The effect of primary mitogens on stem cell mediated regeneration of the rat liver

Doctoral theses

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Budapest
2009

I. INTRODUCTION

The proliferation and cell death of hepatocytes in the adult liver is finely regulated. Damaged liver can be regenerated by three different sources. 1. The proliferation of the remaining hepatocytes is the quickest and most effective way to generate new hepatocytes. 2. If the replication of mature hepatocytes is delayed or entirely blocked, intrahepatic stem cells are activated. These cells form the terminal bile-ductules, the so-called canals of Hering. 3. Extrahepatic stem cells are also able to produce hepatocytes, but the efficiency of this process under physiologycal conditions is so low, that it plays a negligible role in the repair.

Though the criteria of stemness (asymmetric cell division, self-renewal) for the hepatic stem cell compartment could have not been proven yet, the cells constructing the canals of Hering are widely accepted as the tissue/adult stem cells of the liver. The difference from the other established adult stem cells is that the stem cells of the liver do not participate in normal cellular turn over, they become activated only when hepatocytes are compromised (e.g. viral infection, chemical toxicity) and are unable to respond to proliferative stimuli. This is why they are also called facultative stem cells. The oval/hepatic progenitor cells are the progenies of stem cells. Their differentiation potential is more limited, but they are able to form at least two different cell types, hepatocytes and biliary epithelial cells. They represent the transit amplifying compartment of the intrahepatic stem cell system. The oval cells form branching ductules, the extensions of the canals of Hering, spreading from the portal region into the liver parenchyma.

The investigation of stem cells in the adult liver is extremely difficult due to their facultative nature and heterogeneity. Several rodent experimental models have been established for this purpose. One of the most widely-used experimental protocol is the AAF/PH model, which consists of two 4-7 days long 2- acetaminofluorene (AAF) administration periods and a traditional 70 % partial hepatectomy (PH) between. The AAF is metabolized exclusively in the hepatocytes, the adducts bind to the DNA preventing its replication, so the hepatocytes are unable to respond to the proliferative stimuli of the PH. The stem cell compartment becomes activated, the first oval cells appear 2 days after the PH, some of them differentiate into hepatocytes 7-12 days after the PH, depending on the applied dose of the AAF and other experimental conditions.

A number of protein markers have been used to characterize the oval cells. However, these markers are often shared with other cell populations, hepatocytes, biliary epithelial cells, haematopoetic cells. The phenotypic heterogeneity of the oval cells is thought to reflect diverse stages of the differentiation process. There are only two markers, which are generally accepted characteristics exclusively of the oval cells in the adult rat liver: α-fetoprotein (AFP), the most well known marker and the recently recognized delta-like protein (dlk).

The so-called ductular reaction has been observed in several human diseases: viral hepatitis, acute or chronic biliary obstruction, primary biliary cirrhosis, alcoholic liver disease, post-necrotic regeneration, α1 antitrypsin deficiency, Wilson’s disease etc. The ductular reaction in the human liver is regarded as the equivalent of the rodent oval cell proliferation, even if it is morphologically different.

Primary mitogens (PM) are chemicals that induce the proliferation of hepatocytes, without preceding cell loss, leading to the direct hyperplasia of the liver. After a single dose of these agents the enhanced proliferation of liver cells can result in the doubling of the hepatic DNA content within 2-3 days. Their biological effect has been studied in several experimental models, but as far as we know the influence of primary hepatocyte mitogens on oval cells has not been examined *in vivo*. Therefore we have decided to investigate if triiodothyronine (T3) and another primary mitogen lead-nitrate can influence the stem cell driven liver regeneration.

II. OBJECTIVES

Here we ivestigated if the primary mitogens, triiodothyronine and lead-nitrate have an impact on the stem cell mediated regeneration of the rat liver.

Our major questions were:

What kind of impact do primary mitogens have on the oval cell driven liver regeneration?

- Do primary mitogens influence the proliferation of oval cells?
- Are these chemicals able to influence the differentiation process of hepatic progenitor cells?
- If they are able to, do they have an impact on the whole organism?

III. MATERIALS AND METHODS

III. 1. Animal experiments

Male F-344 rats were treated according to the AAF/PH protocol, then with primary mitogens (either with triiodothyronine or with lead-nitrate). 48 hours later the animals were sacrificed. The relative liver mass was measured, liver samples from different lobes were taken for histology, blood was drawn from the right ventricle of the heart for laboratory examinations. The control animals were treated by the AAF/PH protocol but at the time of the mitogen treatment were administered only by the solvent.

