Architectural changes in the rat liver during ontogenesis, regeneration and oncogenesis

Doctoral thesis

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INTRODUCTI ON

Liver has extremely high regeneration ability, accommodating its size to the demands of the body. Normally, in the postnatal period a significant increase in size occurs only during ontogenesis. However, if 2/3 of rats' liver is removed surgically (partial hepatectomy), liver mass regenerates within 7 to 9 days due the compensatory hyperplasia of the remaining lobes. Though hepatocytes have a long lifespan and normally they rarely undergo cell division, they do maintain their proliferative capacity.

Investigations of growth processes in the liver were based on the study of the classical tissue unit of the organ, i.e. the lobule. During our experiments we studied changes of lobules situated just under the surface of the organ. During our measurements we took advantage on the fact that the axis of subcapsular lobules is usually perpendicular on the liver surface, and thus, after visualization of the vascular system, their size can be measured from the surface. Also, these lobules have the same localization within the hepatic structure, while lobules seen in slices display different forms and sizes.

Physiological and regenerative liver growth has already been studied on molecular level by many work teams. However, it is not exactly known what changes occur in the liver structure during the two growth processes.

During our experiments we investigated the following 2 different growth models: 1. Hepatic growth seen during ontogenesis; 2. Regeneration occurring via both hepatocytes and progenitor cells after surgical or chemical hepatectomy.

Changes of hepatic lobules may happen through 3 possible ways in all models:

1. New lobules are created;
2. Lobules size increases;
3. Both the number and size of lobules increase.

Lobules are structural – and also functional – units, and their size and number largely depends on the amount and size of cells they are built up of. Therefore, during our experiments we could not ignore the fact that possible enlargement of lobules may result from both the increase in size and in number of hepatocytes.

During ontogenesis and after simple partial hepatectomy (PH) involving 2/3 of the organ, it is the division of hepatocytes that is responsible for liver enlargement, while stem cells are not activated in the canals of Hering. However, if besides inhibition of liver cell division (by administering acetaminofluoren, AAF), mitogen stimuli are also provided, the proliferation of progenitor cells derived from stem cells is also induced. As an extension of Hering's canals, new structures with a lumen, made of cells with an oval nucleus and reminding of small biliary tracts, grow into the parenchyma. Their cells can be differentiated both into hepatocytes and cholangiocytes. After differentiation n into hepatic cells, initially small hepatocytes with basophilic nuclei cluster into groups, thus creating regenerative foci.

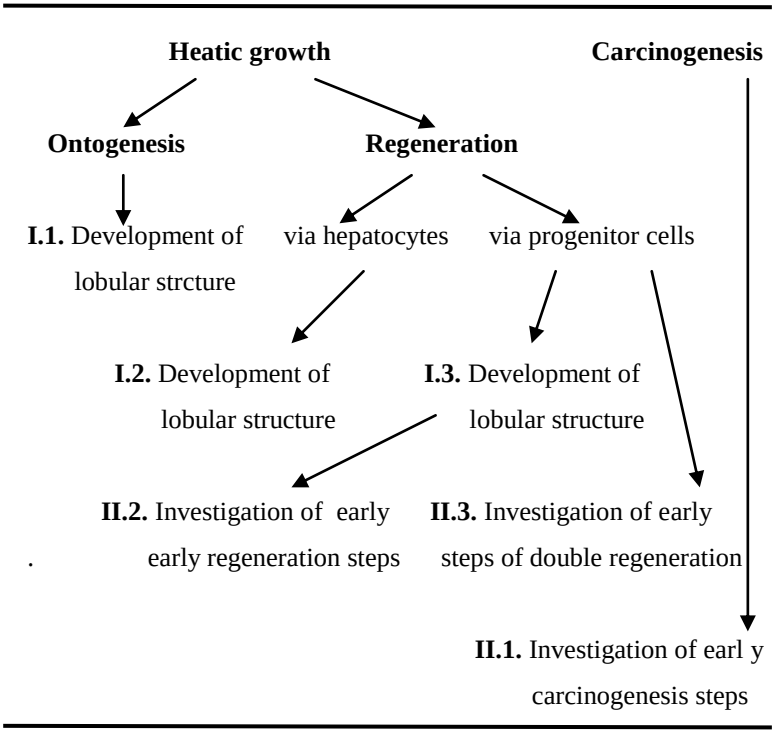
During our prior work, we often observed that when rats received carcinogenic treatment before hepatectomy, regenerative foci developed earlier and the process was completed more rapidly. This phenomenon was associated with the following observation. When

