Architectural and immunohistochemical characterization of small bile ducts harboring hepatic adult stem cells

Doctoral theses

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CONTENTS

I INTRODUCTION 3
I.1. The microanatomy of the liver 3
I.2. Hepatic stem cells 4
I.3. Thy-1 expression 4

II. OBJECTIVES 5

III. MATERIALS AND METHODS 6
III.1. Animal experiments 6
III.2. Human tissues 6
III.3. Morphological analysis 6
III.4. Colocalization analysis 6
III.5. Electronmicroscopy 6
III.6. Gene expression analysis 7

IV. RESULTS 8
IV.1. Architecture and immunophenotype of the bile duct in rat liver 8
IV.2. Architecture and immunophenotype of the bile duct in human livers 8
IV.3 Thy-1 expression in healthy livers 9
IV.4. Thy-1 expression during regeneration 10

V. CONCLUSIONS 11

VI. PUBLICATION RECORD 12

I INTRODUCTION

I.1. The microanatomy of the liver

The mammalian liver seemingly has a simple structure, with only three basic structural components:

(1) The portal triad consists of the following structures: portal vein, hepatic artery and interlobular bile duct,

(2) Central or hepatic veins,

(3) the hepatic parenchyma situated between these vascular structures.

The classical functional unit of the liver is the hepatic lobule. The "classic" liver lobule has a sixsided polyhedral shape with portal triads at each of the corners. The long axis of the lobule is traversed by the central vein. In rats an anastomotic network of the sinusoids at the edge of the lobule is contributing to the uniform blood distribution of the hepatic lobule. In human livers a blood vessel containing structure, the so called „vascular septum" is outlining the hepatic lobules. Reports about the terminal branches of hepatic artery, their entrance into the vascular septum have been disputed without finally conclusion. The bile is collected in bile canaliculi, which merge to form bile ducts. The canals of Hering represent the smallest, most distal tributary of the biliary tree outlined by basement membrane. Although they were recognized as anatomic structures for more than a century, the canals of Hering have not been the subject of systematic study. This may be related largely to the fact that they can not be identified on routine sections and therefore are not easily amenable for study. The cells of these structures are the primary candidates for the liver-residing adult hepatic stem cell, so this could be the so called „hepatic stem cell niche". This explains the tremendously increased interest for these ductules nowadays. Electron microscopy has shown that these ductules have a partial lining of cholangiocytes and hepatocytes, and they are outlined by continuous „U" shaped basement membrane. Unfortunately, they have no specific immunophenotypic or other morphological marker. They can be identified only from their position as an interface between the hepatocytes and larger bile ducts.

I.2. Hepatic stem cells

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 turnover; they become activated only when hepatocytes are compromised and are unable to respond to proliferative stimuli. This is why they are also called facultative 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. In rat livers the progenies of stem cells are called oval cells, in human these cells are the intermediate hepatobiliary cells.
Several rodent experimental models have been established for investigation of oval cells. One of the most widely-used experimental protocols in rats is the AAF/PHx model. In the human livers the so called atypical ductular reaction, even if it is morphologically different, is regarded as the equivalent of the rodent oval cell proliferation.
The oval cell and the intermediate hepatobiliary cells form ductules and are surrounded by laminin-positive basement membrane.
Hepatic progenitor cells in rodents and human liver are well characterized and they express several neuroendocrine, fetal hepatocyte, adult hepatocyte and biliary cell markers.

I.3. Thy-1 expression

Thy-1, a hemopoietic stem cell marker was reported to be present in rat liver on the oval cells in stem cell-mediated liver regeneration. Later, a precursor-product relationship was described between bone marrow stem cells and oval cells in experimental models. Several groups have confirmed the Thy-1 expression on oval cells, resulting in the extensive use of Thy-1 as a cell surface marker to sort out liver progenitor cells.
At the same time, other authors described mesenchymal cells coexpressing Thy-1 and smooth muscle actin (SMA) in similar experimental settings questioning the identity of the Thy-1-expressing cells in the liver.

II. OBJECTIVES

In our study, we set out to find an immunphenotype specific to the canals of Hering making possible their equivocal identification within the biliary tree.

I. Characterization of hepatic stem cell compartment harbouring the canals of Hering in human and rat livers:

I.1. Analysis the architecture of the biliary ductules by confocal microscopy;
I.2. Analysis the immunophenotype of the biliary ductules.

