Liver fibrosis and regeneration in dogs and cats : An immunohistochemical approach

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Introduction

In veterinary medicine, canine and feline liver disease is a common phenomenon. Most dogs are diagnosed with hepatitis, whereas cats often have cholangitis, hepatic lipidosis, and less frequently acute fulminant hepatitis [16]. Chronic hepatitis/ cirrhosis is uncommon in cats [16]. Interestingly, feline hepatic lipidosis shares many morphological similarities with human non-alcoholic steato-hepatitis (NASH) [6] [16]. As outcome of clinical liver disease is often unfavorable [16], extension of current limited therapeutic potential is much sought-after. Present therapeutic options have been derived from human hepatology. Also, in-depth studies into spontaneous liver pathology were restricted to man; therefore species specific knowledge of canine liver pathology is limited. In this respect, improved insight into the species specific reaction pattern of the dog is urgently needed to provide a solid basis for inventing new therapeutic strategies. This review reports about identification of cells taking part in the complex pattern of liver tissue repair in acute and chronic liver diseases of dogs and cats.

Etiology of canine and feline hepatitis

An infectious etiology is often suspected in canine hepatitis, but despite the existence of many known etiologic factors, the etiology of individual canine cases stays largely unknown [6]. Identified infectious agents include viruses (*canine adenovirus-1/ Rubarth virus*: infectious canine hepatitis or ICH and feline *infectious peritonitis virus; Herpesviruses*), bacteria (*Clostridium piliformis*: Tyzzer's disease; *Leptospira spp.*; *Helicobacter canis* and septicemic diseases), protozoa (*Toxoplasma gondii, Neospora, Leishmania chagasi*) or fungi (*Histoplasma capsulatum*) [6] [16]. Several toxic agents are also known to inflict hepatitis, such as toxins synthesized by poisonous mushrooms (*Amanitum spp*.) or blue green algae (*Cyanophyaceae*). Also, idiosyncratic drug reactions to therapeutical drugs can give rise to severe liver necrosis, e.g. benzodiazepine, acetaminophen, trimetoprim sulfonamide, carprofen and amiodrone [16]. Copper toxicosis is a common cause of hepatitis in dogs. Mostly, this is a breed related disease, frequently occurring in Bedlington terriers, Doberman pinschers, Sky terriers, West Highland white terriers, Dalmatians, Anatolian shepherds, American and English Cocker spaniels and Labrador retrievers. This breed-association suggests an underlying genetic defect, which so far has been identified in the Bedlington terrier only, a mutation in the MURR1 (COMMD1) gene associated with reduced bile copper excretion [6]. Copper toxicosis has been reported only once in the cat [6].

Histological changes in canine and feline hepatitis

Hepatitis in veterinary hepatology always includes hepatocellular cell death: necrosis and/or apoptosis, and an inflammatory infiltrate varying in type and extent [16]. Furthermore, acute hepatitis (AH) sometimes features regeneration, whereas chronic hepatitis (CH) always conveys regeneration and fibrosis. Fibrosis is defined as detectable deposit of extracellular matrix [6]. It can develop into cirrhosis, characterized by disruption of the normal hepatic architecture by fibrous septa into structurally abnormal nodules, associated with porto-central vascular anastomosis [16]. Lobular dissecting hepatitis (LDH) is a special form of cirrhosis typically occurring in younger dogs [6] [16]. Its fierce clinical course rapidly progresses to a fatal outcome. Histological changes include complete disruption of the lobular architecture by fine fibrotic septa which encompass individual hepatocytes, or small groups of hepatocytes. LDH bears resemblance to human neonatal hepatitis in morphological distribution and occurrence at young age, but seems to have a poorer prognosis [6]. To date, it is unknown which factors determine the LDH reaction pattern versus "normal" cirrhosis.

Liver tissue repair

Species specific knowledge regarding liver tissue repair in the dog is limited, as most studies were performed in rodents [12]. Following injury, the liver elicits a tissue repair response in order to regain its physiological function. This reaction pattern comprises two balanced actions: a) parenchymal

regeneration (of hepatocytes and/or cholangiocytes) and b) supported by regular wound healing, which is a mesenchymal regenerative process. Generally, the term "liver regeneration" is solely reserved for the parenchymal regeneration; this custom will be followed in the next discussion. When the balance between regeneration and fibrosis is extremely charged, liver function is no longer safeguarded and clinical symptoms develop, in worst case with a fatal outcome. On the one hand, the wound healing process can give rise to adverse fibrosis or even develop into cirrhosis. On the other hand, the replicative capacity of the remaining hepatocytes may fall short [6] [17]. Hepatocellular regeneration in hepatitis can occur by two means: replication of mature hepatocytes and, in case of hampered hepatocytic reproduction, also by proliferation of the local progenitor cell population, in rodents called "oval cells" [6] [12] [15] [17]. A third (theoretical) route by fusion or transdifferentiation of hemopoetic or bone marrow stem cells into hepatocytes, is not regarded clinically important [6]. However, bone marrow stem cells may contribute to repopulation of myofibroblasts or sinusoidal endothelial cells, and thus play a role in liver tissue repair [6].