applying the well-known Solt-Farber model of carcinogenesis, we observed that after induction with diethylnitrosamine (DEN, which has a carcinogenic and mutagen potential), parallel with the regeneration of periportal and pericentral necrosis, small biliary tracts also appeared in the periportal area, then further proliferated and grew into the lobules toward the central vein.

After regeneration via progenitor cells (in the AAF/PH model) and after the lobular structure is restored, structures resembling small biliary tracts still remained in the parenchyma. It was supposed that with these two methods (1.carcinogenic induction, 2.completed regeneration) the cellular material of progenitor cells was increased, since the small biliary tracts were present as an extension of Hering's canals. According to this explanation, the liver that previously has already regenerated; the second time will regenerate more rapidly and in more foci. Structural changes in these two models – i.e. carcinogenic induction and double regeneration – were compared to changes occurring during the first weeks of single regeneration happening via progenitor cells.

During the double regeneration partial surgical hepatectomy could not be performed, thus, for the purposes of comparability and repeatability chemical hepatectomy was performed in all three models by oral administration of high dose carbon tetrachloride (CCl_4). We believed that formation and persistence of the above-mentioned small biliary tracts could be related to the fact that the dynamics of regenerative actions was different if preceded or not by DEN-induction and completed

regeneration. Thus, our experiments were meant to support the hypothesis that structures with lumen and including inactive oval cells may form and persist, which in a possible regeneration process may function as "stem cell pools", speeding up regeneration.
Thus, hepatic growth processes were studied based on the following approach:



AIMS

Our work was designed to answer the following questions:

1. **How does the lobular structure change during ontogenesis and during liver regeneration occurring via hepatocytes or progenitor cells? Is there any difference in the hepatocyte number, size or both at the end of the two different regeneration processes?**

2. **How does the size of hepatic cells change during ontogenesis and during the two different regeneration processes?**

3. **Comparison of the origin and role of foci developing during regeneration and hepatocarcinogenesis.**

METHODS

1. Experimental animals

Experiments were performed in male Fischer 344 rats reared in our institution. After surgical hepatectomy and chemical necrosis, measurement results were obtained from the right liver lobe and from the medial lobe, respectively.

2. Experimental methods

2.1. Animal study protocols

During our investigations we performed the following animal experiments:

- traditional partial hepatectomy involving 2/3 of the liver
- lobule size measurement by filling the central veins with synthetic resins
- simultaneous priming of the central and portal system – assessment of the absolute number of lobules covering the lobe, and of the number of portal branches surrounding the central vein
- AAF/PH experiment
- modified Solt-Farber model with chemical hepatectomy – DEN/AAF/CCl_4 experiment (M-S-F)
- modified AAF/PH experiment with chemical hepatectomy – AAF/CCl_4 experiment (REG-1)

- repeated regeneration - 2 x AAF/CCl_4 experiment (REG-2)

2.2. Morphometric investigations

After completing animal experiments, the following measurements were performed:

- assessment of area, circumference and number of lobules per area unit on the surface of lobes filled with synthetic resin via inferior vena cava
- assessment of hepatocyte size in the pericentral area labelled with pancitocreatin antibodies
- assessment of number and size of regenerative foci in digitalized sections labelled with streptavidin
- percentage of the area occupied by Ov6-positive small biliary tracts was measured on images taken with a confocal microscope

2.3. Immunofluorescence investigations– small biliary tract phenotyping by using different antibody combinations

2.4. Investigations related to lobule zonality – via labelling the cytochrome P450 IIE1 enzyme expressed by hepatocytes in the central and middle zone