II. In order to identify precisely the location of Thy-1 in normal and damaged liver we performed a detailed morphological examination:

II.1. Analysis of Thy-1 expression in healthy human and rat livers;

II.2. Analysis of Thy-1 expression in regenerating livers:

II.2.a. In AAF/PHx model, stem cell mediated liver regeneration in rats
II.2.a. In atypical ductular reaction containing human liver samples.

III. MATERIALS AND METHODS

III.1. Animal experiments

Male F-344 rats were treated according to the AAF/PHx protocol. Groups of three animals were sacrificed in different timepoints after PHx. After resection of the liver, snap frozen liver samples were stored at −70°C.
Animal protocols were approved by the ethical committee of the Semmelweis University.

III.2. Human tissues

Normal human liver specimens were collected from cadavers of spontaneous premature birth neonates without developmental abnormalities and individuals who died suddenly in accidents without morphological signs and anamnestic data of any liver disease. Snap-frozen human liver specimens for immunohistochemical examination were obtained also from two patients who underwent orthotopic liver transplantation because of fulminant liver failure of unknown etiology.
The procedure was approved by the ethical committee of the Semmelweis University (TUKEB 141/2005).

III.3. Morphological analysis

Frozen sections were used for immunohistochemistry. We characterized the canals of Hering in rat and human livers by indirect fluorescent immunohistochemistry.
To examine the architecture of the small bile ducts we used serial sections.
To indentify the exact localisation of the Thy-1 protein we used triple immunofluorescent stainings.

III.4. Colocalization analysis

Colocalization analysis was performed using the Image J program (National Institutes of Health, Bethesda, MD, USA).

III.5. Electronmicroscopy

In order to determine in details the localisation of Thy-1 positive cells we performed electronmicroscopy and immunoelectronmicroscopy.

III.6. Gene expression analysis

To analyse the mRNA expression, oval cell populations were microdissected by PALM MicroBeam laser microdissector.
mRNA was isolated from dissected oval cells, healthy liver samples and whole liver samples of the AAF/PHx experiment. After reverse transcription the cDNA samples were analysed by quantitative real-time PCR, using the ΔC_T method. AFP, SMA, and Thy-1 expression were determined as a ratio to GAPDH expression.

IV. RESULTS

IV.1. Architecture and immunophenotype of the bile duct in rat liver

Analysis of the bile duct immunophenotype has shown that larger interlobular bile ducts surrounded by continuous basement membrane were stained for CK19 and also with CK7. However, certain small biliary structures situated at the edge of the portal area, remained unstained with CK7 antibodies.
The canals of Hering, recognized from the U-shaped laminin staining, were consistently negative for CK7 albeit they were stained by CK19 antibody.
Their unique CK19+/CK7− immunophenotype has made their identification within the biliary tree easier.
Following these CK7-negative ductules through serial sections we could distinguish three main types of these ductules, based on their branching pattern. The 3 types were represented roughly equally. These ductules never entered into the liver lobule through the limiting plate.

IV.2. Architecture and immunophenotype of the bile duct in human livers

In human liver all the biliary structures were positive for CK7 as well as for CK19. Opposed to the rat liver, no CK7-negative biliary cells were observed.
There are several proposed markers for hepatic progenitor cells in human liver, but most of them did not distinguish hepatic ductules of the vascular septa from larger interlobular bile ducts in our hands. Some of the markers (AFP, chromogranin, synaptophysin, DMBT, DLK, CEA, CK20, CK14) did not label any biliary structures, while others (EpCAM, E-cadherin, CK7, CK19) stained the complete biliary tree. Only three markers reacted differentially with bile ducts and ductules. Epithelial membrane antigen (EMA) resulted in a very sharp characteristic linear apical staining in the interlobular bile ducts. Conversely, it was absent in the small ductules even on cross sections. The staining pattern of CD133 and CD56 was opposite.
CD133 and CD56 stainings were strictly confined to the small ductules of the vascular septa.
High power examination of individual biliary ductules showed that these structures showed a kind of perilobular arrangement. The canals of Hering

leave the periportal space and spread into the liver parenchyma along rudimentary interlobular septa outlining the hepatic lobules.
They spread until the half of the porto-portal distances along the vascular septa which resulted in watershed-like gaps in the middle of these stretches. We could demonstrate the presence of interlobular connective tissue septa in a rudimentary form in healthy livers. The canals of Hering run in these septa in line with the terminal branches of the portal vein and hepatic arteries. The canals of Hering can be identified by a unique CD56+/CD133+/EMA– antigen immunophenotype.

