New therapeutic strategies for canine liver disease

Growth factors and liver progenitor cells

Brigitte Arends

Cover illustration: Background: primary liver cells in culture. Sculpture (Hubert von der Golz): picture made by Brigitte Arends in Alphen aan den Rijn .
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New therapeutic strategies for canine liver disease
Growth factors and liver progenitor cells

Nieuwe therapeutische strategieën voor leverziekten bij de hond
Groeifactoren en progenitor cellen
(met een samenvatting in het Nederlands)

Proefschrift

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Contents

General introduction

1. Liver regeneration

The extra-ordinary capacity of the liver to regulate its growth is a long known and widely accepted phenomenon which has been an intriguing topic from Greek legends to medical science nowadays. Together with bone and skin, the liver is a unique organ that regulates its own mass after loss of (or damage to) functional liver cells. After surgical resection of a part of the liver, the original mass will be restored from as little as twenty-five percent remaining liver within two weeks. The tremendous capacity of the liver to regulate its own mass is further illustrated by whole-liver transplantation into larger or smaller recipients, as the liver precisely regulates its size relative to the host organism. For instance, livers from large animals (dogs) transplanted to smaller animals decrease in size, while the opposite is also true as shown with livers from small to large dogs and from baboons to humans [1, 2]. These findings indicate that liver mass is meticulously regulated and that the body produces signals that regulate liver mass until the desired set-point has been reached. The underlying mechanism of liver regeneration is an intricate process involving multiple factors on both the cellular and molecular level. Understanding the molecular mechanisms of liver regeneration could lead to improved therapies for the treatment of acute or chronic liver disease, in which the regeneration capacity is lost. In this chapter, an overview is given of liver regeneration at the cellular and molecular level. Furthermore, therapeutic potential of this knowledge for chronic diseases in a clinical setting is discussed.

1.1 Liver regeneration after a partial hepatectomy (phx)

The most common model to investigate liver regeneration is a partial hepatectomy (phx), a surgical procedure by which one or more lobes of the liver are removed [3]. After phx, the undamaged remnant liver lobes grow in size equivalent to the original liver mass, so that the restored liver is an enlarged version of the remnant liver lobes. The term ‘regeneration’ refers to the hyperplastic response of the liver, because the remaining lobes increase in size and the removed liver lobes will not grow back. A complete restoration of liver mass is seen even after removal of seventy-five percent of the liver. This capacity to grow is practically unlimited as full restoration occurs even after multiple sequential phxs [4]. The phx model is reproducible in terms of mass removed and precision in timing of the sequence of ensuing events. Liver regeneration after toxin-induced liver failure occurs in a similar way, but proliferation of the different cell types is not as synchronized as in the phx model. The phx model is therefore the preferred approach for the experimental study of liver regeneration and has provided invaluable information about the regenerative response of the ‘healthy’ liver.

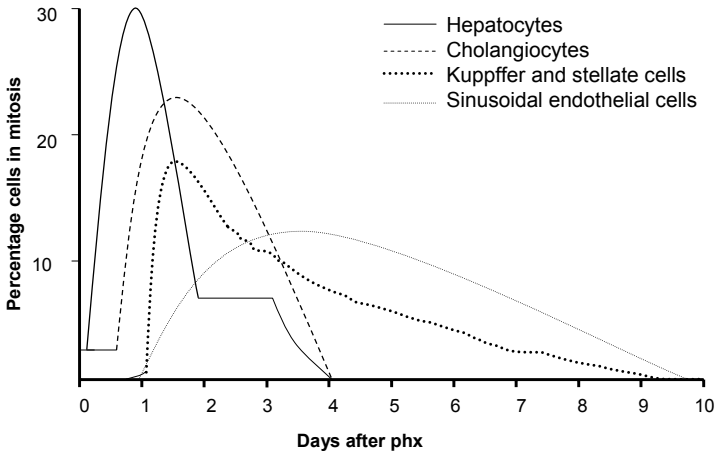

Figure 1. Proliferation of different cell types during liver regeneration after a phx in the rat. The different major cell types undergo DNA synthesis at different times. DNA synthesis peaks at 24 hours for hepatocytes, whereas the other cell types proliferate at later time points [5].

After a phx, a rapid response of cellular division proceeds in an orderly fashion, involving all the mature cell populations of the liver [5]. Normal liver consists of two major epithelial cell types, hepatocytes and biliary epithelial cells (cholangiocytes). The hepatocytes are the parenchymal (functional) cells of the liver and make up around eighty percent of the hepatic cells. Other cell types include mesenchymal cells such as stellate cells and Kupffer cells (macrophages) as well as endothelial cells. During liver regeneration, the different cell types do not divide simultaneously. However, the timing of each cell type to enter DNA synthesis is cell autonomous (Figure 1) [6]. After a phx, hepatocytes are the first cells to enter the DNA synthesis. Proliferation of cholangiocytes and endothelial cells occurs a little later when compared to hepatocytes (Figure 1) [7].

The time at which cell proliferation occurs differs among species. In the rat, hepatocytes simultaneously undergo one round of DNA synthesis which peaks at 24 h. When all hepatocytes in the liver equally participate in growth, the hepatocytes divide on average 1.7 times after a 2/3 phx. A second (smaller percentage) of hepatocytes enter into a second round of DNA synthesis after 36-48 hours and establish the original number of hepatocytes. The peak of hepatocyte proliferation and the subsequent events are frame-shifted 6–12 hours in mouse and 48 hours in man and dog [5]. Generally cellular proliferation begins in the periportal region (i.e. around the portal triads) and proceeds towards the pericentral areas [8]. Whether this is due to a gradient of available growth factors or to other reasons is not fully known.

Three to four days after phx, liver histology is characterized by clumps of small hepatocytes that become rearranged into the typical hepatocyte plates seen in the mature liver. A slow lobular reorganization takes place for several weeks. Liver histology eventually becomes indistinguishable from the original situation [5, 9]. A key endpoint of liver regeneration is the restoration of the total number and mass of hepatocytes. Although the initiation of cellular proliferation is well studied, the termination phase and reorganisation are still under study.

1.2 The contribution of liver progenitor cells in liver regeneration

Tissue specific stem cells, or progenitor cells, are immature cells that are resident in mature organs. These cells remain the capacity to produce daughter cells that are capable to proliferate and differentiate into the required cell type [10]. The existence of progenitor cells has been generally accepted in bone marrow [11], intestines [12, 13], and the epidermis [14, 15] for a long time. However, the existence of such progenitor cells resident in the mature liver has been questioned. Healthy livers have a little cellular turnover of hepatocytes and cholangiocytes and the liver does not operate as a typical stem cell lineage-dependent system like that of other self renewing tissues [16, 17]. Furthermore, liver regeneration after a phx is carried out by proliferation of the mature cellular populations in the liver and does not rely upon tissue specific stem cells.

The excistence of liver specific stem cells that act as a ‘reserve cell compartment’ was first described in mice after severe dietary injury [18]. Subsequently, the proliferation of these ‘unusual’ non-parenchymal epithelial cells with stem cell properties was described in regenerating rat livers after proliferation of mature hepatocytes was inhibited by the treatment of carcinogenic agents [19]. These cells were called oval cells, referring to their typical oval shaped morphology. Oval cells initially have an authentic biliary epithelial appearance with the expression of biliary cytokeratin intermediate filaments and hepatocytic characteristics such as alpha-fetoprotein expression and a low albumin synthesis [16, 17]. Upon activation, oval cells proliferate and migrate into the periportal and midzonal parenchyma, where they differentiate into hepatocytes or cholangiocytes; a process that is called ductular reaction [20].

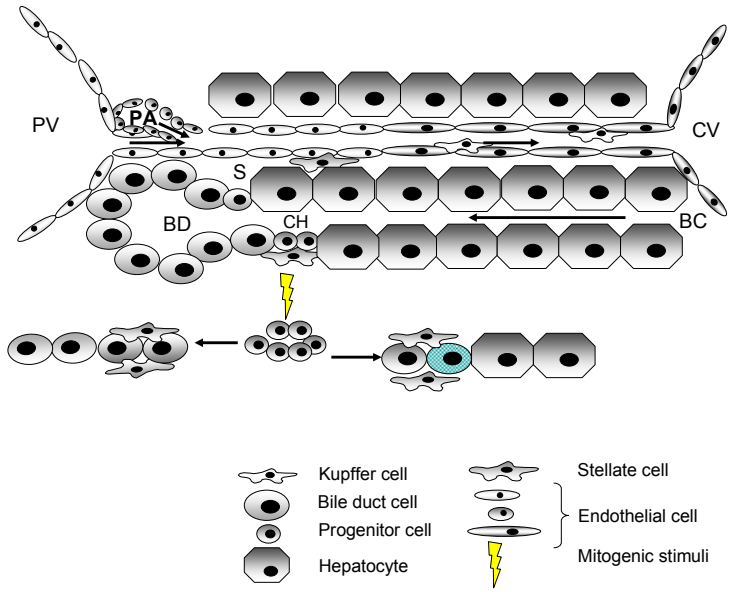

Figure 2. In the liver, the canals of Hering and bile ductuli, both originating from the ductal plates in the embryo, functions as minute local progenitor cell niches. PV: portal vein; BD: bile duct; BC: bile canaliculus; CV: central vein; CH: canal of Hering; S : sinusoid.

The evidence for the existence of a human counterpart of rodent oval cells emerged from studies of liver injury, regeneration, and carcinogenesis in human liver [21-23]. Human oval cells are called liver progenitor cells (LPCs). LPCs remain in a quiescent and undifferentiated state in healthy liver, yet they proliferate to produce daughter cells that will differentiate into the required (damaged) cell type (hepatocyte or cholangiocyte), in diseased liver (Figure 2). Activation of LPCs is observed in liver diseases where the large regenerative capacity of the mature liver cells is inadequate, such as subacute hepatitis with severe hepatocellular necrosis due to chronic viral hepatitis, chronic biliary hepatitis, primary biliary cirrhosis, alcoholic and non-alcoholic fatty liver diseases, and in cirrhotic livers [24, 25]. In healthy livers, LPCs are present in scarce numbers in their stem cell niche; the smallest and the most peripheral branches of the biliary tree (the canals of Hering; Figure 2) [26]. The stem cell niche defines the microenvironment of a stem cell, and is essential for the maintenance and activation of a stem cell population. Stromal cells (such as the stellate cells), and non-cellular components (such as extracellular matrix components) are considered central in the regulation of the stem cell niche of the liver.

2. Molecular mechanisms of liver regeneration

Liver regeneration is a complex interplay of different factors, and many growth factors and cytokines have been implicated in the regulation of liver growth [27]. Most of the genes involved in the regeneration process can be classified into three distinct phases; the initiation phase, progression phase, and the termination phase (Figure 3).

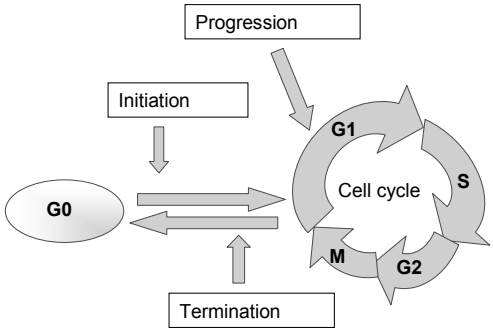

Figure 3. Stages in regeneration in relation to cell cycling [28].

1) During the initiation phase, the liver is being prepared for entering the regeneration process. To allow subsequent cell divisions, extracellular matrix (ECM) is remodelled and degenerated, a process that is associated with changes in the urokinase plasminogen activator (uPA) system and extracellular matrix proteases (metallomatrix-proteases, MMPs) [29, 30]. Due to ECM remodelling, matrix bound mitogens are released and activated. Hepatocytes in intact, healthy livers are in the resting (G0) phase of the cell cycle. During the initiation phase, hepatocytes are prepared to enter the G1 phase of the cell cycle, where they become sensitive to growth factors (priming). Furthermore, gene expressions need to be modified so that hepatocytes remain their homeostatic functions, while rendering in a state of replicative competence [31]. Overall, the initiation phase induces a rapid induction of more than one-hundred immediate early genes not expressed in normal liver within a few minutes after phx. Most of these genes encode for transcription factors, such as c-jun, c-myc, and c-fos [32-34]. Other transcription factors that gained increased attention are nuclear factor kappa B (NFκB), Signal Transduction and Activator of Transcription-3 (STAT-3), and CCAAT/enhancer-binding proteins (C/EBPs) [35, 36]. Important cytokines for the priming process are IL6 (activates multiple intracellular pathways, including STAT-3) and tumour necrosis factor-α (required for a normal proliferative response after a phx) [37].

2) During the progression phase, growth factor levels rise and signalling pathways leading to cell cycle become activated. Growth factors are required for a progression of the cell cycle beyond the initiation phase. Potential mitogens of the liver can be screened in primary hepatocyte cultures. Direct mitogens of hepatocytes in culture are growth factors such as hepatocyte growth factor (HGF) and ligands of the epidermal growth factor receptor (EGFR) such as epidermal growth factor (EGF) and transforming growth factor-α (TGFα) [38]. High levels of HGF, EGF, or TGFα induces hepatocyte proliferation and liver enlargement in intact mice and rats [39-42]. The effect of these growth factors is largely enhanced, when animals are subjected to a relatively small (30%) phx, which is the threshold to affect liver function, indicating the necessity of the initiation phase during liver regeneration [43].

3) During the termination phase, cell growth is suppressed when liver mass is restored. The molecular mechanism by which the regenerative process is halted remains speculative. The most likely candidate is transforming growth factor-β1 (TGF-β1). TGF-β1 binds to the type II receptor, which recruits and phosphorylates a type I receptor. The type I receptor then phosphorylates downstream effectors of the SMAD protein family [44]. SMAD proteins are transcription factors that, once activated, translocate to the nucleus and regulate transcription of target genes that are important for many cellular processes, such as programmed cell death and the accumulation of ECM components [45]. TGF-β1 is a potent inhibitor of hepatocyte growth in culture, and is suggested to play a role in retaining hepatocytes in the G0 phase in quiescent livers. After phx, TGF-β1 levels rise early in the regeneration process and expression is maximal at the time that cell division stops [46]. The TGF-β1/SMAD pathway is antagonized by two transcriptional repressors, SnoN and Ski. Complexes between SnoN, Ski, and the activated SMAD proteins were detected during the proliferative phase in regenerating liver, and decreased after liver mass restitution. This observation suggests that activated SMAD proteins might participate in returning the liver to a quiescent state [47].

2.1 Hepatocyte growth factor (HGF)

Hepatocyte growth factor (HGF) is a multifunctional growth factor, originally identified in the plasma of partially hepatectomized rats as a potent mitogen for hepatocytes in primary culture [48, 49]. After phx, HGF levels in plasma increase 10 to 20 fold, indicating the essential role of HGF in liver regeneration [50]. Indeed, blocking or silencing the receptor for HGF diminishes or completely abrogates the regenerative response of the liver [51, 52]. The regeneration response induced by HGF can not be compensated by other mechanisms. Interestingly, the blockade of other transduction pathways, such as TGFα did not result in a similar inhibition of liver regeneration. Considering the impact of elimination of its receptor, the signalling by HGF appears as a major contributor to liver regeneration [27].

HGF is a large, 90 kDa glycosylated protein consisting of a 56 kDa α-chain and a 34 kDa β-chain. The β-chain contains a hairpin structure and four kringles, whereas the α-chain is a serine protease. The hairpin and the adjacent two kringles are essential structures for the typical functions of the HGF molecule. In the liver HGF is synthesized and secreted by cells of mesenchymal origin [53] and hepatic endothelial cells [54] as an inactive single-chain precursor, that needs to be activated by proteolytic cleavage. The HGF receptor (c-MET) is a tyrosine kinase receptor of 190 kDa with two disulfide-linked subunits, a 50 kDa extracellular α-chain and a 145 kDa β-chain. After HGF binding, autophosphorylation in the kinase domain of c-MET (Tyr^{1234}, Tyr^{1235}) induces a downstream located multifunctional docking site (Tyr^{1349}, Tyr^{1356}) in the C-terminal domain [55-57]. The activation of c-MET mediates signalling to multiple transducers, including the Akt/PKB, STAT-3, and ERK1/2 (one of the Ras-mitogen-activated protein (MAP) kinases) mediated pathways [58]. These transduction pathways are involved in different aspects of liver regeneration, such as mitogenesis, motogenesis, morphogenesis, anti-apoptosis, and induction of angiogenesis (Figure 4). HGF exerts is effect upon epithelial and endothelial cells in an endocrine and/or paracrine manner. The pleiotrophic effects of HGF are mediated via different signalling pathways, which may differ in various epithelial and endothelial cells [59, 60].

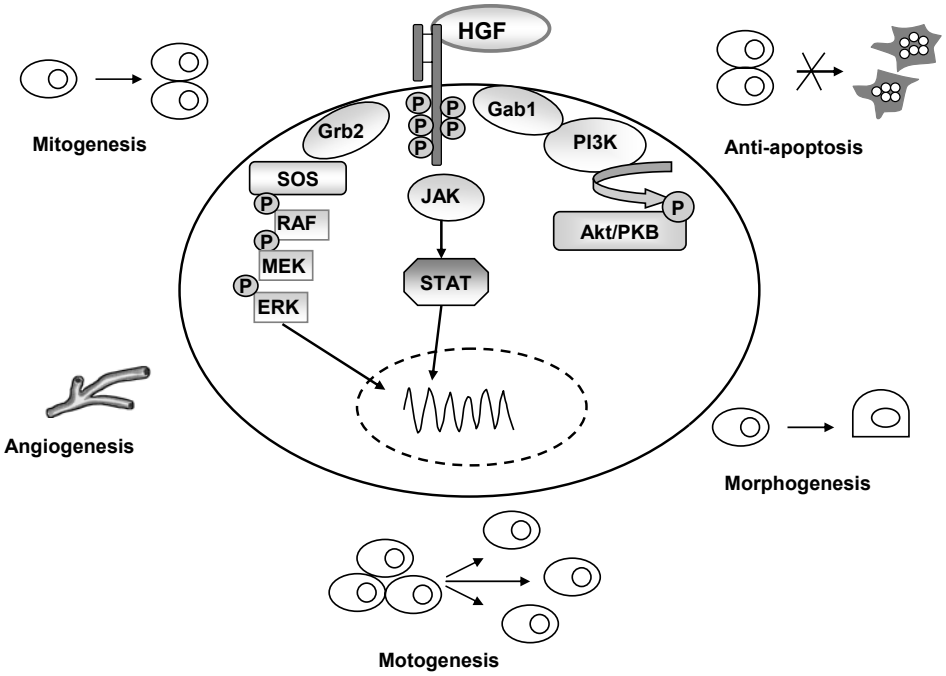

Figure 4. Signal transduction pathways of c-MET. After activation, HGF binds to its receptor c-MET. Autophosphorylation in the kinase domain of c-MET induces a downstream located multifunctional docking site in the C-terminal domain mediating in signalling to multiple transducers, including the Akt/PKB, STAT-3, and ERK1/2 mediated pathways.

3. Tissue repair; combining cellular and molecular knowledge into therapies

Liver injury of any aetiology will lead to the secretion of potent mediators of the inflammatory response (reactive oxygen species (ROS), eicosanoids, nitric oxide, carbon monoxide, TNFα, and other cytokines) by macrophages and infiltrated inflammatory cells. A prolonged elevation of ROS and cytokines ultimately leads to liver injury, and stellate cell activation [61]. Activated stellate cells undergo transdifferentiation to fibrogenic myofibroblast like cells, the main cell type that is responsible for the accumulation of ECM (collagens, proteoglycans, glycoproteins) in liver fibrosis and cirrhosis [62, 63]. Among the molecular mediators involved in fibrogenesis, TGF-β1 and platelet-derived growth factor (PDGF) are thought to play a central role [64-66]. Severe fibrosis of the liver is associated with a delayed, incomplete hepatic regeneration, leading to poor restoration of liver function, and morbidity in the course of a chronic liver disease [67-69]. Liver regeneration could be stimulated by counteracting the negative balance of cytokines that induces oxidative stress or activate stellate cells, by adding growth factors that either reduce oxidative stress and/or inhibit cytokines that lead to the activation of stellate cells. Furthermore, stimulating remaining viable liver cells in severe chronic and in acute hepatitis, such as the liver progenitor cells, would potentially be life-saving and could eventually lead a reduced need for liver transplants.

3.1 HGF for liver diseases

HGF is one of the principal factors stimulating the liver to grow and regenerate and reduces the susceptibility of hepatocytes to toxic and oxidative damage. Furthermore, HGF induces growth inhibition and enhanced apoptosis in portal myofibroblasts, stimulates collagenase expression in hepatic stellate cells, and interferes with PDGF and TGF-β1 signalling [70-72]. The findings with respect to the crucial counteracting effects of HGF and TGF-β1 in liver growth, regeneration, and fibrosis have stimulated researchers to investigate the regeneration potential of HGF in experimental animals after hepatotoxic or fibrogenic drugs were given. In rodent models treated with hepatotoxins remarkable positive effects have been shown. Administration of HGF did nearly or completely prevent or, when given afterwards, reverse the toxic effects. Even chronic damage with already formed disruption of the normal micro-architecture of the liver by fibrous septa (cirrhosis) was largely reversed after HGF treatment [73-78]. In addition, the normally fatal course of fulminant hepatitis (severe acute hepatic necrosis) was prevented by HGF [79, 80]. Therefore, the clinical use of HGF in the treatment of fatal liver disease including fulminant hepatic failure and cirrhosis holds great potential.

3.2 LPC for liver diseases

The discovery of adult progenitor cells and their contribution to liver regeneration in several liver diseases has provided a new option of approach in the treatment of currently untreatable liver diseases. The great potential of LPCs in clinical hepatology is underlined by the fact that the outcome of acute hepatitis is directly related to the amount of progenitor cells and subsequent intermediate hepatocytes (lineage between LPCs and mature hepatocytes) present in the liver [81]. The amount of LPC activation is a sign of disease severity and occurs early (within one week) in the course of disease. However, the development of intermediate hepatocytes is often too late for survival of the patient. Therefore, strategies to reach an increased growth of LPCs and/or faster differentiation could be beneficial in the acute phase after fulminant hepatitis. Liver regeneration could be stimulated by injecting diseased livers with progenitor cells [82, 83], or by stimulating the endogenous progenitor cells to proliferate and/or differentiate. To reach these potential applications the mechanisms of activation (proliferation and differentiation) need to be understood in detail. Interactions between progenitor cells and relevant other cells of the progenitor cell niche, such as stellate cells, could be studied in *in vitro* co-culture systems. Furthermore, cell culture could be used for the expansion of progenitor cells as a strategy to acquire more cells than the patient may generate *in vivo*. However, cell isolation and culturing has been hampered by the lack of LPC specific markers and their scarce numbers in healthy livers. The search for (a specific combination of) markers for LPCs, or to find alternative isolation techniques to permit cell culturing is currently an important research topic in hepatology.

One of the alternative isolation techniques could be achieved by using the increased resistance of LPCs to normally toxic substances, most likely due to the presence of ABC transporters such as ABCG2 (BCRP) [84]. These transporters can be used for cell isolation by their ability to efflux the fluorescent dye Hoechst 33342. Cells that efflux Hoechst 33342 can be visualized in a dual-wavelength emission plot as a characteristic side-branch profile during fluorescence-activated cell sorter (FACS) analysis and are called side-population (SP) cells [85]. SP cells with a greatly enriched haematopoietic stem cell potential were first isolated from mouse bone marrow [86, 87]. Subsequently, SP cells with enriched progenitor cell potential were identified in several other tissues such as skeletal muscle, heart, brain, spleen, kidney, lung, small intestine, and liver [88]. Isolating SP cells from the liver could therefore be a strategy to isolate LPCs from the liver without the use of specific markers.

4. The dog; patient or an animal model?

Liver diseases occur quite frequently in dogs; approximately 1% of the referred population to the university clinic for companion animals in Utrecht suffers from liver disease. Similar to man, the various forms of canine hepatitis vary from acute to chronic hepatitis and cirrhosis, and have a time-course development that is comparable to man, as well as a high similarity at the clinical, and pathological level. Hepatitis is not as well defined in the dog as in human liver disease in terms of the aetiology which, for many cases of canine hepatitis, is unknown. However, a world consensus statement, addressing clinical and histo-pathological diagnostic criteria of canine liver diseases such as fulminant, acute, and chronic hepatitis, as well as cirrhosis, provides researchers with a well defined, uniform terminology and diagnostic criteria that allow a comparison of canine diseases with similar human liver disorders [89].

The application of new therapies developed in experimental rodent models for human clinical liver diseases requires a translational bridge. Dogs would well fulfil this need since canine liver diseases are spontaneous and resemble human liver pathologies in great detail. Domestication of dogs started thousands of years ago [90]. Therefore, both man and dog are exposed to the same environmental and micro-biological stressors that can affect liver function. There are more advantages for using the dog as a model organism in translational physiology. The human genome appeared to have much higher degree of homology with the genome of the dog compared to the mouse genome [91]. Despite the fact that most dog breeds were selected during the last 100 years, the genetic flow is much greater than in laboratory mice which are uniform inbred strains. The complexity in human liver diseases is therefore better reflected in the clinical dog population.

Several publications proposed the use of canine spontaneous diseases as large mammalian models for man [92, 93]. The increased interest in the dog as a model would accelerate the laborious process of designing new therapies which will ultimately benefit both man and dog. Although ethical restrictions still apply, the restrictions would be considered lower in the dog models compared to human clinical practice. Therefore, it is expected that translation from basic research into clinical practice is fairly rapid in veterinary medicine. In the end the use of dog patients can lead to a reduction in the amount of experimental animals.

5. General aims of this thesis

The present thesis is focused on the development of and search for novel therapeutic strategies to stimulate liver regeneration in canine liver diseases which are relevant for comparable human diseases. The specific aims are:

1. To make a molecular characterization of different forms of hepatitis and cirrhosis in dogs in comparison to human hepatitis/cirrhosis with respect to the activation of regenerative and fibrotic pathways.
2. To produce and characterize recombinant HGF and to measure its *in vitro* and *in vivo* activity.
3. To start the first clinical trial in which the effect of recombinant HGF on the liver and the kidneys in dogs with congenital portosystemic shunting is examined.
4. To establish primary cell culture conditions for a long term culture of primary canine liver cells and to characterize colony-forming epithelial liver cells in culture with respect to their differentiation potential
5. To isolate progenitor cells from perfused canine liver cells by using their ability to efflux the fluorescent dye Hoechst-33342.

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Major HGF-mediated regenerative pathways are similarly affected in human and canine cirrhosis.

Major HGF-mediated regenerative pathways are similarly affected in human and canine cirrhosis.

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Abstract

The availability of non-rodent animal models for human cirrhosis is limited. We investigated whether privately-owned dogs (*Canis familiaris*) are potential model animals for liver disease focusing on regenerative pathways. Several forms of canine hepatitis were examined: Acute Hepatitis (AH), Chronic Hepatitis (CH), Lobular Dissecting Hepatitis (LDH, a specific form of micronodulair cirrhosis), and Cirrhosis (CIRR). Canine cirrhotic samples were compared to human liver samples from cirrhotic stages of alcoholic liver disease (hALC) and chronic hepatitis C infection (hHC).

Canine specific mRNA expression of the regenerative hepatocyte growth factor (HGF) signaling pathway and relevant down-stream pathways were measured by semi-quantitative PCR and Western blot (STAT3, PKB, ERK1/2, and p38-MAPK). In all canine groups, levels of c-MET mRNA (proto-oncogenic receptor for HGF) were significantly decreased ($p < 0.05$). Surprisingly, ERK1/2 and p38-MAPK were increased in CH and LDH. In the human liver samples Western blotting indicated a high homology of down-stream pathways between different etiologies (hALC and hHC). Similarly activated pathways were found in CIRR, hALC, and hHC.

In canine hepatitis and cirrhosis the major regenerative downstream pathways were activated. Signaling pathways are similarly activated in human cirrhotic liver samples, irrespective of the differences in aetiology in the human samples (alcohol abuse and HCV-infection). Therefore, canine hepatitis and cirrhosis could be an important clinical model to evaluate novel interventions prior to human clinical trials.

Comparative Hepatology 2007, 6: 8

Introduction

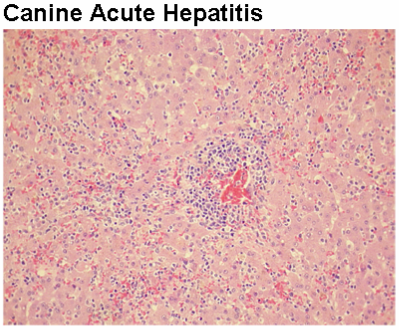
Chronic hepatitis (CH) and end-stage cirrhosis (CIRR) are an increasing medical problem, affecting over 5% of the world population [1, 2]. The best-studied animal model for these liver diseases is tetracarbon-induced fibrotic liver diseases inflicted in rats [3]. Many more models have been devised to mimic liver diseases in man; the time-course of the development however, is not always comparable to the human situation [4-8]. Furthermore, the variability in the affected human population such as age, social factors, eating behaviour, body weight etc. is not fully covered in standardized laboratory conditions.

Dogs have liver diseases which are clinically highly comparable with the human counterparts and both species have a high resemblance at the genetic level [9, 10]. In contrast to rodent models, hepatitis in these dogs is not deliberately induced. Previous studies already showed a high resemblance between man and dogs in the formation of fibrosis during liver diseases [11]. Furthermore dogs share many years in close proximity of humans exposing them to the same environmental and biological stresses. Recently, detailed clinical and histological diagnostic standards have been published for all liver diseases of dogs [12]. Corroborating the histological similarities between human and dogs, examples are provided in Figure 1. Taken together, these similarities suggest the dog as a model animal for liver diseases in man [13].

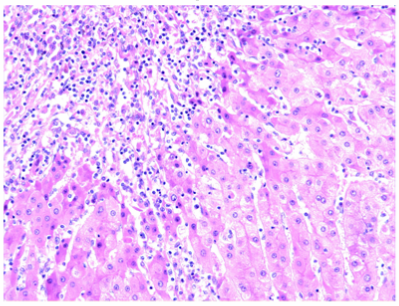
Liver regeneration is a complex interplay of different factors [14]. One of the main growth factors identified in liver regeneration is Hepatocyte Growth Factor (HGF). HGF activates the proto-oncogenic receptor tyrosine kinase c-MET and subsequent down-stream pathways, including the anti-apoptotic protein kinase-B (PKB/Akt) cascade, the proliferative MAP-kinase pathway (ERK1/2 and p38MAPK), and the STAT3 signaling (signal transducers and activators of transcription) [15-17]. At present, a comparison between these regeneration signal transduction pathways in human and canine livers is missing, hampering the application of dogs as (pre-) clinical model animals for human medicine.

We have analyzed HGF-mediated regeneration signaling in canine samples from dogs with Acute Hepatitis (AH), Chronic Hepatitis (CH), and Lobular Dissecting Hepatitis (LDH, a specific form of micronodulair cirrhosis similar to neonatal hepatitis in human hepatology) [18, 19], and CIRR. The cirrhotic samples were compared to two human cirrhotic diseases with different etiologies; HCV-induced (hHC) and alcohol-induced (hALC). This study will elucidate the potential of (non-experimental) dogs to bridge between toxin-induced rodent models and human clinical situation.

Canine Acute Hepatitis



Human Acute Hepatitis



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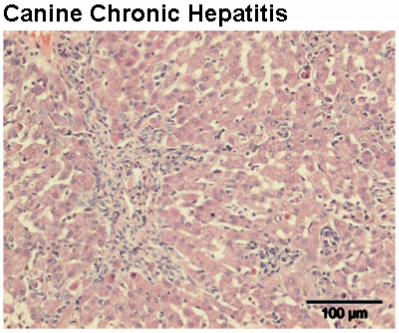
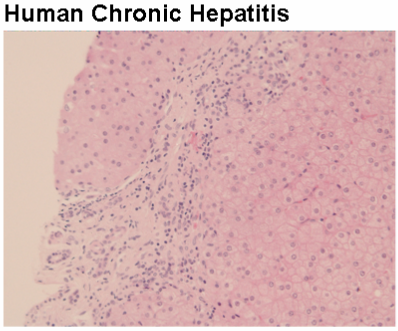
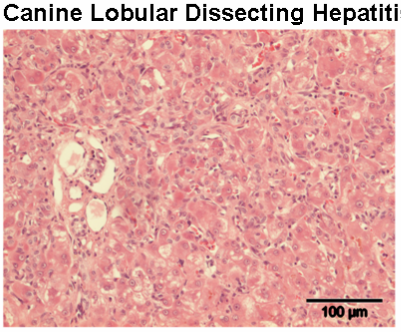


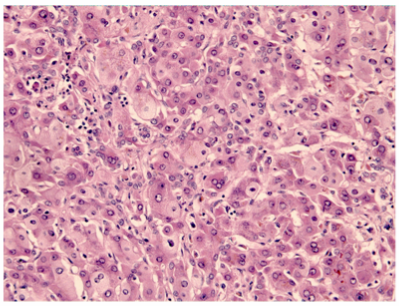
Human Chronic Hepatitis



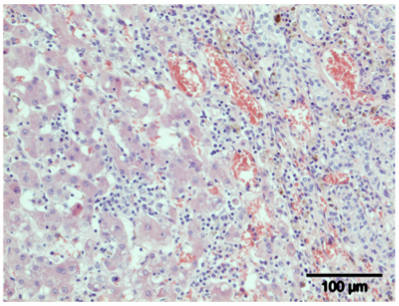
Canine Lobular Dissecting Hepatitis



Human Micronodulair Cirrhosis



Canine Cirrhosis



Human Cirrhosis

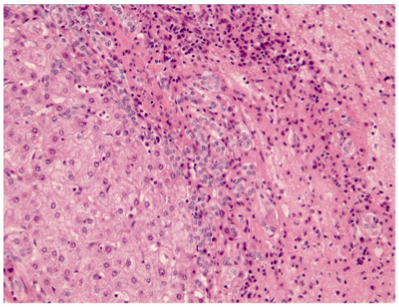


Figure 1. Examples of histological samples (HE) from canine and human liver diseases**.** Canine acure hepatitis: Marked infiltration of mononuclear and granulocytic inflammatory cells and focal necrosis. Canine chronic hepatitis: Small fibrous septum with interface hepatitis as well as lobular hepatitis; several apoptotic bodies. Canine lobular dissecting hepatitis: Lobular dissection by fibroblasts and ductular proliferation and a slight inflammatory infiltrate. Canine cirrhosis: Broad fibrous septum with ductular proliferation and a marked inflammatory infiltrate with interface hepatitis. Human acute hepatitis: Marked infiltration of mononuclear and some granulocytic inflammatory cells and some apoptotic bodies. Human chronic hepatitis: Fibrous septum with some ductular proliferation and slight interface hepatitis. Human micronodular cirrhosis: Dissection of the parenchyma by small fibrous septa, slight ductular proliferation and inflammation; hepatocytic ballooning, Mallory bodies, and apoptosis. Human cirrhosis: Broad fibrous septum with ductular proliferation and a marked inflammatory infiltrate with interface hepatitis.

Methods

Animals.

All samples were obtained from privately owned canines of different breeds referred to our veterinary clinic. All procedures were approved by Utrecht University's Ethical Committee, as required under Dutch legislation. Each disease group (n = 11 dogs) was compared to age-matched healthy control dogs (n = 12), without clinical signs of hepatitis or other disease (histopathology did not reveal any abnormalities). Liver biopsies were obtained (ultrasound-guided) from all dogs under local anaesthesia with a true cut 14G biopsy needle, preceded by ultrasonographic evaluation of the liver to exclude non-homogeneous hepatic changes. Two formalin-fixed biopsies were embedded in paraffin, sliced, and stained with hematoxylin and eosin-, van Gieson-, and reticulin-stain according to Gordon and Sweet. All histological examinations were performed by one experienced, certified veterinary pathologist. Two other biopsies were snap-frozen and stored at -70°C until molecular analysis.

Human patients.

All liver samples were obtained from surgical patients transplanted at the Department of Abdominal Transplantation in the University Hospital Leuven, Leuven, Belgium. The procedures were approved by Leuven University's Ethical Committee, as required under Belgian legislation. Human explant samples were collected directly after surgery and immediately snap-frozen. All patients, predominantly male, were presented with micronodular cirrhosis. The Alcoholic Cirrhosis (hALC) group contained five patients (n = 5) characterized by cirrhosis with neutrophil infiltrations, alcohol related morphological changes (hepatocyte ballooning, Mallory bodies, necrosis), and in some cases steatosis and increased iron deposition. The Hepatitis C (hHC) group contained four patients (n = 4) characterized by

cirrhosis with neutrophil infiltrations, lymphoid follicles and aggregates. All cases were presented with hepatocyte decay, apoptosis/necrosis, regeneration, and fibrosis.

Table 1. Nucleotide sequences of dog-specific primers for real-time quantitative PCR

Gene	F/R	Sequence (5'-3')	Tm (°C)	Product size (bp)	Accession number
GAPDH	F	TGT CCC CAC CCC CAA TGT ATC	58	100	AB038240
	R	CTC CGA TGC CTG CTT CAC TAC CTT			
HPRT	F	AGC TTG CTG GTG AAA AGG AC	56	100	L77488 /
	R	TTA TAG TCA AGG GCA TAT CC			L77489
HGF	F	AAA GGA GAT GAG AAA CGC AAA CAG	58	92	BD105535
	R	GGC CTA GCA AGC TTC AGT AAT ACC			
c-MET	F	TGT GCT GTG AAA TCC CTG AAT AGA AAT	59	112	AB118945
	R	CCA AGA GTG AGA GTA CGT TTG GAT GAC			

Immunoblot analysis.

Twenty micrograms of pooled protein extracts (n = 6 dogs per group, randomly chosen) was separated by SDS-PAGE, and transferred to a Hybond ECL nitrocellulose membrane (Amersham-Biosciences, Cleveland, OH). The procedure for immunodetection was based on an ECL-Western blot analysis system, as previously described [20]. Primary antibodies are depicted in Table 2. Densitometric analysis was performed with a Geldoc2000 system with QuantityOne 4.3.0 software (Biorad). Human samples (protein load twenty micrograms) were analyzed separately; the immunoblot analysis was the same as for the dog protein extracts.

Quantitative PCR.

