

In our next experiment we tested the hypothesis of whether TCPOBOP is able to initiate proliferation in cirrhotic livers.

We induced liver cirrhosis with two different chemicals, carbon tetrachloride (CCl4) and thioacetamide (TAA). The animals were fed their usual chow for 2 weeks after the withdrawal of fibrotic agents, when the development of fibrosis/cirrhosis was confirmed by picrosirius staining.

Similarly to our previous experiment, we recorded the relative liver masses at the time of TCPOBOP treatment and 5 days later. We assessed DNA synthesis at 36 hours after TCPOBOP treatment, and following the 5-day continuous administration of BrdU through the drinking water.
The TCPOBOP treatment resulted in a considerable increase in the relative liver masses of the animals. The change was somewhat more pronounced in the control animals, but the difference between the groups was not significant.
We were able to detect substantial BrdU incorporation in all 3 groups, but it was significantly higher in the control group compared to the cirrhotic groups. The proportion of cells entering the S-phase during the 5-day treatment was significant across all 3 groups, while the highest values were found in the control animals.

Through gene expression studies of Cyp2b10, a target gene of CAR, we investigated the activation of the TCPOBOP CAR signal transduction pathway. We found similar increases in all 3 groups. The Cyclin A expression peaked at 36 hours, and significant differences were found between the control and cirrhotic animals. The levels of TGFβ1 and p27 were consistently higher in the cirrhotic groups, showing increases across all 3 groups.

Our next aim was establish an immunophenotype based classification system of the various types of ductular reactions found in human livers. Among the immunohistochemical

markers found in the literature AFP, chromogranin, CEA, DLK1, DMBT-1 proved to be negative in all samples, while CK19, EpCAM, E-cadherin and claudin 2 gave positive reactions in both the bile ducts and the ductuli, making these antibodies inappropriate for the classification of ductular reactions. CK7 also gave positive reactions with both the bile ducts and the ductuli, and at the same time it vaguely stained the intermediary hepatobiliary cells, therefore we used this antibody for the visualization of the ductuli.
EMA, CD10 and CD56 showed various staining patterns in the ductuli, enabling us to categorize 59 out of the 69 cases into one of the following 3 groups.

In the Primitive (P) type reactions the ductular cells were CD56 positive and CD10 and EMA negative. With CK7 staining we were able to visualize flattened cells forming trabecules or bundles without lumens. Most of the samples belonged to this group, which included all cases of focal nodular hyperplasia and primary biliary cirrhosis, among other less frequent entities.

The next group denominated the Differentiation (D) group, based on simultaneous CD56, CD10 and EMA positivity, included two major entities, namely cirrhosis and samples of fulminant hepatic failure. There were conspicuous morphological differences between these groups. While the ductuli of fulminant hepatic failure were composed of numerous large, vaguely CK7 positive, so called intermediary hepatobiliary cells, the ductuli found in cirrhotic livers showed atypical ductular morphology with only a few intermediary hepatobiliary cells.

In the Obstructive (O) type reactions the ductuli resembled bile ducts, demonstrating CK7 and EMA positivity along with

CD10 and CD56 negativity. Bile duct obstruction was the common link between all entities belonging to this group (acute bile duct obstruction, biliary atresia, secondary biliary cirrhosis.)

Discussion

We examined the effect of TGFβ1 on TCPOBOP induced hepatocyte proliferation. Our results indicate that the primary hepatocyte mitogen TCPOBOP induced hepatocyte proliferation is not sensitive to the mitoinhibitory effect of TGFβ1.

These results may explain the anti carcinogenic effect of some primary hepatocyte mitogens. A theory known as the TGFβ paradoxon claims that the tumor cells loose their TGFβ1 sensitivity, thereby gaining a relative proliferation advantage compared to the healthy cells. The utilization of primary hepatocyte mitogens might moderate this growth advantage, compensating the effects of the TGFβ paradoxon.

The decreased regeneration capacity of cirrhotic livers is also attributed to the increased TGFβ1 production. Our results suggested that the regeneration capacity of cirrhotic livers might be improved by the administration of primary hepatocyte mitogens. We tested this hypothesis in our following experiments.

In our following setup, we investigated whether TCPOBOP is able to induce hepatocyte proliferation in cirrhotic livers. Our results indicated that TCPOBOP was able to induce considerable hepatocyte proliferation in cirrhotic murine livers, but this effect was less pronounced when compared to the control animals.

Data in the literature suggests that the livers of old animals regenerate less effectively than the livers of young animals,

and this effect was explained at least partly by the increased production of p27. The expression difference could be abolished by the administration of TCPOBOP, resulting in decreased p27 levels of old livers. We were unable to show a decrease in p27 expression of the TCPOBOP treated mice, which might explain why TCPOBOP induced a substantial, but significantly less effective hyperplasia in fibrotic livers, compared to the control animals.
Although the reactions observed in the cirrhotic livers as a result of TCPOBOP treatment differed significantly from reactions seen in the control livers, the primary mitogen was still able to induce considerable proliferation in the fibrotic liver, suggesting that appropriate usage of human hepatocyte mitogens would improve the regeneration capacity of cirrhotic livers.

The ductular reactions seen in hepatic injuries of various etiologies are quite heterogeneous in their appearance, origin and function. The lack of a standardized classification and nomenclature system makes it difficult to compare different observations found in the literature. Our attempts were able to classify the majority, 86 percent of the ductular reactions into one of our 3 groups. The great majority of reactions occurring within a given entity fell into the same category; therefore this classification system reflects the etiology and pathogenesis of the ductular reactions. Our classification scheme is by no means final or perfect, but it may serve as a basis for further large scale investigations.

Conclusions:

1. The primary hepatocyte mitogen TCPOBOP was able to induce a similar degree of hepatocyte proliferation in wild type animals as in TGFβ1 over expressing transgenic mice, indicating that this type of cell proliferation is not sensitive to the mitoinhibitory effect of TGFβ1.

2. TCPOBOP was able to induce substantial proliferation in fibrotic/cirrhotic livers, but the extent of this proliferation was less than what was seen in control animals.

3. The ductular reactions seen in human livers can be classified into three groups based on their immunophenotype. The majority of reactions occurring within same disease entities fell into the same group, indicating that this classification reflects the etiology and pathogenesis. Our classification might serve as a basis for further large scale studies.

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