Liver tissue repair is a tightly regulated, very complex process involving orchestrated interplay between all resident liver cell types, stroma and signaling molecules/ growth modulators. These factors cooperatively create a favorable microenvironment which is compulsory for successful regeneration [17]. Liver regeneration features a priming phase, growth phase and growth inhibitory phase, which requires priming factors, growth factors and growth inhibitory factors, respectively [17]. To unravel the discussion regarding the complex pattern of liver tissue repair, the following evaluation is divided as follows: first, major signaling pathways are described (I), followed by cell types (II) and finally, an integrated outline of events is presented (III).

I. Signaling pathways liver regeneration

Hepatocyte growth factor (HGF) and transforming growth factor-β-1 (TGF-β-1) are two main players in the liver tissue repair response. HGF is mainly produced by activated hepatic stellate cells and induces liver regeneration by mitogenic and motogenic effects on hepatocytes [6] [12]. On the other hand, TGF-β-1 which is also produced by activated hepatic stellate cells, exerts opposite effects in promoting fibrosis and hepatocellular apoptosis [6] [12].

Hepatocyte growth factor (HGF)

HGF, also called scatter-factor (HGF/SF), is synthesized and secreted as its single-chain precursor form [6]. This inactive single-chain HGF is bound to hepatic matrix (to the glycosaminoglycan heparin) [12] and is released during matrix remodeling [6]. Metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) play an important role in regulating the release of HGF from the matrix and its availability for activation [6]. Matrix serine proteases modulate inactive single chain HGF into its active two-chain form [6]. These proteases include HGF activator or HGF-converting enzyme, urokinase-type plasminogen activator (uPA) and Factor Xa [6]. Proteolytically activated HGF, consisting of a 69 kDa α-subunit and a 34 kDa β-subunit [6], is locally available, but also overflows into the circulation [6].

All effects of HGF on hepatocytes are mediated by its receptor c-Met [6]. Matrix glycosaminoglycans heparin, CD44 and dermatan sulfate function as co-receptors potentiating c-Met activation *in vitro* [6]. C-Met is a dimeric membrane-spanning tyrosine kinase type receptor composed of an extracellular 50-kDa α-chain and a transmembrane 145 kDa β-chain. Both α and β units are required for high-affinity receptor binding of HGF [6]. Intracellular signaling pathways driven by HGF-c-Met coupling lead to mitogenic (stimulating proliferation), motogenic (stimulation cell movement), morphogenic (stimulating maturation), branching (stimulating formation of branching ductules), angiogenic and anti-apoptotic activities [6]. At least in hepatocytes, c-Met binds and sequesters, thus inactivates death receptor Fas [6]. These different effects of c-Met activation are mediated by different intracellular transducers [6]. Noteworthy, c-Met was initially identified as an oncogene [6] and has attracted considerable interest in tumor biology ever since, due to its control of growth, invasion and metastasis [6].