Quantitative real-time PCR (Q-PCR) was performed on HGF and c-MET. The abundance of mRNA was determined by reverse transcription followed by real-time quantitative PCR using appropriate primers (Table 1), as described previously [21]. For each experimental sample, two endogenous reference genes (GAPDH and HPRT) were included. The use of two reference genes is sufficient for reliable data [22]. Results were normalized according to the average amount of the endogenous references. The relative gene-expression of each gene-product was used as the basis for all comparisons. The results were assessed for normality and homogeneity of variances using, respectively, the Kolmogorov Smirnoff and the Levene tests. The data were normally distributed and variances complied; therefore differences between means were determined by One Way Analysis of Variance (ANOVA) followed by

the (2-sided) Dunnett post-test analysis of all groups towards control (healthy) samples. A *P*-value < 0.05 indicated significant changes.

Table 2. Primary antibodies used in Western blot experiments

Antibody	Manufacturer	Product size (kDa)	Dilution	Secondary antibody	dilution
HGF	Santa Cruz (sc-1357)	80	1:1,000	Anti-goat	1:20.000
c-MET	Santa Cruz (sc8307)	145	1:1,000	Anti-goat	1:40.000
p-c-MET [a]	Abcam (ab5662)	169	1:1,000	Anti-rabbit	1:20.000
STAT3	BD Biosciences 610189)	86	1:2,500	Anti-mouse	1:20.000
p-STAT3 [b]	Cell Signaling (9134)	86	1:1,000	Anti-rabbit	1:20.000
p-STAT3 [c]	Cell Signaling (9138)	86	1:1,000	Anti-mouse	1:20.000
PKB	BD Biosciences 610876)	60	1:250	Anti-mouse	1:20.000
p-PKB [d]	Cell Signaling (9275)	60	1:1,000	Anti-rabbit	1:20.000
Erk1/2	Cell Signaling (9102)	42/44	1:1,000	Anti-rabbit	1:20.000
p-ERK1/2 [e]	Cell Signaling (9101)	42/44	1:1,500	Anti-rabbit	1:20.000
p38-MAPK	Abcam (ab19329)	38	1:500	Anti-rabbit	1:20.000
p-p38-MAPK [f]	Abcam (ab4822)	38	1:1,000	Anti-rabbit	1:20.000
Beta-actin	Neomarkers (pan Ab-5)	42	1:2,000	Anti-mouse	1:20.000

[a] Phospho Tyr1230, Y1234, Y1235.
[b] Phospo Ser727
[c] Phospho Tyr705
[d] Phospho Thr308.
[e] Phospho Thr202, Y204
[f] Phospho Thr180/Tyr182

Results

HGF/c-MET signaling pathways involved in liver regeneration in dogs with AH, CH, CIRR, and LDH.

Statistically significant differences (ANOVA) were identified in the expression of mRNA encoding HGF ($P < .001$) and c-MET ($P < .001$). Towards healthy control samples HGF mRNA levels were significantly induced in CH, LDH, and CIRR, three-, five-, and five-fold, respectively (Figure 2A). In AH, HGF mRNA levels remained unchanged toward healthy control. Western blot analysis on HGF protein showed the presence of a HGF band (80 kDa) in all canine samples. There is a clear quantitative correlation between HGF protein and mRNA levels, as both HGF mRNA and protein levels are increased in CH, LDH, and CIRR. The c-MET mRNA levels in all groups were significantly decreased towards control, with a maximum four-fold reduction in AH (Figure 2B). Total c-MET protein levels are similarly reduced as the mRNA levels. Analysis on phosphorylated c-MET showed an immuno-reactive band in CH, LDH, and CIRR, which was almost absent in AH.

Western blot analysis on STAT3, PKB/Akt, ERK1/2, and p38MAPK in canine liver homogenates.

In Figure 3 important downstream signaling proteins of HGF/c-MET (STAT3, PKB/Akt, ERK1/2, and p38-MAPK) are depicted. The total STAT3 was detected as an immuno-reactive 86 kDa band in all hepatic diseases, with slightly lower expression in CH, LDH, and CIRR. Tyrosine (Tyr)-phosphorylated STAT3 was strongly reduced in AH and was increased in LDH. Healthy controls, CH, and CIRR groups had comparable levels of Tyr-phosphorylated STAT3. Serine (Ser)-phosphorylated STAT3 was slightly decreased in CIRR. PKB/Akt plays a pivotal role during regeneration and growth. Total PKB/Akt was detected in all hepatic diseases as a single 60 kDa protein. Threonine (Thr)-phosphorylated PKB/Akt was less present in AH and CIRR, whereas CH and LDH showed no apparent quantitative differences toward healthy controls. Analysis of total ERK1/2 (42/44 kDa), a principle kinase in growth factor signaling, showed a slight reduction in AH, and moderate increases in CH, LDH, and CIRR. The phosphorylated form threonine/tyrosine (Thr/Tyr)-phosphorylated ERK1/2 was strongly increased in CH and LDH. A similar effect can be seen in p38-MAPK, an increased expression of total p38-MAPK in CH, LDH, and CIRR and decreased levels in AH. The threonine/tyrosine (Thr/Tyr)-phosphorylated p38-MAPK form is increased in CH, LDH, and CIRR.

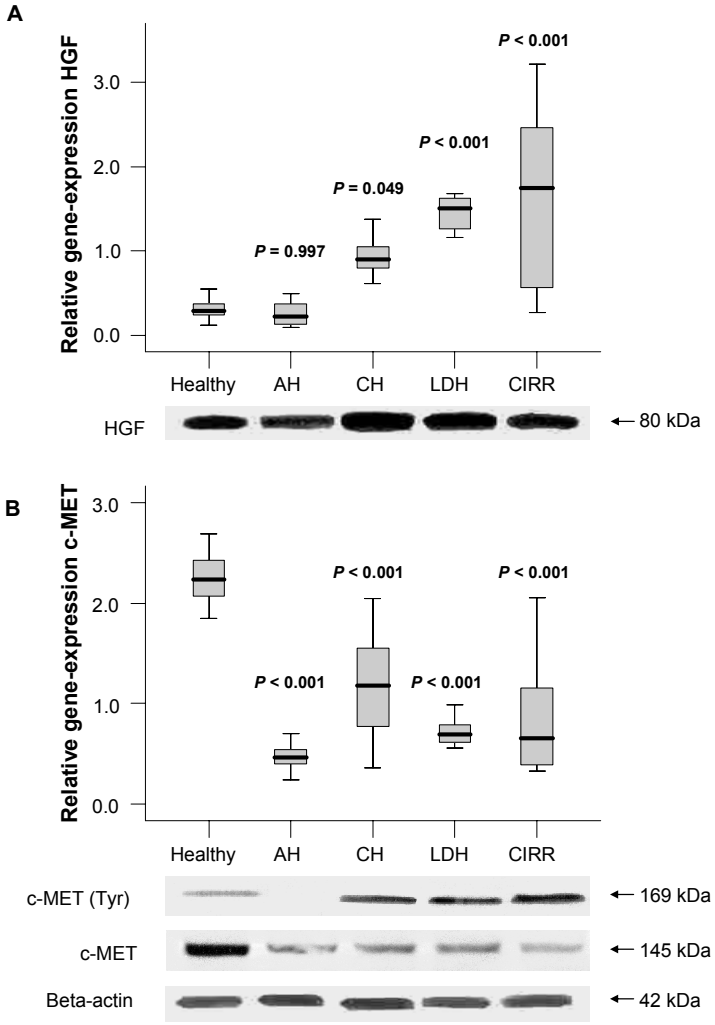


Figure 2. Quantitative Real-Time PCR of genes involved in regeneration and growth**.** Expression analysis of acute hepatitis (AH), chronic hepatitis (CH), lobular dissecting hepatitis, cirrhotic samples (CIRR). HGF expression and detection of the 80 kDa HGF protein is shown in (A). c-MET gene expression and detection of c-MET including phosphorylated (Tyr) form is shown in (B). Relative gene expression data represent mean + SE. A Kolmogorov-Smirnov test was performed to establish a normal distribution and a Levenes test for the homogeneity of variances. All samples included in this study were normally distributed. The statistical significance of differences between diseased and control animals was determined by using the two-sided Dunnetts post-test analysis. A *P*-value < 0.05 was considered statistically significant. Analysis was performed using SPSS software (SPSS Benelux BV, Gorinchem, the Netherlands).

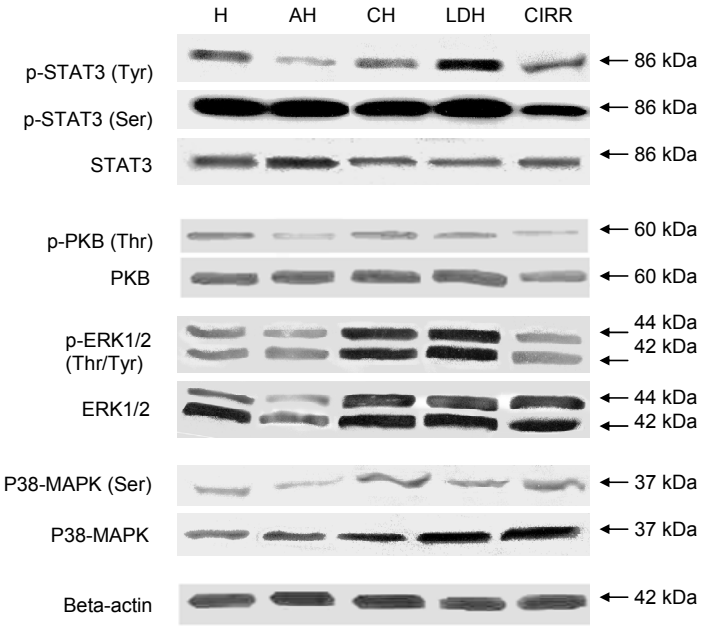


Figure 3. Western blot analysis of pooled canine liver homogenates of acute hepatitis (AH), chronic hepatitis (CH), lobular dissecting hepatitis (LDH), and cirrhotic samples (CIRR). Detection of the 86 kDa STAT3 protein, the 60 kDa PKB protein, the 44/42 kDa ERK 1/2 protein, and the p38-MAPK protein are shown. Beta-actin was used as a loading control.

Western blot analysis on human cirrhotic explant samples after alcohol abuse (hALC) and after hepatitis C virus infection (hHC).

Western blot analysis on HGF in hALC and hHC samples showed a detectable 80 kDa HGF in all samples with minor quantitative differences (Figure 4). The 145 kDa c-MET was also detected in all samples. The phosphorylated form of c-MET (Tyr) was detected in all samples. Total PKB/Akt was detected in all samples. The phosphorylated PKB (Thr) indicated moderate to high levels in most cirrhotic samples. Total STAT3 was detectable in all individual samples. STAT3 (Tyr) was detected in all groups although two samples in the hHC group were less phosphorylated. In general the serine phosphorylated STAT3 is lower in the hALC group compared to the hHC group. Total ERK1/2 was detected in all samples with no apparent quantitative differences. Phosphorylated ERK1/2 was detectable in all samples with different degrees of phosphorylation. Both total p38MAPK as well as the serine phosphorylated form was detected in all samples with no apparent quantitative differences.

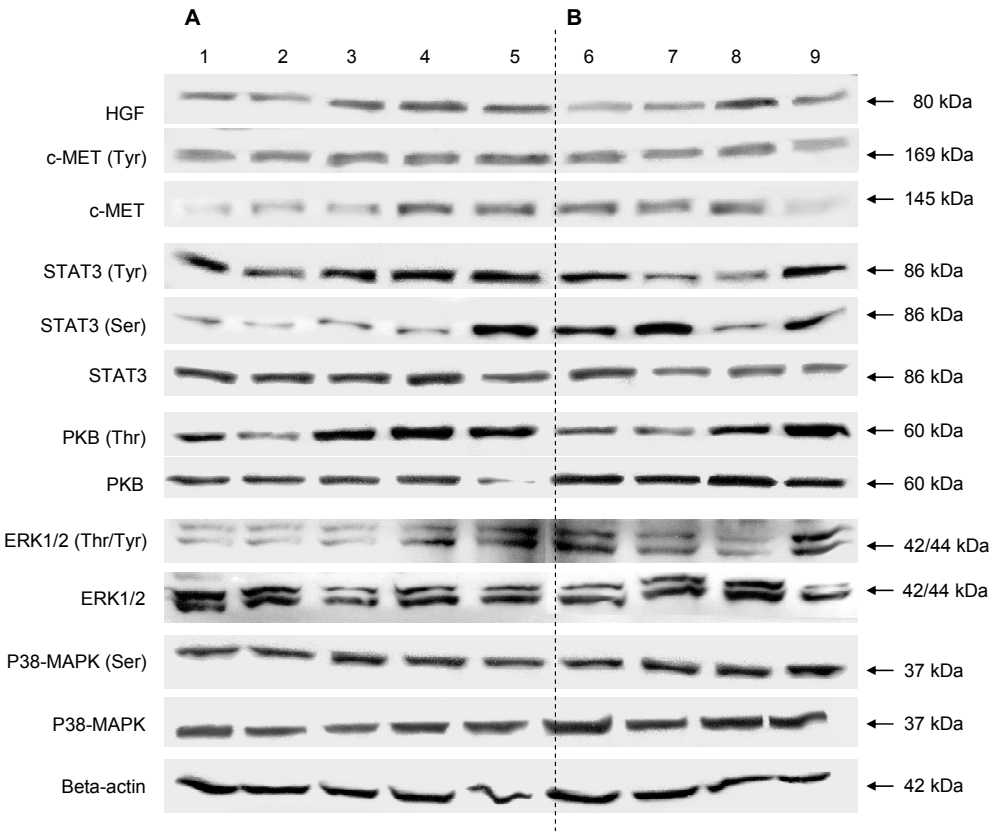


Figure 4. Western blot analysis of human liver homogenates of several diseases (n = 6)**.** Samples 1 to 5 represent individual alcoholic cirrhosis (hALC) shown in (A), samples 6 to 9 represent individual hepatitis C induced cirrhotic samples (hHC) shown in (B). Beta-actin was used as a loading control.

Discussion

To investigate if hepatic regenerative signal transduction pathways are similarly affected in liver diseases between man and dogs, the expression of HGF and c-MET was measured. Furthermore, Western blot analysis was used to show (de)activation of important signaling pathways of liver regeneration. This provided insight into major regeneration pathways in AH, CH, LDH, CIRR. A comparison between canine cirrhosis and human cirrhotic samples with different etiologies provided additional information on the suitability of the canine-model.

The increased mRNA levels of HGF and the decreased levels of c-MET mRNA in fibrotic canine diseases (CH, LDH, and CIRR), were in line with publications from human samples [24]. Together this suggests that HGF-mediated regeneration in human cirrhosis is similarly affected in canine CH, LDH, and CIRR. The reduced c-MET protein levels indicate a utilization of c-MET which is degraded through endocytosis after phosphorylation. Intracellular degradation is dependent on the interaction with Cbl ubiquitin ligases [25]. Semi-quantitative hepatocyte proliferation studies on canine samples with a Ki67 antibody indicate a moderate hepatocyte proliferation in CH; whereas cirrhotic samples (CIRR and LDH) as well as healthy samples are virtually negative (data not shown). Similar results have been described in human chronic and cirrhotic liver samples, irrespective of the etiology [26, 27]. This indicates the ability of hepatocytes to proliferate after c-MET activation in more chronic stages while cirrhotic samples do not complete the cell-cycle of which the cause remains to be elucidated.

To further substantiate the molecular comparison between human and canine fibrotic livers the activation status of HGF/c-MET downstream signaling components involved in regeneration was analyzed. HCV cirrhotic samples had high levels of Ser-phosphorylated STAT3 compared to alcohol induced cirrhotic samples. This HCV-induced up regulation of STAT3 phosphorylation was shown before in man and rodent models *in vivo* as well as *in vitro* studies on cell-lines [28, 29]. In other studies the levels of STAT phosphorylation indicated phosphorylated STAT3 protein in HCV affected cirrhotic livers compared to primary biliary cirrhotic samples and healthy tissue [30]. In the canine samples including the healthy control biopsies STAT3-Ser phosphorylation was strongly present. The levels of STAT3-Tyr phosphorylation were the lowest in AH and strongest in LDH. As the amount of STAT3-Tyr phosphorylation indicates the DNA binding capacity of the protein, the pathways seems to be activated in fibrotic diseases and not in acute hepatitis.

Next to STAT3 phosphorylation, HCV induces ERK1/2 phosphorylation [31]. In the canine samples, an up regulation of the levels of phosphorylated ERK1/2 was observed in CH and LDH. Finally, the increase in p38MAPK phosphorylation as observed in CH, LDH, and CIRR has been described in fibrotic tissues in man [32]. In general, HGF/c-MET downstream signaling in CH, LDH, and CIRR is to a high degree comparable with the molecular data obtained from human clinical samples.

Surgical animal models for liver regeneration, such as partial hepatectomy (PH), represent an over-simplification by the absence of inflammation or overperfusion; furthermore, all hepatocytes are stimulated by PH to enter the G1 phase simultaneously. Toxic models induced by dimethylnitrosamine, CCl_4, acetominophen, or thioacetamide can represent chronic as well as acute/fulminant hepatitis [33-35]. Toxic models are better clinical models as hepatotoxins can be used to selectively induce centrolobular and periportal necrotic

lesions and thus mimic clinical liver diseases. However, toxin-induced models do not represent the full range of changes seen in human liver diseases [36].

Comparison between canine and human diseases was obtained by using cirrhotic human samples derived from alcohol abuse or hepatitis C infection, two of the most common causes of hepatitis in the Western world [37, 38]. As in humans, chronic hepatitis in dogs is associated with progressive fibrosis, reduction in liver size and regeneration, and finally disruption of the liver architecture (cirrhosis), which may cause portal hypertension, ascites, and portosystemic encephalopathy [39]. Although histologically highly comparable to their human counterparts, the etiology of canine hepatitis is largely unknown [40]. However, human samples (hALC and hHC) showed the same degree of activation in the signal transduction pathways, irrespective of the different underlying etiology. Therefore, despite unknown etiology in dogs the underlying mechanisms are similarly activated.

Conclusion

This study is the first to measure expression profiles of crucial pathways of liver regeneration in canine liver diseases in comparison with man. Previously, a high similarity of affected fibrotic pathways between human- and canine-liver diseases was found [11]. Combining these measurements on fibrotic- and regenerative-signaling pathways, privately owned dogs may help to fill in the gap between toxin-induced rodent models and human diseases. Furthermore, this study provides the basis to analyze more acute forms of hepatitis such as (sub)acute hepatitis in dog. Taken together, these results indicated that CH, LDH, and CIRR are suitable spontaneous large animal models to evaluate the clinical application of therapies such as cell transplantation or the administration of growth factors.

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Transforming growth factor β-1 signalling in canine hepatic diseases: new models for human fibrotic liver pathologies.

Transforming growth factor β-1 signalling in canine hepatic diseases: new models for human fibrotic liver pathologies.

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Abstract

The purpose of this study was to validate spontaneous chronic hepatitis and cirrhosis in dogs as a potential large animal model for fibrotic liver disease in humans by evaluating their molecular pathophysiology. Transforming growth factor-β1 (TGF-β1) signalling was analysed in liver samples of dogs with acute hepatitis (AH), chronic hepatitis (CH), cirrhosis (CIRR), and a specific form of cirrhosis, lobular dissecting hepatitis (LDH), in comparison with human cirrhotic samples from alcohol abuse (hALC) and hepatitis C (hHC). Canine samples were investigated with quantitative real-time PCR (Q-PCR) and Western blotting on TGF-β1 signalling including Smad2/3 phosphorylation. Immunohistochemistry on collagens I and III was performed. Q-PCR showed an increase in TGF-β1 levels and downstream effector gene products in CH, LDH, and CIRR. The same fibrotic diseases also showed an increase in phosphorylated Smad2/3 and a higher deposition of collagens I and III. In contrast, in AH neither active TGF-β1 signalling nor collagen deposition was observed. Western blot analysis on human hALC and hHC indicated a high similarity with canine samples in TGF-β1 expression and Smad2/3 phosphorylation. Our results demonstrate that fibrosis in spontaneous dog liver diseases is highly comparable to their human counterparts and might serve as models for anti-fibrotic strategies.

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Introduction

Chronic hepatitis is characterized by hepatocellular apoptosis or necrosis, inflammation, and fibrosis. Fibrosis is the major factor causing morbidity and mortality because of the development of cirrhosis [1, 2]. Chronic hepatitis may be the sequel of various causes such as (viral) infections, exposure to toxins and drugs, or immunological and metabolic disorders [3]. Dogs have chronic liver diseases that are clinically and pathologically highly comparable with their human liver disease counterparts; they may thus fulfill a role as a spontaneous (non-experimental) animal model, in between induced toxic or surgical models in rodents, and diseases in humans [4]. To become fully accepted as a model for human diseases, molecular pathways must be similarly affected. We chose four different spontaneous forms of hepatitis and cirrhosis in dogs for comparative analysis of fibrosis. They ranged from acute hepatitis (AH) without fibrosis to chronic hepatitis (CH), and cirrhosis (CIRR), including lobular dissecting hepatitis (LDH). The last disease is a specific form of cirrhosis with severe fibrosis and complete disruption of the lobular architecture and is comparable to neonatal hepatitis in humans [5, 6]. Although the aetiology of canine hepatitis is largely unknown, the reaction patterns (clinical and pathological) of the liver to injury are uniform and therefore mimic their human counterparts [7]. Like in humans, cirrhosis is the end stage of chronic hepatitis, which typically needs 1–5 years to be reached. Dogs with cirrhosis develop portal hypertension, and in the decompensated stage, this may be complicated by formation of ascites and development of hepatic encephalopathy and coagulopathies [5-7].

It has already been established that a transient increase of transforming growth factor β1 (TGF-β1) in the liver promotes fibrosis with the formation of extracellular matrix (ECM) components and suppresses hepatocyte proliferation [8, 9]. The major components of the ECM are interstitial collagens (types I and III), membrane collagen (type IV), and non-collagenous glycoproteins, such as laminin and fibronectin [10]. In several toxin-induced liver fibrosis models, fibrotic lesions are associated with an increase in procollagens type I, III, IV, and TGF-β1 mRNAs [11, 12]. Intracellular TGF-β1 signaling involves interaction of Smad proteins [13]. Smad-2 and 3 are phosphorylated at the carboxy terminus by activated TGF-β receptor kinases. When bound with co-Smads, the Smad-complex translocates into the nucleus and acts as a TGF-β1-induced transcriptional activator of target genes [14].

The present study was undertaken to investigate the molecular pathophysiology of canine liver disease and compare it with the current paradigm of liver fibrosis in humans [15-17]. Therefore, our emphasis was on the TGF-β1 signaling routes, that are involved in the crucial pathways of fibrosis. We analyzed TGF-β1 signaling in liver tissues obtained from dogs with different forms of hepatitis, encompassing acute to chronic hepatitis and cirrhosis. In contrast with chemically induced rodent models, these spontaneous dog diseases are

expected to be very well comparable with chronic human liver disease. If TGF-β1 signaling is similarly affected in canine and human diseases, these spontaneous dogs could form a good model for evaluating the clinical effects of new therapies, such as anti-fibrotic factors, before their application in humans. Then, these animals can bridge the gap between basic physiology and clinical patient care.

Materials and methods

Animals.

All samples were obtained from dogs of different breeds examined at our faculty of veterinary medicine. The procedures were approved by Utrecht University's Ethical Committee, as required under Dutch legislation. Each disease group contained eleven dogs (*n*=11 dogs), and was compared with a group of 12 age-matched healthy control dogs (*n*=12 dogs), without clinical signs of hepatitis or other disease. Liver tissue was obtained from all dogs under local anaesthesia by ultrasound-guided biopsy with a true-cut 14-G biopsy needle, preceded by ultrasonographic evaluation of the liver to exclude biopsy artefacts in case of non-homogeneous hepatic changes. Two formalin-fixed biopsies were embedded in paraffin, sliced, and stained with haematoxylin and eosin, Van Gieson stain, and reticulin stain according to Gordon and Sweet. Fibrosis scoring was performed according to Scheuer, a defined scoring method for fibrosis in hepatitis [18]. The slides were examined by one certified veterinary pathologist. Two other biopsies were snap frozen in liquid nitrogen, and stored at −70°C until molecular analysis. Dogs with hepatitis presented with apathy, anorexia, vomiting, and/or jaundice, and, in cases of CIRR and LDH, often with signs of liver decompensation with ascites and hepatic encephalopathy. The presence of a liver disease was confirmed by finding high plasma levels of AP, ALT, and/or BA. The AH, CH, CIRR, and LDH diseases were diagnosed according to the criteria of the World Small Animal Veterinary Association (WSAVA) Liver Diseases and Pathology Standardization Research Group [19]. AH was clinically characterized by acute onset of the disease and histologically by inflammation, hepatocellular apoptosis, and necrosis. CH was characterized histologically by mononuclear or mixed inflammation, apoptosis and necrosis of hepatocytes, fibrosis, and regeneration. CIRR, the end stage of chronic hepatitis, was characterized by bridging fibrosis, shunting of afferent and efferent vessels, and conversion of the normal liver architecture into abnormally structured parenchymal nodules. LDH, a rapidly progressive form of cirrhosis, was characterized by fibrosis surrounding individual hepatocytes with complete disruption of the lobular architecture. Although the aetiology of canine hepatitis is largely unknown, several known aetiological agents associated with hepatitis in other species have been

excluded [20]. Furthermore, two known causes of hepatitis in the dog, CAV-1 and copper toxicosis, have been excluded by PCR and (immuno)histochemistry.

Human patients.

All samples were obtained from surgical patients who were transplanted at the department of Abdominal Transplantation in the University Hospital Leuven, Leuven, Belgium. The procedures were approved by Leuven University's Ethical Committee, as required under Belgian legislation. Human explant samples were collected directly after surgery and immediately snap- frozen. All patients were presented with micronodular cirrhosis and were predominantly male. The alcoholic cirrhosis (hALC) group contained five patients (n=5) characterized by cirrhosis with neutrophil infiltrations, alcohol-related morphological changes (hepatocyte ballooning, Mallory bodies, necrosis), and, in some cases, steatosis and increased iron deposition. The hepatitis C (hHC) group contained four patients (n=4) characterized by cirrhosis with neutrophil infiltrations, lymphoid follicles, and aggregates, and in one case in combination with hepatocellular carcinoma (HCC, sample 6). All cases were presented with hepatocyte decay, apoptosis/necrosis, regeneration, and fibrosis.

Quantitative PCR and statistical methods.

Quantitative real-time PCR (Q-PCR) was performed on a total of nine gene products involved in fibrosis; e.g., TGF-β1, TGF-β receptor type I (TGF-β RI), TGF-β receptor type II (TGF-β RII), uPA, collagen-I, collagen-III, collagen-IV, fibronectin, and hypoxia-induced factor (HIF)-1α. The abundance of mRNA was determined by reverse transcription (RT), followed by real-time Q-PCR using appropriate primers (Table 1), as previously described [21]. In short, total cellular RNA was isolated from each frozen canine liver tissue in duplicate, using the Qiagen RNeasy Mini Kit (Qiagen, Leusden, the Netherlands) according to the manufacturer's instructions. The RNA samples were treated with DNase-I (Qiagen Rnase-free DNase kit). In total, 3 µg of RNA was incubated with poly(dT) primers at 42°C for 45 min, in a 60 µl reaction volume, using the RT System from Promega Benelux (Leiden, the Netherlands). Q-PCR was based on the high-affinity double-stranded DNA-binding dye SYBR® green I (BMA, Rockland, ME). For each experimental sample, the amount of the gene of interest and of the two independent endogenous references (glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyl transferase (HPRT)) was determined from the appropriate standard curve in autonomous experiments. If relative amounts of GAPDH and HPRT were constant for a sample, data were considered valid and the average amount was used as a normalization factor in the study (data not shown). Results were normalized according to the average amount of the endogenous references. The

normalized values were divided by the normalized values of the calibrator (healthy group) to generate relative expression levels [22].

A Kolmogorov–Smirnov test was performed to establish a normal distribution and Levene's test for the homogeneity of variances. All samples included in this study were normally distributed. The statistical significance of differences between diseased and control animals was determined by using Student's *t*-test. A *P*-value <0.05 was considered statistically significant. Analysis was performed using SPSS software (SPSS Benelux BV, Gorinchem, the Netherlands).

Table 1. Nucleotide sequences of dog-specific primers for real-time quantitative PCR

Gene	F/R	Sequence (5'–3')	Tm (°C)	Product size (bp)	Accession number
GAPDH	F	TGT CCC CAC CCC CAA TGT ATC	58	100	AB038240
	R	CTC CGA TGC CTG CTT CAC TAC CTT			
HPRT	F	AGC TTG CTG GTG AAA AGG AC	56	100	L77488/
	R	TTA TAG TCA AGG GCA TAT CC			L77489
TGF-β1	F	CAA GGA TCT GGG CTG GAA GTG GA	66	113	L34956
	R	CCA GGA CCT TGC TGT ACT GCG TGT			
TGF-β R I	F	CAG TCA CCG AGA CCA CAG ACA AAG T	59	101	AY455799
	R	TGA AGA TGG TGC ACA AAC AAA TGG			
TGF-β R II	F	GAC CTG CTG CCT GTG TGA CTT TG	61	116	AY455800
	R	GGA CTT CGG GAG CCA TGT ATC TTG			
uPA	F	CTG GGG AGA TGA AGT TTG AGG TGG	64.5	105	AY455801
	R	TGG AAC GGA TCT TCA GCA AGG C			
Collagen I	F	GTG TGT ACA GAA CGG CCT CA	61	111	AF056303
	R	TCG CAA ATC ACG TCA TCG			
Collagen III	F	ATA GAG GCT TTG ATG GAC GAA	65	134	AB042266
	R	CCT CGC TCA CCA GGA GC			
Collagen IV	F	CAC AGC CAG ACA ACA GAT GC	67	151	U07888
	R	GCA TGG TAC TGA AGC GAC G			
Fibronectin	F	AGG TTG TTA CCA TGG GCA	61	91	U52106
	R	GCA TAA TGG GAA ACC GTG TAG			
HIF-1α	F	TTA CGT TCC TTC GAT CAG TTG TCA	61	106	AY455802
	R	GAG GAG GTT CTT GCA TTG GAG TC			

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyl transferase; TGF, transforming growth factor; HIF, hypoxia-induced factor.

Immunoblot analysis.

Pooled liver tissues (*n*=6 dogs per group, randomly chosen from the original group) were homogenized in RIPA buffer containing 1% Igepal, 0.6 mM phenylmethylsulphonyl fluoride, 17 µg/ml aprotinine, and 1 mM sodium orthovanadate (Sigma-Aldrich, Zwijndrecht, the Netherlands). Protein concentrations were obtained using a Lowry-based assay (DC Protein Assay, BioRad, Ueenendaal, The Netherlands). Detection of TGF-β1 was performed under non-denaturing conditions with 20 µg of protein electrophoresed on 15% Tris-HCl polyacrylamide gels (BioRad). For the detection of Smad's, 20 µg of protein of the supernatant was denatured for 3 min at 95°C and electrophoresed on 7.5% Tris-HCl polyacrylamide gels (BioRad). The procedure for immunodetection was based on an ECL Western blot analysis system, performed according to the manufacturer's instructions (Amersham-Biosciences, Roosendaal, The Netherlands). The membranes were incubated with 4% ECL blocking solution in TBS for 1 h under gentle shaking. The incubation of the primary antibody was performed at 4°C overnight in TBST with 4% BSA (Table 2). After washing, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h in TBST with 4% BSA. Exposures were made with Kodak BioMax Light-1 films (Sigma-Aldrich). Densitometric analysis of immunoreactive bands was performed with a Gel Doc 2000 system using Quantity One 4.3.0 Software (BioRad).

Immunohistochemistry.

Collagens I and III were evaluated from at least four sections per group (*n*=4 dogs, randomly chosen) and compared with sections from normal control dogs (*n*=4 dogs, randomly chosen). For antigen retrieval, the sections (3 µm) were heated in 10 mM citrate buffer (pH 6.0) in a microwave oven (850 W) at full power until boiling point, after which the sections were heated for 15 min at half-power. Sections were incubated in 0.3% hydrogen peroxide in PBS for 30 min at room temperature to quench endogenous peroxidase activity. After washing with PBS buffer containing 0.1% Tween-20, background staining was blocked in 10% normal horse serum in PBS for 30 min at room temperature. Sections were incubated overnight at 4°C with the primary antibody mouse anti-collagen-I (Abcam, Cambridge, UK) diluted 1:100 in PBS with 2% bovine serum albumin (BSA). The primary antibody mouse anti-collagen III (Chemicon, Victoria, Australia) was diluted 1:50 in PBS. After washing in PBS-Tween, slides were incubated with the biotinylated secondary antibody horse anti-mouse IgG (Vector Laboratories, Peterborough, UK) diluted 1:125 in PBS for 1 h at room temperature. After washing in PBS buffer, slides were treated with the ABC peroxidase complex (Vectastain,Brunschwig, Amsterdam, the Netherlands) for 1 h at room temperature. The peroxidase activity was visualized by applying 0.5 mg/ml diaminobenzidine chromogen

containing 0.035% hydrogen peroxide in PBS for 10 min at room temperature. The DAB solution contained 0.3% (w/v) di-ammonium nickel (II) 6-hydrate (Brunschwig) to improve contrast. The sections were counterstained with haematoxylin, dehydrated, and mounted. They were examined and photographed using an Olympus BX41 light microscope equipped with an Olympus DP50 digital camera (Olympus, Zoeterwoude, the Netherlands). The amount of positive staining was semi-quantitatively assessed by one certified veterinary pathologist examining the sections (original magnification of × 450) and the sections were also evaluated with respect to localization of the staining. Negative controls were performed as described above, yet without incubation of the primary antibody; these sections showed no positive staining in any of the diseases under study (data not shown).

Table 2. Used antibodies in Western blot experiments

Antigen	Manufacturer	Product size (kDa)	Dilution	Secondary antibody	Dilution
TGF-β1	Santa Cruz (sc-146-G)	12.5	1:1000	Anti-goat	1:20 000
p-Smad2/3 [a]	Cell-Signaling (3101)	58	1:500	Anti-rabbit	1:20 000
Smad2/3	BD Biosciences (610842)	58	1:500	Anti-mouse	1:20 000
Beta-actin	Neomarkers (pan Ab-5)	42	1:2000	Anti-mouse	1:20 000

[a] (Ser 465/467)

Results

To gain an insight into the activity of the TGF-β1 pathway in spontaneous canine liver diseases, we first measured mRNA levels by means of Q-PCR. Second, Western blotting was performed to confirm mRNA quantities and to show activation at the protein level. Third, Western blots were used to compare canine samples with human cirrhotic samples from hALC and hHC. Finally, immunohistochemistry was used to determine ECM deposition.

TGF-β1 cascade signalling pathway involved in fibrosis.

In AH (Fig. 1A), none of the mRNA levels analysed were significantly changed. In contrast, in CH (Fig. 1B) we found that all analysed mRNA levels were significantly induced. An induction was seen in the TGF-β1 mRNA levels (twofold), whereas the receptors type I (signalling) and type II (binding) were induced three- and twofold, respectively. The proteolytic enzyme involved in activation of TGF-β1, urokinase-type plasminogen activator (uPA), was induced twofold in the CH group. In the LDH group (Fig. 1C), TGF-β1 mRNA levels were induced fourfold, type I receptor threefold, and type II receptor fourfold. The uPA mRNA levels were highly induced (sixfold). In the CIRR group (Fig. 1D), an increase in TGF-β1 mRNA levels was also seen (twofold). The highest induction of TGF-β RI mRNA levels was seen (12-fold) in this group, but type II mRNA levels were not significantly changed. The uPA mRNA level was induced fivefold.

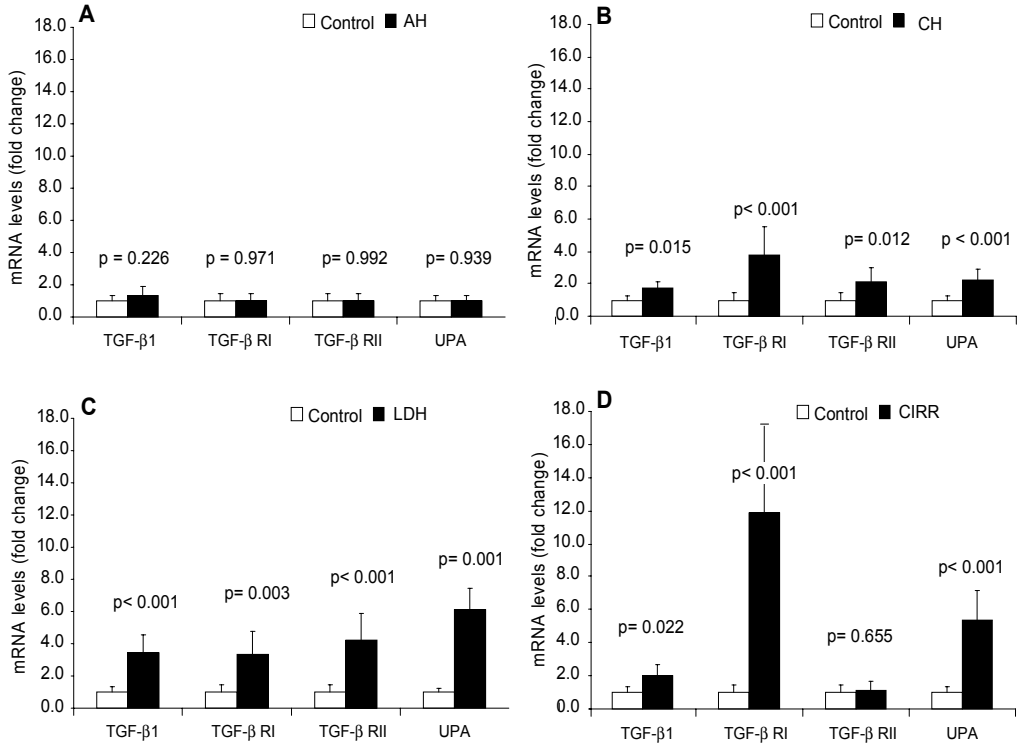

Figure 1. Quantitative real-time polymerase chain reaction (PCR) of gene products involved in fibrosis. The relative expression of mRNA levels in acute hepatitis (AH) shunt is shown in (A), in chronic hepatitis (CH) in (B), in lobular dissecting hepatitis (LDH) in (C), and in cirrhotic samples (CIRR) in (D). Data represent mean±SD.