III. 2. Morphological analysis

Frozen sections were used for immunohistochemistry. We examined markers that are characteristic for oval cells (OV-6, dlk, AFP), and for mature hepatocytes (HNF-4, CYP-450, connexin 32, α1 integrin, CD26). The sections were analysed by confocal scanning microscope.

III. 3. Determination of the labeling index (LI) of oval cells and small hepatocytes

To measure the proliferative rate of oval cells and newly formed small hepatocytes, the DNA replicating cells were labeled with the pulse BrdU (bromdeoxyuridine) method (the animals are sacrificed 1 hour following one

dose of BrdU administration). We measured the labeling index 18, 24, 48 and 96 hours after the mitogen treatment.

To identify the labeled oval cells, the basal membrane around the oval cells formed ductules were labeled with laminin antibody. The incorporated BrdU was marked with BrdU antibody, the nuclei were stained by DAPI. We counted the BrdU positive and total cell nuclei within the randomly chosen oval cell ductules.

Small hepatocytes were identified with HNF-4 antibody (they can be easily distinguished from the HNF-4 positive old hepatocytes by their smaller size) followed by BrdU immunostaining. The BrdU positive and total small hepatocytes were counted, their ratio was calculated.

III. 4. Retroviral transduction

To prove that the progenitor cells are able to differentiate into hepatocytes two different pulse chase methods were used. We selectively labeled the proliferating cells either with BrdU, or with an amphotropic retroviral vector, which contains the E. coli β-galactosidase gene coupled to the nuclear localization signal of the SV 40 large T antigen. Proliferating cells were labeled before the PM treatment. One group of the rats were sacrificed before the mitogen treatment (*pulse*), to identify labeled cells. The chase animals were sacrificed 48 hours after the PM treatment. Since the BrdU and the bacterial gene were incorporated into the DNA of the proliferating cells, they were passed to the progenies of these cells during mitosis. Therefore the positive cells in the chase livers were the progenies of the positive cells in the pulse rats.

Cells infected by the retrovirus were idendified by ß-gal antibody, incorporated BrdU was labeled with anti – BrdU antibody.

III. 5. Gene expression analysis

To analyse the mRNA expression, different cell populations were microdissected by PALM MicroBeam laser microdissector: (i) oval cells from T3 treated and (ii) control livers, (iii) small hepatocytes from T3 treated livers and (iiii) normal hepatocytes from healthy livers. Following standard RNA isolation and reverse transcription the cDNA samples were analysed by quantitative real-time PCR, using the $\Delta\Delta C_T$ method. AFP, albumin, a tyrosine aminotransferase (TA), tryptophan 2,3-dioxygenase (TO2) expression were determined.

IV. RESULTS

IV. 1. The effect of primary mitogens on the progenitor cell mediated liver regeneration

The livers of the thyroid hormon treated rats were significantly enlarged, the relative liver weight (liver/body weight) increased in 48 hours compared to the control (2,6 % ± 0,41 vs. 2 ± 0,25, $p< 0,05$). The histological structure also changed dramatically at this time point. In the T3 treated livers most of the oval cells disappeared and small, sometimes trabecularly arranged polygonal cells emerged in the periportal zone. They had round nuclei and looked like small hepatocytes. In our AAF/PH model oval cells appear 2 days after the PH, the first small hepatocytes are formed 9 days later, that is 11 days after the PH. In the T3 treated rats the first small, hepatocyte looking cells appeared 7 days following the PH and concomitantly the number of oval cells sharply decreased.

IV. 2. The effect of T3 in the proliferation of the oval cells and small hepatocytes

The rate of DNA synthesis was measured by BrdU pulse labeling. There was no labeling at all in the hepatocytes. The unresponsiveness of the hepatocytes could be explained by the AAF administration, which inhibited the DNA replication in these cells. BrdU incorporation was significantly higher in the oval cells of the rats at 18 hours after administration of T3 compared to the solvent treated rats (30,2 ± 0,7 vs. 17 ± 1,7, $p < 0,05$). Later the proliferation

rate of the oval cells returned to the control level. The BrdU was also taken up by the newly formed polygonal cells, although their labeling index was lower, than that of the oval cells. The large hepatocytes remained unlabeled.

IV. 3. Characterization of the differentiation process of the oval cells

Our imunohistochemical studies proved that the oval cell derived small polygonal cells are functional hepatocytes indeed. Markers characteristic of mature hepatocytes (HNF-4, connexin 32, α1 integrin and Cytochrom P450) were upregulated, while the oval cell specific OV-6 and the basement membrane staining disappeared. The CD 26 reaction demonstrated the formation of bile canaliculi among the small polygonal cells.