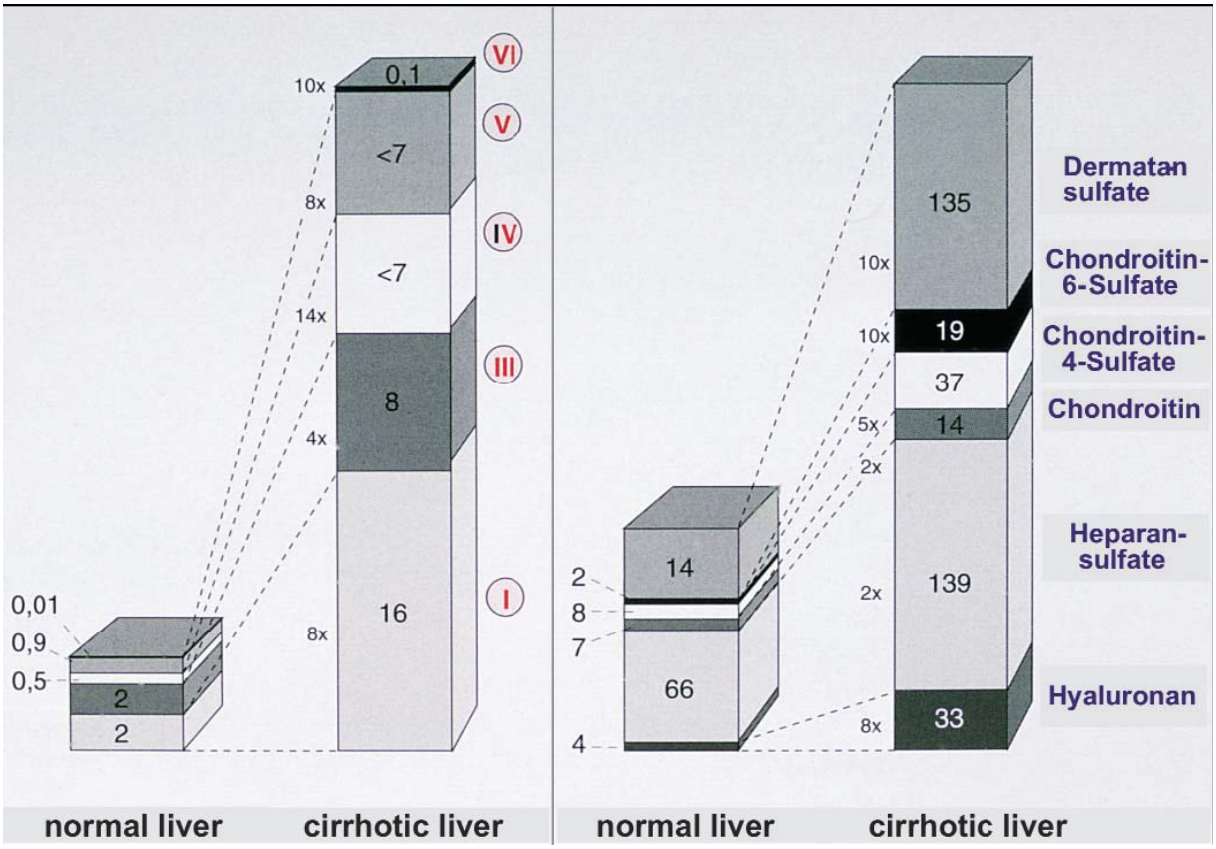

Figure 1. Changes in the amount and composition of collagens I, III, IV, V and VI (left) and of glycosaminoglycans (right) from normal to cirrhotic human liver. Numbers give the concentrations for collagen in mg/ wet weight and for glycosaminoglycans in mol hexosamine/ 100g dry weight. (Reproduced with kind permission from J Cell Mol Med 10: 76-99, 2006).

Transforming growth factor β-1 (TGF-β-1)

The three closely related isoforms (TGF-β-1, TGF-β-2 and TGF-β-3) of the TGF-β family elicit distinct biological responses *in vivo* [5]. Of these, TGF-β-1 is considered of main importance in liver tissue repair [5] [12]. TGF-β's are synthesized as inactive precursors. The biologically active form is a disulfide linked 25 kDa dimer [12]. In normal liver, TGF-β-1 is stored in matrix by binding to decorin [6]. It exercises competing tonic effects on hepatocytes, opposing effects of other matrix-bound growth factors, thus keeping hepatocytes in quiescent state [6]. Besides, latent TGF-β-1 is also stored in extracellular matrix by complexes of latent TGF-β with binding glycoproteins (fibronectin and latent TGF-β binding protein) which is mediated by heparan sulfate proteoglycans (HSPGs) [6]. Collagen type IV, fibrinogen and urokinase type plasminogen activator activate latent TGF-β-1 [6]. At matrix remodeling during tissue injury and repair TGF-β-1 is released from matrix deposits by proteases thrombospondin, tissue plasminogen activator (tPA) and MMPs [6]. When TGF-β-1 is released into the circulation, it is bound and inactivated by α-2-macroglobulin [6].

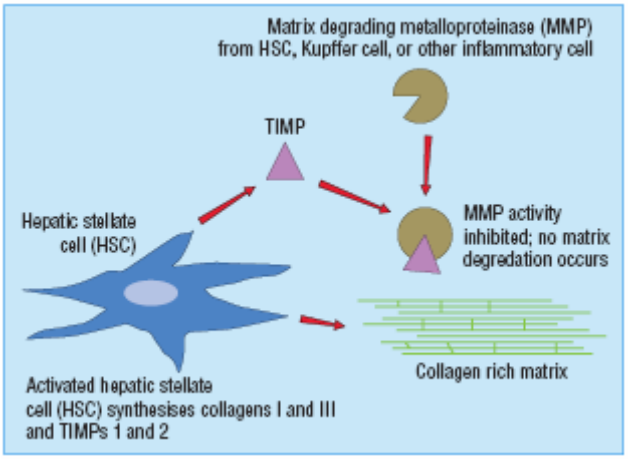

Figure 2. Tissue inhibitors of metalloproteinase (TIMPs) secreted by activated hepatic stellate cells prevent matrix degradation

by inhibiting enzymatic activity of matrix degrading metalloproteinases (MMPs) (Reproduced with kind permission from B.M.J. 327: 143-147, 2003).