Gene expression of ECM gene products and HIF-1α.

Collagen I levels are commonly associated with severe forms of hepatic fibrosis [23]. The mRNA levels of collagens I, III, and IV were decreased three-, six-, and fourfold, respectively, in AH, although fibronectin and HIF-1α mRNA levels did not change significantly (Fig. 2A). In the CH group, the only significant change was seen in the collagen III mRNA level, which was elevated twofold (Fig. 2B). All mRNA levels were significantly elevated in the LDH group (nine-, four-, four-, three-, and threefold, for collagens I, III, and IV, fibronectin, and HIF-1α, respectively) (Fig. 2C). In the CIRR group (Fig. 2D), the mRNA levels of collagen I were induced threefold, but the mRNA levels of collagen IV and fibronectin were significantly decreased (two- and fourfold, respectively). The highest increase in HIF-1α mRNA levels was found in this group, with a sevenfold induction compared with the healthy group.

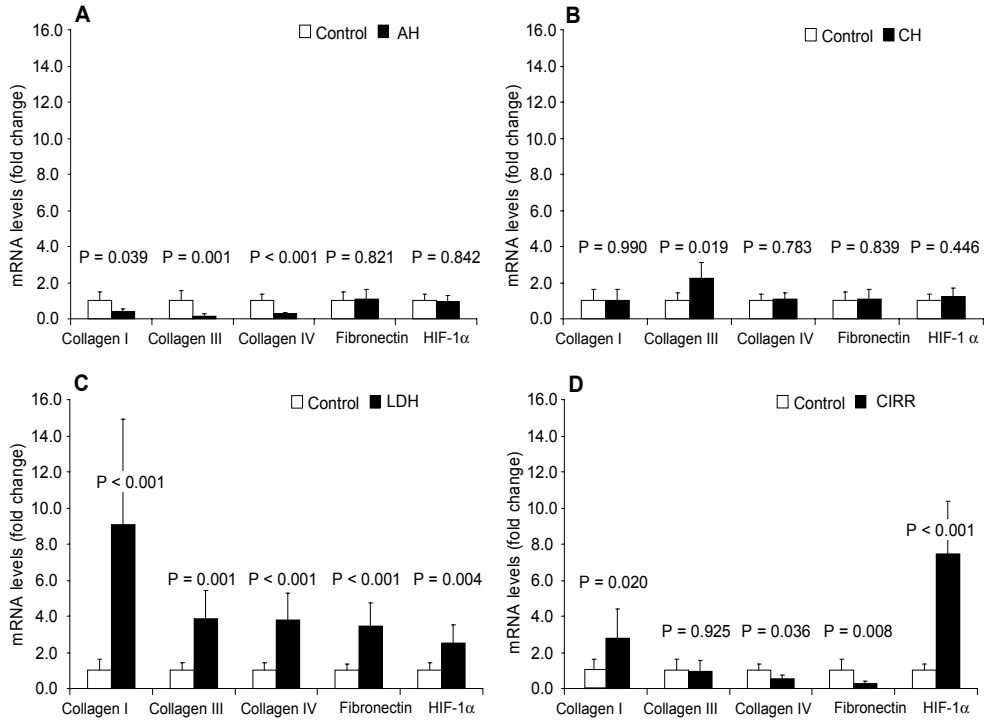

Figure 2. Quantitative real-time polymerase chain reaction (PCR) of collagens, fibronectin, and hypoxia-induced factor-1α. The relative expression of mRNA levels in acute hepatitis (AH) shunt is shown in (A), in chronic hepatitis (CH) in (B), in lobular dissecting hepatitis (LDH) in (C), and in cirrhotic samples (CIRR) in (D). Data represent mean±SD.

Western blot analysis on canine AH, CH, CIRR, and LDH.

TGF-β1 was detected in all diseases as a peptide of 25 kDa (Fig. 3). TGF-β1 seemed to be induced in all fibrotic diseases compared with the healthy group, with the highest increase in CIRR. The non-phosphorylated Smad2/3 (58 kDa) was detected in fibrotic disease types, with a slight quantitative increase in CH. Thus, the activated thus phosphorylated Smad2/3 was detected in CH, LDH, and CIRR, where it was present as a single band of 58 kDa. Interestingly, the quantity of phosphorylated Smad2/3 was very low in healthy samples and AH, the latter emphasizing the absence of fibrogenesis in this type of disease. Furthermore, the phosphorylated Smad2/3 protein showed the highest increase in LDH.

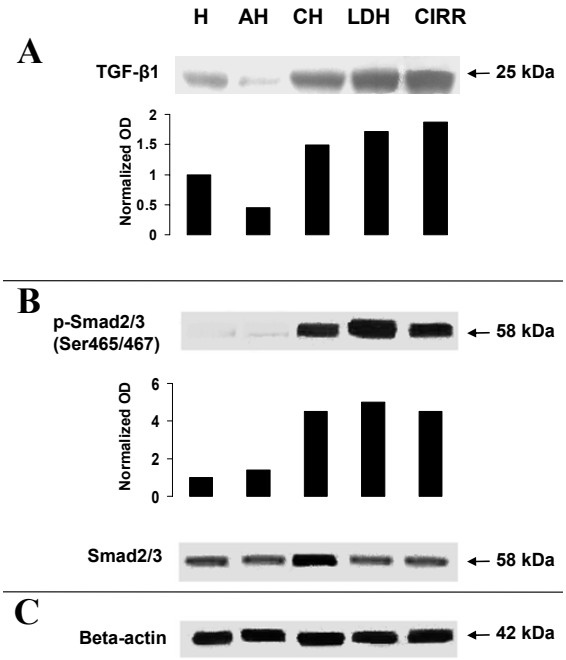

Figure 3. Western blot analysis of diseased and control canine liver homogenates. Detection of the 25 kDa TGF-β1 protein is shown in (A). Detection of the 58 kDa Smad2/3 protein is shown in (B). β-actin served as a loading control. Quantitative analysis of immunoreactive bands by means of optical density (OD) measurements corrected for the amount of β-actin (loading control, shown in (C)).

Western blot analysis on human hALC and after hHC.

Western blot analysis on human hALC and hHC samples showed a detectable 25 kDa TGF-β1 in all samples with minor quantitative differences (Fig. 4). The 58 kDa Smad2/3 was also detected in all samples, although two samples in the hHC group (Fig. 4B, lanes 7 and 8) were present to a lesser extent. The phosphorylated form of Smad2/3 (58 kDa) was detected in all samples; however, two samples in the hHC group had a diminished phosphorylation of the Smad2/3 protein (Fig. 4B, lanes 7 and 8).

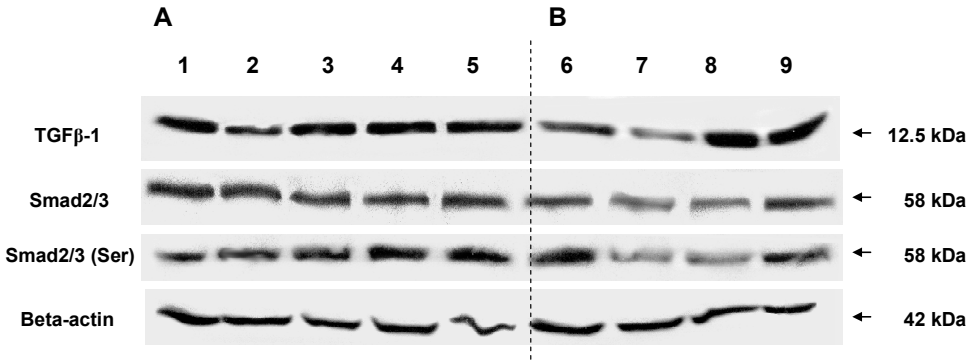

Figure 4. Western blot analysis of human liver homogenates. Analysis of the (25 kDa) TGF-β1, (58 kDa) Smad2/3 protein, and (42 kDa) β-actin. Human cirrhotic samples from alcohol abuse (hALC, lanes 1–5) are shown in (A); cirrhotic samples after hepatitis C infection (hHC, lanes 6–9) are shown in (B).

Immunohistochemical observation of collagen levels.

Collagens I and III showed a similar pattern in the normal canine liver. Marked positive staining was seen in the stroma of the larger portal areas and around the larger hepatic veins, whereas mild staining was seen in the smaller portal and perivenous areas. Moreover, a fine fibrillar staining was often observed in the perisinusoidal space in the centrolobular areas (Fig. 5A). In the AH group, the collagens I and III staining showed a pattern comparable with that of the control dogs, although the fibres in the centrolobular areas were less recognizable. In the CH group (Fig. 5B), the stroma of the portal areas and around the hepatic veins stained as in the normal dogs. The parenchyma showed an increased presence of both collagens I and III in the periportal and centrolobular parenchyma, regularly surrounding individual liver cells or groups of them, and associated with inflammation. In the CIRR group (Fig. 5C), there was marked staining for both collagens I and III in the fibrous septa separating large parenchymal hyperplastic nodules. Staining within these nodules varied depending on the activity of the disease as evidenced by the degree of inflammation and necrosis. In nodules without inflammation, only the preexistent stroma of the portal areas and around the hepatic

veins stained positive, whereas in nodules with inflammation and necrosis, an increased staining was often seen encircling single liver cells or small groups of them. An increased staining for collagen I and III surrounding individual and small groups of hepatocytes was also observed in larger areas between nodules with a mixture of hepatocytes, inflammation, and fibrosis. In the LDH group (Fig. 5D), a marked increase in collagens I and III was seen throughout the parenchyma encircling individual liver cells and small groups of hepatocytes. In some dogs, small hyperplastic parenchymal nodules were seen surrounded by thin fibrous septa containing both collagens I and III; in these nodules, only slight staining for collagens I and III was present.

Discussion

The results of this study showed that the molecular pathophysiology of canine fibrotic liver diseases is highly comparable to the pathophysiology of their human counterparts. This emphasizes the potential of spontaneous dog diseases as models for human clinical investigation. We have analyzed liver fibrosis using several molecular and biochemical techniques (Q-PCR, Western blotting, and immunohistochemistry on ECM proteins). This provided insight into the TGF-β1 pathway in four spontaneous canine hepatic diseases: AH, CH, LDH, and CIRR.

Prolonged overexpression of TGF-β1 suppresses cell proliferation, is pro-apoptotic, and induces a deposition of ECM proteins, resulting in fibrosis in major organs such as the liver [15]. By independent techniques, we showed that in fibrotic diseases (CH, LDH, and CIRR), the TGF-β1 pathway was activated. First, we found an increase in mRNA levels of the TGF-β1 ligand and the two receptors. Second, uPA (Fig. 1), the activator of TGF-β1, was expressed at a higher level [24]. Third, Western blotting confirmed increased TGF-β1 (Fig. 3A) in these diseases. Taken together, increased levels of TGF-β1 signaling may very well explain the activation of Smad2/3 and subsequently the formation of collagens. Comparing Western blot analysis with cirrhotic samples derived from human explant samples indicated a high similarity in protein quantity and activity of TGF-β1 signaling components. In both analyzed human cirrhotic groups (alcohol-induced (hALC) or because of hHC infection), TGF-β1 was detected (Fig. 4). More importantly, phosphorylated Smad2/3 was detected in all samples, although somewhat reduced in two patients as shown in lanes 7 and 8 as shown in Fig. 4B. This observation is probably due to a decrease in the total amount of Smad2/3, whereas β-actin indicated an equal amount of protein loading.

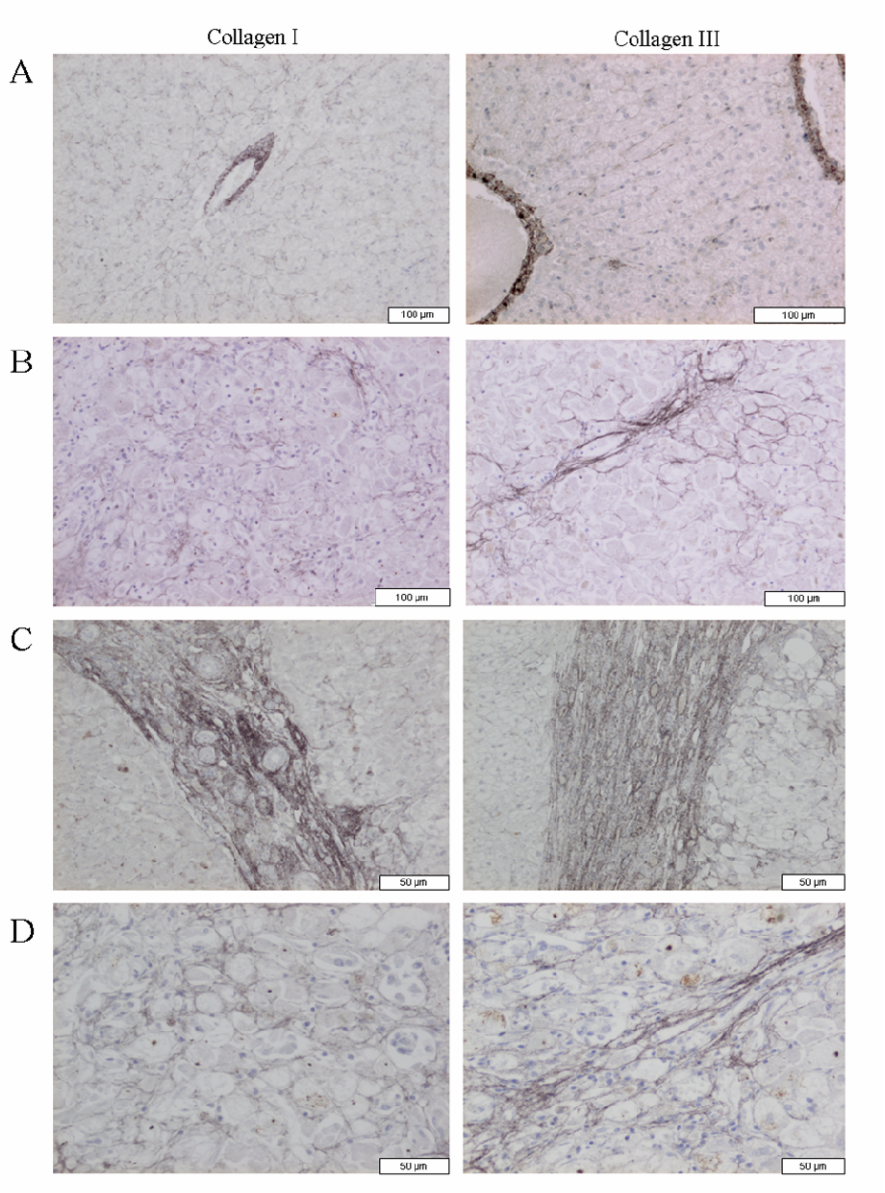

Figure 5. Immunohistochemistry on collagens I and III in liver samples. Collagens I and III in healthy liver tissue are shown in (A). Collagens I and III in chronic hepatitis are shown in (B). Collagens I and III in cirrhotic liver tissue are shown in (C). Collagens I and III in lobular dissecting hepatitis are shown in (D). The size bar is indicated in the figures.

Immunostaining against collagens I and III on normal liver tissues showed slight staining of the collagens in portal tracts, around the hepatic veins, as well as in the perisinoidal space (particularly centrolobular). Our observations of an increase in collagen III mRNA levels in dogs with CH and the increased presence of collagen I mRNA levels in CIRR were surprising. These data indicate that the chronic accumulation of collagens could begin with collagen III and gradually form broad septal tracts composed of collagens I and III in CIRR. However, immunostaining against collagens I and III in CH and CIRR showed increased presence of both collagens. This indicates that the mRNA levels for collagen do not always reflect the presence of the protein product. This observation can be ascribed to the chronic stage of our cirrhotic samples and most likely is due to the long half-life of these ECM proteins. The corresponding results of mRNA levels and immunostaining in LDH can be explained by the rapid clinical course of the disease in LDH. The different inflammatory activities of our cirrhotic samples, which varied from very active to almost inactive, are associated with rather small standard deviations at the mRNA level. Thus, it seems that the number of patients in each group, combined with different biopsies per individual, greatly averages the differences between active and non-active CIRR. In CCl_4-induced rodent models of liver fibrosis, TGF-β1 and procollagen mRNAs were increased, including procollagens I, III, and IV [25, 26]. The fact that not all the collagens were induced in our spontaneous dog liver diseases stresses the importance of studying spontaneous chronic models in comparison with toxin-induced models [27].

The liver is comprised of several cell types of both mesenchymal and parenchymal origin. Therefore, the observed differences in gene expression in our measurements could be because of changes in the amount of cell-types and the relative ratios between cell types present. For instance, the observed increase in TGF-β1 mRNA and protein level could be because of an increase in (myo)fibroblasts (activated stellate cells) that express TGF-β1 in the perpetuation phase of fibrosis [28]. Immunohistochemistry and micro-dissection can answer which specific cell types are involved in the pathogenesis of these hepatic diseases. Furthermore, the discrepancy between TGF-β1 mRNA and protein level as seen in AH could be contributed to the lack of activation of TGF-β1 in this group, which could be still be non-covalently bound to the latency associated peptide (LAP), which occurs after expression [15].

The mechanisms underlying progressive fibrosis are unknown, but hypoxia is a known fibrogenic stimulus [29]. On the other hand, it is conceivable that increased collagen deposition leads to reduced oxygen levels in the surrounding tissue and consequently up-regulates HIF-1α. Indeed, the two major fibrotic diseases, LDH and CIRR, clearly showed an induction of HIF-1α mRNA levels of three- and sevenfold, respectively, indicating the correlation between extensive fibrosis and the presence of HIF-1α.

The use of TGF-β1 intervention to halt the progression of liver fibrosis and aid regeneration has been applied successfully in animal models [30-33]. Although many of these techniques are based on rodent models [34], they could be further evaluated in these spontaneous canine liver diseases.

Conclusion

Canine liver diseases are spontaneous models representing the entire complexity of human liver diseases so that they provide natural models for comparison with human diseases. The variations in age, body weight, and feeding behaviour, as presented by the dogs visiting our clinic, resemble the variations in a human population well. Moreover, dogs are large animals permitting procedures that would also be relevant for human medicine. Finally, the use of canine patients can lead to a reduction in the amount of experimental animals. Proven effects in dogs may predict potential effects in human medicine with much greater power than effects in artificial rodent models. Our spontaneous dog models may therefore help to bridge the gap between basic physiology in rodent models and the patient.

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In vitro and *in vivo* bioactivity of recombinant canine HGF.

In vitro and *in vivo* bioactivity of recombinant canine HGF.

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Abstract

Hepatocyte growth factor (HGF) is crucial for the development and regeneration of the liver and is a possible new therapeutic strategy for the treatment of canine liver diseases. In this study, the *in vitro* and *in vivo* bioactivity of recombinant canine HGF (rcHGF) produced with a baculoviral expression system in insect cells was measured. *In vitro* rcHGF induced mitogenesis, motogenesis, and phosphorylated the HGF receptor c-MET and its downstream mediators PKB, and ERK1/2 in two canine epithelial cell lines. After a partial hepatectomy (phx) in dogs, rcHGF increased phosphorylation of c-MET, PKB, and ERK1/2. A moderate increase was seen with the cell cycle protein PCNA in rcHGF treated livers, but no HGF-induced increase in liver weight was seen at seven days after phx. Furthermore, rcHGF treated livers showed lower levels of the key mediator of apoptosis, caspase-3 at seven days after phx. In conclusion, rcHGF is an *in vitro* and *in vivo* biologically active protein and the baculoviral expression system supplies sufficient amounts of rcHGF for future clinical studies in dogs.

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Introduction

Hepatocyte growth factor (HGF) is a multifunctional heterodimeric glyco-protein, synthesized and secreted by cells of mesenchymal origin and targeting epithelial and endothelial cells in an endocrine and/or paracrine fashion [1]. The biological activities of HGF are triggered by the phosphorylation of tyrosine residues of a heterodimeric membrane-spanning tyrosine kinase receptor that is encoded by the proto-oncogene c-MET [2, 3]. Activation of the c-MET signalling pathway induces a number of cellular responses, including mitogenic, motogenic, morphogenic, and anti-apoptotic activities [4, 5]. These activities play an important role in embryological development and regeneration of many tissues such as the liver [6, 7], kidneys [8], heart [9], and neural tissues [10].

Serum levels of HGF are increased in many liver diseases [11, 12]. Furthermore, exogenous administration of recombinant HGF or induction of an increased expression of the HGF gene has been found to be protective, even lifesaving in toxin-induced fulminant and chronic liver failure in rodent models [13-16]. Therefore, HGF is considered the most important factor for growth and regeneration of the liver and may be a potential therapeutic agent for the treatment of liver diseases in which growth or regeneration is hampered.

The overall incidence of liver disease in dogs has been estimated at around 1-2% of clinical cases [17, 18]. In hepatic under-development, such as congenital portosystemic shunt and primary portal vein hypoplasia, and liver diseases in which regeneration is hampered, such as in chronic inflammation and cirrhosis, HGF or c-MET pathways are altered [19, 20]. These altered regeneration pathways provide a rationale for the therapeutic use of HGF. However, no therapeutic evaluation of HGF has been performed in dogs, possibly reflecting the high cost and the lack of sufficient quantities of ready available recombinant HGF.

In the present study, rcHGF was produced using a baculoviral expression system in insect cells [21]. The bioactivity of rcHGF was measured *in vitro* using two canine epithelial cell lines. The effect of rcHGF on the activation of c-MET and downstream pathways was measured in dogs that had received a 30% partial hepatectomy (phx). The phx model is a widely used technique to study liver regeneration *in vivo*. After a phx, the remaining hepatocytes enter the G1 phase of the cell cycle, which makes the normally quiescent hepatocytes more responsive towards growth factors, such as HGF [22]. These results provide the basis for future application of rcHGF in naturally occurring canine liver diseases.

Materials and methods

Cell lines and cultures.

Canine bile duct epithelial (BDE) cells were acquired from the Amsterdam Medical Centre, Experimental Liver cell bank [23]. BDE cells have a hepatocyte-like differentiation profile, because they express albumin and ceruloplasmin, as determined by quantitative polymerase chain reaction (Q-PCR) and immunodetection. Madin-Darby canine kidney (MDCK) cells were purchased from the American Type Culture Collection. Both cultures were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (FCS, PAA Laboratories) and standard antibiotics at 37 ºC with 5% CO_2 and 95% air in a humidified atmosphere. High-5 cells were cultured in SF900 medium (Invitrogen) containing 3% FCS.

Expression and purification of rcHGF.

The rcHGF was produced by infecting High-5 cells with recombinant baculovirus (Invitrogen) expressing the complete coding region of canine HGF [24]. Three days after infection, rcHGF-containing supernatant was harvested and concentrated by ultra microfiltration (NORIT Membrane Technology). The concentrate was centrifuged at 100,000 *g* for 1 h to remove the baculovirus particles, and gamma irradiated (25 kGray) to inactivate residual baculovirus.

The rcHGF was purified using a heparin-sepharose CL-6B column (Amersham Biosciences). After loading and washing the column according to the manufacturer's instructions, rcHGF was eluted with a linearly increasing gradient of 0.4–1.5 mM NaCl in 25 mM Tris-HCl. Elution samples (0.5 mL) were collected for further protein determination. To show the presence of rcHGF in different elution samples, Western blotting was performed with a specific HGF antibody (Table 1). The degree of rcHGF purification was estimated using densitometry analysis (QuantityOne software, Geldoc2000 system) of a silver stained Tris-HCl polyacrylamide gel. The rcHGF concentration was measured in an ELISA based on capturing with a mouse monoclonal antibody against human HGF (Lab Vision Corporation) and detection with a biotin labelled, goat polyclonal antibody against human HGF (Lab Vision Corporation). The results from the ELISA and the bioactivities of rcHGF were validated towards a standard line of recombinant human HGF (rhHGF, R&D systems) with known concentrations.

In vitro biological activity of rcHGF.

The mitogenic *in vitro* activity of rcHGF was measured using the colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) proliferation assay [25]. Cells were seeded in each well of a 96-well culture plate at a density of $1.5x10^3$ cells per well in DMEM medium containing 1% FCS. After an overnight culture starvation period, rcHGF or rhHGF was added in the first three wells to a final concentration of 300 ng/mL. The HGF containing medium was serially diluted three-fold each step, except for the last column (control cells). After 24 h under culture conditions, 0.5 mg/mL MTT (Sigma) were added to each well for 3 h. Cell growth was calculated as percentage increase in absorbance of treated cells compared to non-treated cells. MTT assay data are represented as mean values ± standard deviation of two independent experiments performed in triplicate.

The motogenic *in vitro* activity of rcHGF on BDE and MDCK cells was evaluated by HGF-induced cell scattering [26]. Cells were seeded in 8-well chamber slides (Labtek II) in a concentration of $3x10^3$ cells per well in DMEM with 1% FCS in the presence of 0, 10, or 100 ng rcHGF or rhHGF per mL. The experiment was performed in triplo. After a 10 h culture period, cells were fixed in Boonfix (Finetec) for 5 min and stained with haematoxylin for 1 min. Photomicrographs were taken from cells of the respective wells at 20x magnification.

Activation of the HGF receptor c-MET and the downstream mediators PKB and ERK1/2 were shown in BDE and MDCK cells by Western blotting. Cells were seeded in 6-well tissue culture plates at a density of $1.5x10^5$ cells per well in DMEM supplemented with 1% FCS. After an overnight starvation period, cells were treated with 0, 1.5, 15, or 150 ng/mL rcHGF for 5 min. Protein isolation and Western blotting was performed as described below.

In vivo biological activity of rcHGF.

This animal experiment was approved by and performed according to the standards of the Ethics Committee of Animal Experimentation of the Utrecht University. To study biological activity *in vivo*, twelve 10-12 month old female beagle dogs (Harlan Winkelmann), weighing 8.5-11 kg, were used. Before surgery, dogs were premedicated with 0.03 mg/kg acepromazine (Vetranquil, CEVA Santé Animale), 0.5 mg/kg methadone (Methadon HCl, Eurovet Animal Health) and 0.02 mg/kg atropine (Atropinesulfaat, Pharmachemie). Anaesthesia was induced with 5 mg/kg propofol (Propovet, Abbott) and maintained with inhalation anaesthesia with nitrous oxide, oxygen, and isoflurane (IsoFlo, Abbott). Additional intraoperative analgesia was provided by an initial dose of 15 µg/kg sufentanil (Sufenta forte, Janssen Cilag) at time of incision and thereafter the same dose was given every 30 min. Postoperative analgesia was provided with repeated intramuscular injections of 18 µg/kg buprenorphine (Temgesic,

Schering-Plough) every 8 h over a period of at least three days. Phx was performed by excision of the left lateral lobe by using a Cavitron Ultrasonic Surgical Aspirator. To permit injections of rcHGF or NaCl directly into the portal vein, the superior mesenteric vein was cannulated with a catheter (UNO Roestvaststaal). Directly after phx and thereafter every 24 h for 7 days, 10 μg/kg rcHGF (dissolved in 0.2 M NaCl) was injected into the catheter. The dose of 10 μg/kg was based on the literature of several rodent studies and one dog study. [22, 27-29]. Prior to HGF injection ultrasound guided liver biopsies were taken with a 14G tru cut needle (Medical device technologies) under local anaesthesia with 2 mL of 2% Xylocaine and 5% adrenaline at 2, 3, 4, and 5 days following phx. Seven days after phx, the dogs were euthanized using an overdose of pentobarbital via the cephalic vein. Weights of the liver, kidneys, heart, and spleen were measured and organ weight/bodyweight ratios were calculated. Significant differences in organ weight were analysed with a Student's *t* test by using SPSS software (SPSS Benelux). A *P*-value < 0.05 was considered to be statistical significant. Normal distribution was measured by the Kolmogorov Smirnov test. Formalin-fixed biopsies from the liver were embedded in paraffin, sliced, and stained with haematoxylin and eosin. The slides were examined by one certified veterinary pathologist.

Table 1. Antibodies used in Western blot experiments

Antigen	Manufacturer	Product size	Dilution	Secondary antibody	Dilution
HGF	Lab Vision (4c12.1)	82	1:100	Anti-mouse	1:20,000
c-MET	Sigma (H97861)	169	1:750	Anti-goat	1:20,000
p-c-MET [a]	Abcam (Ab5662)	169	1:750	Anti-rabbit	1:20,000
PKB	BD Bioscience (610876)	58	1:250	Anti-mouse	1:20,000
p-PKB [b]	Cell Signaling (9275)	58	1:1000	Anti-rabbit	1:20,000
ERK 1/2	Cell Signaling (9102)	44/42	1:1000	Anti-rabbit	1:20,000
p-ERK1/2 [c]	Cell Signaling (9101)	44/42	1:1500	Anti-rabbit	1:20,000
PCNA	DAKO (M0879)	36	1:1000	Anti-mouse	1:20,000
Caspase-3	Calbiochem (235412)	34/20/18	1:500	Anti-rabbit	1:40,000
Beta-actin	Lab Vision (pan Ab-5)	40	1:2000	Anti-mouse	1:20,000

[a] Phospho Y1230, Y1234, Y1235.
[b] Phospho T308.
[c] Phospho T202, Y204

Protein electrophoresis, silver staining, and Western blotting.

Proteins were obtained by homogenizing cells or liver biopsies in Radio-Immunoprecipitation Assay (RIPA) buffer containing 1% Igepal, 0.6 mM phenylmethylsulfonyl-fluoride, 17 µg/mL aprotinine, and 1 mM sodium-orthovanadate (Sigma). Twenty micrograms of pooled protein extracts was electrophoresed on Tris-HCl polyacrylamide gels (Biorad), using a Mini Trans-Blot Cell blot-apparatus (Biorad). To detect the total amount of proteins, gels were stained with a Bio-Rad Silver Stain kit, according to the manufacturer's instructions. For Western blotting, proteins were transferred to Hybond-C Extra Nitrocellulose membranes (Amersham Biosciences Europe). The procedure for immunodetection was based on an ECL Western blot analysis system (Amersham Biosciences Europe). Antibodies and used dilutions are described in Table 1. Densitometric quantification was performed in triplicate with a Geldoc2000 system with QuantityOne 4.3.0 software (Biorad). Differences in the optical densities (OD) of the Western blot immunoreactive bands were analysed with a Mann-Whitney U test using SPSS software (SPSS Benelux BV, Gorinchem, the Netherlands). A *P*-value < 0.05 was considered to be statistical significant.

PCNA immunohistochemistry.

Immunohistochemistry was performed on Proliferating Cell Nuclear Antigen (PCNA, DAKO), a cell-cycle dependent protein that is synthesized in early G1 and S phases of the cell cycle. Formaldehyde (4%) fixated, paraffin embedded sections were mounted on poly-L-lysine coated slides, post-fixed in ice-cold acetone for 10 min, air dried and stored at 4 °C until use. For antigen retrieval, slides were boiled in 10 mM citrate buffer (pH 6.0) in a microwave oven for 20 min. Endogenous peroxidase activity was blocked by 0.3% H_2O_2 in methanol for 30 min. Background staining was blocked by incubating the sections with normal goat serum 1:10 diluted in Tris-buffered saline (TBS; 0.01M Tris, 0.9% NaCl, 0.1% Tween-20, pH = 7.6) for 30 min. Sections were incubated overnight at 4ºC with the primary antibody PCNA (1:5,000 in TBS). Sections were incubated in goat anti-mouse IgG (EnVision, DAKO) for 45 min at room temperature. The peroxidase activity was visualised by applying 0.5 mg/mL 3,3-diaminobenzidine chromogen containing 0.035% hydrogen peroxide in PBS, for 10 min at room temperature. Sections were counterstained with haematoxylin for 1 min. The average amount of PCNA positive nuclei of six independent microscopic fields at 40x magnification were counted. Differences in the average amount of PCNA positive nuclei between NaCl and HGF treated livers was analysed with a Student's *t* test using SPSS software. A *P*-value < 0.05 was considered to be statistical significant. A normal distribution of the data and homogeneity of variances were confirmed by the Kolmogorov Smirnov and the Levene's test.

Results

Expression and partial purification of rcHGF.

Three days after infecting High-5 cells with recombinant baculovirus, rcHGF levels in the supernatant were between 10 to 20 mg/l. The rcHGF was subsequently purified using a heparin-sepharose column. Protein-containing fractions that were eluted from the column with 0.7 to 1.2 M NaCl and a conduction of 50-70 ms/cm were collected and analysed using silver staining (Figure 1a) and Western blotting (Figure 1b). Densitometric analysis of the silver staining showed that the purity of rcHGF was >85%. As shown with Western blotting, HGF immunoreactive bands were seen at 82 kDa. After purification, rcHGF was diluted in 0.2 M NaCl in a stock concentration of 0.5 mg/mL and stored in aliquots at –20 °C.

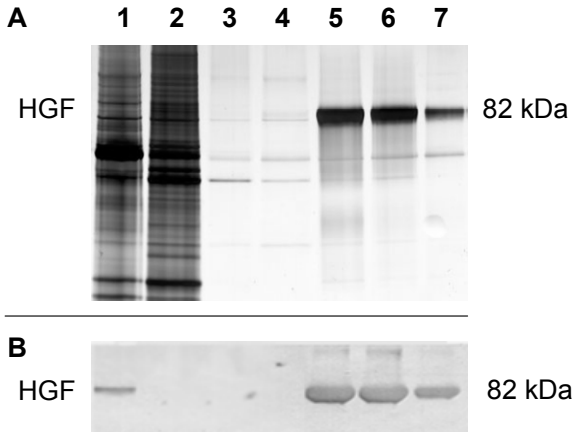

Figure 1. Protein fractions collected during heparin sepharose purification of rcHGF. (A) The detection of the total amount of proteins by means of silver staining. (B) The detection of HGF specific bands by means of Western blot analysis. Lane 1: rcHGF after gamma irradiation, lane 2: rcHGF flow through, lane 3-7: rcHGF fractions eluted with 0.5 (lane 3), 0.6 (lane 4), 0.7 (lane 5), 1.0 (lane 6), and 1.2 (lane 7) M NaCl. As shown in b, rcHGF was present in the fractions eluted from 0.7 to 1.2 M NaCl.

In vitro activity of rcHGF.

Both rcHGF and rhHGF stimulated cell growth of BDE cells in a similar dose-dependent manner. Cell growth started at a concentration of 11 ng/mL HGF and the maximum proliferation activity was seen at 100 ng/mL HGF (Figure 2). As described before by Tajima et al., HGF did not stimulate cell growth in MDCK cells [30]. rcHGF and rhHGF induced cell scattering of BDE cells and MDCK cells at a concentration of 10 ng/mL and 100 ng/mL HGF. Cells were considered to scatter when they lost cell-cell contact and obtained a more fibroblastic appearance as described in previous studies [31, 32]. Representative examples of rcHGF and rhHGF induced cell scattering are shown for MDCK and BDE cells in Figure 3.

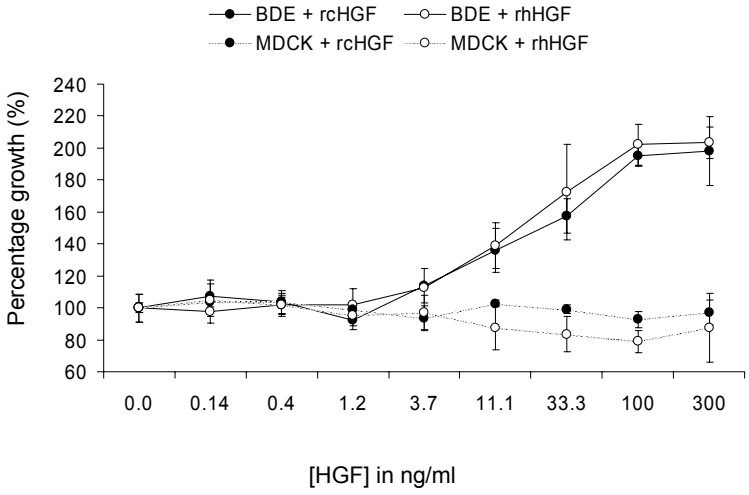

Figure 2. *In vitro* mitogenic effect of rcHGF and rhHGF upon BDE and MDCK cells. Cells were grown in a 96-well culture plates in the presence of 0 to 100 ng/mL rcHGF or rhHGF. After a 24 h culture period, cell growth was measured by using the MTT proliferation assay. Results are presented as means ± standard deviation. HGF induced cell growth in BDE cells in a dose-dependent manner. HGF did not induce cell growth in MDCK cells.

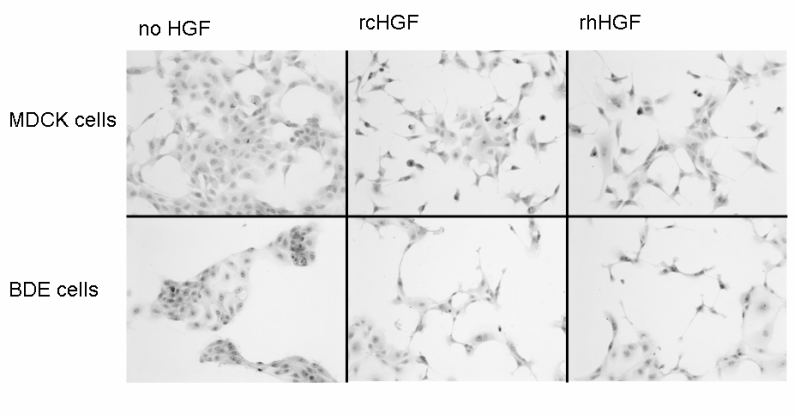

Figure 3. *In vitro* motogenic effect of rcHGF or rhHGF upon BDE and MDCK cells. Cells were grown in an 8-well chamber with or without 100 ng/mL rcHGF or rhHGF. After 10 h culture period, cells were fixed in Boonfix and stained with haematoxylin for 1 minute. Pictures were made at 200x original magnification.