3. Experimental systems

The above-mentioned methods were used to create the following experimental systems:

3.1. Study of changes in the lobular structure during ontogenesis and regeneration via hepatocytes or via progenitor cells

In animals with different body weights (20–250 g), after traditional partial hepatectomy involving 2/3 of the liver and 3 months after application of AAF/PH experiment (on day 84 after PH) we assessed:

- the circumference and area of lobules situated under the surface of the liver
- the number of lobules,
- the circumference and area of pericentral hepatocytes

Furthermore, in the last 2 experiments the number of portal branches surrounding one central vein was also determined, and zonality assessments were also performed.

3.2. Comparison of early steps of hepatocarcinogenesis and liver regeneration

Three experimental groups were created to compare the hepatocarcinogenesis model (modified Solt-Farber model) and the early steps of regeneration occurring via progenitor cells:

1. Modified Solt-Farber model with chemical hepatectomy, M S-F (DEN/AAF/CCl_4)
2. Modified AAF/PH experiment with chemical hepatectomy – REG-1 (AAF/CCl_4)
3. Repeated regeneration, REG-2 (2xAAF/CCl_4)

After CCl_4-probing the following parameters were assessed in all three models:

- area occupied by small biliary tracts,
- number and area of regenerative foci,
- characterization of ductule immunophenotypes
- tumour formation after 12 months from the start of experiment

In the first model, the area occupied by small biliary tracts was also measured after DEN injection, as well.
In the third model animals have rested for 3 months between the two AAF/CCl_4 treatments.

RESULTS

1. Changes in lobular structure of the, liver during ontogenesis and during liver regeneration occurring via hepatocytes or progenitor cells

During **ontogenesis** the size of lobules was measured in animals of different ages. During the investigated period, their body weight and the volume of the right lateral liver lobe increased by a factor of about 12, compared to animals with a body weight of 20 g. Lobule circumference and area showed an increase of more than 2,5 times and more than 5 times, respectively. The number of lobules situated under the surface increased by about 30%, while the number of portal branches did not change when comparing the two investigated time points (animal weighing 50 g and 160 g, respectively). In this period, the size of hepatocytes also increased; while during the investigated later stages of ontogenesis the size of liver cells did not change.

During the liver regeneration process occurring after PH, and thus with participation of hepatocytes both the area and circumference of lobules gradually increased during the first 7 days, while in the following 3 weeks increase was insignificant. The number of lobules covering the surface of the liver did not change significantly compared to the controls weighing 160 g, while the number of portal branches surrounding the lobule did increase. The size of liver cells did not change.

Regeneration occurring via progenitor cells was characterized 3 months after the AAF/PH experiment. Compared to the control organ the volume of the investigated lateral lobe increased more than 3 times and almost 5 times on day 28 after PH, and after AAF/PH, respectively. Parallel with increase in volume, the size of lobules and hepatocytes slightly exceeded the size seen after PH. The number of lobules covering the surface did not change, while the number of portal branches surrounding the lobule increased in the same manner as after PH.
In both cases, lobule structure has also changed during regeneration: central veins situated under the surface became longer and displayed more branches, while the prior zonal distribution of the citP450 enzyme took a segmented character.

2. Investigation of early histological events during regeneration and hepatocarcinogenesis

In all three models, the extent and phenotype of Ov6-positive biliary tracts present before and appearing after mitogen stimulation, as well as the number and size of regenerative foci were investigated during the comparative investigation of regeneration and hepatocarcinogenesis.
In our experiments we found that the amount of small intralobular biliary tracts significantly increased both after DEN treatment and in the liver of animals who have already undergone an AAF/CCl_4 cycle (REG-2). (2 times and more than 1.5 times, respectively.) In the REG-1