IV. 4. The small hepatocytes derive from the oval cells

Although the dynamics of histomorphological changes indicated the oval cell origin of the newly formed small polygonal cells, this relationship was verified by two independent pulse chase experiments.
(I) BrdU was given to the rats two hours before the T3 treatment. The first set of animals was killed 1 hour later, that is before the T3 administration. BrdU was incorporated in addition to the non-parenchymal cells into the nuclei of the oval cells, no staining was present in the "old" hepatocytes. The second set of rats was sacrificed at 48 hours following the T3. BrdU decorated the nuclei of the small, hepatocytes which were also positive for HNF-4. The "old" hepatocytes remained negative in this later timepoint as well.

(II) The proliferating oval cells were also exclusively marked in another experiment by bacterial ß-gal expressing retroviruses 2 days after the PH, that is 3 days before the T3 administration. When the labeled oval cells were traced 2 days after the T3 treatment, groups of the small polygonal cells were found with ß-gal positive nuclei without any labeling in "old" hepatocytes. These two experiments strongly indicated that the "new" polygonal cells were derived from the oval cells.

IV. 5. Gene expression analysis

The gene expression of oval cells was compared to normal hepatocytes and small polygonal cells appearing in the liver 48 hours after the T3 treatment. The mRNA level of oval cell specific AFP, and three hepatocyte specific proteins: albumin, TAT and TO2 were investigated. The AFP expression level of the control oval cell was taken as 100 %, in case of the hepatocyte specific proteins, the mRNA level of the healthy hepatocytes was 100%. The highest AFP expression was measured in the control oval cells, followed by the oval cells of the T3 treated rats (70 %), and the small hepatocytes (27 %). The expression of the three hepatocyte specific proteins changed inversely. The highest level was detected in the small hepatocytes (albumin 8,5 %, TAT 102,3 %, TO2 33,9 %), and the lowest in the control oval cells (albumin, 3,1 %, TAT 23 %, TO2 11,3 %). Consequently the expression of AFP decreased during the differentiation of oval cells into hepatocytes, while albumin, TAT and TO2 increased.

IV. 6. The effect of the mitogen treatment on the metabolic function of the liver

Forty-eight hours after the mitogen treatment blood samples of the rats were analysed. The mitogen treated livers showed better metabolic functions, the serum bilirubin concentration decreased (17,6 ±16,9 v.s. 5,4 ± 4,9, $p<0,05$), the prothrombin level increased (49,2 ±25,5 vs. 21,3 ± 18,5, $p<0,05$) compared to AAF/PH, but not T3 treated animals.

IV. 7. The effect of lead-nitrate on oval cell mediated liver regeneration

The lead-nitrate, another primary hepatocyte mitogen had similar effect on the stem cells. Functional experiments have not been performed, but according to our morphological observations, lead nitrate also accelerated the oval cell mediated liver regeneration in our model

V. DISCUSSION

Triiodothyronine and lead nitrate are primary hepatic mitogens. Their single dose induces the proliferation of hepatocytes, which leads to liver hyperplasia. Triiodothyronine (T3), has been reported to enhance the regenerative capacity of the liver following PH. Another mitogen, induced the differentiation of oval cells *in vitro*. Here we investigate if two primary mitogens: triiodothyronine and lead-nitrate can influence the stem cell driven liver regeneration. Oval/progenitor cell proliferation was generated by the widely used AAF/PH model.

A single dose of the primary hepatocyte mitogens accelerated the proliferation and differentiation of oval cells into hepatocytes. The differentiation could be verified by morphological, immunophenotypical and functional characterization of the oval cells and their progenies.

The livers of the T3 treated rats were enlarged, the relative liver weight (liver/body weight) increased in 48 hours compared to the control (solvent treated) rats. BrdU incorporation was significantly higher in the oval cells of the rats at 18 hours after administration of T3 compared to the control rats. Later the proliferation rate of the oval cells returned to the control level.

The course of the accelerated differentiation was the same as it had been described earlier in this model. However, the spontaneous differentiation of the oval cells occured later on the 11th day after the PH. The expression of the oval cell specific OV-6 and alpha-fetoprotein (AFP) was lost, the supporting basal membrane was degraded. While differentiating cells started to express hepatocyte specific markers like HNF-4, CYP450, cx 32, α1 integrin, CD 26

positive bile canaliculi were formed between the newly differentiated small hepatocytes.