Western blot analysis were used to visualize the phosphorylation and the total amount of c-MET, PKB, and ERK1/2 in MDCK and BDE cells after treatment with 0, 1.5, 15, and 150 ng/mL rcHGF. Figure 4 depicts the immunoreactive bands and the calculated ratios of the ODs of the phosphorylated protein towards the OD of the total protein. In BDE and MDCK cells, rcHGF induced c-MET phosphorylation (Y1230, Y1234, Y1235) and PKB (T308) phosphorylation in a dose dependent manner. The phosphorylation of PKB (T308) and ERK1/2 (T202/Y204) occurred in the presence of 15 ng/mL rcHGF in BDE cells, and in the presence of 1,5 ng/mL rcHGF in MDCK cells. ERK1/2 phosphorylation did not occur in a dose dependent manner. The total amount of proteins of c-MET, PKB, or ERK1/2 did not change when BDE and MDCK were treated with rcHGF. Western blotting against beta-actin (40 kDa) confirmed an equal amount of protein loading on the gel (data not shown)

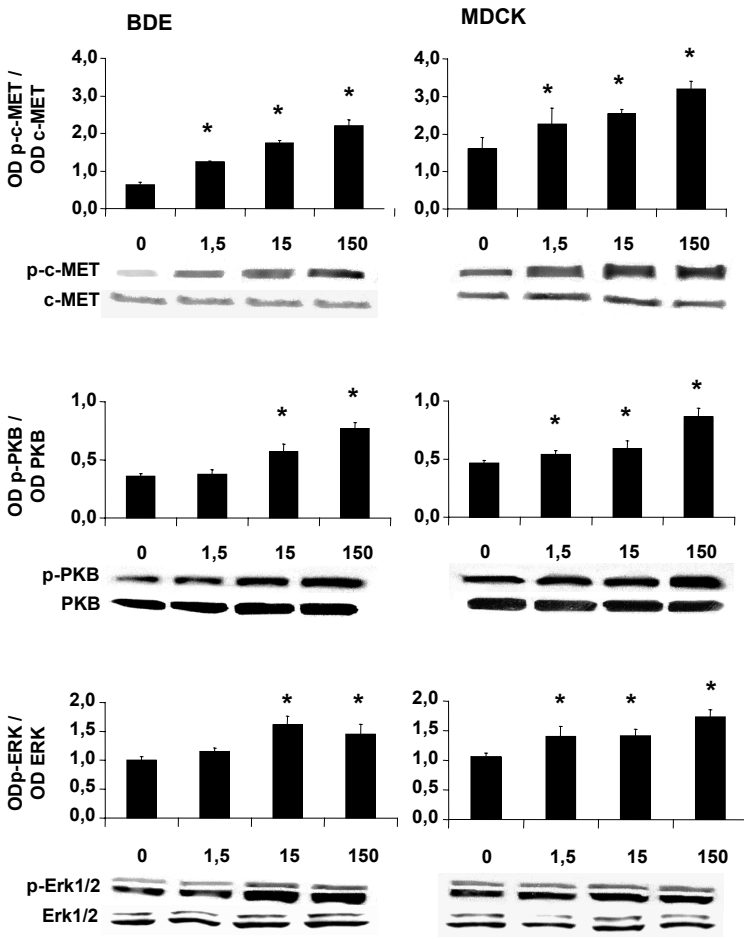

Figure 4. Western blot analysis of phosphorylated and non-phosphorylated c-MET, PKB and ERK1/2, induced by 0, 1.5, 15, or 150 ng/mL rcHGF in BDE and MDCK cells. Optical densities (OD) of immunoreactive bands were measured in triplicate and presented as means with standard deviation. Differences in means were considered significant when $P<0.05$ and are indicated with an asterix (*).

In vivo activity of rcHGF, organ weights, (gross) pathology, and PCNA immunohisto-chemistry.

No differences were found in organ weight/bodyweight ratio between the two groups for the liver (average ratio ± standard deviation; rcHGF: 0.292 ± 0.05; NaCl: 0.316 ± 0.05), kidneys (average ratio ± standard deviation; HGF: 0.051 ± 0.01, NaCl: 0.053 ± 0.01), heart (average ratio ± standard deviation; HGF: 0.079 ± 0.01, NaCl: 0.071 ± 0.03), or spleen (average ratio ± standard deviation; HGF: 0.084 ± 0.02; NaCl : 0.084 ± 0.01). Histopathological assessment of H/E stained slides indicated a normal architecture of the liver without any sign of hypertrophy, hyperplasia, or severe apoptosis at all time points measured.

No macroscopic abnormalities were found during necropsy 1 week after phx in any of the dogs that received rcHGF or NaCl. Immunohistochemistry confirmed a difference in the average amount of PCNA positive cells in HGF treated livers at 5 days after phx (Figure 5), however no differences were found between HGF and NaCl treated dogs at days 0, 2, 3, 4, and 7.

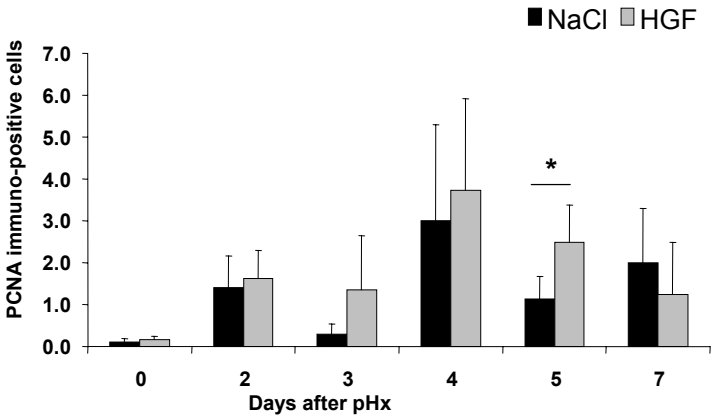

Figure 5. Immunohistochemistry with a PCNA specific antibody on biopsies of NaCl (n=6), or HGF (n=6) treated livers at 0, 2, 3, 4, 5, and 7 days after phx. The Figure presents the average amount of PCNA positive nuclei in six different microscopic fields at 400x original magnification with standard deviations. Differences were considered significant when $P<0.05$ and are indicated with an asterix

In vivo activity of rcHGF: Western blot analysis of c-MET, PKB, and ERK1/2.

Western blot analysis were used to visualize the phosphorylation and the total amount of c-MET (Figure 6A), PKB ((Figure 6B), ERK1/2 (Figure 6C), and PCNA (Figure 6D) in pooled biopsies of NaCl and HGF treated livers at 0, 2, 3, 4, 5, and 7 days after phx. The ODs of the immunoreactive bands were measured and corrected with the ODs of the non-phosphorylated protein, or Beta-Actin. The treatment of rcHGF increased the phosphorylation of c-MET (Y1230, Y1234, Y1235) at days 3, 4, and 5 after phx, the phosphorylation of PKB (T308) at days 2, 3, 4, and 5 after phx, and the phosphorylation of ERK1/2 (T202/Y204) at 4, and 5 days after phx. The amount of the cell cycle protein PCNA was higher in rcHGF treated livers at days 3, and 5 after phx, and higher in the NaCl treated livers at 2 days after phx.

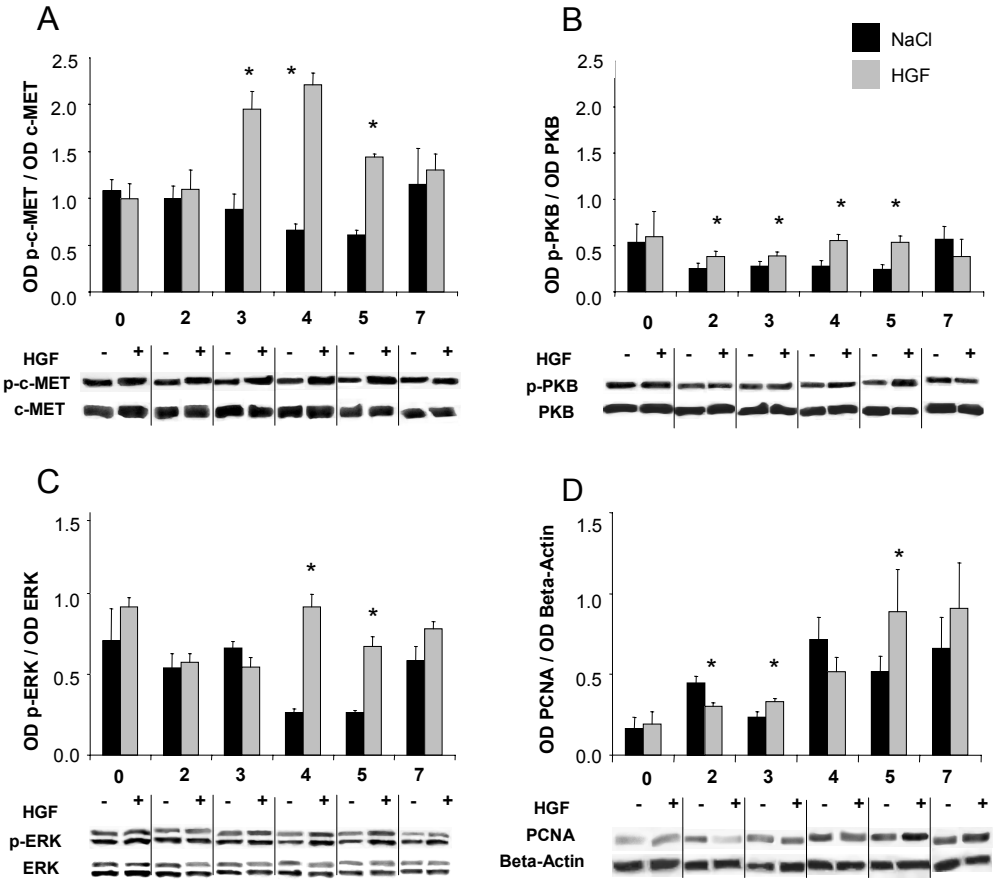

Figure 6. Western blot analysis on pooled liver biopsies of NaCl (n=6), or HGF (n=6) treated livers at 0, 2, 3, 5, and 7 days after phx. Immunoreactive bands were detected for (A) c-MET, (B) PKB, (C) ERK1/2, (D) PCNA, and beta-actin. Optical densities (OD) of the immunoreactive bands were measured in triplicate and presented as means with standard deviation. Differences in means were considered significant when $P<0.05$ and are indicated in with an asterix (*).

In vivo *activity of rcHGF: Western blot analysis of caspase-3.*

Immunoreactive bands were found for inactive caspase-3 (34 kDa) and two cleaved (activated) caspase-3 subunits (20 and 18 kDa) in the individual samples at day 7 after phx (Figure 7A). The ODs of the immunoreactive bands were measured and normalized with the ODs of Beta-Actin (Figure 7B). When compared to NaCl treated livers, HGF treated livers showed similar levels of inactive caspase-3, and lower levels of the two cleaved subunits of caspase-3 at 7 days after phx.

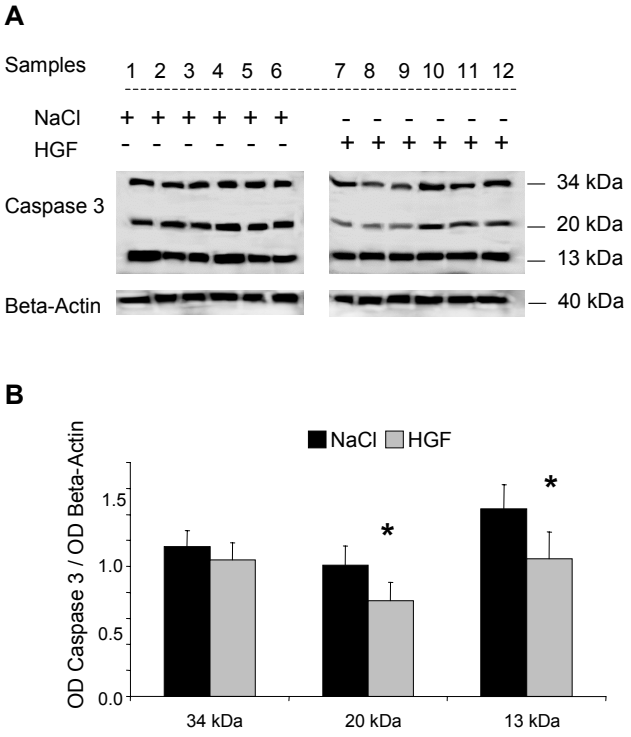

Figure 7. (A) Western blot analysis for inactive (34 kDa) caspase-3, two active caspase-3 subunits (20 and 13 kDa), and Beta-Actin (40 kDa) on liver biopsies of NaCl (n=6), or HGF (n=6) treated livers at 7 days after phx. (B) The optical density (OD) of the caspase-3 immunoreactive bands were corrected with the OD of Beta-Actin and presented as mean ratio with standard deviation for NaCl (n=6) and HGF (n=6) samples. Differences in means were considered significant when $P<0.05$ and are indicated with an asterix (*).

Discussion

HGF is considered the most important factor for the development and regeneration of the liver and the use of HGF may potentially be useful to stimulate liver regeneration in currently untreatable fatal liver diseases [13, 16]. Despite the discovery of HGF more than 20 years ago, the therapeutic use has never been evaluated in naturally occurring liver diseases. As a step toward performing first clinical studies in dogs, biologically active rcHGF was produced using a baculoviral expression system in High-5 cells.

The described production and purification strategy produces sufficient biological active rcHGF to start first clinical trials in dogs. However, the obtained rcHGF product should be further purified before the first clinical studies could start. The production level of rcHGF was higher than the previously reported production of rhHGF using insect cells [33, 34] or mammalian cells [35]. The use of other production systems, for instance the yeast Pichia pastoris [36] has been proposed to yield even higher quantities of more than 500 mg/l recombinant HGF. When the effects of post-transcriptional modifications such as glycosylation [37] are fully known *in vivo*, the use of other production systems, such as non-eukaryotic cells may be a tool to yield mass productions of rcHGF.

In the present study, rcHGF increased the phosphorylation of c-MET, PKB, and ERK1/2 *in vitro* and *in vivo*. Biological activities of HGF are mediated by the phosphorylation of tyrosine residues of the c-MET receptor. Upon HGF-induced phosphorylation, c-MET binds and phosphorylates several intracellular signal transduction molecules that are important for the regeneration of the liver, such as PKB and ERK1/2, one of the Ras-mitogen-activated protein (MAP) kinase cascade [6]. In general, PKB plays a major role in cell viability, while MAP kinases are important in the regulation of cell growth, motility, and differentiation [38]. On the other hand, altered expressions of receptor tyrosine kinases have been shown to play a role in a variety of malignancies [4]. Regarding the sustained phosphorylation of c-MET and downstream pathways of c-MET in the present study, the potential of rcHGF to induce malignancies needs to be evaluated before the beginning of clinical applications.

In the present study, rcHGF moderately increased the amount of PCNA positive cells; however no increase in liver weights were seen at 7 days after phx. This may be explained by the relatively low amount of rcHGF (10 µg/kg/day) that was given when compared to similar experiments in the rats and mice [22, 28]. In rodent experiments, the amount of HGF that is used is highly variable and range between 30 µg - 5 mg/ kg/day [15, 16, 22, 28, 29, 39]. Under consideration that the degree of HGF activity is dose dependent, a stronger effect of rcHGF on liver cell proliferation might be expected with an increased amount of HGF. In contradiction, Kobayashi et al. showed that a very low dose of 0.5

µg/kg/day of HGF was sufficient to increase liver weight in dogs at 3 days after a phx [27]. Therefore, another explanation may be that differences in liver weight may have been found in the present study at earlier time points then at 7 days after phx. This is corroborated by an increased ERK1/2 phosphorylation at day 4 and 5 and an increased amount of PCNA positive cells at day 5, while no differences were found at day 7.

HGF may prevent apoptosis by transcriptionally inducing the Bcl2 family member Mcl-1 and FLIPs through the PKB pathway [40, 41]. Furthermore, altered caspase-3 levels during liver regeneration were suggested to play a role in remodelling of the regenerating liver [42]. In the present study, the administration of rcHGF decreased active caspase-3 levels at 7 days after phx. Additional studies are required to examine the exact role and contribution of caspase-3 during liver regeneration.

Clinical studies using rcHGF in veterinary medicine in dogs are feasible because clinical and clinicopathological examination, diagnostic imaging (ultrasonography, scintigraphy, CT scanning etc.), and sampling of liver biopsies are routinely available techniques in dogs. Furthermore, a world consensus statement was recently reported, addressing clinical and histopathological diagnostic criteria of canine liver diseases such as fulminant, acute, and chronic hepatitis, as well as cirrhosis [43]. This consensus provides researchers with a well defined, uniform terminology and diagnostic criteria that will allow comparison of canine diseases with similar human liver disorders and results between different studies.

In conclusion, both *in vitro* and *in vivo* studies showed a biologically active rcHGF and the High-5 expression system produced sufficient amounts for further applications of rcHGF in naturally occurring canine liver diseases.

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HGF improves viability after H_2O_2-induced toxicity in bile duct epithelial cells.

HGF improves viability after H_2O_2-induced toxicity in bile duct epithelial cells.

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Abstract

Intracellular defence mechanisms against oxidative stress may play an important role in the progression of liver diseases, including cholangiopathies. The multifunctional anti-apoptotic hepatocyte growth factor (HGF) has been suggested to have antioxidant functions.The effect of HGF upon cell viability, the generation of ROS, the expression of genes that play a role in ROS defence, and the activation of caspase-3 were measured in bile duct epithelial (BDE) cells in the presence or absence of H_2O_2. HGF reduced H_2O_2-induced loss of viability, diminished H_2O_2-mediated ROS generation and abrogated H_2O_2-triggered changes in GSH/GSSG ratio. Furthermore, HGF increased the gene-expression of gamma-glutamylcysteine synthetase (GCLC) and glutathione reductase (GSR), while no effect was seen upon the gene-expression of superoxide dismutase 1 (SOD1), catalase (CAT), glutathione peroxidase (GPX1), and glutathione synthetase (GSR). Finally, HGF diminished the proteolytical activation of the key mediator of apoptosis (caspase-3) after H_2O_2 exposure.Together, HGF may improve viability in bile duct epithelial cells after H_2O_2 induced toxicity by proliferation, strengthening the intrinsic antioxidant defences, and/or by an attenuation of apoptosis. These *in vitro* results support the evaluation of HGF as antioxidative factor in hepatobiliary pathologies.

Comparative Biochemistry and Physiology 2008 147, 3, Pages 324-330

Introduction

One of the main functions of the liver is nutrient and xenobiotic handling. Consequently liver cells are exposed to high levels of reactive oxygen species (ROS). Not surprisingly, oxidative stress is involved in the pathogenesis and progression of liver diseases in which inflammation is present, including cholangiopathies [1]. Cholangitis results from a failure in cholangiocyte function or from the reaction of cholangiocytes to acute or chronic liver damage [2]. Although cholangiocytes only account for 3 to 5 percent of the total liver cell population, injury to cholangiocytes and progressive bile-duct loss can result in considerable morbidity and mortality. End-stage liver disease such as primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) account for approximately one third of human patients referred for liver transplantation.

Decreases in oxidative defence mechanisms lead to an accumulation of ROS [3]. An accumulation of these highly reactive molecules may subsequently lead to further cell damage, or cause a cascade of events that are involved in the progression of the disease, such as apoptosis or the formation of extracellular matrix components, and could even contribute to the accumulation of malignant mutations [4, 5].

The scavenging of free radicals in the cell occurs by both enzymatic and non-enzymatic reactions. Enzymes involved in ROS protection include superoxide dismutase 1 (SOD1), catalase (CAT), glutathione peroxidase (GPX1), glutathione reductase (GSR), gamma-glutamylcysteine synthetase, also known as glutamate-cysteine ligase (GCLC), glutathione synthetase (GSS). Antioxidant nutrients (vitamine C and E) and glutathione (GSH) are the most important non-enzymatic antioxidants [6]. Reduced GSH is rapidly oxidized to GSSG by radicals and other reactive oxygen species [7]. The redox ratio of GSH/GSSG decreases in the development of chronic liver disease [3]. Insights into the regulation of antioxidant enzyme expression are, therefore, of crucial importance.

Hepatocyte growth factor (HGF) is a mesenchymal derived multifunctional growth factor that plays an important role in liver development and regeneration by inducing cell proliferation, migration, differentiation, and survival [8, 9]. HGF is found to be protective in chronic liver disease by decreasing the accumulation of extracellular matrix components [10]. Recent papers provided evidence for another mechanism of HGF-mediated cellular protection. HGF acts as an antioxidant factor and decreases oxidative stress-induced apoptosis in different cell types such as primary hepatocytes [11]. Whether HGF acts as an antioxidant factor and decrease oxidative stress-induced apoptosis in cholangiocytes is not fully understood.

In the present study the effect of HGF, in the presence or absence of H_2O_2 was examined upon the cell viability of a bile duct epithelial cell line. To gain insight into the

ROS (SOD1, CAT, GPX1, GSR, GCLC, and GSS) and apoptosis (proteolytical activation of caspase-3) were analyzed before and after exposure to H_2O_2. Furthermore HGF-mediated effects on the GSH/GSSG ratio in absence or presence of H_2O_2 were included.

Material and methods

Chemicals.

H_2O_2 was purchased form Boom (Meppel, the Netherlands). Recombinant canine HGF was produced and purified as described previously [12]. Stock concentrations of 50 µg/ml HGF, dissolved in elution buffer (1M NaCl in 25 mM Tris-HCl), was stored at -20°C.

Cell culture.

The canine bile duct epithelia (BDE) cell line was acquired from the Amsterdam Medical Center (Amsterdam, the Netherlands [13]) and maintained in 10% FCS-supplemented DMEM (Invitrogen, Breda, the Netherlands) as described before [14]. All experiments were performed in DMEM supplemented with 1% FCS. Furthermore, all experiments were started after an overnight starvation period in 1% FCS-supplemented DMEM.

MTT assay.

Cell viability was determined by means of the colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay (MTT, Sigma, Zwijndrecht, the Netherlands) [15]. The formed dark blue formazan crystals were dissolved in 100 µl DMSO and absorbances were measured at 595 nm with a reference at 650 nm. Viability was calculated as a percentage of absorbance in comparison to non-treated cells. MTT assay data are represented as mean values ± standard deviation of three independent experiments that were performed in triplicate.

Determination of reactive oxygen species.

The formation of oxygen radicals was measured after cells were incubated cells with 20 µM of the fluorescent probe 2',7'-Dichlorofluorescein diacetate (H_2DCFDA; Biotium, Breda, the Netherlands) for 30 minutes under standard culture conditions in the dark. The fluorescence was measured at an extinction of 485 nm and an emission of 530 nm.

Measuring GSH and GSSG levels.

GSH and GSSG levels were determined by a colorimetric microplate assay as described before [16] and was based upon the original Tietze assay [17]. For the determination of GSSG levels, GSH was scavenged by adding 3mM 1-methyl-2-vinylpyridinium trifluoromethanesulfonate (M2VP; Sigma-Aldrich) in the cell lysis buffer. In short, cell lysates (1 mg/ml) were incubated with 0.325 mM DTNB (Sigma), 1.5 U/ml GSH reductase, and 0.175 mM NADPH (Sigma) for 5 minutes. The rate of TNB production was measured at 450 nm, and is directly proportionate with the amount of GSH in the samples. The amount of GSH and GSSG was calculated by comparison to a calibration curve.

RNA isolation, revers transcriptase reaction and QPCR.

RNA isolation and QPCR were performed as described before [18]. In short, total cellular RNA was isolated using the RNeasy Mini Kit (Qiagen, Leusden, the Netherlands) and treated with DNase-I (Qiagen, Rnase-free DNase kit). Reverse transcriptase-PCR was performed using iScript™ cDNA Synthesis Kit (BioRad laboratories, Veenendaal, the Netherlands). QPCR was performed in duplicate in a spectrofluorometric thermal cycler (iCycler®, BioRad) using iQ™ SYBR® Green SuperMix (BioRad). Gene-expressions were normalized with the average gene-expressions of the endogenous references beta-2 microglobulin (B2MG), Ribosomal Protein S5 (RPS5) and hypoxanthine phosphoribosyl transferase (HPRT). Primer pairs are indicated in table 1.

Western blot analysis.

The amount of inactive and active caspase-3 protein in the cell was determined by Western blot analysis [19]. In short, twenty µg of denatured protein was electroferesed on 15% Tris-HCl polyacrylamide gels (BioRad) and transferred to Hybond-C Extra Nitrocellulose membranes (Amersham Biosciences Europe, Roosendaal, the Netherlands) using a Mini Trans-Blot® Cell blot-apparatus (BioRad). Immunodetection was based on an ECL Western blot analysis system (Amersham Biosciences Europe). Proteins were visualized with a caspase-3 primary antibody (1:500; Calbiochem/Merck Chemicals Ltd. Nottingham, UK), and a horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:40,000; R&D systems, Europe). A Beta actin primary antibody (1:2.000 dilution; Lab Vision, Fremont, CA) was used to confirm equal protein loading on the gel. Densitometric quantification was performed with a Geldoc2000 system with QuantityOne 4.3.0 software (BioRad), in triplicate.

Statistical analysis.

A Kolmogorov-Smirnov test was performed to establish a normal distribution and a Levene's test for the homogeneity of variances. If values were not normally distributed, the equality of medians among the different groups was compared with the non-parametric Kruskall Wallis test. When there was a difference ($p<0.05$) the Mann Whitney U test was performed to examine the differences between the individual groups. A P value < 0.05 was considered statistically significant. Analysis was performed using SPSS software (SPSS Benelux BV, Gorinchem, the Netherlands).

Table 1. Nucleotide Sequences of Dog-Specific Primers for Real-Time Quantitative PCR

Gene	F/R	Sequence (5'-3')	Tm (^{o}C)	Product size (bp)	Accession number
B2MG	F	TCC TCA TCC TCC TCG CT	61.2	85	XM535458
	R	TTC TCT GCT GGG TGT CG			
RPS5	F	TCA CTG GTG AGA ACC CCC T	62.5	141	XM533568
	R	CCT GAT TCA CAC GGC GTA G			
HPRT	F	AGC TTG CTG GTG AAA AGG AC	56.0	100	AY283372
	R	TTA TAG TCA AGG GCA TAT CC			
SOD1	F	TGG TGG TCC ACG AGA AAC GAG ATG	64	99	AF346417
	R	CAA TGA CAC CAC AAG CCA AAC GAC T			
CAT	F	TGA GCC CAG CCC TGA CAA AAT G	62	119	AB012918
	R	CTC GAG CCC GGA AAG GAC AGT T			
GSS	F	CTG GAG CGG CTG AAG GAC A	62	131	AY572226
	R	AGC TCT GAG ATG CAC TGG ACA			
GCLC	F	TTA TTT GCA AAC CAT CCT GAC	63	92	XM847752.1
	R	ATT TGC AAT TTG GTT ACA CTT CA			
GPX1	F	GCA ACC AGT TCG GGC ATC AG	62	123	AY572225
	R	CGT TCA CCT CGC ACT TCT CAA AA			
GSR	F	TTC AAC CAC CTT TAC CCC AAT GTA TC	61	102	XM532813.2
	R	GAT CCC AAC CAC CTT TTC CTC CA			

Results

Modulating effect of HGF upon cell viability before and after incubation with H_2O_2.

BDE cells were starved for an overnight period (1% FCS-supplemented DMEM) and exposed to different concentrations of HGF with or without H_2O_2 in the presence of 1% FCS. Results are depicted in Figure 1. In the absence of H_2O_2, HGF increased viability around 50%. Cell viability was strongly reduced by H_2O_2 in a concentration-dependent manner (ED_{50} of 10 µM H_2O_2). An HGF-induced increase in viability remained up to 30 µM H_2O_2 when a high HGF dose was given (100 ng/ml), and up to 3 µM H_2O_2 when a low HGF dose was given (10 ng/ml).

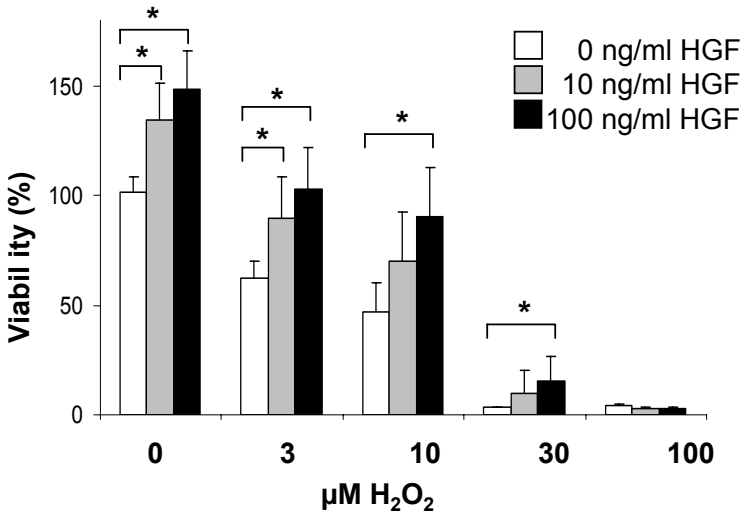

Figure 1. Effect of HGF and H_2O_2 upon cell viability. Cells were pre-treated with 0, 10, or 100 ng/ml HGF for 24 hours. Thereafter 0, 3, 10, 30, or 100 µM H_2O_2 was added for an additional 24 hours. Cell viability was measured using a MTT assay. Results are presented as means ± standard deviation of three independent experiments. Differences in means were considered significant when $P<0.05$ and are indicated with an asterix (*).

Modulating effect of HGF on the amount of ROS before and after incubation with H_2O_2.

The amount of intracellular ROS was determined by using the fluorescent H_2DCFDA probe. This probe detects intracellular ROS in the cytoplasm and cellular organelles such as the mitochondria, by oxidizing into a fluorescent green product which can be measured. The amount of intracellular ROS increased when cells were exposed to 10 µM H_2O_2 for more then 4 hours (Figure 2). This increase in ROS was reduced when cells were pre-incubated with 100 ng/ml HGF. No differences between HGF treated and untreated cells were seen when cells were exposed to higher H_2O_2 concentrations.

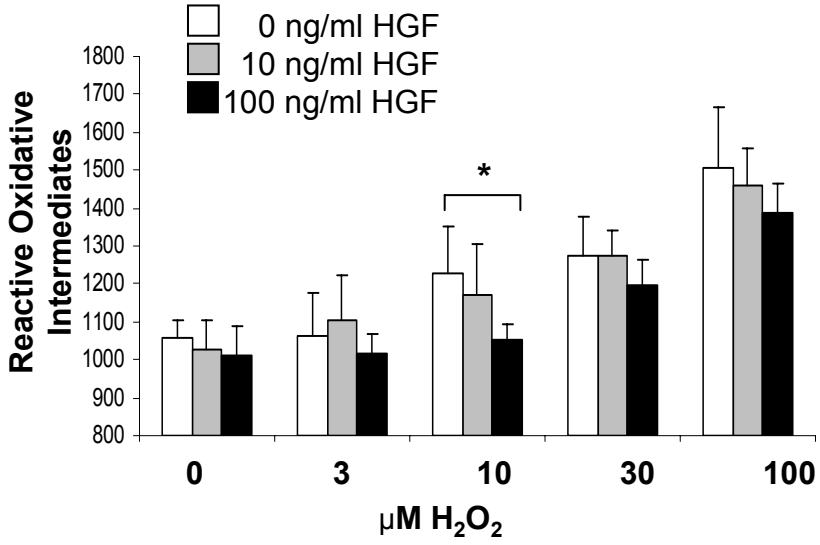

Figure 2. Effect of HGF upon intracellular ROS before and after H_2O_2 exposure. Cells were pre-treated with 0, 10, or 100 ng/ml HGF for 24 hours. Thereafter 0, 3, 10, 30, or 100 µM H_2O_2 was added for an additional 4 hours. Reactive oxygen intermediates were detected using a H_2DCFDA probe, and fluorescence was measured at an extinction of 485 nm and an emission of 530 nm. The results are presented as means ± standard deviation of three independent experiments. Differences in means were considered significant when $P<0.05$ and are indicated with an asterix (*).

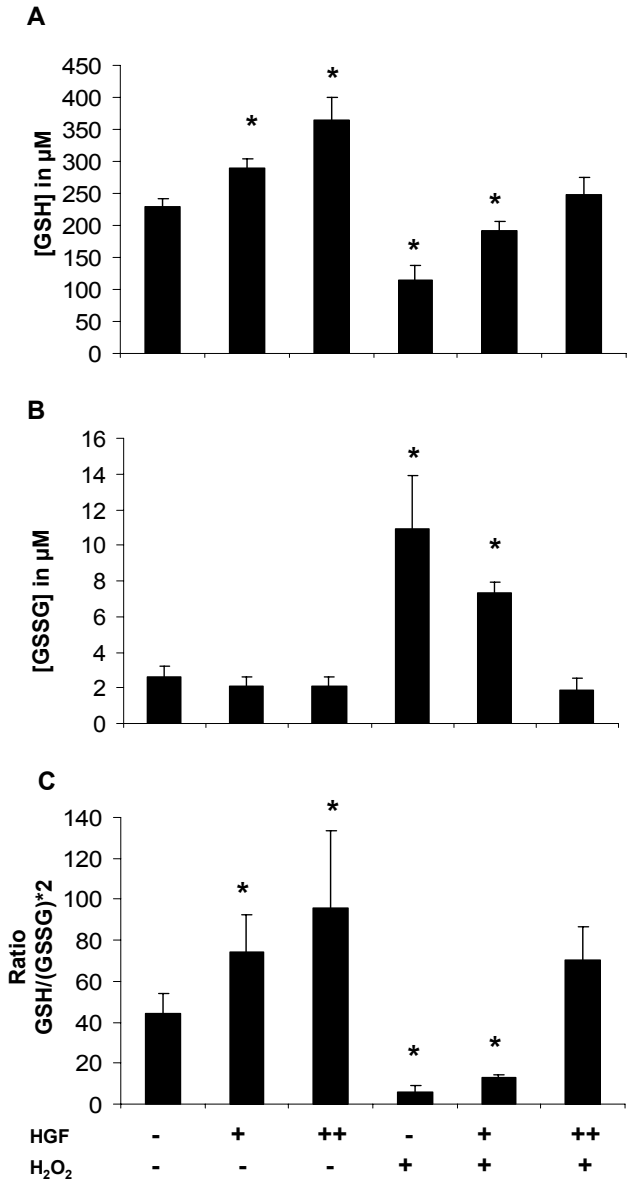

Figure 3. Effect of HGF and H_2O_2 on reduced and oxidized GSH levels. Cells were pre-treated with 0 (-), 10 (+), or 100 (++) ng/ml HGF for 24 hours. Thereafter, 0 (-), or 10 (+) µM H_2O_2 was added for an additional 24 hours. Reduced and oxidized GSH levels were measured and depicted in A (GSH levels), and B (GSSG levels).The ratio of (GSH/GSSG)*2 is depicted Figure C. The results are presented as means ± standard deviation of three independent experiments. Differences in means were considered significant when $P<0.05$ and are indicated with an asterix (*).

The effect of HGF on intracellular GSH and GSSG levels and gene-expression before and after exposure to H_2O_2.

GSH levels increased when cells were treated with 10 ng/ml, or 100 ng/ml HGF, while GSH levels decreased after 10 µM H_2O_2 exposure (Figure 3A). GSSG levels were not altered by 10, or 100 ng/ml HGF, however GSSG levels were strongly increased after H_2O_2 exposure (Figure 3B). The decreased GSH levels, and increased GSSG levels, as triggered by oxidative stress, were diminished by 10 ng/ml HGF and abrogated by 100 ng/ml HGF. Consequently, the GSH/GSSG ratio was increased after HGF treatment and decreased when cells were exposed to H_2O_2. The altered GSH/GSSG ratios by H_2O_2 were again diminished by 10 ng/ml HGF, and abrogated by 100 ng/ml HGF (Figure 3C).

HGF-mediated gene-expression of defence mechanisms that are involved in ROS scavenging after exposure to H_2O_2.

No effect of HGF or H_2O_2 exposure was seen upon the mRNA expression of SOD1, CAT, GSS (synthesis of GSH), and GPX1 (GSH oxidation). GCLC (synthesis of GSH) mRNA expression was increased by 100 ng/ml HGF, and after 10 µM H_2O_2 exposure. However, no additive effect was observed when cells were exposed to HGF and H_2O_2 simultaneously. GSR levels were increased when cells were exposed to 100 ng/ml HGF, or to 10 µM H_2O_2. However, this increase was not observed when cells were simultaneously treated with HGF and H_2O_2. All results are depicted in Figure 4.

HGF reduced the proteolytical activation of caspase-3 after H_2O_2 exposure.

Western blotting against inactive caspase-3 (34kDa) and an active cleavage product (20kDa) was performed after cells were exposed to 10uM H_2O_2 with or without the presence of 10, or 100 ng/ml HGF (Figure 5). No effect of HGF was seen upon the amount of active and inactive caspase-3. The exposure to 10 µM H_2O_2 induced an activation of caspase-3, thereby increasing the active/inactive caspase-3 ratio. The increase in active/inactive caspase-3 ratio was reduced when cells were simultaneously treated with 100 ng/ml HGF.