IV.3 Thy-1 expression in healthy livers

In healthy rat livers Thy-1 expression was detectable and confined to the periportal region by immunohistochemistry. Thy-1 antibody decorated intensely and sharply the cross sections of peripheral nerves around the portal area elements. There was some faint cloudy staining around the major interlobular bile ducts. This cloudy staining related with one layer of Thy-1 positive cells, situated outside the basement membrane, and it was not present around small bile ducts.
Serial sections stained for Thy-1 desmin and smooth muscle actin showed: desmin antibody reacted with nonparenchymal cells inside the liver lobule in addition to the muscular wall of the blood vessels, peribiliary vascular plexus and scattered single cells in the periportal connective tissue. Conversely, no SMA-positive cells were seen inside the liver lobule; only the blood vessels and the peribiliary vascular plexus were stained. Thy-1 positive staining observed around larger interlobular bile duct could not be seen by desmin and SMA antibodies. There were scattered exclusively Thy-1-positive cells in the portal area. The co-localization of desmin Thy-1 and smooth muscle actin were seen only in the wall of peribiliary vascular plexus.
Thy-1 expression was analysed in healthy human liver samples at different age groups. The examined bile ducts were always Thy-1 negative.

IV.4. Thy-1 expression during regeneration

In rat liver treated according to the AAF/PHx protocol oval cell formed ductules invaded the liver lobules. These ductules were surrounded by continuous basement membrane and Thy-1 reaction was observed consistently outside the basement membrane.
Immunoelectronmicroscopic examination also revealed Thy-1 positive long cell processes running clearly outside the basement membrane.
Thy-1 molecule is bound weakly to the cell membrane. The so called „shedding" characteristic of this protein is well known. To exclude the oval cell origin of the Thy-1 positivity outside the basement membrane we performed QRT-PCR analysis. When microdissected oval cells from AAF/PHx-treated animals were examined, no expression of Thy-1 or SMA mRNA was detectable despite higy level of AFP transcripts which are the most reliable markers of oval cells.
However, Thy-1 and SMA mRNA expression could be demonstrated in RNA extracted from whole liver sections.
The pattern of Thy-1 imunostaining was reminiscent of the localization of stellate cell/myofibroblast, which also could be found outside the basement membrane. Therefore, in the rat liver we performed co-staining of Thy-1/SMA and Thy-1/desmin, the two most widely used stellate cell/myofibroblast markers. In the co-staining experiments, Thy-1 showed frequent colocalization with SMA. Eighty-one percent of the Thy-1-positive areas stained with SMA, and 58% of the SMA-positive field was decorated by Thy-1. Thy-1 positivity hardly overlapped with desmin; the value of the colocalization index was 6.8%.
Thy-1 antibody also decorated cellular elements and long processes outside the basement membrane in human livers with extensive ductular reactions due to fulminant hepatic failure.

V. CONCLUSIONS

I. The canals of Hering are the primary candidates for the liver residing adult hepatic stem cell compartment. They can be identified in rat/human livers due to their special arrangement and their unique immunophenotype.

I.1. In rats a CK19+/CK7– cholangiocyte population is present in the smaller branches of the biliary tree including the canals of Hering. These structures are strictly confined to the periportal space.

I.2. In human livers the canals of Hering outlining the hepatic lobules, run in rudimentary vascular septa in line with the terminal branches of the portal vein and hepatic arteries. The canals of Hering can be identified by the unique CD56+/CD133+/EMA– immunophenotype.

II. Thy-1 is expressed in healthy as well as in regenerating rat and human liver:

II.1. In healthy rat and human livers Thy-1 antibody decorated scattered single SMA and desmin negative cell in the periportal space, which most likely correspond to the portal fibroblast; Peripheral nerves branching were also Thy-1 positive.

II.2.a. No Thy-1 expression can be detected in oval cells during the stem cell-mediated rat liver regeneration. Therefore, Thy-1 can not be used as a cell surface marker for isolation of oval cells from liver. Instead, Thy-1 is produced by a subpopulation of periductal cells, most likely myofibroblasts.

II.2.b. The intermedier hepatobiliary cells are also Thy-1 negative in ductular reaction of human liver and Thy-1 can be demonstrated in myofibroblast like cells.

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