model, after chemical hepatectomy we found the same proportion of Ov6-positive ductuli as in control livers.
In the M S-F model, the highest number of oval cells was seen on day 6 after carbon tetrachloride treatment, after which their number continuously decreased, reaching the baseline value on day 14.
In the REG-2 model, the dynamic of oval cell formation was similar as in the M S-F model, but the area occupied by oval cells after treatment was larger throughout.
In the REG-1 model, structures formed by oval cells started to proliferate significantly only on day 12, and on day 14, proliferation was still present and continued further on.
In the M S-F and REG-2 models foci appeared first on day 4 after chemical hepatectomy, while in the REG-1 model the appeared only on day 10. The number of foci was the highest in the M S-F model. Also, during double regeneration about two times as many foci developed by day 14 as in REG-1 model.
In the M S-F and REG-2 model, a parallel increase of focus diameter was seen, while in the REG-1 model foci apparently developed slower and remained smaller in size during the investigated period.

2.1. Further characterization of immunophenotypes of cytokeratin (Ov6) positive ductuli

Cells of intraparenchymal ductuli developing on DEN treatment did not express DLK (delta-like protein), AFP-(α-fetoprotein) and CK7 (citokeratin-7). Initially, they were accompanied by SMA (α-smooth muscle actin) and

desmin positive activated myofibroblasts. SMA positivity gradually disappeared, and after 3 months it was no more detectable. Desmin positive cells were always present around the small biliary tracts.
In the ductuli forming after AAF/CCl_4 treatment – just before the second chemical hepatectomy – AFP, DLK, CK7 negative epithelial cells were surrounded by desmin positive cells, which however did not express SMA.
In all three models, intraparenchymal ductuli developing after chemical hepatectomy were AFP, DLK positive and CK7 negative, while accompanying myofibroblasts proved to be SMA and Des positive.

3. Tumour formation in the investigated models

In the modified Solt-Farber model, after 12 months tumour formation was observed in most animals, while in the REG-2 model that proved to be almost identical as regarding the early histological events and the regeneration process, cell proliferation occurred only in 1 single case. In the REG-1 model livers remained intact.

DISCUSSION

1. Investigation of hepatic lobular structure during ontogenesis

As a result of a complex process, liver volume increases more than ten times by reaching adulthood compared to its birth value. We developed a new method for assessment of size and number of lobules occupying identical places in the liver hierarchy, which is based on the observation that axes of “surface lobules” situated under the liver capsule are parallel with each other. By priming sinusoid veins via central veins that are running through the middle of lobules, we were able to visualize lobule borders.

According to our investigations, in the early stages of ontogenesis the number of lobules situated under the surface increases, since new lobules develop. Afterwards however, although liver volume increases significantly, the number of lobules does not change, additional liver growth is possible only by modification of their size.

The presence of pancytokeratin antibody in hepatocytes results in a characteristic membrane staining, which helps to visualize cell borders, especially on using immunofluorescence technique, and thus enables accurate evaluation of cell size. According to our measurements, the size of hepatocytes also increases in rat livers in the early stages of ontogenesis; later however, no additional growth can be seen.

2. Investigation of lobule size and number in rat liver after partial hepatectomy

After surgical removal of certain lobes of the clearly segmented rat liver (partial hepatectomy), liver mass restores through growth of the remaining lobes. The original lobular structure of the liver is definitely maintained during regeneration, as well. Since during regeneration the volume of remaining lobes increases by many times, this process has to be accompanied by formation of new lobules and/or by increase in lobule size. Three months after partial hepatectomy, when liver volume and histological structure were already restored, an increase in lobule size on the surface of the studied lobe was seen, lobule number however, did not change. Thus, during traditional regeneration occurring with participation of hepatocytes, liver volume increased only through the increase in lobule size. However, on cell level the situation is exactly the opposite, liver growth occurs through the division of liver cells and not via their enlargement.

However, when comparing regenerated lobules with the intact liver some subtle changes were seen: in regenerated livers central veins showed a branching pattern under the surface; the number of portal branches surrounding one central vein increased; and the distribution of the studied (cytochrome P450 IIE 1) enzyme that was produced zonally has also changed.