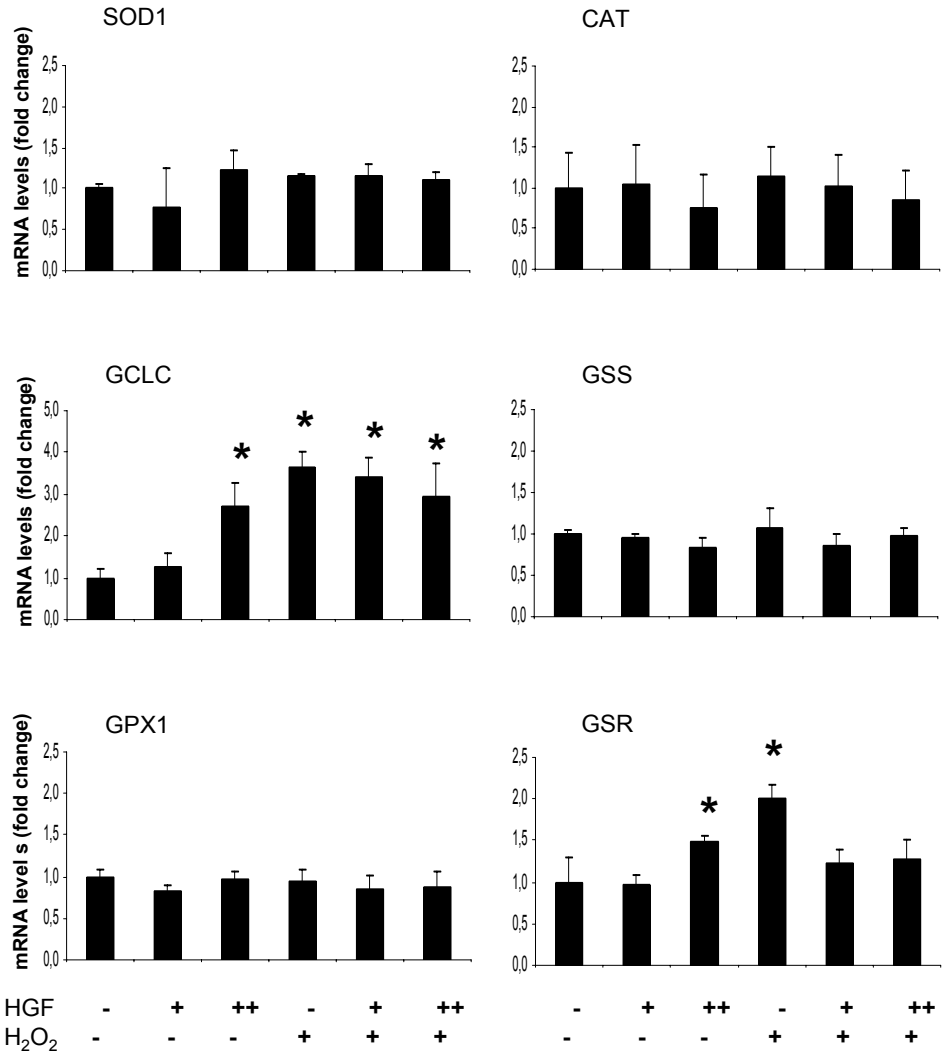

Figure 4. Effect of HGF and H_2O_2 on the gene-expression of SOD1, CAT, GCLC, GSS, GPX1, and GSR. Cells were pre-treated with 0 (-), 10 (+), or 100 (++) ng/ml HGF for 24 hours. Thereafter, 0 (-), or 10 (+) μM H_2O_2 was added for an additional 24 hours. The results are presented as fold change in mRNA (when compared to untreated cells) ± standard deviation of three independent experiments. Differences in means were considered significant when $P<0.05$ and are indicated with an asterix (*).

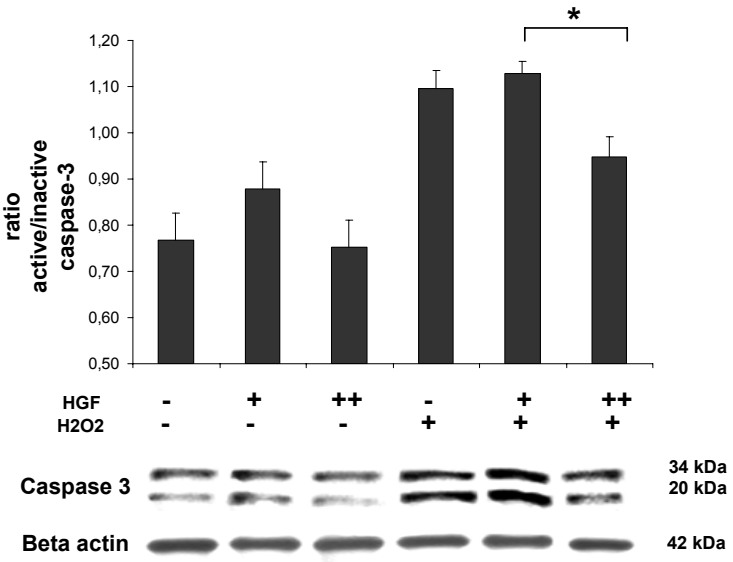

Figure 5. Effect of HGF and H_2O_2 on the amount of inactive (34 kDa), and active (20 kDa) caspase-3. Cells were pre-treated with 0 (-), 10 (+), or 100 (++) ng/ml HGF for 24 hours. Thereafter, 0 (-), or 10 (+) μM H_2O_2 was added for an additional 24 hours. Caspase-3 was measured with Western blotting and the optical density of the bands was measured. The ratio of active / inactive caspase-3 was calculated and depicted in the Figure. Beta actin was used to confirm equal protein loading on the gel. The results are presented as means ± standard deviation of three independent experiments. Differences in means were considered significant when $P<0.05$ and are indicated with an asterix (*).

Discussion

In the present study, we showed that HGF improves viability of bile duct epithelial cells after H_2O_2 exposure. This increase in viability can be explained by several means; I) HGF induced cell proliferation, and replicating cells may be more resistant to toxins than quiescent cells [20]. II) HGF decreased intracellular ROS and may therefore have acted as an antioxidant factor (Figure 2). III) HGF triggered increased production of GSH; consequently the GSH/GSSG ratio had improved (Figures 3, and 4). IV) HGF reduced the amount of the H_2O_2-triggered activation of the pro-apoptotic protein caspase-3, and may therefore have acted as an anti-apoptotic factor (Figure 5). V) HGF may restore the pro/anti-apoptotic balance through other intracellular pathways that were not included in the present study. Finally, these different explanations are not mutually excluding, in fact they can be causally related.

In the present study, increased GSH levels after HGF exposure may be explained by an increased gene-expression of GCLC (the rate limiting enzyme in GSH synthesis) and a

slight increase in GSR, the enzyme that is responsible for the conversion of GSSG to GSH. Although we measured mRNA levels, the altered GSH/GSSG ratio indicted a similar change of GCLC and GSR activity. These results are in line with previous studies. In primary hepatocytes for instance, a dose- and time-dependent increase in intracellular glutathione levels was explained by an HGF induced increase and activation of GCLC [21]. Furthermore, in retinal pigmented epithelial cells, HGF upregulated cellular redox status after oxidative stress was induced by GSH deficiency [22]. Although the exact mechanism can not be explained from these data, HGF may upregulate GCLC expression indirectly via the PI3K pathway [23-25]. The PI3K-PKB pathway is one of the major intracellular signal transduction pathways that is activated by c-MET, the receptor of HGF, and is important for cell survival, growth, proliferation, angiogenesis, metabolism, and migration [26]. An HGF-induced phosphorylation of PI3K-PKB in BDE cells was recently described [12].

Next to an increase of defence mechanisms against oxidative stress, HGF diminished the proteolytical activation of the final executer of apoptosis (caspase-3) after H_2O_2 exposure. An anti-apoptotic role of HGF during oxidative stress was described before in hepatocytes, and again an important role was described for the PI3K-PKB pathway [11]. Furthermore, several studies have investigated protection with HGF against oxidative stress in non-hepatic tissues and cell systems such as mesengial cells [25], hippocampus [27], beta-cells [28], cardiac myocytes [29], and small airway epithelial cells [30]. In these studies, HGF was effective as an antioxidant, either directly affecting ROS production and scavenger expression, or indirectly by modification of pathways leading to apoptosis. In the present study, HGF-mediated protection against oxidative stress in bile duct epithelial cells may be mechanistically similar to previous reports on other cell types. However, additional experiments should be performed to clarify the mechanism in which HGF protects against oxidative stress.

Several studies reported that ROS may be generated by the membrane-localized NADPH oxidase (Nox) triggered by receptor binding of numerous growth factors such as HGF [31]. Although in the present study no such increase in ROS were seen in BDE cells, the generation of moderate levels of ROS cannot be excluded. High levels of ROS are toxic to cells, nevertheless moderate levels of ROS are important for the regulation of cellular functions such as gene expression and signal transduction [32, 33]. For example, ROS may stimulate the gene expression of HGF and the activity of c-MET and it's downstream pathways [34, 35]. This mutual interaction between ROS and HGF needs to be elucidated for a more complete understanding of diseases in which oxidative stress plays an important role.

Cholangiopathies are usually associated with inflammation and bile duct injury which ultimately could lead to bile duct loss and function and an increased chance of developing cholangiocarcinoma. Although the etiology of several cholangiopathies is known

(e.g., inherited disorders, autoimmune, drug-induced, ischemia, infections), for the most part the pathogenesis is poorly defined. One common feature is the formation of ROS which is generally associated with inflammation [3, 36]. Even though the exact mechanism needs to be clarified in additional studies, our data suggests that HGF exerts its protective effect by strengthening the intrinsic antioxidant defences of BDE cells through modulation of intracellular GSH and thus protects by acting on the redox status. The present study provides a basis for further studies in order to develop therapies directed against severe hepatobiliary diseases.

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First clinical trial of HGF treatment in a dog with a congenital portosystemic shunt.

First clinical trial of HGF treatment in a dog with a congenital portosystemic shunt.

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Abstract

A congenital portosystemic shunt (CPSS) of the liver is characterized by a nearly complete diversion of the portal blood flow past the liver resulting in strongly reduced liver growth and function. Conversely, volume and function of the kidneys are increased. The aim of the present study is to examine the effect of hepatocyte growth factor (HGF) administration on liver and kidney size and function, and portal blood flow in dogs with CPSS. This chapter describes the results of the first dog that was treated with HGF.

Treatment with recombinant HGF was given twice daily during three weeks by intravenous injection of 200 µg/kg HGF through a catheter in the jugular vein. Liver and kidney volumes were determined with CT scanning before and at 1, 2, 3, and 7 weeks after starting treatment. At these time points portosystemic shunting was evaluated with an ammonia tolerance test and liver portal perfusion was measured by scintigraphy of the liver and lungs obtained by injection of ^{99m}Tc albumin macroaggregates in a splenic vein. Simultaneously, blood parameters for liver and kidney function were assayed and liver and kidney biopsies were taken for histology and immunohistochemistry.

During three weeks of HGF treatment, hepatocyte proliferation and liver volume increased. Kidney volume remained unaltered, while a slight increased kidney cell proliferation was observed. The abnormal high glomular filtration rate, which is frequently seen in dogs with CPPS, and was also present in this dog, almost normalized during HGF treatment. HGF treatment did not affect portal blood flow, and liver volume decreased to its original size within four weeks after the last HGF administration. Although more dogs should be treated with HGF for a reliable conclusion, the present results indicate that HGF induced substantial liver parenchymal growth. However, within the examined time frame an inadequate expansion of the portal vein branches and unchanged portal blood flow could have caused a reversion of liver volume to pre-treatment level.

Introduction

A congenital portosystemic shunt (CPSS) of the liver is an abnormal vascular communication between the hepatic portal vein and the caudal vena cava, or the vena azygos [1, 2]. Consequently, portal blood flow from the gastrointestinal tract is diverted past the liver limiting the liver's vital functions in metabolism and detoxification. CPSS is a rare condition in man, however the abnormality affects 1-6% of the population in around 20 different dog breeds [3-5]. Dogs with CPSS have a reduced hepatic volume and renal enlargement [6-9]. Hampered liver growth after birth results in an increase of the clinical signs when dogs grow up to maturity. A CPSS will never close naturally and elevated blood levels of toxic compounds, such as ammonia, severely disturb brain function which leads to chronic and severe impairment of the quality of life and to death.

Complex and invasive therapies have been developed that force the portal blood flow into the liver by surgical attenuation of the shunt [10-12]. After a successful therapy, liver and kidney size and function will normalize within 3-5 weeks. However, due to the potential risk of portal hypertension after surgical narrowing of the shunt, complete closure is almost never possible [13, 14]. Furthermore, recurrence or persistence of clinical signs has been documented with all techniques used for shunt attenuation. Overall the mortality rate at 30 days after surgery ranges from 2.1% to 29%, depending on the type of shunt (extra- or intra-hepatic) and the degree of portal vein hypoplasia within the liver [15-18].

A stimulation of the liver to grow to its normal size may be sufficient for a remission of the clinical signs, because clinical problems in dogs with CPSS arise from the specific combination of both shunting and decreased liver function. The most promising candidate to stimulate the liver to grow is Hepatocyte Growth Factor (HGF). HGF is a multifunctional protein that plays an important role in many physiological processes, such as mammalian development, angiogenesis and tissue regeneration [19-21]. The therapeutic potential of HGF is proven in numerous toxin-induced acute and chronic liver failure models in rodents [22-25]. However, HGF has never been used in clinical patients with spontaneous liver disease, such as dogs with CPSS. The presence of the receptor of HGF (c-MET) on a molecular level as well as functional down-stream regulators in dogs with CPSS indicated that they are potentially good candidates for treatment with HGF [26].

The aim of the present study is to examine the effect of HGF treatment on liver and kidney size and function, and portal blood flow in dogs with a CPSS. This chapter describes the results of the first dog that was treated with HGF.

Materials and methods

Materials.
Recombinant feline HGF (fHGF) was provided by Zenoaq (Fukushima, Japan). Recombinant canine HGF (cHGF) was provided by Intervet (Boxmeer, the Netherlands). Recombinant human HGF (hHGF) was purchased from R&D (Oxon, UK).

In vitro activity of recombinant feline, canine, and human HGF.
Canine bile duct epithelial (BDE) cells were acquired from the Amsterdam Medical Centre, Experimental Liver cell bank [27]. Madin-Darby Canine Kidney Cells (MDCK) were purchased from the American Type Culture Collection (ATCC). Culturing and experimental conditions are similar to conditions as described before [28]. The *in vitro* mitogenic activity was measured by using the colorimetric MTT assay using BDE cells. Cell growth was calculated as percentage increase in absorbance (595 nm) of HGF treated cells compared to non-treated cells. The phosphorylation of c-MET was visualized by Western blotting in BDE and MDCK cells after incubation with 100 ng/ml fHGF, cHGF or hHGF for 5 minutes [28]. The antibodies used are listed in Table 1.

In vivo activity of recombinant feline HGF.
Activity of feline HGF *in vivo* was measured by Western blotting in liver biopsies that were taken exactly at 15 minutes after the first HGF injection.

Dog patient and HGF treatment.
This animal procedure was approved by and performed according to the standards of the Ethics Committee of Animal Experimentation of the Utrecht University. Furthermore, the experiment was conducted with informed consent of the owners. One female dog (5,5 years; 3,2 kg) with an extrahepatic congenital portocaval shunt was treated with fHGF (200 μg /kg; two times a day) for a period of 3 weeks by injection through a central venous catheter (Cavafix®Certo®, B.Braun, Oss, the Netherlands) in the jugular vein. The time points of examinations (see below), are scheduled in Figure 1. The dog was fed the Royal Canin hepatic diet (Veghel, the Netherlands).

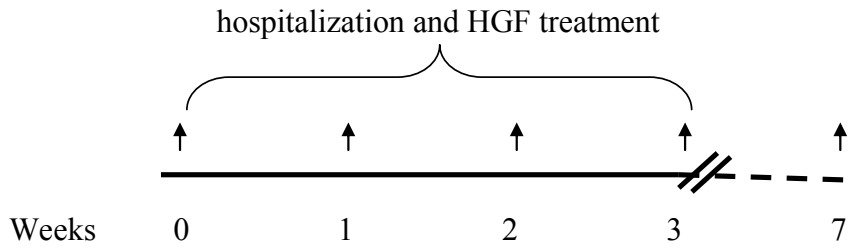

Figure 1. Schedule of hospitalization, HGF treatment. An ↑ indicates time points of blood and urine analysis, ammonia tolerance test, GFR, CT scan, perfusion scan, and liver kidney biopsies. Kidney biopsies were not taken at week 2.

Blood and urine analysis.

Blood was sampled through the jugular catheter and plasma was analyzed for the following parameters: activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), concentration of bile acids, total proteins, albumin, urea, creatinine, and fibrinogen. Urine was sampled by cystocenthesis and was routinely analyzed for total proteins and creatinine.

Ammonia tolerance test.

To measure the degree of portosystemic shunting, a dose of 2 ml/kg NH_4Cl (5%) was administered rectally to the colon via a 2.0 mm Arnolds® catheter (AUV, Cuijk, the Netherlands). The amount of ammonia in the blood was measured at 0, 20, and 40 minutes after the injection using the Ammonia Test Kit II (Arkray, Amstelveen, the Netherlands) combined with the Ammonia Checker II (Menarini, Florence, Italy).

Glomular filtration rate (GFR).

In a volume of 1 ml, 28 MBq ^{99m}Tc-DTPA Diethelene Triamine Pentacetic Acid (TechneScan® DTPA, Mallinckrodt Medical B.V., Petten, The Netherlands) was injected in the vena cephalica. Blood samples were collected at 5, 30, 60, 90, 120, 180, 240, and 300 minutes after the injection. The radioactivity was measured in 0.5 ml plasma using an automated gamma well-scintillation counter (Packard BioScience Benelux). The GFR was calculated with the two-compartment model described in Figure 2.

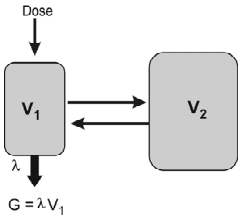

Figure 2. Two-compartment model.

General anaesthesia.

For premedication, 0,3 mg/kg methadone IV (Eurovet, Bladel, the Netherlands) and 0.02 mg/kg atropine IM (Eurovet, Bladel, the Netherlands) were used. For induction, 1-5 mg/kg propofol IV (PropoVet™, AST Farma, Oudewater, the Netherlands) was used. For maintenance isoflurane (IsoFlo, AST Farma, Oudewater, the Netherlands) was given via an endotracheal tube (0.5-2% Et). During recovery 10-20 µg/kg buprenorphine was given i.m. (Temgesic®, Schering-Plough, Utrecht, the Netherlands).

Determination of the liver and kidney volumes using computed tomography (CT) scanning.

CT was performed in the anaesthetized dog with a single slice spiral CT scanner (Secura, Philips NV, Eindhoven, the Netherlands). CT of the liver and the kidneys was performed using 120 kV, 280 mA, 0,7 s scan time and a pediatric filter. Section thickness was 3 mm, with a 1 mm pitch and a reconstructed section thickness of 2 mm. Liver volume was calculated using EasyVision software (Philips NV, Eindhoven, the Netherlands) with a window width of 200 and a window level of 50 for all measurements. On each transverse section of a series, a Region of Interest (ROI) was drawn from which the area was calculated by the software. For the liver, the ROI included the liver parenchyma and the internal blood vessels that were completely surrounded by the liver parenchyma. Adjacent blood vessels, such as some parts of the caudal vena cava, and the gallbladder were excluded. For the kidneys, the ROI included the kidney parenchyma and excluded the pelvis and ureters. Volume was calculated for the entire series by multiplying the area of the ROI with section thickness [29].

Perfusion scintigraphy.

Perfusion scintigraphy was performed with an Integrated ORBITER Gamma Camera System with open ICON Workstation (Siemens Medical Systems, the Netherlands) equipped with a low-energy high resolution parallel-hole collimator. The first injection of 30 MBq ^{99m}Tc-MAA Macro aggregates (Technescan® LyoMAA, Mallinckrodt Medical B.V., Petten, the Netherlands) was injected in the splenic vein under ultrasound guidance. After the aggregate injection the dog was positioned with the right lateral side on top of the collimator (turned 180^{o}) and the first image was obtained during a period of 4 minutes. In the same position, the second injection of 30 MBq ^{99m}Tc-MAA Macro aggregates was injected in the vena cephalica and after 1 minute the second image was obtained during 4 minutes. The calculations were done with an open ICON Workstation computer using the Siemens ICON™ computer system software connected to the gamma camera. The shunt index (SI) was calculated with the following formula: SI = cpm lungs / (cpm lungs + cpm liver).

Molecular analysis and histology of liver and kidney biopsies.

Ultrasound guided liver- and kidney-biopsies were taken with a fine needle (B.Braun, for Western blotting) or a 14G tru cut needle (ACN™, Medicor, Nieuwegein, the Netherlands, for immunohistochemistry). For Western blot analysis, liver biopsies were snap frozen and stored at -70°C until use. For (immuno) histochemistry, biopsies were fixed in 4% neutral buffered formalin for 4 hours, embedded in paraffin, and stored at 4°C until use. Western blot and immunohistochemistry for KI67 were performed as described before [28]. Used antibodies are listed in Table 1. For histological evaluation, tissue sections were stained with HE and PAS.

Results

In vitro activity of recombinant feline, canine, and human HGF.

Cell proliferation of BDE cells was measured with an MTT assay. Cellular proliferation was induced at a concentration of 11 ng/mL HGF and a maximal proliferative activity was seen around 100 ng/mL HGF (Fig. 3A). No differences were seen in proliferative activity between recombinant, fHGF, cHGF, and hHGF after an incubation period of 48 hours. Phosphorylation of the receptor of HGF (c-MET) was visualized in BDE and MDCK cells with Western blotting (Fig. 3B). A phosphorylation of c-MET was observed in both cell lines after 5 minutes of incubation with HGF. No differences were seen in c-MET phosphorylation after recombinant fHGF, cHGF, or hHGF treatment.

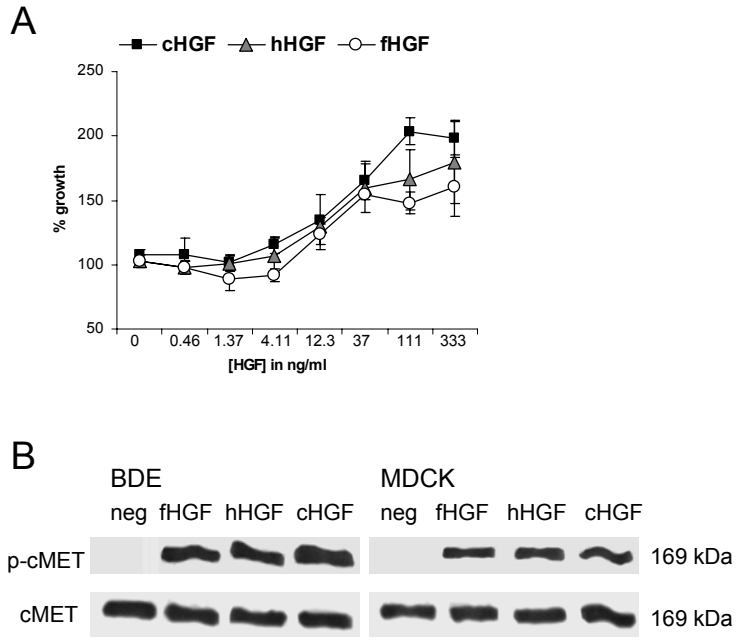

Figure 3. *In vitro* activity of recombinant HGF. (A) Line graph representing the mean percentage growth of bile-duct epithelial cells (BDE) after recombinant HGF treatment, as measured by the MTT assay. (B) Effect of recombinant HGF treatment on the c-MET protein in BDE or Madin-Darby Canine Kidney cells (MDCK) after 5 minutes of treatment with HGF. fHGF; feline recombinant HGF, cHGF; canine recombinant HGF, hHGF; human recombinant HGF.

In vivo activity of recombinant fHGF.

To measure the *in vivo* activity of fHGF, the phosphorylation of c-MET and downstream ERK1/2 was measured in aspiration biopsies of liver tissue at 0, 5, 10, 15, 30, and 60 minutes after fHGF injection (Fig. 4). An increased phosphorylation of c-MET and ERK1/2 was seen 5 minutes after fHGF injection. Phosphorylation reached maximum levels at 10 minutes and a sustained phosphorylation of c-MET and ERK1/2 was present for at least 1 hour after fHGF treatment.

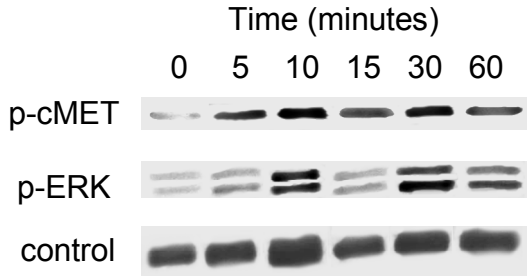

Figure 4. *In vivo* activity of recombinant feline HGF at different time points after an intravenous injection (200 µg/kg).

Histological examination of liver and kidney biopsies.

(I) Liver. Before HGF treatment (week 0), no recognizable portal veins were observed in the portal areas, they showed slight proliferation of arterioles as well as bile ducts. In both portal areas and throughout the parenchyma many lipogranulomas were seen. Binucleated hepatocytes were counted in the periportal parenchyma and on average 1.3 were present per portal area. During HGF treatment (weeks 1, 2, and 3) no changes were seen in liver histology, except for the presence of double-lined hepatocytic cords and an increased presence of binucleated hepatocytes ranging from 2.1 per portal area in weeks 1 and 2, and 1.6 per portal area in week 3.

(II) Kidneys. Before HGF treatment (week 0), a normal aspect of kidney histology was found. During (week 3) and at four weeks after HGF treatment (week 7), slight glomerular changes were observed with slight protein deposition in Bowman's space and podocyte swelling. Furthermore, protein casts were found in some tubular lumina at week 7.

Blood analysis.

Results are depicted in Table 1. Liver enzymes (ALT, AST, and AP) ranged within reference values before (week 0) during (weeks 1, 2, and 3), and 4 weeks after fHGF treatment (week 7), except for an increase of AP in week 3. Bile acids were higher than reference values at all time points, as is usually seen in dogs with CPSS. The increased amount of bile acids at weeks 1 and 2 is probably due to feeding before blood sampling. Total protein and albumin levels were slightly lower than reference values at all time points measured, reflecting the small size of the liver and an impaired liver function. Fibrinogen levels ranged within reference values at week 0, 1, and 7 and an increased fibrinogen level was measured at week 3. Urea and creatinine levels were lower than reference values, which could be explained by decreased urea formation in the liver and a high glomerular filtration activity of the kidneys. No changes in urinary protein/creatinine ratio were found during HGF treatment, except for week 7 which was caused by hematuria induced by a kidney biopsy. Overall, no effect of HGF treatment was seen on measured blood and urine parameters.

Table 1. Blood parameters and protein/creatinine ratio in the urine before (week 0), during (weeks 1, 2, and 3), and 4 weeks after HGF treatment (week 7). – No fibrinogen was measured at week 2.

	Reference values	Week 0	Week 1	Week 2	Week 3	Week 7
ALT (U/L)	< 54	23	22	19	22	28
AST (U/L)	< 34	36	37	31	29	47
AP (U/L)	< 73	59	58	60	100	61
Bile acids (μmol/L)	<10	60	240	270	62	118
Total protein (g/L)	55-72	53	49	50	52	49
Albumin (g/L)	26-37	21	19	20	18	18
Urea (mmol/L)	3.0-12.5	< 0.3	0.4	0.8	0.6	0.6
Creatine (μmol/L)	50-129	33	34	39	33	38
Fibrinogen (g/L)	1.0-2.8	1.6	2.1	-	3.3	1.6
Protein/creatinine ratio urine		4.0E-5	4.6E-5	4.3E-5	1.9E-5	2.1E-4

Liver volume and liver cell proliferation.

Liver was scanned by CT and liver volume was calculated before (week 0), during (weeks 1, 2, and 3) and 4 weeks after (week 7) HGF treatment (Fig. 5A). Compared to the liver volume of week 0, liver volume was increased 3% at week 1, 11% at week 2, and 44% at week 3, while no increase was found at week 7. The amount of KI67 immunopositive hepatocytes was calculated after immunohistochemistry (Fig. 5B). When compared to week 0, the amount of immunopositive hepatocytes was increased to 218% in week 1, 239% in week 2, and 377 % in week 3, while no increase was found in week 7.

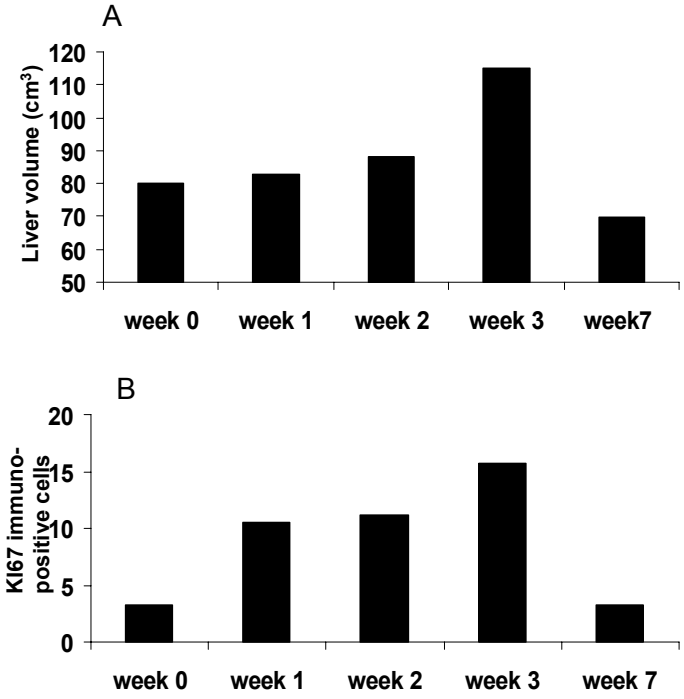

Figure 5. Liver volume and hepatocyte proliferation before (week 0), during (weeks 1, 2, and 3) and after HGF treatment (week 7). (A) Volume determination of the liver using computed tomography (CT) scanning. (B) Average number of counted KI67 immunoreactive cells in the liver per microscopic field at 200x original magnification.

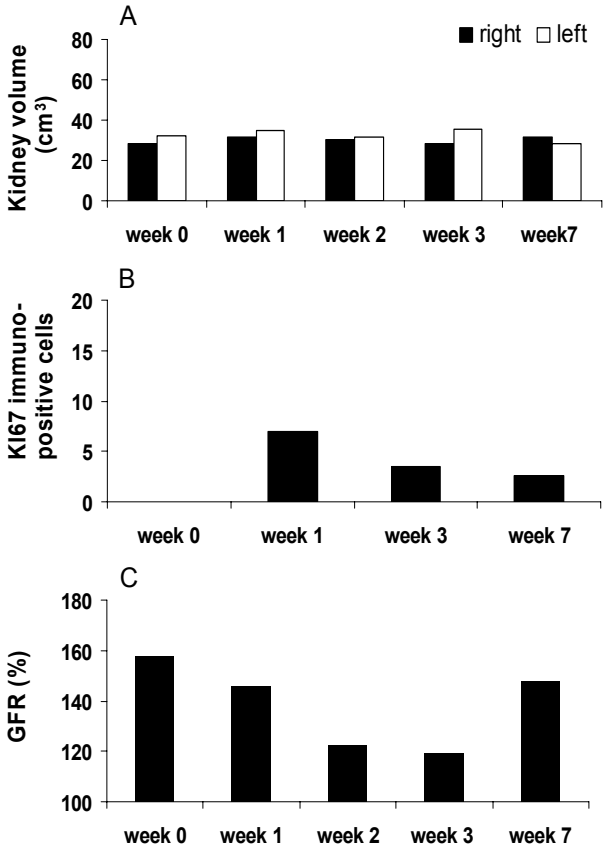

Figure 6. Kidney volume, kidney cell proliferation, and glomerular filtration rate (GFR) before (week 0), during (weeks 1,2, and 3) and 4 weeks after HGF treatment (week 7). (A) Volume determination of the kidney using computed tomography (CT) scanning. (B) Average number of KI67 immunoreactive cells in the kidney per microscopic field at 200x original magnification. (C) Glomerular filtration rate (GFR) as percentage (%).

Kidney volume, kidney cell proliferation, and GFR.

The kidneys were scanned by CT and kidney volume was calculated before (week 0), during (weeks 1, 2, and 3) and 4 weeks after (week 7) HGF treatment (Fig. 6A). Total kidney volume (left and right) varied from 60 cm^3 (week 0), to 67 cm^3 (week 1), 62 cm^3 (week 2), 64 cm^3 (week 3), and 60 cm^3 (week 7). The amount of KI67 immunopositive nuclei was counted after immunohistochemistry (Fig 6B). No immunopositive cells were observed in week 0. However, 7 immunopositive cells per microscopic field were observed in the proximal and distal tubular epithelium in week 1. Thereafter, immunopositive tubular epithelial cells

gradually declined to 3.5 per field in week 3 and to 2.6 per field in week 7. No positive nuclei were detected in glomerular cells before, during or at 4 weeks after HGF treatment.

The GFR before, during and 4 weeks after HGF treatment is given in Fig. 6C. A filtration rate of 100% is considered normal, although this percentage may vary as filtration rate depends on different factors, such as age, weight, and breed of the dog. Furthermore, dogs with CPSS normally show a remarkable renal enlargement combined with a renal/glomerular hyperfiltration which both normalize after surgical correction of the shunt. In the present dog GFR was 158% (week 0), and declined during HGF treatment to 146% (week 1), 122% (week 2), and 119% (week 3). At 4 weeks after HGF treatment (week 7), GFR was 148%.

Perfusion scintigraphy.

The percentage of portosystemic shunting was determined by an ultrasound-guided percutaneous injection of ^{99m}Tc-MAA in the splenic vein. Without shunting of the liver, the macroaggregates lodge in the small hepatic capillaries, showing radioactivity in the liver. When a shunt is present macro aggregates will bypass the liver and lodge in the pulmonary capillaries, showing radioactivity in the lungs. In the present dog, the shunt fraction was 98% in week 0, and did not change during (weeks 1, 2, and 3), or 4 weeks after HGF treatment (week 7).

Discussion

HGF is well known as an organotrophic factor and has therefore been suggested as potential therapy to stimulate tissue regeneration in a wide variety of diseases, including liver diseases. HGF treatment was recently suggested for dogs with CPSS, a condition characterized by insufficient hepatic growth. An increase in functional liver mass by HGF may be sufficient for a remission of the clinical signs, because portosystemic shunting only causes clinical problems when combined with failing liver function. In addition, the reported angiogenic effects of HGF could be beneficial for increasing the portal perfusion of the expanding liver, and concurrent hemodynamic changes could reduce shunting to a subclinical level. In the present study the results of the first HGF treatment of a dog with CPSS is described.

After three weeks of HGF treatment, liver volume and hepatocyte proliferation were increased; however, a remission of liver volume was seen at four weeks after HGF treatment. This remission could be explained by the unaltered portal blood flow to the liver. The portal blood flow plays an important role in liver size and regeneration, which can be mutually explained by the delivery of hepatotrophic factors (derived from the intestine, pancreas, and

spleen) by the portal vein and portal blood pressure [30, 31]. The importance of the portal blood flow is illustrated by several experimental models. For instance, an obstruction of the portal blood flow in one part of the liver (portal vein embolization) induces atrophy of the affected part and hypertrophy of the other unaffected part. Conversely, a reduction of the portal flow weakens hepatocyte proliferation and delays regeneration of the liver after a partial hepatectomy [32, 33]. This physiology is also apparent when surgical ligation of a portosystemic shunt is performed, after which liver volume increases to normal size within weeks. Considering these studies, an HGF-induced increase in liver volume in dogs with CPSS would therefore not sustain without an increase in the portal blood flow.

Most knowledge concerning the tremendous regeneration capacity of the liver is gained from partial hepatectomy (phx) studies in rodents. After a phx tissue remodelling, such as the proliferation of endothelial cells, occurs later than the proliferation of epithelial cells. The replication stimulus induced by HGF alone is weaker compared to a phx, and could require more time for a complete regeneration of the liver which involves all mature cell-types of the liver, including endothelial cells. In the present study, merely proliferation of hepatocytes was observed, indicating an inadequate effect on the portal veins and the portal hepatic perfusion. Therefore, a prolonged HGF treatment or an extra stimulation of vascular development (for example by vascular endothelial growth factor, VEGF) could be considered if the lack of portal blood flow proves to be a constant phenomenon in more dogs [34]. Furthermore, age could influence the response to HGF treatment, as it is known from surgical ligation that a higher age is a risk factor for survival. Considering the age (5,5 years old) of the present dog, the HGF treatment of other (younger) dogs might induce changes in portal perfusion next to liver growth.

The availability of recombinant feline HGF and the high homology of amino acid sequence with canine HGF (97.5 %) prompted the use of feline HGF in this clinical trial [35]. As expected, *in vitro* activity of feline HGF was highly comparable to that of canine HGF in two epithelial canine cell lines. Furthermore, one intravenous injection of feline HGF in the present dog induced a sustained c-MET and ERK1/2 phosphorylation in the liver for at least 1 hour, confirming the activity of fHGF in dogs. However, a prolonged treatment with a recombinant feline-based protein in dogs could trigger the formation of antibodies. In addition, repeated administrations of HGF could have an adverse effect on kidney function [36], for instance by suppressing the expression of the podocyte-specific nephrin gene (an important protein that is involved in retaining the structure and function of the slit diaphragm). In the present dog, no indications of antibody formation were found. However, slight glomerular changes were observed at 3 and 7 weeks. Even though a decreased GFR is associated with an increase in liver function after surgical correction of a shunt, extra attention should be given to the detection of antibodies against HGF and to the function of the

kidneys in the next dogs to be treated with fHGF. In addition, further studies are required to clarify the tubular proliferation following repeated doses of HGF in clinical patients.

In conclusion, HGF treatment induced substantial growth of the liver in a dog with CPSS within three weeks. However this treatment was not sufficient in the present dog to increase blood flow to the liver, which is obviously required for a sustained liver size after HGF treatment. The treatment of future dogs of different breed and age will show the possible need for a longer HGF treatment, and/or the co-administration of other growth factors such as VEGF.

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In vitro differentiation of liver progenitor cells derived from healthy dog livers.

In vitro differentiation of liver progenitor cells derived from healthy dog livers.

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Abstract

Naturally occurring liver disease in dogs resemble human liver disease in great detail; including the activation of liver progenitor cells (LPC) in acute and chronic liver disease. The aim of the present study was to isolate, culture, and characterize progenitor cells derived from healthy mature dog livers. A hepatocyte fraction was isolated and cultured in Hepatozyme-serum-free media (SFM) to stimulate the growth of colony-forming small epithelial cells. After two weeks of culturing, clonal expansion of keratin 7 immunopositive small cells with a large nucleus/cytoplasm ratio emerged in the hepatocyte monolayer. These colonies expressed genes of several hepatocyte (CYP1A1, ALB, and KRT18), cholangiocyte/LPC (KRT7 and KRT19), and progenitor cell markers (AFP, CD44, PROM1, KIT, THY1, and NCAM1), indicating their immature and bi-potential nature. Gene-expression profiles indicated a more pronounced hepatic differentiation in Hepatozyme-SFM compared to William's Medium E (WME). Furthermore, colony-forming cells differentiated towards intermediate hepatocyte-like cells with a more pronounced membranous keratin 7 immunostaining.

In conclusion, colony-forming small epithelial cells in long-term canine liver cell cultures express liver progenitor cell markers and have differentiating capacities. These cells may therefore be considered as progenitor cells of the liver.