The results of the gene expression analysis also confirmed the hepatocytic differentiation, hepatocyte specific mRNAs e. g. albumin, tyrosine aminotransferase (TAT) and tryptophan 2, 3-dioxygenase (TO2) could be detected by real-time PCR in microdessected small hepatocytes, while the oval cell specific AFP expression sharply dropped.

Liver function tests improved concurretly with the accelerated differentiation, the bilirubin concentration decreased, while the prothrombine level increased.

Although the dynamics of histomorphological changes indicated the oval cell origin of the newly formed small polygonal cells, this relationship was verified by two independent pulse chase experiments.

Proliferating oval cells were labeled either with BrdU or with a retroviral vector which contained the gene of the bacterial ß-galactosidase. When the labeled cells were traced 2 days after the T3 treatment, groups of the small polygonal newly formed hepatocytes were found to contain these markers, without any labeling in “old” hepatocytes. These two experiments strongly indicated that the “new” polygonal cells were derived from the oval cells.

Based on our studies we conclude that applied primary mitogens accelerate the proliferation of the oval cells. They differentiate into small, polygonal cells which are functionally mature hepatocytes. The mechanism of the accelerated differentiation is the same as it has been described earlier in this model, but the spontaneous differentiation of the oval cells occurs later. This spontaneous differentiation int he AAF/PH model occurs on the 11th day after the PH, in the mitogen treated rats the first small hepatocytes appear on the 7th day after the

partial hepatectomy. Since T3 receptor can be detected in the nuclei of oval cells, we suppose that the triiodothyronine can influence directly the oval cells.

Intrahepatic progenitor cells have been observed in several human diseases. Their regenerative capacity is still enigmatic, but complete regeneration of the liver by progenitor cells has been documented in a patient receiving auxiliary liver transplant after massive hepatic necrosis. Intermediate hepatobiliary cells (the human equivalents of the rodent oval cells) are frequently observed in the livers of patients dying of liver failure or recipients of liver transplants, indicating that though the stem cell compartment is activated, it fails to regenerate the liver.

Our study suggests a novel therapeautic strategy for liver disease. T3 and lead-nitrate are naturally inappropriate for human treatment. However, both of them accelerate the stem cell mediated liver regeneration, it is probably a common feature of primary hepatic mitogens. Triiodothyronine agonists without cardiologic effects have already been described. Application of primary mitogens might be an option in for treatment of failing liver.

PMs are able to reduce the incidence of hepatocellular carcinomas in a rodent model. The mechanism of this inhibition is not clear yet, but, based on our results we assume that the forced differenciation of the oval cells – the precursors of the tumors – is responsible for the decreased tumorigenesis. In this case primary mitogens might have a therapeautic potential in the secondary prevention of hepatocellular carcinomas as well.

VI. CONCLUSIONS

1. Primary mitogens (triiodothyronine, lead-nitrate) accelerate the proliferaton and hepatocytic differentiation of intrahepatic progenitor cells, accordingly they support the stem cell mediated liver regeneration.

2. The newly-formed hepatocytes are the progenies of the oval cells.

3. The course of accelerated differentiatiation is identical with the spontaneous process.

4. The newly formed small hepatocytes are functionally mature hepatocytes, they express enzymes and proteins, characteristic for the liver parenchyme.

VII. PUBLICATION RECORD

VII. 1. Publications in the subject of the dissertation

László V, Dezso K, Baghy K, Papp V, Kovalszky I, Sáfrány G, Thorgeirsson SS, Nagy P, Paku S. (2008) Triiodothyronine accelerates differentiation of rat liver progenitor cells into hepatocytes. Histochem Cell Biol, 130 (5): 1005-14.
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Szabó E, Lódi C, Korpos E, Batmunkh E, Rottenberger Z, Deák F, Kiss I, Tokés AM, Lotz G, *László V*, Kiss A, Schaff Z, Nagy P. (2007) Expression of matrilin-2 in oval cells during rat liver regeneration. Matrix Biol, 26 (7): 554-60.
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Papp V, Dezsö K, *László V*, Nagy P, Paku S (2009) Architectural changes during regenerative and ontogenic liver growth in the rat. Liver Transpl. 15 (2): 177-83.
IF: 2.559

VII. 2. Publications in different subject

Füle T, Csapó Z, Máthé M, Tátrai P, László V, Papp Z, Kovalszky I. (2006) Prognostic significance of high-risk HPV status in advanced cervical cancers and pelvic lymph nodes. Gynecol Oncol, 100 (3): 570-8.
IF: 2.614

Nagy P, László V, Schaff Z. (2006) [Hepatocarcinogenesis in human liver] Magy Onkol, 50 (2): 107-13.

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IF: 1.597

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