3. Characterization of the regenerated liver in the AAF/PH experiment

If for certain reasons hepatocytes cannot contribute to the replacement of the destroyed liver mass, progenitor cells

in the liver are activated, and the original structure is restored through the resulting differentiation process. In one of the most widely used experimental systems (i.e. the AAF/PH model), a complex tissue reaction occurs in the days following hepatectomy, during which lobular structure is blurred, because of the intensive proliferation of oval cells.

28 days after the experiment the same changes were seen in the regenerated liver as in the regeneration process described above, occurring with the participation of hepatocytes.

However, some subtle differences were seen in livers regenerating differently. 28 days after the AAF/PH experiment, ductular structures with narrow lumen were seen in the parenchyma of the enlarged lobules. Their localization was reminding of bunches of oval cells that were present in large numbers in earlier stages of the experiment, but cell morphology was rather consistent with that of smaller biliary tracts of intact livers. The reason for this phenomenon is probably that not all oval tubes become differentiated.

4. Characterization of intraparenchymal ductular structures

Ductular structures described above were also seen three months after single DEN treatment, as well as in the AAF/CCL_4 model, at a similar late time point. Unlike oval cells, ductuli proved to be AFP and DLK negative, and were not surrounded by SMA positive, activated ITO cells/myofibroblasts, they showed however, strong Ov-6 positivity, just like the epithelial cells of biliary tracts. The canals of Hering that were accommodating stem

cells were differentiated from other biliary tracts by their CK7 negativity. The CK7 reaction proved to be negative in this case too. Accordingly, intraparenchymal ductuli that were present in regenerated livers after single DEN treatment or following the AAF/CCL_4 experiment were similar to canals of Hering seen in intact livers.

5. Effects of the amplification of the progenitor cell compartments on liver regeneration

In order to investigate the role of larger numbers of parenchymal ductular structures in the regeneration process, they were induced with the above-mentioned two methods (DEN treatment and progenitor cell mediated regeneration) and then liver was forced to regenerate (repeatedly in the second case). Surgical hepatectomy was replaced with a single high dose CCl_4-treatment causing central necrosis. Then these two models were compared with animals that did not have parenchymal ductuli at the beginning of experiment. Both investigated parameters, i.e. oval cell proliferation and foci have appeared earlier in animals in which ductuli number was increased with prior treatment. Thus, by extending and amplifying the stem cell compartment liver regeneration can be accelerated in rats.

Of the 3 models, after 12 months survival, significant tumour formation (90%) was seen only in the modified Solt-Farber experiment. Thus, amplification of stem cell compartment in itself does not represent a carcinogen risk.

To our knowledge this is the first *in vivo* experimental model to demonstrate that liver regeneration can be made more effective by increasing the number of stem cells.

CONCLUSIONS

I. During ontogenesis, it is both the increase in number and enlargement of hepatic lobules that contributes to the increase in liver volume. In early stages of ontogenesis an increase in hepatocyte size also promotes the enlargement of lobules.

II. Also, during regeneration occurring with the participation of hepatocytes and progenitor cells, liver volume restores only by an increase in lobule size. New lobules do not form.

III. During regeneration triggered by administration of necrotising doses of diethylnitrosamine or by using the AAF/CCl_4 experimental system, some ductular structures appear in the hepatic parenchyma, the cells of which are similar to liver stem cells as regarding their phenotype and function.

IV. The regeneration process is accelerated by the amplification of liver stem cell compartment.

PUBLICATIONS

1. According to the dissertation

1. 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.

2. 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.

3. Dezső K, Papp V, Bugyik E, Hegyesi H, Sáfrány G, Bödör C, Nagy P, Paku S. (2012) Structural analysis of oval cell mediated liver regeneration in rats. Hepatology 56:1457-67.

2. Other publications

1. Dezső K, Paku S, Papp V, Turányi E, Nagy P. (2009) Architectural and immunohistochemical characterization of biliary ductules in normal human liver.
Stem Cells Dev. 18(10):1417-22.

2. Dezso K, Bugyik E, Papp V, László V, Döme B, Tóvári J, Tímár J, Nagy P, Paku S. (2009) Development of arterial blood supply in experimental liver metastases.
Am J Pathol. 175(2):835-43.