Submitted

Introduction

Liver progenitor cells (LPCs) are bi-potential cells of the liver that are located in the smallest and the most peripheral branches of the biliary tree; the canals of Hering [1-3]. In healthy livers these cells are present in scarce numbers. However, LPCs proliferate and invade the parenchyma in several pathological conditions such as (sub)acute hepatitis with severe hepatocellular necrosis, chronic biliary hepatitis, (non)alcoholic fatty liver diseases, and cirrhosis [4, 5]. Depending on which cell type is damaged the most, activated LPCs differentiate into mature hepatocytes or cholangiocytes to reconstitute the damaged liver. LPCs coexpress hepatic markers such as albumin (ALB) and cytokeratin 18 (K18), biliary markers such as cytokeratins 7 and 19 (K7/K19), fetal hepatoblast markers such as α-fetoprotein (AFP), and hematopoietic/mesenchymal stem cell markers such as prominin1 (PROM1), T-cell antigen (THY1), CD44 antigen (CD44), KIT oncogene (KIT), and neural cell adhesion molecule 1 (NCAM1) [6-11]. The bi-potential nature and the high resistance to toxins make these cells of great (pre)clinical importance for currently untreatable liver diseases. A tool to gain insight into the molecular mechanisms and regulatory events leading to LPC activation (proliferation and differentiation) is provided by culturing these cells in vitro.

By setting specific culture conditions, a subpopulation of liver epithelial cells with a immature morphology and a high growth potential can be obtained from adult livers [12, 13]. This 'plate and wait' technique for adult progenitor cells was first described for rat liver cell cultures [14, 15]. These cells share hepatocytic, biliary, and progenitor cell markers, and differentiate towards mature hepatocytes under specific culture conditions *in vitro* [16]. Recently the equivalent of rodent progenitor cells in culture was described from diseased and healthy human livers [17-19]. These recently described cultures of human progenitor cells and the demonstrated LPC activation in clinically occurring liver diseases in dogs [20-22] prompted us to explore the potential to grow liver progenitor cells derived from healthy dog livers under different culture conditions.

Dogs referred to veterinary clinics are gaining increased attention to bridge rodent models to human medicine, because naturally occurring liver diseases of dogs have a high homology with human liver diseases at the molecular and pathological level [23-26]. In the present study, a hepatocyte fraction was isolated from healthy dog livers and cultured in hepatocyte-specific medium. After two weeks of culturing, clonally expanding epithelial cells were cultured in different media to investigate the effect on differentiation potential based on morphology, immunocytochemistry and gene-expression profiles. The described protocol for the isolation and culturing of liver cells derived from healthy dog livers is a reproducible method for obtaining progenitor cells with differentiation potential in culture.

Materials and methods

A*nimals.*

Livers were obtained from six healthy surplus dogs that were euthanized in liver-unrelated research projects. All these projects were approved by and performed according to the standards of the Ethics Committee of Animal Experimentation of the Utrecht University. Before euthanasia, dogs received an intravenous injection of 10.000 IE heparin (Leo Pharma BV, Breda, the Netherlands). HE stained slides from formaldehyde (4%, Klinipath B.V., Duiven, the Netherlands) fixed paraffin embedded liver biopsies were histo-pathologically examined. In all livers under study no indication of liver pathology was found.

Isolation and culturing of canine liver cells.

The left medial hepatic lobe was excised *ex vivo*, and perfused with wash solution (0.14 M NaCl, 6.7 mM KCl, and 10 mM HEPES (pH 7.4)) with 0.5 mM EGTA (15 minutes, 30ml/min, 37ºC), subsequently with wash solution without EGTA (15 minutes, 30ml/min, 37ºC), and finally with wash solution with 4.8 mM $CaCl_2*2H_2O$ and 0.14 Wunsch units/ml Liberase Blendzyme 3 (Roche Diagnostics, Almere, the Netherlands, 25ml/min, 37ºC). Released cell suspension was filtered through a sterile 70 μm nylon mesh filter (Millipore, Amsterdam, the Netherlands). Percoll (Sigma-Aldrich, Zwijndrecht, the Netherlands) was added to a final concentration of 55% and cells were spun down to discard large hepatocytes (50g, 10 minutes, 4ºC). The supernatant contained hepatocytes (>95%) that were small in size and other non-parenchymal cells. Cells in the supernatant were diluted with HBSS to a Percoll concentration of 15%. The pellet was washed (200g, 5 minutes, 4ºC) two times with HBSS with 10% bovine serum albumin (Sigma-Aldrich) and resuspended in Hepatozyme (Invitrogen, Breda, the Netherlands) supplemented with 2 mM glutamine, 0.5 mg/ml gentamicin (Invitrogen), 10% FCS, and standard anti-biotics or in William's Medium E (WME, Gibco, Breda, the Netherlands) supplemented with 10 Lg/ml insulin, 2 mM glutamine, 10 mM nicotinamide, 50 ng/ml EGF (Cell Science (CRE100A), Canton, MA), 30 ng/ml IGF2 (Cell Science (CR1502B)), 10% FCS, and standard anti-biotics. Cell viability was determined by using the Trypan Blue Exclusion test (Invitrogen). Cells were plated in 58.95 cm^2 primaria dishes in a concentration of $9*10^4$ viable cells/cm^2 (BD Bioscience, Alphen a/d Rijn, the Netherlands). Media was replaced with Hepatozyme-Serum Free Medium (SFM), or WME-SFM after three hours when most cells were attached. Emerged colonies were marked on the bottom of the culture dish to permit serial observation.

Keratin 7 (K7) immunocytochemistry of cultured cells.

K7 immunocytochemistry procedure was derived from an optimized protocol for paraffin embedded dog liver biopsies. This antibody showed specific immunostaining of cholangiocytes and ductular reactions in canine fibrotic diseases [22]. For immunocytochemical staining in vitro, cells were fixed in 4% formaldehyde (Klinipath B.V.) for 5 minutes. Endogenous peroxidase activity was blocked for 30 minutes in 0.3 % H_2O_2 in methanol and background staining was blocked for 30 minutes in 10% normal goat serum. The primary antibody Keratin 7 (K7; DakoCytomation (M7018), Heverlee, Belgium, dilution 1:50 in PBS) was incubated overnight at 4ºC. The horseradish peroxidase-labelled, goat-anti-mouse secondary antibody (DakoCytomation, K4001) was used for 45 minutes. The peroxidase activity was visualized by incubating the cells for 10 minutes in 0.5 mg/ml 3, 3-diaminobenzidine chromogen containing 0.035% H_2O_2. Negative staining was performed by omitting the primary antibody (data not shown).

RNA isolation, reverse transcription, and quantitative RT-PCR (QPCR).

Clone cylinders (Sigma-Aldrich) were used to isolate RNA from colonies and surrounding hepatocytes separately. RNA was isolated from six separate plates (n=6) derived from two individually perfused livers. Colonies of the same plate were pooled. Total cellular RNA was isolated using the RNeasy Mini Kit (Qiagen, Leusden, the Netherlands) and treated with DNase-I (Qiagen, RNasefree DNase kit). Reverse transcriptase-PCR was performed with 2 µg of total RNA in a total volume of 40 µl using iScript™ cDNA Synthesis Kit (Biorad, Veenendaal, the Netherlands). Quantitative RT-PCR (QPCR) was performed in triplicate in a spectrofluorometric thermal cycler (iCycler®, BioRad). For each PCR reaction, 0.5 µl of cDNA was used in a reaction volume of 25 µl containing 13 µl iQ™ SYBR-Green SuperMix (Biorad) and 11.7 pmol of both primers on 96-well iCycler iQ plates (BioRad). Standard QPCR conditions were used as described before [27]. Sequences of the primers and the used annealing temperature (Tm) are depicted in Table 1. Gene expression of each sample was normalized with the average gene-expression of the endogenous references beta-2 microglobulin (B2MG) and hypoxanthine phosphoribosyl transferase (HPRT) [27]. Difference between means was determined by the non-parametric Mann-Whitney U test. A p-value < 0.05 was considered to indicate significant changes.

Table 1: Nucleotide sequences of dog-specific primers for QPCR.

Gene	F/R	Sequence (5'-3')	Tm (°C)	Product size (bp)	Accession number
B2MG	F	TCCTCATCCTCCTCGCT	61.2	85	XM_535458
	R	TTCTCTGCTGGGTGTCG			
HPRT	F	AGC TTG CTG GTG AAA AGG AC	56.0	100	L77488/
	R	TTA TAG TCA AGG GCA TAT CC			L77489
CYP1A1	F	CACCATCCCCCACAGCACAACAAA	59.7	140	AB094348
	R	GCTCTGGCCGGAATGCAAATGGAT			
KRT18	F	TTGCTACCTACCGTCGCCTGTTGG	63.5	109	XM_534794/
	R	ATCTTGCGGGTGGTGGTCTTCTGG			AB090854
ALB	F	TGTTCCTGGGCACGTTTTTGTA	63.8	92	AB090854
	R	GGCTTCATATTCCTTGGCGAGTCT			
KRT7	F	GCGTGGGAGCCGTGAACATC	55.0	109	XM_534795
	R	CCGCCGCCGCTGGAGAA			
KRT19	F	GCCCAGCTGAGCGATGTGC	63.8	86	EU107521
	R	TGCTCCAGCCGTGACTTGATGT			
AFP	F	GCTGCTCCGCCATCCATCC	62.9	123	AB089789
	R	GGGGTGCCTTCTTGCTATCTCAT			
THY1	F	CAGCATGACCCGGGAGAAAAAG	63.5	134	XM_546483
	R	TGGTGGTGAAGCCGGATAAGTAGA			
CD44	F	CGCTCCTGGCCTTGGCTTTGATT	65.7	110	Z27115
	R	CCCCACTGCTCCATTGCCATTGTT			
CCND1	F	ACTACCTGAACCGCT	58.0	150	NM_001005757
	R	CGGATGGAGTTGTCA			
KIT	F	GCCTTCTCTGTGATGTGCTT	61.2	88	AY313776
	R	GTGCATTAGTCTGCTGGCTGT			
PROM1	F	CTGGGGCTGCTCTTTGTGAT	60.4	115	XM_545934
	R	AGGCCCCATTTTTCTTCTGTC			
NCAM1	F	GCTCATGTGCATCGCTGTCAACCT	76.2	136	AY860628
	R	CTCCTCCTCAGTCCGCACCTCCAC			

Results

Liver cell isolation and culturing.

After iso-density Percoll centrifugation, the viability of the cell suspensions was more than 98%. The use of WME-SFM medium resulted in a low cell attachment and a rapid growth of cells with a fibroblastic appearance that did not survive in culture for more than two weeks. The use of Hepatozyme-SFM resulted in a high attachment efficiency of cells with a surface confluence of around 70-80%. Hepatozyme-SFM inhibited the growth of non-parenchymal cells, resulting in a 100% confluent monolayer consisting of predominantly hepatocytic morphology as indicated by the cell size and the presence of binucleated cells [28]. After two weeks of culturing in Hepatozyme-SFM, small epithelial cells with a high nucleus/cytoplasm ratio consistently emerged within the monolayer of hepatocytes (Figure 1A). These small epithelial cells grew exponentially into colonies (40-60 colonies per plate) during one week after their first appearance (Figures 1B and C). Thereafter, the morphology of the colony-forming cells changed towards a bigger cell type with a smaller nucleus/cytoplasm ratio (Figure 1D and E). After a culture period of 6 weeks, colony-forming cells were differentiated toward hepatocyte-like cells. Several binuclear cells were observed among these differentiated cells (Figure 1F). Passaging of individual colonies could be obtained by trypsinization, however long-term culturing and proliferation of the colony-forming cells was only successful in the presence of the surrounding hepatocytes or upon culturing in Matrigel™ matrix (BD Bioscience), see supplemental file 1.

Immunocytochemical characterization of individual colonies.

Colony-forming cells were observed after culturing in Hepatozyme-SFM for two weeks. Cells within these colonies were strongly immunopositive for K7 (Figure 2A), while hepatocytes surrounding the colonies were clearly K7 immunonegative (B). Four weeks after the colonies emerged, several large K7 immunopositive cells were observed at the borders of the colonies (Figure 2C). Upon higher magnification, these larger cells showed a weaker cytoplasmatic and a more pronounced membranous immunocytochemical staining for K7, indicating the presence of intermediate hepatocytes, a differentiation stage between progenitor cells and hepatocytes (Figure 2D).

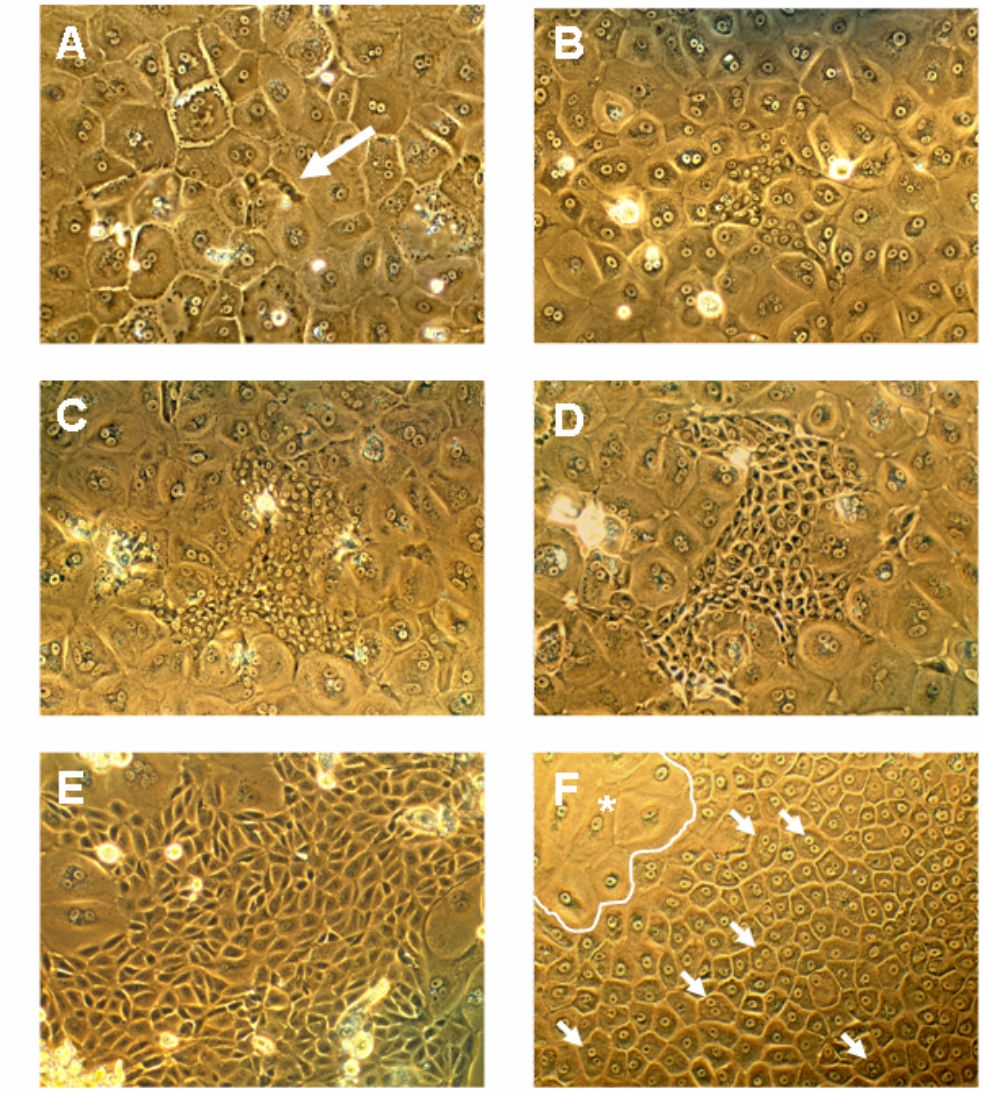


Figure 1. Phase contrast microphotographs of colony-forming cells (long white arrow) that emerged between the hepatocyte monolayers after 2 weeks of cell culturing (A). Colony-forming cells proliferated for two days, while no differentiation was seen in cell morphology (B and C). However, after 1 week cell size increased and a higher cytoplasm/nucleus ratio was seen (D and E). At 6 weeks after their first appearance, cells were differentiated in hepatocyte-like cells with the presence of binuclear cells (arrows) (F). In the left corner, the remaining of the hepatocyte monolayer is seen (asterix). Original magnification 200x.

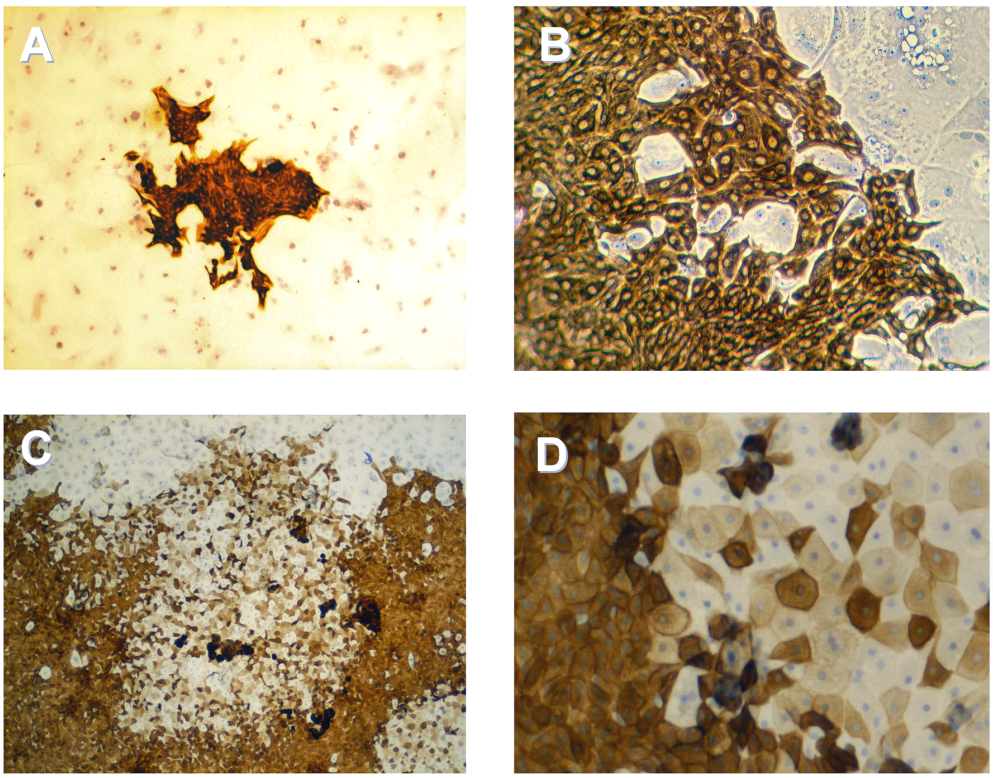


Figure 2. Immunohistochemical staining for K7 of colony-forming cells at 3 days after their first appearance (original magnification 200x (A)). Colony forming cells were immunopositive for K7, while the hepatocytes surrounding the colony were immunonegative for K7 (original magnification 400x (B)). Immunocytochemical staining for K7 of colony-forming cells at 4 weeks after their first appearance (C). A decreased cytoplasmatic and a more pronounced membranous immunostaining for K7 is seen in larger colony-forming cells (original magnification 400x (D)).

Gene expression profile of colony-forming cells in different media.

After colonies emerged in Hepatozyme-SFM, cells were cultured in either in Hepatozyme-SFM or WME-SFM for two weeks before gene-expression profiles were determined. Within the colonies, the gene expression of several hepatocyte, cholangiocyte and progenitor cell markers was measured and compared to the gene expression of the surrounding hepatocytes (Figure 3). In Hepatozyme-SFM, colony-forming cells showed a higher gene expression of the hepatocyte markers CYP1A1 (3.2 fold), KRT18 (2.1 fold), and ALB (2.0 fold) compared to the surrounding hepatocytes, indicating a differentiation of the colonies toward hepatocyte-like cells. The expression of the LPC/cholangiocyte markers KRT7 and KRT19 was higher in

the colonies compared to the surrounding hepatocytes in Hepatozyme-SFM (KRT 7: 14.3 fold, KRT19 22.9 fold). Furthermore, colony-forming cells showed a higher expression of the progenitor cell markers AFP (3.4 fold), CD44 (2.2 fold), and PROM1 (2.9 fold) indicating the immature character of these cells compared to the surrounding hepatocytes. No differences were found for the progenitor cell markers KIT, THY1, and NCAM1. Colony-forming cells showed a higher expression of the cell-cylce marker Cyclin D1 (CCND1), indicating a higher proliferation when compared to the surrounding hepatocytes.

Interestingly when cells were cultured in WME-SFM, colony-forming cells and surrounding hepatocytes showed a lower expression of hepatocyte markers and a higher expression of progenitor cell markers compared to the cells that were cultured in Hepatozyme-SFM. In WME-SFM, no difference between colony-forming cells and hepatocytes in the expression of hepatocyte (CYP1A1, KRT18, and ALB) and progenitor cell markers (AFP, CD44, KIT, THY1, NCAM) was found, except for an increased expression of PROM1 (1.8 fold). Furthermore, the cholangiocyte/LPC markers KRT7 (15.7 fold), and KRT19 (6.6 fold) were increased in the colonies when compared to the surrounding hepatocytes. A higher gene expression of CCND1 in colony-forming cells and in hepatocytes indicated a higher proliferation of these cells in WME-SFM when compared to Hepatozyme-SFM.

Discussion

In the present study we described a reproducible protocol to obtain colony-forming epithelial cells from healthy dog livers. These colonies emerged within the monolayer of hepatocytes after two weeks of culturing in hepatocyte-specific medium (1 colony per 4.500 to 80.000 initial plated cells). The gene expression of several hepatocyte (CYP1A1, ALB, and KRT18), cholangiocyte/LPC (KRT7 and KRT19), and progenitor cell markers (AFP, CD44, PROM1, KIT, THY1, and NCAM1) indicated the immature and bi-potential phenotype of these colony-forming cells. Furthermore, gene expression analysis and immunocytochemistry for K7 indicated a more pronounced hepatic differentiation in hepatocyte-specific medium. Although these independent measurements (QPCR, immunocytochemistry, cell culture) together are suggestive for the existence of progenitor cells in long-term hepatocyte cultures further experiments e.g. single cell isolation and in vivo differentiation are needed to confirm the bi-potential nature of these cells.

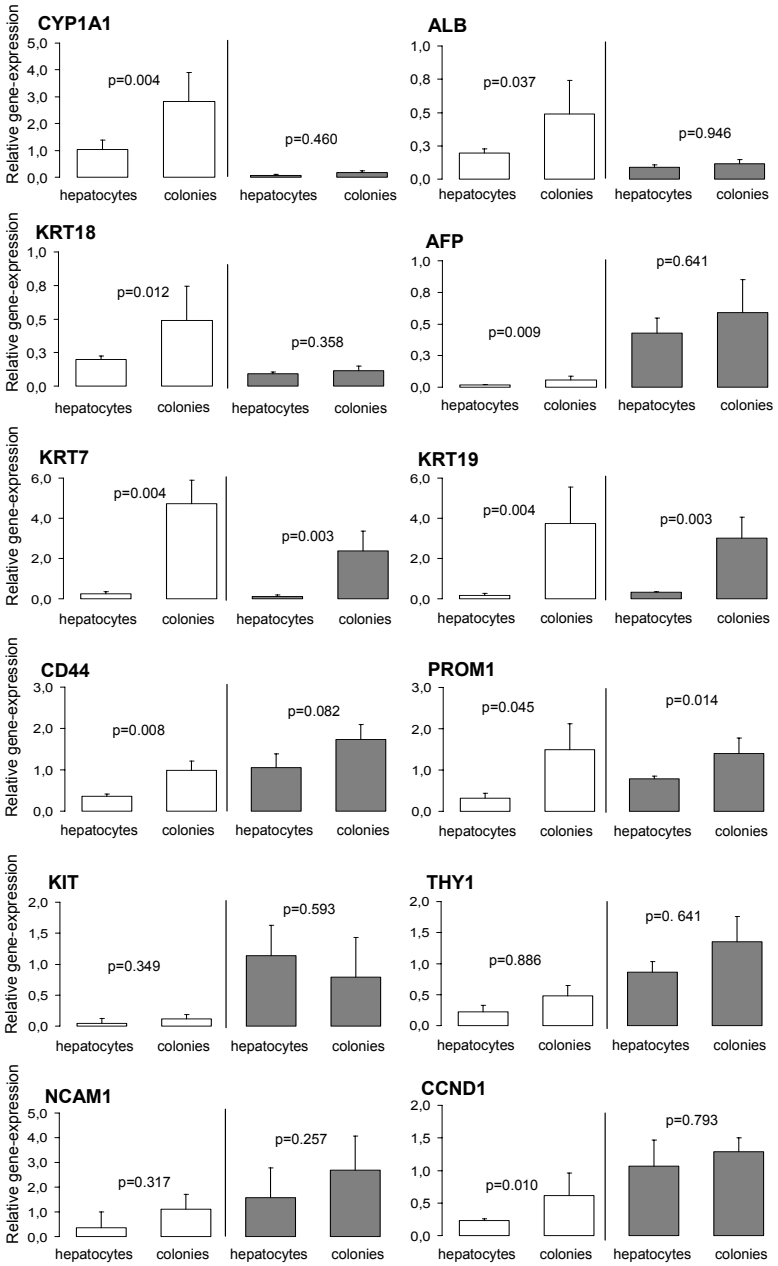


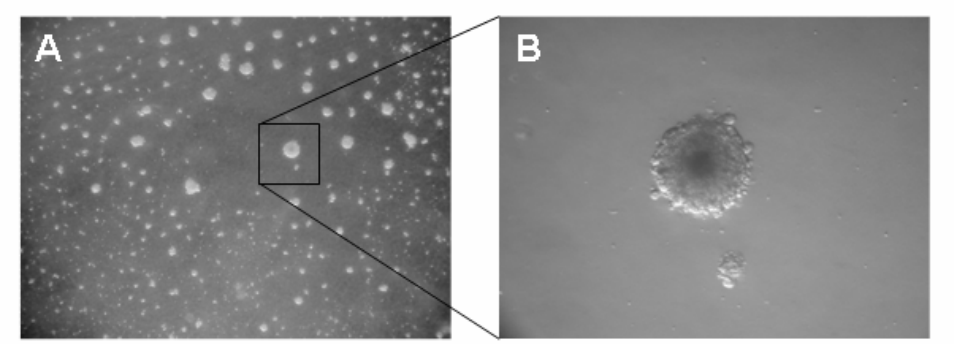
Figure 3. Relative gene-expressions of colony-forming cells (n=6) compared to surrounding hepatocytes (n = 6) for hepatocyte related markers (CYP1A1, ALB, and KRT18) and progenitor cell markers (AFP, KRT7, KRT19, CD44, PROM1, KIT, THY1, NCAM1), and the cell cycle marker CCND1. Gene-expression was measured after two weeks culturing in Hepatozyme-SFM (white bars) or WME-SFM medium (grey bars).

The colony-forming cells derived from healthy canine livers showed morphological and phenotypical similarities with progenitor cells that were described previously for rodent and human livers [12, 15, 29]. These cells show progenitor cell features, such as the co-expression of hepatocyte (ALB) and cholangiocyte/LPC markers (K7, K19, and AFP) [30]. These colony-forming cells were derived from liver cell fractions and grow within the monolayer of other (non)parenchymal liver cells. These co-cultures could represent a cooperative interaction between hepatocytes and stellate (or other non-parenchymal) cells as occurs in the micro-environment of progenitor cells in vivo. The expression of THY1 is recently demonstrated to be expressed in a subpopulation of activated stellate cells [31-33]. The presence of activated stellate cells in our culture system could have activated the colony-forming cells to proliferate and differentiate in culture. Non-parenchymal cells produce growth factors such as HGF and extra-cellular matrix components such as type I collagen fibres that are important for progenitor cell proliferation and differentiation [13, 19, 34, 35]**.** By further defining these co-culture systems the interactions between progenitor cells and other relevant cells constituting the progenitor cell niche can be further explored.

Using co-cultures to induce the proliferation and differentiation of progenitor cells in culture may raise the question whether the emerged colonies were isolated as a part of the isolated cell fraction directly after liver cell perfusion or whether these colonies are merely a result of a de-differentiation of hepatocytes. Indeed, hepatocytes acquired a high expression of most measured progenitor markers when cultured in non-hepatocyte-specific WME-SFM medium. This de-differentiation of hepatocytes in culture and the expression of cholangiocyte/progenitor cell markers such as KRT19, THY1, and KIT has been described previously, indicating the adaptation of cultured cells to the culture media [36-38]. In the present study however, hepatocytes did not acquire the expression of K7. Furthermore, no cells with a K7 immunopositive intermediate hepatocytes were seen before the colonies emerged between the hepatocyte monolayer. Therefore, these results support the hypothesis that the colony-forming cells are a unique cell population that can be isolated directly from healthy livers.

The discovery of progenitor cells and their contribution to liver regeneration in several liver diseases has provided a new option of approach in the treatment of currently untreatable liver diseases. Liver regeneration could be stimulated by injecting diseased livers with progenitor cells [39, 40], or by stimulating the endogenous progenitor cells to proliferate and/or differentiate. The availability of high numbers of suitable spontaneous liver diseases comparable to man makes the dog very relevant to perform such clinical trials before application in man. In vitro expansion of undifferentiated progenitor cells may be a strategy to acquire more cells than the patient generates in vivo. The colony-forming cells that consistently appeared in long-term hepatocyte cultures derived from livers of healthy mature

dog livers expressed hepatocyte, LPC, and biliary markers and were capable to differentiate towards the hepatocytic lineage. The characterization of these cells in the present study is a first step towards performing the first clinical trials in spontaneous canine liver diseases.



Supplemental Figure. Phase contrast microphotographs of speroids that were formed by colony-forming small cells cultured in matrigel (BD Biosciences, Alphen aan den Rijn, the Netherlands). Magnification 200x (A), and 400x (B).

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The dog liver contains a 'side population' of cells with hepatic progenitor-like characteristics.

The dog liver contains a 'side population' of cells with hepatic progenitor-like characteristics.

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Abstract

The aim of the present study was to isolate and characterize potential progenitor cells from healthy dog livers. Stem/progenitor cells can be prospectively isolated from a diversity of tissues using their ability to efficiently pump out the dye Hoechst33342, thereby portraying a side population (SP) in dual-wavelength flow cytometry. We here describe the detection of a SP in dog liver, constituting ~3 % of the non-parenchymal enriched cell fractions. A subpopulation of the SP (~30 %) was immunonegative for the pan-hematopoietic marker CD45, and consisted predominantly of small, mononuclear, keratin 7-immunoreactive cells, characteristics suggestive of a liver progenitor cell phenotype. Both the CD45- and CD45+ SP showed upregulated expression of progenitor/cholangiocyte marker genes, but also low-level expression of hepatocyte markers, suggesting the presence of progenitor cells committed to the hepatic lineage in both SP fractions. Our findings demonstrate that healthy canine liver contains a small population of cells with progenitor-like characteristics that can be isolated on the basis of efficient Hoechst33342 expulsion.

Submitted

Introduction

The adult liver contains a small number of primitive epithelial cells that are present in the smallest branches of the biliary tree, the Canals of Hering [1]. These so-called liver progenitor cells (LPCs) are quiescent in healthy liver, but activated in certain liver diseases in which the regenerative capacity of mature hepatocytes and/or cholangiocytes is impaired [2-4]. Activated LPCs participate in liver regeneration by proliferating and differentiating towards the hepatocytic and/or biliary lineages [5].

Dogs referred to veterinary clinics gain increasing attention as a new model to bridge rodent experimental models to human diseases in a clinical setting [6, 7]. Liver diseases of dogs have a time-course development that is comparable to humans, as well as a high similarity with human liver diseases at the clinical, molecular and pathological level [8, 9]. Interestingly, LPC activation has recently been demonstrated in spontaneously diseased canine livers, making the dog a suitable model for the evaluation of new LPC-based therapies in a clinical setting [10-12]. A first step towards a better understanding of the molecular machinery and regulatory network of LPC activation is the isolation and characterization of LPCs. However, purification of LPCs is complicated due to their scarce number in healthy livers and the lack of specific LPC markers.

A method increasingly used for the isolation of putative tissue stem/progenitor cells is based on the efflux of the fluorescent dye Hoechst33342 [13]. Cells with high efflux capacity are called side population (SP) cells, referring to their characteristic side-branch profile in a dual-wavelength emission plot during fluorescence-activated cell sorter (FACS) analysis [13]. The prominent Hoechst33342 efflux capacity of SP cells is due to their high expression of the conserved ABC (ATP-Binding Cassette) transporters, and in particular of ABCG2 (also known as BCRP1) [14, 15]. SP cells were first isolated from mouse bone marrow, and displayed a greatly enriched haematopoietic stem cell potential *in vitro* and haematopoietic reconstitution activity *in vivo* [16, 17]. Subsequently, a SP was identified in multiple tissues and typically found to be enriched for authentic or potential tissue stem/progenitor cells. Recently, a SP was also detected in murine and human liver [13, 18, 19]. These SP cells showed hematopoietic as well as hepatocytic differentiation capacities *in vitro* and *in vivo*, indicating that at least part of the SP may consist of cells committed to the hepatic lineage.

In the present study, we detected for the first time a SP in the liver of normal, clinically healthy dogs (non-diseased). SP cells were examined by FACS for expression of the pan-hematopoietic marker CD45, and by qRT-PCR for expression of genes characteristic for different liver cell types, including hepatocytes, stellate cells, and LPC/cholangiocytes. Cell morphology and keratin 7 (K7) immunocytochemistry was further

examined to evaluate the presence of LPCs. We found that the dog liver SP is composed of a CD45+ and a CD45- subfraction, and that both SP subsets display LPC-associated characteristics.

Materials and methods

Cell isolation.

Livers were obtained from healthy dogs that were participating in non-liver related experiments. All experiments were approved by the Ethics Committee of Animal Experimentation of the Utrecht University. Microscopic abnormalities of the liver were excluded by histopathological examination of formalin-fixed, paraffin-embedded and haematoxylin/eosin-stained liver sections by one board certified veterinary pathologist. Immediately after euthanasia, the left medial hepatic lobe was excised ex-vivo and perfused with perfusion buffer (0.14 M NaCl, 6.7 mM KCl and 10 mM HEPES; pH 7.4) containing 0.5 M EGTA (15 minutes; 30ml/min), followed by perfusion buffer without EGTA (15 minutes, 30ml/min), and finally with perfusion buffer containing 4.8 mM $CaCl_2.2H_2O$ and 0.14 Wunsch units/ml Liberase Blendzyme 3 (20 minutes; 25ml/min; Roche, Woerden, The Netherlands). Liver lobe was placed in Hank's balanced salt solution (HBSS; Invitrogen, Breda, The Netherlands) and gently shaken for 15 minutes to liberate the liver cells. Cell suspension was filtered through a sterile 70 µm nylon mesh filter (BD Biosciences, Breda, The Netherlands). Percoll (Amersham Biosciences, Roosendaal, The Netherlands) was added to a final concentration of 55% and tubes were spun at 50g for 10 minutes (4ºC). The supernatant was diluted in HBSS to a final concentration of 20% Percoll, cells were pelleted by centrifugation at 150g for 10 minutes (4ºC). After washing two times in HBSS (200g, 10 minutes, 4ºC), cells were finally resuspended in Hepatozyme-Serum Free Media (Hepatozyme-SFM; Invitrogen) at a concentration of $10x10^6$ cells/ml. Cells were cryopreserved in the presence of 10% DMSO by using a controlled freezing container (Mr. Frosty, Wessington Cryogenics, Houghton-le-Spring, UK). Cells were stored in liquid nitrogen for up to four months.

Cell staining with Hoechst and anti-CD45 antibody, and analysis by FACS.

Cells were thawed in a 37°C water bath and immediately placed on ice. Cells were slowly diluted 1:1 in Hepatozyme-SFM (4°C), which was repeated five times with three minute intervals. To exclude non-viable cells, Percoll was added to a final concentration of 25% and cells were spun down at 200g for 10 minutes at 4°C. Cell viability was measured using Trypan Blue staining (Invitrogen). Pellets were resuspended in Hepatozyme-SFM to a final

concentration of $1x10^6$ viable cells/ml. Cells were incubated with 5 µg/ml Hoechst33342 (Sigma-Aldrich, Zwijndrecht, The Netherlands) for exactly 90 min at 37°C with regular mixing. Cells were then immediately placed on ice and spun (200g, 5 min, 4°C). The cell pellet was resuspended in ice-cold PBS and stained with a RPE-labelled rat anti-canine CD45 antibody (1:10 dilution; AbD Serotec; Dusseldorf, Germany) for 30 min on ice. Cells were washed twice in excess PBS. The final cell pellet was resuspended in PBS containing 2% foetal calf serum and 2 µg/ml propidium iodide (PI; Sigma-Aldrich), and kept on ice before and during FACS analysis. To assess active efflux and side-population (SP) phenotype, cells were incubated with the transport blockers reserpine (100 µM), or verapamil (100 µM), 20 minutes before Hoechst33342 incubation. Furthermore, a RPE-labelled isotype control antibody (mouse IgG2b negative/isotype control, RPE labelled; AbD Serotec) was used to set the gates for CD45 immunopositivity (data not shown).

Cells were analysed using a FACSVantage (BD Biosciences). The SP was visualized after UV excitation on the basis of blue emission through a BP 424/44 filter and of red emission through a BP 630/22 filter (Omega Optical, Brattleboro, VT). Within the living (PI^-) cell population, CD45-positive (CD45+) SP cells, CD45 negative (CD45-) SP cells, and cells of the main population (MP) were sorted, and separately collected in 900 µl extraction buffer (from the PicoPure RNA Isolation Kit; Arcturus, Molecular Devices, Sunnyvale, CA) for gene-expression analysis, or in 900 µl Hepatozyme-SFM for cytospin.

RNA isolation, amplification including reverse transcription, and quantitative PCR (QPCR). Total cellular RNA was isolated using the PicoPure RNA Isolation Kit (Arcturus) and treated with DNase-I (RNase-free DNase kit from Qiagen, Venlo, The Netherlands). The quantity and quality of the purified RNA was measured with the lab-on-chip capillary gel-electrophoresis system using an Agilent Bioanalyzer 2100 (Agilent Technologies, Amstelveen, The Netherlands). Agilent software calculates the RNA Integrity Number (RIN), which is a classification of total RNA quality on a numbering system from 1 representing the most degraded RNA, to 10 representing the most intact RNA [20]. All RNA Integrity Number (RIN)-values fell between 7.1 and 7.4 (not shown). The RNA quality was therefore considered sufficient for QPCR experiments [21]. Amplified ssDNA was made in duplicate from 5 µl of total RNA using the WT-Ovation Pico RNA Amplification System (NuGen Technologies, Bemmel, The Netherlands) according to the manufacturer's instructions. QPCR was performed in duplicate in a spectrofluorometric thermal cycler (iCycler, BioRad, Veenendaal, The Netherlands) using 96-well iCycler iQ plates (BioRad). For each PCR reaction, 0.5 µl of cDNA was used in a reaction volume of 25 µl containing 13 µl iQ SYBR Green SuperMix (Biorad) and 11.7 pmol of both primers. Primers (Isogen, IJsselstein, The Netherlands, Table 1) were designed for genes known to be

markers of LPCs/cholangiocytes (ATP-Binding Cassette, Subfamily G, Member 2 (ABCG2); Tumor Necrosis Factor Receptor Superfamily, Member 12A (FN14/TNFRSF12A); CD44 Antigen (CD44) [22]; Keratin 19 (KRT19); Keratin 7 (KRT7); and v-KIT Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog (KIT)), and alpha-fetoprotein (AFP)), endothelial progenitor cells (CD34), macrophages (Monocyte Differentiation Antigen CD14 (CD14)), stellate cells (Desmin (DES) and Neural Cell Adhesion Molecule 1 (NCAM1)), and hepatocytic markers (Cytochrome P450, Subfamily 1, polypeptide 1 (CYP1A1); Hepatocyte Nuclear Factor 4-Alpha (HNF4A); ATP-Binding Cassette, Subfamily C, Member 2 (ABCC2/MRP2), and Albumin (ALB)). The expression level of each gene was normalized to the averaged level of the three endogenous reference genes, beta-2 microglobulin (B2MG), hypoxanthine phosphoribosyl transferase (HPRT), and ribosomal protein L8 (RPL8)[23].

Cytospin and immunocytochemistry.

Cells were spun down onto Superfrost Plus glass slides (Menzel-Gläser, Braunschweig, Germany) at 800 rpm for 10 minutes. The cytospin slides were dried for ten minutes, fixed in 4% paraformaldehyde and stored at 4°C under humidified conditions. For immunohistochemistry, slides were first washed in TBST (0.01M Tris-Buffered Saline solution, 0.9% NaCl (pH = 7.6) with 0.1% Tween-20). Slides were then incubated for ten minutes in 0.3% H_2O_2 in methanol to inhibit endogenous peroxidase activity, and for ten minutes in normal goat serum (1:10) to reduce background staining. Slides were incubated overnight at 4°C with a mouse anti-human Keratin 7 antibody (Dako, Heverlee, Belgium; 1:25 dilution in TBS) [12]. Slides were washed twice with TBST and incubated with a horseradish peroxidase-labelled, goat-anti-mouse secondary antibody (EnVision+ reagent, Dako) for 35 minutes at room temperature. Finally, the peroxidase activity was visualized by incubating the cells for eight minutes in 0.5 mg/ml 3,3-diaminobenzidine chromogen containing 0.035% hydrogen peroxide. Cells were counterstained with Hematoxylin for three seconds, dehydrated, and mounted in Eukitt (Boom, Meppel, The Netherlands). A positive control for K7 immunohistochemistry (LPCs/cholangiocytes) was performed on frozen sections of healthy canine livers.

Statistical analysis.

The equality of medians among the different groups was compared with the non-parametric Kruskall Wallis test. When there was a difference ($p<0.05$) the Mann Whitney U test was performed to examine the differences between the individual groups. A P value < 0.05 was considered statistically significant. Analysis was performed using SPSS software (SPSS Benelux, Gorinchem, The Netherlands).

Table 1. Nucleotide Sequences of Dog-Specific Primers for Real-Time Quantitative PCR

Gene	F/R	Sequence (5'-3')	Tm	Product size	Accession number
B2MG	F	TCCTCATCCTCCTCGCT	61.2	85	XM535458
	R	TTCTCTGCTGGGTGTCG			
HPRT	F	AGC TTG CTG GTG AAA AGG AC	56.0	100	L77488 /
	R	TTA TAG TCA AGG GCA TAT CC			L77489
CYP1A1	F	CACCATCCCCCACAGCACAACAAA	59.7	140	AB094348
	R	GCTCTGGCCGGAATGCAAATGGAT			
HNF4A	F	GACCGGGCCACAGGGAAACACTAC	65.0	122	XM543008
	R	TCCACGACGCATTGCCGACTAAAC			
ABCC2	F	GGCGTCTATGGAGTTCTGGGATTA	65.7	134	NM001003081
	R	ATGGGTGCTTGAAGGATGTTGTTT			
ALB	F	TGTTCCTGGGCACGTTTTTGTA	63.8	92	AB090854
	R	GGCTTCATATTCCTTGGCGAGTCT			
DES	F	GACCGCTTCGCCAACTACATC	61.3	135	NM001012394
	R	CTCGCGCAGCTCCTCCTC			
NCAM1	F	GCTCATGTGCATCGCTGTCAACCT	76.2	136	AY860628
	R	CTCCTCCTCAGTCCGCACCTCCAC			
CD14	F	CCCGGCGCTCACCACCTTAGAC	60	98	EU263365
	R	CCTGGAGGGCCGGGAACTTTTG			
ABCG2	F	TGCTGTCCTTTTGCTCCTG	57.9	191	NM001048021
	R	GCTCACAATGGTAACCCACTG			
FN14	F	AACACCAGGCCCCACCCACTC	65.7	190	EU63365
	R	TTCTCCCTCCCCTCCAAACTCTCC			
CD44	F	CGCTCCTGGCCTTGGCTTTGATT	65.7	110	Z27115
	R	CCCCACTGCTCCATTGCCATTGTT			
KRT19	F	GCCCAGCTGAGCGATGTGC	63.8	86	EU107521
	R	TGCTCCAGCCGTGACTTGATGT			
KRT7	F	GCGTGGGAGCCGTGAACATC	55.0	109	XM534795
	R	CCGCCGCCGCTGGAGAA			
KIT	F	GCCTTCTCTGTGATGTGCTT	61.2	88	AY313776
	R	GTGCATTAGTCTGCTGGCTGT			
CD34	F	TCAGGGCCCCCGACATCTC	65.7	115	NM00100341
	R	TCTCTGCTCACCCCTCTGGAAAAA			

Results

Characterization of a SP in the non-parenchymal enriched cell fraction of dog liver.

Cells were isolated from 4 healthy dog livers and cryopreserved. After thawing and Percoll centrifugation, non-parenchymal enriched liver cells showed a viability of 80% ± 20% (mean ± SD). This non-parenchymal enriched fraction was incubated with Hoechst33342 and found to contain a SP of 2.8 ± 1.2% (n=4, Figure 1A). Pre-incubation of the cells with ABC transporter inhibitors reduced the SP with 36.7 ± 11.4% (reserpine, Figure 1B) and 37.9 ± 16 (verapamil, Figure 1C). Staining with a RPE-labelled anti-CD45 antibody showed that about 70 % of the SP cells were positive for CD45 (n=4, Figure 1D and E). Thus, about 30 % (27.8 ± 5.9%) did not express CD45.

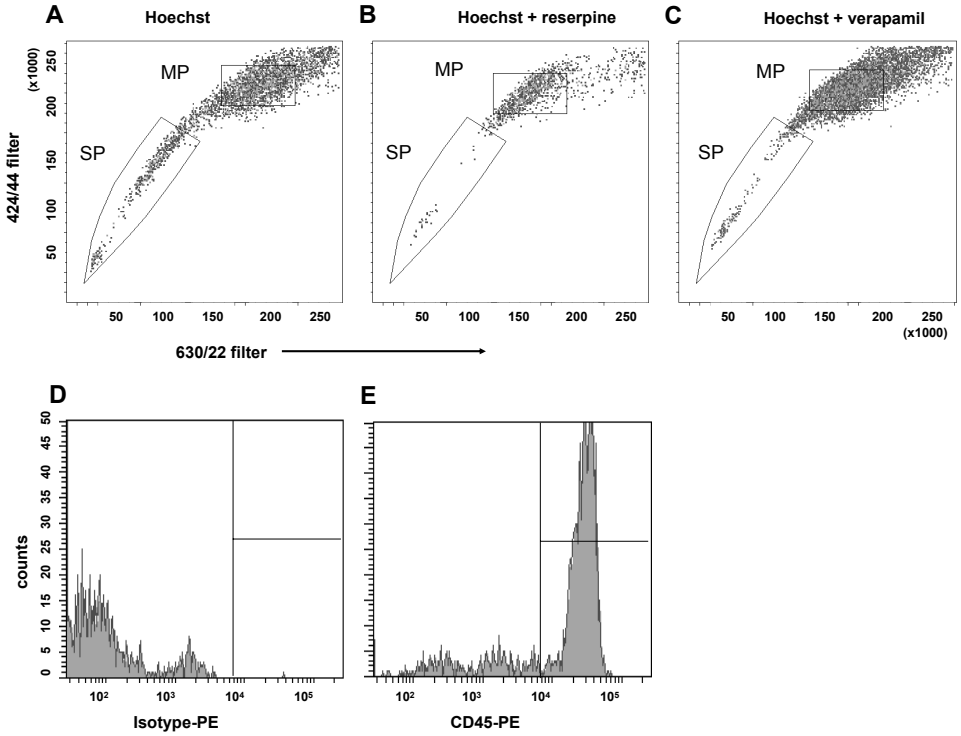


Figure 1. Flow-cytometric analysis of non-parenchymal enriched liver cells after incubation with Hoechst33342. The SP fraction comprises 3% of the total (living) cells analyzed (A). Hoechst dye efflux is significantly reduced in the presence of reserpine (B), or verapamil (C). To determine the amount of CD45+ cells within the SP, the gates were set by using a RPE-labelled isotype control antibody (D). Within the SP, 70 % of cells were positive for CD45 (E).

Overall morphology and Keratin 7 immunoreactivity of CD45- and CD45+ SP cells, and of MP cell.

The great majority (>97%) of the CD45- SP fraction consisted of small (8 - 10 µm) K7-immunoreactive mononuclear cells (Figure 2A). K7 is described as a marker for LPCs in different species including the dog [12]. A small number (<3%) of larger (10 – 24 µm) K7-immunoreactive cells were present within the CD45- SP fraction (Figure 2B). The morphology in the CD45+ SP fraction varied from small to larger, mononuclear (8 – 24 µm), and binuclear (20 – 26 µm) cells. The majority (> 80 %) of these CD45+ SP cells was immunonegative for K7 (Figure 2C-D). The MP fraction consisted of predominantly larger (16 – 30 µm) mononuclear and binuclear cells. As expected, the vast majority of the MP cells (>90%) was K7-immunonegative (Figure 2E-F).

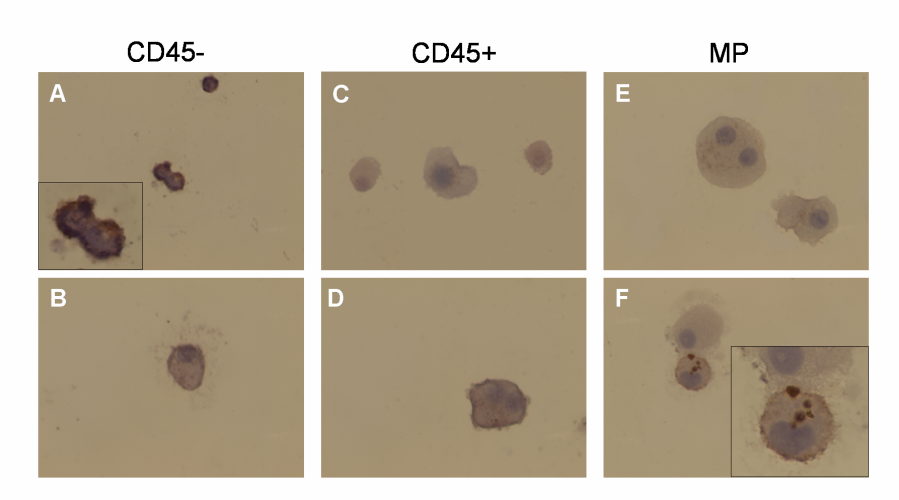


Figure 2. Morphology and K7 immunoreactivity of CD45+ and CD45- SP cells, and of MP cells. CD45- SP cells are small (8 - 10 µm), mononuclear, and predominantly K7-immunoreactive cells (A). Some larger (10-24 µm) K7-immunoreactive cells are found in the CD45- SP fraction, likely representing cholangiocytes (B). The morphology in the CD45+ SP fraction varies from small to larger, mononuclear (8 – 24 µm), and binuclear (20 – 26 µm) cells that are mainly K7immunonegative (C). Scarce numbers of K7-immunopositive cells found in the CD45+ SP fraction (D). The MP fraction consists of larger (16 – 30 µm) mononuclear and binuclear cells that are predominantly K7-immunonegative (E). K7-immunopositive cells are very rare in the MP fraction. Original magnification, 200x.

Gene-expression analysis of CD45+ and CD45- SP, and of MP cell fractions. For RNA isolation (n=2) the amount of cells per sort ranged from 3500-5000, 6500-8000, and 32000-200000 cells for respectively CD45-, CD45+ and MP fractions. Expression of the late-developmental markers of hepatocytes (CYP1A1, HNF4, and ABCC2 (MRP2)) was higher in MP cells when compared to CD45- or CD45+ SP cells. Albumin (ALB) mRNA expression, considered a mid-late developmental marker for hepatocytes, was also

significantly higher in the MP than in the CD45- and CD45+ SP. Expression of the stellate cell markers Desmin (DES) and NCAM1, and the macrophage marker CD14 was undetectable, or very low in both SP fractions, indicating the absence of stellate cells and macrophages in these groups. When compared to MP and CD45+ SP cells, higher expression levels were observed for ABCG2 and CD44 in the CD45- SP fraction. No differences in ABCG2 and CD44 expression were seen between CD45+ SP cells and MP cells. When compared to the MP, mRNA levels of the LPC/cholangiocyte markers FN14, KRT19 and KRT7 were increased in both the CD45- and CD45+ SP, whereas a small but significant increase of KIT expression was only seen in the CD45- SP. The endothelial progenitor cell marker CD34 was undetectable in the CD45- and CD45+ SP, whereas prominent expression of CD34 was found in the MP. No AFP expression was found in CD45-, CD45+ SP cells or in the MP fractions.

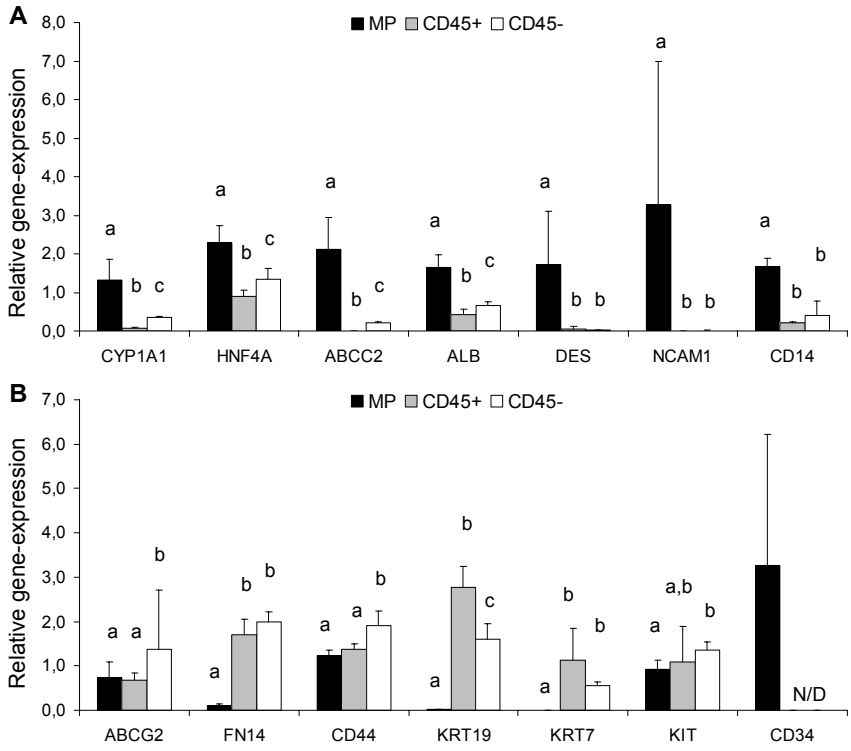


Figure 3. Gene-expression levels of markers of (A) hepatocytes (CYP1A1, HNF4, ABCC2, ALB), stellate cells (DES, NCAM1), and macrophages (CD14), and (B), progenitor cell markers (ABCG2, FN14, CD44, KRT19, KRT7, KIT, CD34). Results are presented as relative gene expression levels normalized to the average expression of three reference genes ± standard deviations (n=2). Different letters above bars indicate statistically significant differences as analysed with the Mann Whitney U test. N/D indicates none detected gene expression.

Discussion

Potential progenitor cells can be isolated from various tissues of humans and rodents by their ability to expel the fluorescent dye Hoechst33342, which clusters them in a so-called side population (SP) during FACS analysis [24-27]. In the present study, a SP was detected in non-parenchymal enriched cells from healthy dog livers. Similar to SP cells isolated from human and mouse livers, the SP represents a small fraction of the total liver cell population [18, 19]. Furthermore, the percentage of CD45+ cells in the SP fraction (70 %) was highly similar to the previously described percentage found in rodents (76 %) [19]. The dog liver SP cells show high gene expression of the (liver) progenitor cell markers ABCG2, FN14, CD44, KRT19, KRT7, and KIT, indicating the presence and enrichment of a progenitor-like cell type in the SP. The absence of AFP expression is in line with the recent findings of Schmelzer et al. in which no AFP expression was found in hepatic stem cells present in healthy human livers throughout life [28]. In contrast, the SP is depleted from stellate cells and macrophages given the absence of DES, NCAM1, and the only very low mRNA level of CD14.

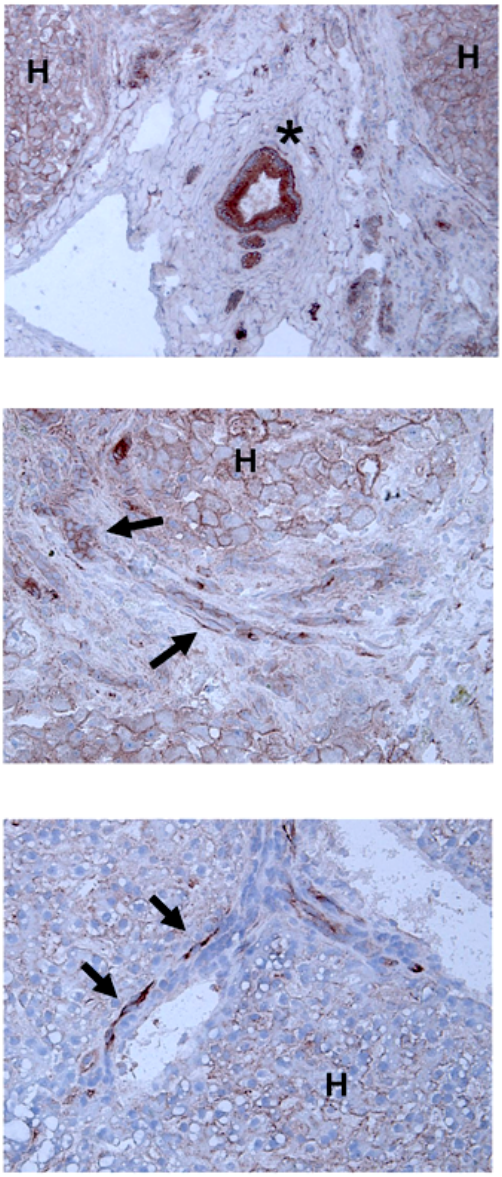
Within the SP of the dog liver, a CD45- and a CD45+ cell fraction was observed. CD45 is a cell surface tyrosine phosphatase that is expressed on all nucleated cells of haematopoietic origin, including haematopoietic stem cells [29]. CD45+ cells are found in the SP of various tissues and may be responsible for the hematopoietic stem cell activity of the SP from these tissues [30]. Some general stem/progenitor cell-associated markers (ABCG2, CD44 and KIT) were detected at higher levels in the CD45- than the CD45+ SP of the dog liver, whereas the LPC markers FN14, KRT19 and KRT7 (also reported in cholangiocytes) were found in both SP subsets. Together, these findings suggest that both the CD45- and the CD45+ SP subpopulation contain progenitor cells that are committed to the hepatic lineage. This suggestion is in line with previous reports, that showed hepatic differentiation capacity of both CD45- and CD45+ SP cells derived from mouse or human livers [18, 19].

However, an alternative explanation is that differentiation of CD45+ SP cells towards the hepatic lineages may have started during the procedures before FACS analysis when cells are incubated in hepatocyte culture medium with specific growth factors such as Hepatocyte Growth Factor (HGF). A (partial) differentiation of CD45+ cells to epithelial cells has been observed in multiple cell systems [31, 32], and bone marrow-derived stem cells may differentiate into parenchymal or non-parenchymal cells during liver regeneration [33, 34]. Furthermore, incubation of the cells in hepatocyte growth medium may have induced differentiation towards the hepatocytic lineage which may explain (low) expression levels of mature hepatocyte markers in the CD45- and CD45+ SP fractions. Thus, both SP

subsets may have the ability of differentiating into hepatocytes, by simple incubation in HGF-supplemented medium. Unfortunately, technical limitations, and in particular the paucity of SP cells that can be obtained, precluded so far the exploration of differentiation capacity of the SP cells.

The ATP-binding cassette (ABC) transporter superfamily is one of the largest protein families involved in a wide variety of transport processes, and at least partly responsible for the resistance of cells to cytotoxic insults [35, 36]. Several studies have indicated that the Hoechst33342 efflux capacity is mainly based upon the functional expression of ABC transporters, and in particular of ABCG2. Higher expression of ABCG2 was found in CD45- SP cells when compared to main population (MP) cells. Nonetheless, expression was clearly measured in the liver MP, which is in line with the detection of ABCG2 immunoreactivity in hepatocytes, of canine and human livers (see supplemental Figure 1) [37]. Moreover, the use of ABC transporter inhibitors (reserpine and verapamil) did not result in complete disappearance of the SP from the FACS plot, suggesting either that the efflux process is not entirely blocked because of involvement of additional transporters, or that part of the SP cells is simply refractory to Hoechst uptake. If present, other transporters likely play a more prominent role in the CD45+ SP fraction, given the non-dissimilar expression levels of ABCG2 in this SP subset and in the MP.

In conclusion, we detected a SP in the non parenchymal-enriched liver cells of healthy adult dogs, consisting of a CD45- and a CD45+ cell subset. Gene expression analysis showed enrichment of both SP fractions for cells that express progenitor-linked markers, suggesting that putative stem/progenitor cells can be isolated from healthy canine livers by using their ability to excrete Hoechst.



Supplemental Figure. 1. ABCG2 immunohistochemistry on frozen sections of diseased livers of dogs with lobular dissecting hepatitis, a disease with massive progenitor cell activation. Positive canalicular and basolateral membranous staining of hepatocytes for ABCG2. Indicted are hepatocyte area (H), bile duct (*) and activated LPCs (black arrows) stained positive in membranes as well as cytoplasm.

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General discussion

9.1 HGF production

A critical stage for the development of a HGF therapy was an efficient production system to provide sufficient yields for *in vivo* application. High amounts of recombinant HGF could be produced in a bacterial expression system, however the lack of post-translational modification (e.g. glycosylation) and incorrect folding in these systems could have lead to the production of biological inactive HGF. Even though the glycosylation is previously shown to be less required for the activity of HGF *in vitro*, specific interactions of sugar chains could be important for tissue distribution and circulating levels [1]. Mammalian cells are therefore an attractive host for the production of recombinant HGF, however previously reported production yields of these cell systems was too low for future *in vivo* applications [2]. Baculoviral expression systems in insect cells are known for their eukaryotic protein processing capabilities that result in highly authentic, soluble end-products and are suitable for the mass-production of recombinant proteins [3]. This system was therefore anticipated to produce recombinant HGF as an alternative for using mammalian cells. The expression systems of baculovirus infected insect cells or silkworms produced biological active recombinant HGF (chapers 4-6). Both systems are easy to use, and readily amenable to scale-up production for *in vivo* application [4].

Although the delivery of HGF is easily accomplished by injecting the recombinant protein itself, there are several drawbacks. Considering the rapid clearance by the liver and the short half-life of the HGF (2.5 minutes) repeated injections are required at short intervals to maintain a constantly high level of exogenous HGF in circulation. For the treatment of chronic liver disease that progresses over long periods ranging from years to decades a long-term HGF treatment of several weeks, or even months could be required [5]. Such a long-term therapy of recombinant HGF is not only inconvenient, but also extremely costly. Furthermore, long-term treatment with a recombinant protein could trigger the formation of antibodies.

Sustained HGF levels in the blood circulation could be accomplished by gene transfer strategy allowing persistent expression of HGF protein *in vivo*. Several studies have reported HGF based gene-therapies, in which HGF was delivered using viral based-vector methods (including retrovirus and adenovirus) as well as liposomes containing the hemagglutinating virus of Japan (HJV) [6-9]. The efficiency of these systems is high; however the construction and preparation of these procedures are labour-intensive. Furthermore, the use of viruses for therapeutically applications is still under debate [10]. A direct administration of plasmid vector containing the HGF genes is another strategy which has been proven to be safe *in vivo*. An efficient and sustained expression was achieved by using a naked plasmid DNA encoding hepatocyte growth factor [11]. An alternative approach

for a slow release of the HGF protein was provided by implanting biodegradable microspheres in which the HGF protein is saturated [12]. For a long-term treatment of dogs, the most appropriate delivery system should be examined in the near future.

9.2 HGF treatment

Although the first clinical trial with HGF was conducted and discussed in dogs with a congenital portosystemic shunt (chapter 6), a number of additional (liver) diseases may benefit from the organotrophic effects of HGF. In the following section, several of these diseases are discussed.

9.2.1 Potential of HGF treatment in chronic and acute liver disease

Cirrhosis cannot be treated nowadays; the only curative treatment for end stage liver cirrhosis in man is liver transplantation. However the number of donor organs available and the clinical condition of the potential recipient limit the applicability of this technique. Despite new surgical techniques (split liver, and living related donor transplant) the limit of donor organs has been reached and calculations indicate that 25% of the patients waiting for transplantation die (Euro Liver Transplant Registry and UNOS data). In dogs, liver cirrhosis is treated symptomatically in an attempt to stabilize the clinical situation. In general, the removal of the causative agent is the most effective intervention in most aetiologies of chronic liver diseases [13, 14]. Unfortunately, most aetiologies of liver diseases in dogs are unknown [15, 16]. HGF may therefore provide a more generalised therapeutic approach to reduce primary and/or secondary effects of chronic liver diseases, by reducing/inhibiting fibrosis, reducing oxidative stress/inflammation, and enhancing the regenerative response. The use of HGF in an experimental setting was promising in several toxin-induced models of cirrhosis in rodents [5, 7, 12, 17-19]. However the use of HGF in a clinical setting is still disputed and needs to be examined. Several fundamental questions remain to be answered about the reversibility of liver cirrhosis in the course of natural occurring disease. For instance, in the course of liver fibrosis ECM components may become more resistant to degradation, attributable to more extensive cross-linking of collagen fibres and different composition of the ECM [20, 21]. The removal or inactivation of activated stellate cells should be accomplished before recovery (remodelling and breakdown of multiple ECM components) could occur [20]. The process of regression of liver fibrosis by HGF can be elucidated in naturally occurring chronic liver diseases of dogs in a clinical setting.

Fulminant hepatitis is a very severe form of acute hepatitis, which is always fatal within 2-5 days without liver transplantation. In patients with fulminant hepatitis, extremely

high serum levels of HGF are observed. HGF levels can even be used for early diagnosis of acute liver failure and the prediction of clinical outcome in acute hepatic injury [22, 23]. However, these high HGF levels may raise the question whether HGF treatment could have an additional effect in liver regeneration. In previous reports, HGF treatment did strongly suppress the extensive progress of hepatocyte apoptosis and increase survival of mice with toxin-induced fulminant liver failure [6, 24]. This beneficial effect of HGF treatment in acute hepatitis could be explained due the presence of inactive single-chain HGF precursors. Therefore, the treatment of biological active HGF (or HGF activator) could increase the potential for liver regeneration [25]. In addition, the co-treatment with other cytokines such as IL-6 could be considered because STAT-3 and downstream mediators of c-MET were not activated in acute liver diseases in the dog (chapter 2). The treatment with recombinant IL-6 enhances priming pathways and could increase the cellular sensitivity towards HGF [26].

9.2.2 Potential of HGF treatment in other tissues

Next to the well reported hepatotrophic actions, HGF plays an important role as a general organotrophic factor. Serum HGF levels are raised in patients suffering from a wide variety of diseases, including renal, lung, and heart disease [27-29]. These observations indicate the importance of HGF in a wide variety of diseases. Although endogenous HGF levels are regulated in response to injury, in some cases HGF levels do not reach sufficient levels to accelerate tissue regeneration. In such cases, an exogenous supplementation of HGF may attenuate disease progression and support the regeneration and remodelling of injured organs. Potential diseases for the therapeutic application of HGF in diverse organs are listed in Table 1.

Table 1: proposed diseases for HGF treatment.

organ	proposed disease for HGF treatment
kidney	acute renal failure[30, 31] chronic renal failure [32, 33] support during surgical treatment (transplantation, ischemia) [34] diabetic nephropathy [35]
lung	acute pneumonia [36] pulmonary fibrosis [37] support during surgical treatment (transplantation, partial resection, ischemia) [38]
cardiovascular system	Angina pectoris [39] Cardiac infarction [40, 41] Cardiomyopathy [42, 43]
digestive tract	gastric ulcer [44] diabetes mellitus [45] pancreatitis [46]
nervous system	cerebrovascular diseases [47-49] neurodegenerative disease [50-53] Spinal cord injury [8]
bone and joint	osteoarthritis [54] rheumatoid arthritis [55]
skin	skin ulcer [56] scleroderma [57]
whole body	Crush syndrome [58]

9.2.3 HGF: the other side of the medal

The multiple cellular responses that are activated by c-MET could be utilised by malignant tissues for their invasive and metastatic behaviour. Increased HGF levels and a deregulation of the c-MET signalling transduction pathway have been observed in many tumours [59]. Involvement of HGF in tumour invasion was first implicated by the finding that scatter factor, identified as a fibroblast-derived cell motility factor for epithelial cells, was identical to HGF. HGF potently stimulates invasion of cancer cells and aberrant expression by the activation of c-MET which is closely associated with the progression of tumours toward metastatic tumours [60]. Furthermore, serum HGF levels reveal high carcinogenic states in chronic hepatitis and liver cirrhosis type C [61]. Due to the role of aberrant HGF/c-MET signalling in oncogenesis (invasion/metastasis) therapies directed against c-MET are developed. One of the best studied HGF antagonists is NK4. This is a bifunctional molecule that acts as an antagonist of HGF [62, 63]. In experimental models of distinct types of cancers, NK4 gene therapy inhibited c-MET receptor activation and this was associated with inhibition of tumour invasion, metastasis, and tumour angiogenesis. Other therapeutic strategies that inhibit c-MET/HGF signalling include c-MET peptide inhibitors, geldamycin family- or anisamycin-

antibiotics, small molecule inhibitors (such as the kinase inhibitors SU5416, STI571, and K252a), and c-MET targeted-ribozyme inhibition [64-67].

However, HGF is not involved in all types of cancer and in some cases HGF can reduce cell growth in tumour cell lines *in vitro*, such as cell lines derived from hepatocellular carcinomas [68]. Furthermore, the over-expression of HGF did not have any effect on tumour growth in mice [69]. In other studies, HGF is even suggested to function as a tumour suppressor during early stages of liver carcinogenesis because a co-expression of HGF and c-Myc delayed the appearance of preneoplastic lesions and prevented malignant conversion in mice [70]. Therefore, the inititiation of tumour formation by HGF treatment is unlikely.

9.3. Liver progenitor cells (LPCs)

The recognition of LPCs has opened a new field in regenerative medicine. LPCs may be ideally suited for future cell therapies. A further possibility could be to stimulate the patient's own LPC-compartment. To reach these potential applications, the factors or signals that determine the LPC involvement in tissue repair need to be understood in detail. In rat and mouse models mechanisms begin to unravel, but in man and dog the understanding of these mechanisms is largely unknown [71-77]. Gene expression analysis upon microdissected resting and activated LPC niches provides a powerful tool to explore a wide variety of genes that are involved in LPC activation. Furthermore, cell culture of LPCs provides proof-of-principle of newly found activating signals. The following sections focus on liver cell isolation and culturing of LPCs.

9.3.1 Liver cell isolation and culturing

Culturing of primary cells of the liver has been shown in various species [78-80]. Most of the techniques involve isolation of cells after dissociation of liver tissue using collagenase [81]. The differences in the protocols between the various species indicate the importance of optimising such a specialised technique. The sensitivity of cells to perfusion buffer flow through speed (and time) as well as collagenase type (and activity) influence the yield and viability of isolated liver cells. The results of chapters 7 and 8 are based on an optimized protocol for the isolation of canine liver cells after a three step collagenase perfusion. For these isolations liberase III (Roche) was used, a highly purified collagenase with low endotoxin levels. For the perfusion of dog livers, several perfusion protocols for cows, pigs and rodents were combined and optimized for the dog. Highly viable liver cells were acquired from intact dog livers that were kept cold for several hours in a physiologic salt solution. For longer storage of livers before dissociation the use of special storage media, such as the

University of Wisconsin (UW) solution, is recommended [82]. The initial cell suspension obtained after liberase perfusion contains a highly viable heterogeneous cell population which could be stored in liquid nitrogen allowing an entire range of experiments to be performed on the same batch of isolated liver cells. These cells can be used for several experimental techniques such as culturing and cell sorting (isodensity centrifugation (Percoll), FACS, MACS).

Acquired canine liver cells can be cultured for up to several months on specialized dishes for primary cells, using specific hepatocyte medium. After two weeks of culturing, colonies of LPC-like cells with bipotential characteristics grow within the monolayers of primary liver cells (chapter 7). These co-culture systems can be used to explore the interaction of progenitor cells and other relevant cells constituting the progenitor cell niche *in vivo*. However, for controlled proliferation and differentiation experiments a homogenous LPC culture is preferred. A more homogenous population can be acquired by subculturing the colonies, since colonies take longer to detach during trypsin treatment than their surrounding cells. Unfortunately, homogenous culture of LPCs is complicated, since the presence of non-parenchymal cells apparently play an important role in the proliferation and differentiation of LPCs in culture [83, 84]. This could be explained by cell-cell contact, the secretion of essential growth factors, and/or the presence of specific ECM components. Cell-cell contact can be achieved by co-cultures with growth-inhibited mouse embryonic fibroblasts (treated with mitomycin c) [85]. Furthermore, LPCs can be cultured in diverse coating matrices (e.g. fibronectin, gelatin, collagen, laminin, hyaluronic acid)[86]. Furthermore, the presence of integrins on LPCs could also allow culturing on other coated surfaces such as osteopontin and thrombospondin. When LPCs could be cultured in an undifferentiated state, the proliferation, and differentiation capacity of additional (growth) factors (e.g. HGF, TGF, EGF, FGF, LIF, insulin, glucagon, hydrocortisone, sodium selenite, ascorbic acid, and linoleic acid) can be explored.

The lack of a well defined specific LPC marker and the wide variation of cell culturing techniques make the comparison of results from different laboratories highly complicated. In chapter 8, an alternative method to isolate LPCs was performed based upon the Hoechst fluorescent dye efflux technique. These findings demonstrate that healthy canine liver contain a small population of cells (SP cells) with progenitor-like characteristics that can be isolated on the basis of efficient Hoechst33342 expulsion. A subpopulation of these cells (~30 %) was immunonegative for the pan-hematopoietic marker CD45, and consisted predominantly of small, mononuclear, keratin 7-immunoreactive cells with an upregulated expression of progenitor/cholangiocyte and hepatocyte marker genes. These characteristics are suggestive for the presence of progenitor cells committed to the hepatic lineage. Isolating

a SP from healthy livers may therefore represent a good alternative to isolate progenitor cells from healthy livers.

9.3.2 Remaining questions concerning LPCs

It remains to be proven how many primitive cell types (hepatic and extrahepatic) reside in the liver. This question arose in chapter 8 where CD45 positive side-population cells, expressing various markers of the hepatic lineage, indicated the presence of haematopoietic (stem) cells with hepatic traits in healthy livers. Early in the embryogenesis, the liver functions as a foetal blood producing organ by haematopoietic stem cells (HSC) derived from the yolk sac. Later in the development these HSCs migrate from the liver to the bone marrow. However, the adult liver retains the capacity to produce blood (haematopoiesis) in certain diseases. This haematopoietic ability and the hepatic markers on haematopoietic cells in the side-population suggest that healthy livers contain remnant cells from foetal haematopoietic stem cells. One could further hypothesize that there are different types of progenitor cells resident in the adult liver, or that the LPC compartment originally stems from bone marrow stem cells circulating through the body. *In vitro* studies showed that bone marrow derived stem cells (BMSC) can be induced to differentiate into hepatocyte-like cells, given the appropriate medium containing HGF [87]. *In vivo*, however, current evidence suggests that BMSCs do not play a large role in the repopulation of the hepatic parenchyma, and cell fusion seems to be the predominant process when this does occur. BMSCs may support liver repair, however this is more likely achieved through the delivery of growth factors that promote liver regeneration, resolution of fibrosis or (new) blood vessel formation. Furthermore, subsets of BMSCs contribute considerably to fibrogenesis within the liver by differentiating towards hepatic myofibroblasts [88]. Theoretically this could have massive negative effects providing a new source of (ECM producing) mesenchymal cells towards functional epithelial cells. Taken together, there are several remaining questions to be resolved concerning LPCs, highlighting difficulties in defining clear boundaries between the different compartments of progenitor cells derived from adult tissue.

9.4. Overall conclusion

In the field of canine hepatic diseases we have made great advances in understanding the biological mechanisms underlying regeneration and fibrosis, and the use of HGF was proposed for the treatment of canine liver disease. The mass production, purification, and characterization of HGF allow clinical trials in dogs with naturally occurring liver disease. A first step towards these clinical studies was made in dogs with CPSS. Furthermore, the use of LPCs was proposed as a candidate for the treatment of canine liver disease, and crucial steps in the isolation, characterisation, and culturing of canine LPCs were taken. Cell culturing could provide proof-of-principle of newly found LPC activating signals in the future. Overall the results that are described in the present thesis can be mutually beneficial for canine and human biomedical research and provide possible novel means of therapy in the struggle against liver disease.

9.5. References

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Summary
&
Nederlandse samenvatting

Summary

Background

The liver is one of the few organs that have the unique capacity to regulate its mass after a part of the functional liver mass is lost due to liver disease, injury, and/or toxicity. The process of liver regeneration is a well described phenomenon and illustrated in several experimental models in rodents. The liver apparently has an almost infinite replication capacity, as sequential partial hepatectomies could be performed in rats withour loss of regeneration potential. However in the course of chronic liver disease the large regeneration capacity of the liver is lost. The presence of inflammatory cells, toxic compounds, and/or causes a deregulation of cytokine and growth factor lead to a disturbed regeneration and an excessive excretion of extracellular matrix (ECM) components. A therapeutic strategy is based upon the restoration of the imbalanced regeneration process by treating livers with specific factors that are important for liver regeneration. Depending on the aetiology and the course of the disease, different cell types (mature or immature) could be triggered or inhibited by these factors. A summary of the cellular and molecular background concerning liver regeneration is given in **Chapter 1.** One of the most important growth factors for the liver is hepatocyte growth factor (HGF). HGF is a direct mitogen for hepatocytes, and has mitogenic, motogenic, morphogenic, and anti-apoptotic activities by binding to its receptor (c-MET). *In vivo*, HGF reduces the stimulus for fibrosis and the susceptibility of the liver to toxic and oxidative damage. HGF has been suggested as promising therapy for the treatment of a variety of liver disease. The present thesis is focused on the development of and search for such novel therapeutic strategies to stimulate liver regeneration in canine diseases which are relevant for comparable human diseases. The specific aims are:

1. To make a molecular characterization of different forms of hepatitis and cirrhosis in dogs in comparison with human hepatitis/cirrhosis with respect to the activation of regenerative and fibrotic pathways.
2. To produce and characterize recombinant HGF and to measure its *in vitro* and *in vivo* activity.
3. To start the first clinical trial in which the effect of recombinant HGF on the liver and the kidneys in dogs with congenital portosystemic shunting is examined.
4. To develop techniques for the isolation and culturing of liver specific stem cells.

1. Molecular characterization of different forms of hepatitis and cirrhosis in dogs

Canine liver diseases are not as well described as their human counterparts. Therefore, molecular pathways of regeneration (**chapter 2**) and fibrosis (**chapter 3**) were analyzed in several canine liver diseases (cirrhosis (CIRR), chronic hepatitis (CH), lobular dissecting

hepatitis (LDH), and acute hepatitis (AH)). These diseases were compared to human liver samples from cirrhotic stages of alcoholic liver disease (hALC) and chronic hepatitis C infection (hHC). In **chapter 2,** the role of HGF and downstream signalling of c-MET were evaluated. HGF mRNA and protein levels were induced in CIRR, CH, and LDH. Furthermore, the presence of phosphorylated c-MET, PKB, STAT3, and ERK1/2 indicated that these regenerative pathways could be used as therapeutic targets. These molecular studies provided a base for the treatment of canine liver disease with HGF. A defective regeneration pathway was present in AH, as shown by the absence of phosphorylated c-MET and downstream PKB. The effect of HGF as a possible therapy in this disease could be questioned and requires further research. For further analysis of canine liver disease, the biochemical pathways of fibrosis were described in **chapter 3**. Transforming growth factor-β1 (TGF-β1) gene expression and protein levels, and downstream transcription factors (Smad 2/3) were increased in canine liver diseases (CH, LDH, and CIRR) that are characterized by the excessive deposition of extracellular matrix (ECM). Regenerative and fibrotic pathways of canine cirrhosis resembled human liver samples (hALC and hHC) reinforcing the current paradigm of a highly comparable pathophysiology of human and canine hepatic fibrosis. The investigated canine liver diseases can therefore be considered relevant animal models for evaluation of novel therapeutic approaches such as the use of HGF for the translation to human clinical medicine.

2.Poduction and activity of recombinant HGF

Therapeutic intervention could be achieved by the application of the HGF protein. Therefore, we have cloned canine HGF, produced it in a baculoviral expression system in insect cells, and purified it with a heparin-sepharose column **(chapter 4).** Standardized *in vitro* experiments in dog derived liver (bile duct epithelia (BDE)) and kidney (Madin-Darby canine kidney (MDCK)) epithelial cell lines were established to measure the *in vitro* activity of recombinant HGF. Results indicated that recombinant canine HGF induced c-MET signalling and activated proliferation and scattering that were highly similar to commercially available human recombinant HGF. Furthermore, canine HGF induced the viability of BDE cells after H_2O_2 exposure **(chapter 5)**. This increase in viability was explained by (a combination of) an increase in cell proliferation, scavenging of reactive oxygen species (ROS) by increasing the GSH/GSSG ratio, and cell viability (by increasing the inactive/active caspase-3 ratio). We have thereafter produced and purified canine HGF in sufficient quantities to permit *in vivo* activity studies in dogs **(chapter 4)**. After a 30% partial hepatectomy (phx), recombinant HGF increased the phosphorylation of c-MET, PKB, and ERK1/2, and moderately increased the cell cycle protein PCNA. However, no differences were seen between HGF and NaCl liver weights at seven days after phx. This observation could be explained by the low HGF

dose, in combination with the time-point of termination. In conclusion, these *in vitro* and *in vivo* studies showed the successful production, and purification of biologically active recombinant HGF derived from a baculoviral expression system in insect cells.

3. First clinical trial in dogs with a congenital porto systemic shunt

Dogs with CPSS have a diversion of the portal blood flow past the hepatic parenchyma by an abnormal vascular connection between the hepatic portal vein and the systemic venous blood circulation. Consequently, the vital functions of the liver are limited and liver growth is hampered after birth. Due to the hampered growth of the liver, fatal liver dysfunction occurs when the animal reaches maturity, resulting in encephalopathy due to circulating toxic compounds such as ammonia. We anticipated that an increase in functional liver mass by HGF might be sufficient for a remission of the clinical signs. In addition, the reported angiogenic effects of HGF could be beneficial for increasing the portal blood flow to the growing liver. Results of the first HGF treatment in a dog with CPSS were described in **chapter 6**. During three weeks of HGF treatment, hepatocyte proliferation and liver volume increased. Kidney volume remained unaltered, while a slight increased kidney cell proliferation was observed. The abnormal high glomular filtration rate, which is frequently seen in dogs with CPPS, and was also present in this dog, almost normalized during HGF treatment. HGF treatment did not affect portal blood flow, and liver volume decreased to its original size within four weeks after the last HGF administration. Although more dogs should be treated with HGF for a reliable conclusion, the present results indicate that HGF induced substantial liver parenchymal growth. However, within the examined time frame an insufficient restauration of the portal blood flow could have caused a reversion of liver volume to pre-treatment level. The treatment of future dogs of different breed and age will show the possible need for a longer HGF treatment, and/or the co-administration of other growth factors such as VEGF for an extra stimulation of vascular development.

4. Isolation and culturing of liver progenitor cells

When the meticulously regulated regeneration process of the liver is imbalanced and the proliferation of mature liver cell types is inhibited, the activation of normally quiescent immature liver cells are observed. These are the so-called liver progenitor cells (LPC). Stimulation of liver progenitor cells (LPCs) is another approach to excite liver regeneration. LPC activation has recently been demonstrated in spontaneous canine liver diseases, making the dog a suitable model for the evaluation of new LPC-based therapies in a clinical setting. A first step towards a better understanding of the molecular mechanisms and regulatory network of LPC activation is the isolation and characterization of these cells. In **chapter 7**, a plate and wait method is described in which colony-forming small epithelial cells emerge within the

monolayer of non-parenchymal enriched cells. These colony-forming cells expressed hepatocytic, biliary, and progenitor cell markers indicating their immature and bi-potential nature. Cell morphology, immunocytochemistry, and gene-expression profiles indicated a more pronounced hepatic differentiation in hepatocyte-specific medium. In conclusion, colony-forming small epithelial cells in long-term canine liver cell cultures have hepatic differentiating capacities and may therefore be considered as progenitor cells of the liver. These cells can be used for *in vitro* investigation of different pathways that may contribute to LPC growth and differentiation. In **chapter 8,** a method is described to isolate cells with a progenitor cell phenotype directly from healthy canine livers by using their Hoechst 33342 expulsion capacity. These cells are called side population (SP) cells, referring to their unique profile on a scatter plot during FACS analysis. SP cells were examined by FACS for expression of the pan-hematopoietic marker CD45, and by qRT-PCR for expression of genes characteristic for different liver cell types, including hepatocytes, stellate cells, and LPC/cholangiocytes. A subpopulation of the SP cells was $CD45^-$ and consisted of small, mononuclear keratin 7 immunoreactive cells, suggestive for liver progenitor cells. However, both $CD45^-$ and $CD45^+$ SP cells expressed genes of progenitor cell-. cholangiocyte, and hepatocyte-markers, suggesting the presence of progenitor cells committed to the hepatic lineages in both SP fractions. These studies provide a fundamental basis for further LPC research and contributed to the overall knowledge of primary liver cell isolation and culturing of dog liver cells.

Conclusion.

In the field of canine hepatic diseases we have made great advances in understanding the biological mechanisms underlying regeneration and fibrosis, and the use of HGF was proposed for the treatment of canine liver disease. The mass production, purification, and characterization of HGF allow clinical trials in dogs with naturally occurring liver disease. A first step towards these clinical studies was made in dogs with CPSS. Furthermore, the use of LPCs was proposed as a candidate for the treatment of canine liver disease, and crucial steps in the characterisation and culturing of canine LPCs provide proof-of-principle of newly found LPC activating signals in the future. These results can be mutually beneficial for canine and human biomedical research and provide possible novel means of therapy in the struggle against liver disease.

Nederlandse samenvatting

Achtergrond

De lever is een belangrijk en veelzijdig orgaan met veel verschillende functies zoals o.a. het produceren, opslaan en uitscheiden van gal. Verder speelt de lever een belangrijke rol in vele andere processen, zoals de productie van stollingsfactoren, de koolhydraatstofwisseling, vetstofwisseling en eiwitstofwisseling. De lever is opgebouwd uit verschillende celtypen; hepatocyten voor o.a. stofwisselingsprocessen, galgangcellen die de galgangen vormen, stellaatcellen voor vitamine A- en vetopslag, endotheelcellen die de bloedvaten vormen en Kupffer die een rol hebben in het ontstekingsproces. Via de poortader krijgt de lever alle componenten aangeleverd die via het spijsverteringskanaal (maag, darmen en alvleesklier) zijn opgenomen. Dit kunnen voedingsstoffen zijn, maar ook schadelijke stoffen. De lever is in staat om schadelijke stoffen direct weer uit te scheiden in de gal of de werking van schadelijke stoffen te verzwakken. De lever fungeert dus als een belangrijk filterorgaan, maar staat daardoor zelf ook bloot aan al deze schadelijke stoffen. Gelukkig is de lever één van de weinige organen met de unieke capaciteit om zijn eigen massa te herstellen. Dit gebeurt nadat een deel van de functionele levermassa verloren is gegaan wegens ziekte, beschadiging en/of toxiciteit. Dit herstelvermogen, of regeneratief vermogen, van de lever is een bekend en goed beschreven fenomeen.

De onderliggende moleculaire processen van leverregeneratie zijn veelvuldig bestudeerd in rat- en muismodellen waarbij één of meerdere kwabben van de lever worden verwijderd. Deze chirurgische procedure wordt een partiële hepatectomie (phx) genoemd. Na een phx groeien de overgebleven leverkwabben binnen twee weken aan totdat de originele functionele massa van de lever is bereikt. Het vermogen van de lever om te regenereren is spectaculair en vindt zelfs plaats nadat 75% van de totale levermassa is verwijderd. Bijna alle levercellen dragen bij aan het herstel van het levervolume. Deze regeneratiecapaciteit gaat echter verloren in de loop van een chronische leverziekte. Een overmatige productie van bindweefsel en de aanwezigheid van ontstekingscellen en schadelijke stoffen beletten de functionele levercellen om deel te nemen aan het regeneratieproces. Wanneer deze levercellen worden geremd om te delen kan een ander celtype van de lever worden geactiveerd. Dit zijn de stamcellen van de lever die kunnen differentiëren naar functionele cellen van de lever. De activatie (vermeerdering (proliferatie) en verandering (differentiatie)) van deze cellen is geconstateerd bij veel acute en chronische ziekten van de lever bij hond en mens.

Afhankelijk van de oorzaak en het verloop van de ziekte kunnen met de kennis van alle betrokken factoren, cytokines en groeifactoren nieuwe therapieën ontwikkeld worden waarbij verschillende celtypes worden geactiveerd, of worden tegengehouden, om deel te nemen aan het regeneratieproces. Één van de belangrijkste groeifactoren voor de lever is

hepatocyten groeifactor (HGF). HGF is een multifunctioneel eiwit dat veel processen in de cel activeert door te binden aan zijn receptor (c-MET) die aanwezig is op het celmembraan van veel epitheel- en endotheelcellen. HGF kan cellen aanzetten om te delen (mitogeniteit), te verplaatsen (motiliteit) en/of te veranderen. Verder verhoogt HGF de viabiliteit van cellen door celdood tegen te gaan en door intracellulaire afweermechanismen tegen schadelijke stoffen en moleculen te verhogen. Al deze functies zijn erg belangrijk voor het regeneratieproces. Daarom wordt HGF gezien als een potentiële behandelingsmethode voor veel verschillende leverziektes. Het doel van dit proefschrift is om nieuwe therapeutische strategieën te bedenken en te onwikkelen voor leverziekten bij honden welke ook toepasbaar kunnen zijn voor humane leverziekten. Om dit te realiseren zijn verschillende doelen opgesteld:

1. Een biochemische analyse van regeneratie en fibrose bij verschillende vormen van hepatitis en cirrose bij de hond en deze resultaten vergelijken met verschillende vormen van hepatitis en cirrose bij de mens.
2. De productie en karakterisatie van recombinant HGF en het meten van de activiteit van recombinant *in vitro* en *in vivo*.
3. Het opzetten van de eerste klinische studie waarbij het effect van recombinant HGF wordt gemeten bij honden met een aangeboren levershunt.
4. Isoleren en kweken van leverstamcellen.

1. Moleculaire karakterisatie van leverziekten

Leverziekten komen bij de hond frequent voor; 1-2% van alle zieke honden die naar de faculteit diergeneeskunde doorverwezen worden hebben een lever aandoening. Net als bij mensen zijn deze leverziekten in de meeste gevallen chronisch en worden ze veroorzaakt door ziekteverwekkers en/of toxische stoffen uit hun omgeving. Leverziekten bij honden zijn echter niet zo goed beschreven als bij mensen. In dit project is daarom gestart met een moleculaire analyse van acute en chronische leverziekten bij honden. In **hoofdstuk 2** is de functionaliteit van de receptor van HGF (c-MET) aangetoond in leverbiopten die jarenlang routinematig van hondenpatiënten zijn afgenomen en bewaard. De resultaten van dit hoofdstuk wezen uit dat de onderliggende intra-cellulaire mechanismen van c-MET geactiveerd kunnen worden door middel van een therapeutische behandeling met HGF. Verder is gebleken dat er een vergelijkbaar beeld bestaat bij leverziekten bij de mens ongeacht de oorzaak van de leverziekte.

Tijdens een chronische leverziekte vindt een sterke toename plaats van het aantal en de activiteit van de stellaatcel. Inactive en met vetbeladen stellaatcellen verliezen hun vetopslag capaciteit en gaan grote hoeveelheden bindweefsel (littekenweefsel) produceren. De opstapeling van bindweefsel wordt fibrose genoemd en kan leiden tot het eindstadium

levercirrose. De leverfuncties worden hierdoor ernstig belemmerd wat uiteindelijk zal leiden tot leverfalen. Dit zijn ernstige leverziekten die gekenmerkt worden door een hoge mortaliteit. In **hoofdstuk 3** zijn biochemische processen van littekenvorming in chronische leverziekten onderzocht. De onderliggende processen van regeneratie en fibrose bij chronische leverziekten van de hond kwamen sterk overeen met die van de mens en dit duidt op een vergelijkbare pathofysiologie op een moleculair niveau. Door honden te behandelen met nieuwe therapieën kan dus ook een vertaalslag gemaakt worden naar de humane klinische geneeskunde.

2. Productie en activiteit van recombinant HGF

Uit de resultaten van de vorige hoofdstukken is geconcludeerd dat HGF als mogelijke therapeutische behandeling gebruikt kan worden voor verschillende vormen van leverziekten. Door een overstimulatie met dit eiwit zou namelijk de balans van fibrosering om kunnen slaan naar regeneratie. Hierop anticiperend is een synthetisch (recombinant) HGF geproduceerd en gezuiverd. Om de activiteit van recombinant HGF aan te tonen, zijn verschillende testen opgezet waarbij gebruik werd gemaakt van gekweekte hondencellen (cellijnen). Deze *in vitro* testen kunnen nu standaard gebruikt worden om de activiteit van recombinant HGF te bepalen. Uit deze testen is gebleken dat het recombinante HGF dezelfde activiteit heeft als het commercieel te verkrijgen humane recombinante HGF (**hoofdstuk 4**). Verder verhoogde recombinant HGF ook de levensvatbaarheid van cellen nadat schade was toegebracht door oxidatieve stress (**hoofdstuk 5**). De activiteit van recombinant HGF is ten slotte *in vivo* aangetoond bij honden na een 30% phx. Dit onderzoek heeft o.a. belangrijke informatie gegeven over de dosering van recombinant HGF in honden (**hoofdstuk 4**). Samengevat kan gezegd worden dat het geproduceerde recombinant HGF biologisch actief is en gebruikt kan worden voor een therapeutische toepassing bij honden.

3. Start van de eerste klinische studie met recombinant HGF

Honden met een aangeboren levershunt (CPSS) hebben een abnormaal bloedvat dat een kortsluiting veroorzaakt tussen de poortader (het bloedvat vanuit de darmen naar de lever) en de systemische circulatie (de bloedsomloop die het hele lichaam bereikt). Door dit extra bloedvat ontvangt de lever bijna geen bloed meer, waardoor vitale functies van de lever grotendeels uitvallen, zoals stofwisseling van eiwitten en de verwijdering van giftige stoffen die vanuit de darm in de poortader komen. Giftige stoffen, zoals ammoniak, die zeer schadelijk zijn voor de hersenen, komen vanuit de darmen door de shunt direct in het lichaam terecht. Geleidelijk aan ontstaat er een steeds verdergaande vergiftiging van het lichaam. Verder groeit de lever met een shunt na de geboorte niet of nauwelijks meer. Dit kan veroorzaakt worden doordat groeifactoren de lever via de poortader niet kunnen bereiken.

Doordat de lever na de geboorte niet met de hond meegroeit zal de mate van leverfalen toenemen met de leeftijd. Een behandeling met HGF kan de functionele levermassa vergroten en tevens de ontwikkeling van de bloedvaten stimuleren. In **hoofdstuk 6** is de eerste HGF behandeling van een hond met CPSS beschreven. Na een HGF behandeling van 3 weken was de lever met 40% gegroeid. Dit effect was echter niet blijvend waardoor de lever 4 weken na de beëindiging van de therapie weer dezelfde omvang had als bij aanvang. Dit kan verklaard worden doordat de HGF behandeling geen effect had op de bloedtoevoer naar de lever. Dit kan betekenen dat de HGF behandeling niet lang genoeg heeft geduurd om de ontwikkeling van de bloedvaten te stimuleren. Of dit inderdaad het geval is, of dat de bloedvatontwikkeling gestimuleerd moet worden met extra groeifactoren, zal blijken na afloop van deze klinische studie.

4. Isolatie en karakterisatie van leverstamcellen

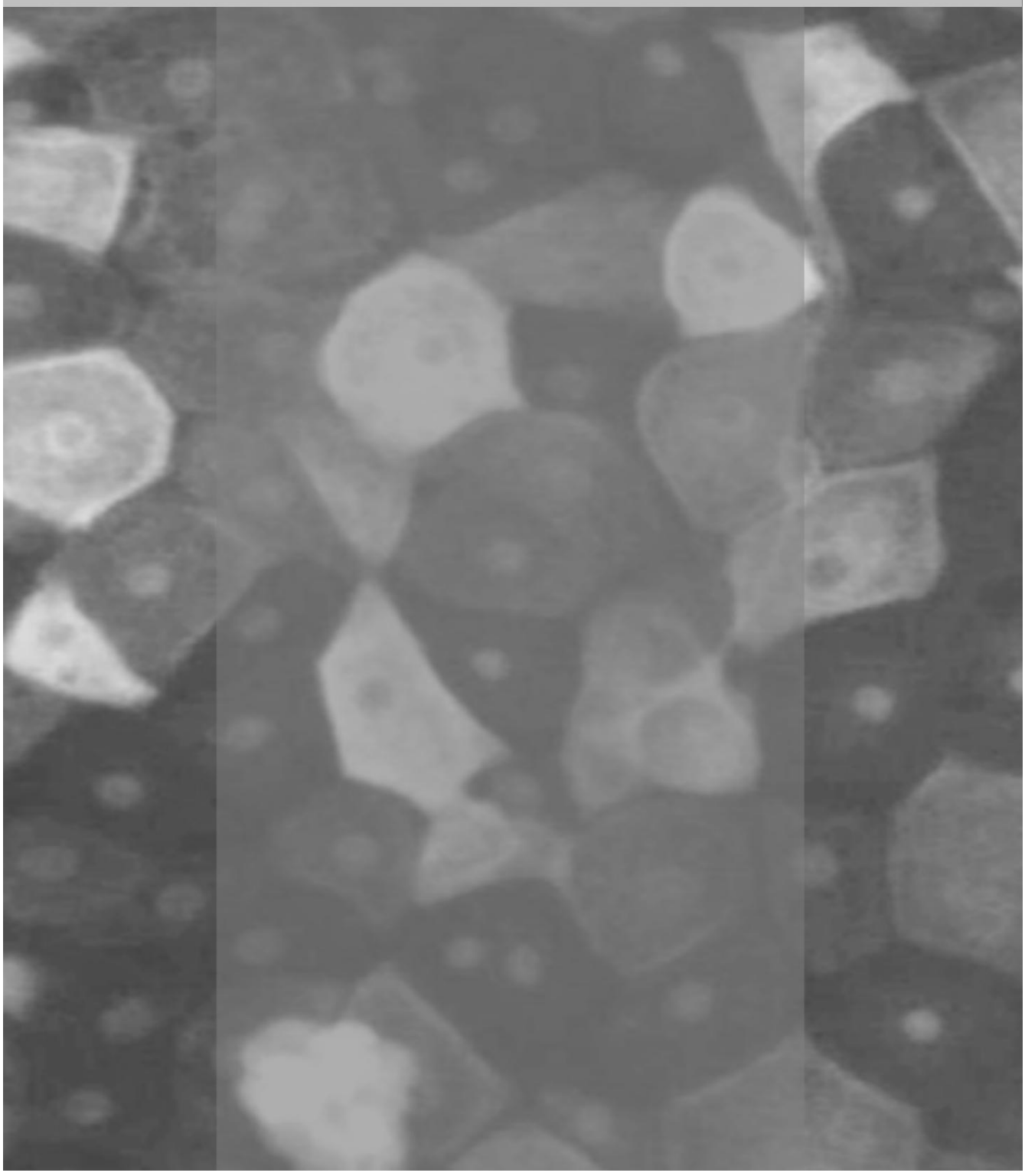
Een andere benadering om leverregeneratie te stimuleren is door gebruik te maken van de aanwezigheid van leverstamcellen. Deze cellen zijn beter bestand tegen veel schadelijke stoffen dan andere functionele levercellen en zijn daarom een veelbelovend alternatief. De aanwezigheid en de activatie van leverstamcellen in hondenlevers is recentelijk aangetoond. Leverstamcellen zijn in staat om te veranderen (differentiëren) naar hepatocyten en galgangepitheelcellen en kunnen zo beschadigde cellen vervangen. Welke factoren betrokken zijn bij deze activatie is nog grotendeels onbekend. Door leverstamcellen uit gezonde levers te isoleren en daarna te kweken kunnen deze factoren uitgezocht worden. Leverstamcellen kunnen gekweekt worden door gebruik te maken van de grote delingscapaciteit van deze cellen **(hoofdstuk 7)**. Hiervoor is een techniek ontwikkeld waarbij levercellen van een intacte leverlob van elkaar worden gescheiden door middel van een enzym. Deze cellen zijn vervolgens in kweek gebracht. Wanneer deze cellen onder de juiste omstandigheden gekweekt worden groeien leverstamcellen na twee weken tussen de andere cellen in. Deze kolonies hebben leverstamcel eigenschappen en zijn daardoor geschikt voor verdere *in vitro* experimenten. Verder kan de interactie tussen de verschillende celtypes in kweek onderzocht worden wanneer deze kweken goed gekarakteriseerd zijn. Voor een gecontroleerde differentiatie van leverstamcellen zal een zuivere celpopulatie geïsoleerd moeten worden. De moeilijkheid van het isoleren van deze cellen is dat er nog geen specifieke marker is gevonden. Daarom is in **hoofdstuk 8** gewerkt aan een techniek om leverstamcellen te isoleren zonder gebruik te maken van een specifieke leverstamcel marker. Hierbij is gebruik gemaakt van de speciale eigenschap van deze stamcellen om een specifieke stof (Hoechst33342) uit te scheiden; deze stof is fluorescerend wanneer deze gebonden is aan DNA. Door middel van een celsorteerapparaat (FACS) kunnen deze fluorescerende cellen worden gedetecteerd en geïsoleerd. Deze cellen worden side population cellen genoemd (refererend naar de selectie

methode). De levercellen die door middel van deze methode zijn geïsoleerd beschikken over morfologische en moleculaire eigenschappen van leverstamcellen en kunnen gebruikt worden voor celkweek. Deze studies vormen een fundamentele basis voor verder leverstamcel onderzoek en hebben bijgedragen aan de kennis over het isoleren en kweken van leverstamcellen uit hondenlevers.

Conclusie

Er is de afgelopen jaren grote vooruitgang geboekt in het onderzoeksveld van leverziekten bij de hond. In dit proefschrift zijn leverziekten bij de hond onderzocht en vergeleken met leverziekten bij de mens. Op basis van deze resultaten is het gebruik van leverspecifieke groeifactoren voorgesteld. Deze groeifactoren kunnen functionele levercellen of leverstamcellen stimuleren om te delen en beschadigde levercellen te vervangen. Deze resultaten hebben geleid tot de productie en zuivering van biologisch actief HGF. Tevens is een start gemaakt met de eerste klinische trial waarbij honden met CPSS worden behandeld met HGF. Ten slotte is een basis gelegd voor nieuwe therapeutische strategieën, zoals het isoleren, kweken en karakteriseren van leverstamcellen. Doordat het verloop en de moleculaire achtergrond van leverziekten bij de hond sterk overeenkomt met leverziekten bij de mens kunnen nieuwe therapieën (zoals de behandeling van zieke levers met groeifactoren) vertaald worden naar de mens.

Curriculum Vitae
and
list of publications



Curriculum Vitae

Brigitte Arends was born on July 26, 1978 in Groningen, the Netherlands. She attended secondary school at the Nienoord College in Leek (1989-1995) and at the Noordelijk Avond College (NAC) in Groningen (1995-1996). After completing her bachelor grade at the nursery school (Hanzehogeschool, Groningen) she started her study Biology at the University of Groningen in 1998. During her study she worked several months as a teaching assistant in the undergraduate course histology and anatomy. From September 2001 to February 2002 she performed her first lab rotation at the department of Molecular Neurobiology of the University of Groningen. Under supervision of Prof. PG Luiten she studied the influence of 'postnatal handling' on neurodegeneration in aging female rats. From April 2002 to September 2002 she performed her second lab rotation at the department of Medical Pharmacology of the VU University Medical Center in Amsterdam. Under supervision of Dr. AN Schoffelmeer she studied the effect of serotonergic anxiolytic drugs on the motivation to self-administered addictive substances. In December 2002, she obtained her Masters degree in Medical Biology at the University of Groningen. In March 2003 she started her research as a PhD-student at the department of Clinical Sciences of Companion Animals of the Utrecht University. In this research new therapeutic options for canine liver diseases were studied. This research resulted in the present thesis which she will defend on May 15th, 2008 in Utrecht.

Peer reviewed articles in this thesis

- **Arends B,** Spee B, Schotanus BA, Roskams T, Penning LC, Rothuizen J. *In vitro* differentiation of liver progenitor cells derived from healthy dog livers. Submitted.
- **Arends B,** Vankelecom H, Roskams T, Penning LC, Rothuizen J, Spee B. The dog liver contains a 'side population' of cells with hepatic progenitor-like characteristics. Submitted.
- **Arends B,** Spee B, Hoffmann G, Jansen GEG, Slump E, Auriemma E, IJzer J, Hemrika W, Romijn RA, van der Heijden-Liefkens KHA, Sondermeijer PJA, van den Ingh TSGAM, Penning LC, Rothuizen J. In vitro and in vivo bioactivity of recombinant canine hepatocyte growth factor. Veterinary Journal, In Press.

- **Arends B,** Slump E, Spee B, Rothuizen J, and Penning LC. Hepatocyte Growth Factor improves viability after H_2O_2-induced toxicity in bile duct epithelial cells. Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology, 2008. Apr;147(3): 324-30.
- **Arends B**, Spee B, van den Ingh TS, Roskams T, Rothuizen J, Penning LC. Major HGF-mediated regenerative pathways are similarly affected in human and canine cirrhosis. Comparative Hepatology, 2007. Jul;31:6:8.
- Spee B, **Arends B**, van den Ingh TS, Brinkhof B, Nederbragt H, IJzer J, Roskams T, Penning LC, Rothuizen J. Transforming growth factor beta-1 signalling in canine hepatic diseases: new models for human fibrotic liver pathologies. Liver International, 2006. Aug;26(6):716-25.

Peer reviewed articles not in this thesis

- Spee B, **Arends B**, van Wees AM, Bode P, Penning LC, Rothuizen J. Functional consequences of RNA interference targeting COMMD1 in a canine hepatic cell line in relation to copper toxicosis. Animal Genetics, 2007. Apr;38(2):168-70.
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- Spee B, Jonkers MD, **Arends B**, Rutteman GR, Rothuizen J, Penning LC. Specific down-regulation of XIAP with RNA interference enhances the sensitivity of canine tumor cell-lines to TRAIL and doxorubicin. Molecular Cancer, 2006. Sep 5;5:34.
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- Spee B, Mandigers PJ, **Arends B**, Bode P, van den Ingh TS, Hoffmann G, Rothuizen J, Penning LC. Differential expression of copper-associated and oxidative stress related proteins in a new variant of copper toxicosis in Doberman pinschers. Comparative Hepatology, 2005. Mar 24;4(1):3.
- Homberg JR, **Arends B**, Wardeh G, Raasø HS, Schoffelmeer AN, de Vries TJ. Individual differences in the effects of serotonergic anxiolytic drugs on the motivation to self-administer cocaine. Neuroscience, 2004.128(1);121-30.

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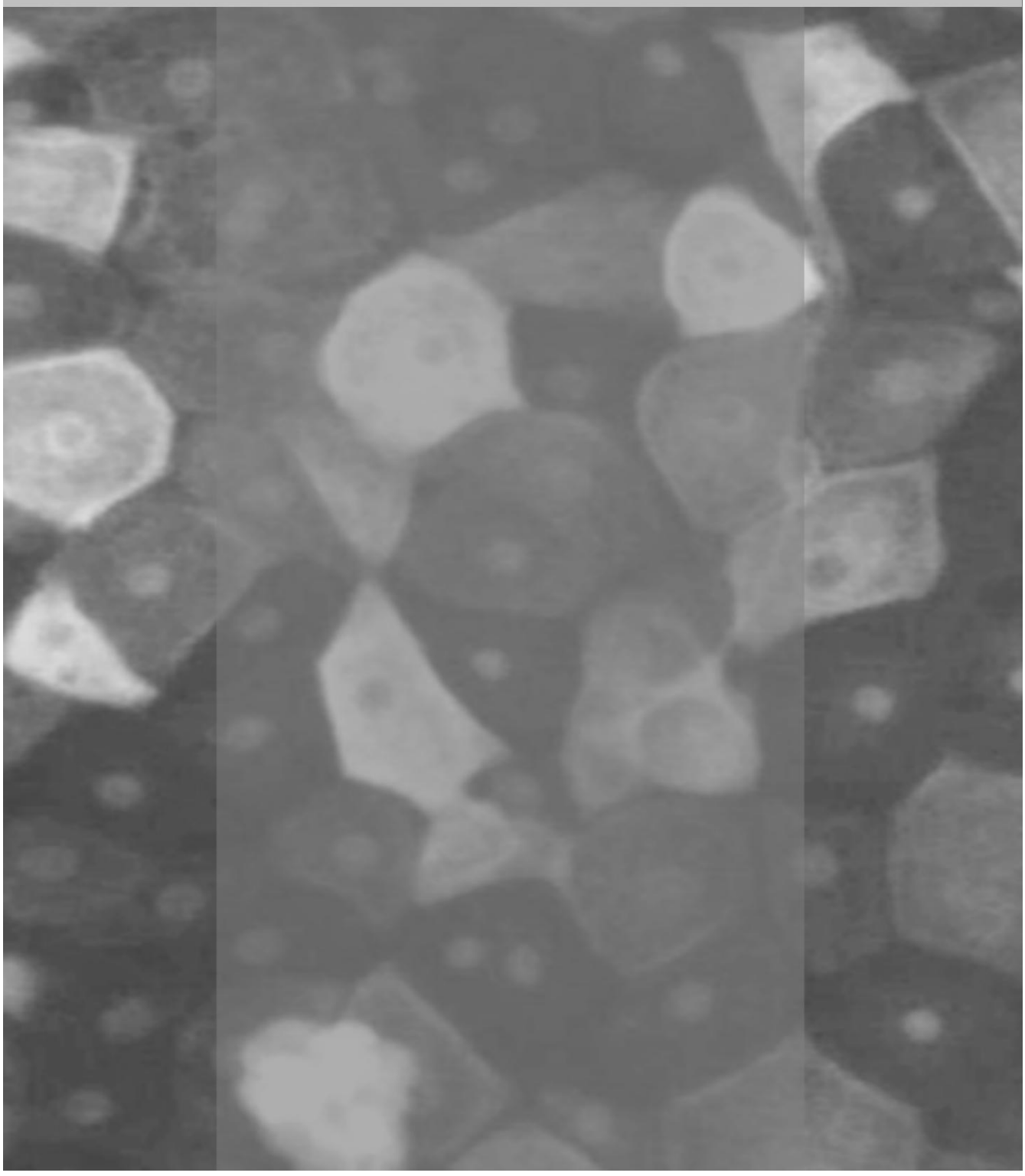
(dankwoord)



Het is zover, na een periode van 5 jaar kan ik dan eindelijk beginnen aan het dankwoord. Het stukje tekst van een proefschrift wat vaak het eerste en ook het meeste gelezen wordt. Met het schrijven van het dankwoord sluit ik niet alleen mijn proefschrift af, maar ook een periode van vijf jaar waarin ik veel heb geleerd; over de wetenschap en over mezelf. En nu terugdenkend aan de afgelopen jaren kan ik alleen maar zeggen dat ik blij ben dat ik heb doorgezet. Dit heb ik te danken aan veel mensen die tijdens mijn onderzoek in meer of mindere mate voor mij hebben klaargestaan. Deze laatste woorden zijn voor jullie!

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Brigitte

List of abbreviations

- ABCC2/MRP2, ATP-Binding Cassette, Subfamily C
- ABCG2, ATP-Binding Cassette, Subfamily G, Member 2
- AFP, alpha-fetoprotein
- AH, acute hepatitis 3
- ALB, albumin
- ALT, alanine aminotransferase
- AP, alkaline phosphatase
- AST, aspartate aminotransferase
- BDE, cells bile duct epithelial cell line
- B2MG, beta-2 microglobulin
- BMSC bone marrow derived stem cells
- CAT, catalase
- CPSS, congenital portosystemic shunt
- CH, chronic hepatitis
- CIRR, cirrhosis
- CYP1A1, Cytochrome P450, Subfamily 1
- DES, Desmin
- ECM, extracellular matrix
- ERK1/2, one of the MAP kinases
- FN14/TNFRSF12A, Tumor Necrosis Factor Receptor Superfamily, Member 12A
- GAPDH, glyceraldehyde-3-phosphate dehydrogen
- GCLC, gamma-glutamylcysteine synthetase
- GFR, glomular filtration rate
- GPX1, glutathione peroxidase
- GSR, glutathione reductase
- hALC, alcohol-induced cirrhotis in human
- hHC, hepatitis C- induced cirrhotis in human
- HGF, hepatocyte growth factor
- hHC, chronic hepatitis C infection
- HNF4A, Hepatocyte Nuclear Factor 4-Alpha
- HPRT, hypoxanthine phosphoribosyl transferase
- KIT, v-KIT Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog
- K(RT), cytokeratin
- LDH, lobular dissecting hepatitis
- LPC, liver progenitor cell
- PHX, partial heptectomy
- MAPK, Mitogen activated protein kinase
- MDCK, Madin-Darby Canine Kidney Cells
- MP, main population
- MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
- NCAM1, neural cell adhesion molecule 1
- p38MAPK, one of the MAP kinases
- PCNA Proliferating Cell Nuclear Antigen
- PKB, Protein Kinase B
- PROM1, prominin 1
- Q-PCR, quantitative real-time polymerase chain reaction
- ROS, reactive oxygen species
- RPL8, ribosomal protein L8
- RPS5, Ribosomal Protein S5
- SOD1, superoxide dismutase 1
- SP, side population
- STAT3, Signal Transducer and Activator of Transcription 3.
- TGF-β1, transforming growth factor β1
- TGF- βRI, transforming growth factor β receptor type I
- TGF-β RII, transforming growth factor β receptor type II
- THY1, T-cell antigen
- uPA, urokinase plasminogen activa