A network of three types of TGF-β receptors (TGF-β-R) and several intracellular signaling mediators (Smad proteins) mediate the biological effects of TGF-β [6]. The most important receptors are TGF-β-R1 and TGF-β-R2, transmembrane serine/threonine kinases. TGF-β first attaches to TGF-β-R2 which subsequently activates TGF-β-R1. This eventually leads to translocation of receptor-regulated Smad (Smad 2 or 3) protein complexes from the cytoplasm to the nucleus, followed by direct binding to DNA sequences [6]. Smad3 is a main fibrogenic mediator in hepatic stellate cells (HSCs) [5]. In the nucleus, target genes are either suppressed or stimulated (like Smad7). By association with activated TGF-β-R1 Smad7 prevents phosphorylation of receptor regulated Smads thus supplying a negative feedback loop [5].

TGF-β-1 stimulates fibrosis by simultaneously inducing upregulation and release of many extracellular matrix (ECM) components: collagen type I, III, IV, V and VI; glycosaminoglycans (hyaluronan, heparan sulfate, dermatan sulfate, chondroitin sulfate: see Figure 1) and other glycoconjugates like the structural glycoproteins tenascin, laminin, fibronectin and proteoglycan core-protein decorin; as well as tissue inhibitors of metalloproteinases (TIMPs), which prevent ECM breakdown [6], (Figure 2). Additionally, integrin expression is also modulated resulting in increased cell-matrix adhesion [11].

HGF and TGF-β-1 signaling in the dog

In the dog, the role of HGF and TGF-β-1 pathways in canine liver disease was investigated [6] [18]. c-Met levels were reduced in canine hepatitis and cirrhosis, but major downstream regenerative pathways were still activated. This pattern proved highly comparable to man. Also, fibrosis in spontaneous dog liver diseases (AH, CH, cirrhosis, LDH) was found highly comparable to man. There was upregulation of the TGF-β pathway with enhanced deposition of collagen I and III.

Other key regulators

Interleukin-6 (IL-6) binding to its receptor Glycoprotein 80 (Gp80), which is either soluble or located on the hepatocellular surface, and Gp130 results in activation of Janus tyrosine kinases (JAKs) and downstream activation of signal transducer and activator of transcription-3 (STAT3) [6] [17]. Subsequently, proliferation promoting factors are upregulated [17]. Interestingly, other members of the IL-6 cytokine family, such as leukemia inhibitory factor (LIF) and oncostatin M (OSM) may also be implicated in progenitor cell-mediated liver regeneration [17]. Transforming growth factor-α (TGF-α) is synthesized by hepatocytes [17]. TGF-α is related to epithelial growth factor (EGF) that is produced continuously in Brunner´s glands in the duodenum. Both growth factors use EGFR, a transmembrane tyrosine kinase receptor to induce proliferative effects on target cells, e.g. hepatocytes [6] [17]. In the regulation of liver regeneration more factors do play a role, such as norepinephrine, bile acids, xenobiotics, serotonin (from platelets), leptin and insulin levels, or Notch and Jagged expression on hepatocytes and cholangiocytes [12]. Platelet derived growth factor (PDGF) and connective tissue growth factor (CTGF) have emerged as additional mediators for fibrogenesis [8].

II. Cells and matrix in liver disease

Kupffer cells are resident macrophages, located in the sinusoidal space (Figure 3). Activation of these cells is an early key issue in liver tissue repair (reviewed in [6]). They are either directly activated by portal vein delivered lipopolysaccharids (LPS) from the gut or by activated complement factors 3a and 5a (C3a, C5a). LPS binds to a complex of toll-like receptor 4 (TLR4) and CD14 receptor. This results via translocation of nuclear factor κB (NF-κB) to the nucleus in elevated output of signaling molecules such as tumor necrosis factor alpha (TNFα), interleukins 1 and 6 (IL-1, IL-6) and nitric oxide (NO). Signal transduction for C3a and C5a is attributed to G-proteins which regulate phospholipase C and eventually leads to production of prostaglandins D_2, E_2 and $F_{2\alpha,}$ thromboxane A_2 and free radical superoxide anion [6]. Furthermore, Kupffer cells also excrete PDGF and matrix degrading metalloproteinase enzymes (MMPs) [1] [6]. **Platelets** store HGF, TGF-β and serotonin. The latter may have an effect on release of HGF and TGF-β [6][12]. **Endothelial cells** do produce HGF. In rats after PHx, proliferating endothelial cells upregulate their vascular endothelial growth factor (VEGF) receptors 1 and 2, platelet derived growth factor (PDGF) receptor and angiopoetin receptors Tie-1 and Tie-2 [12].

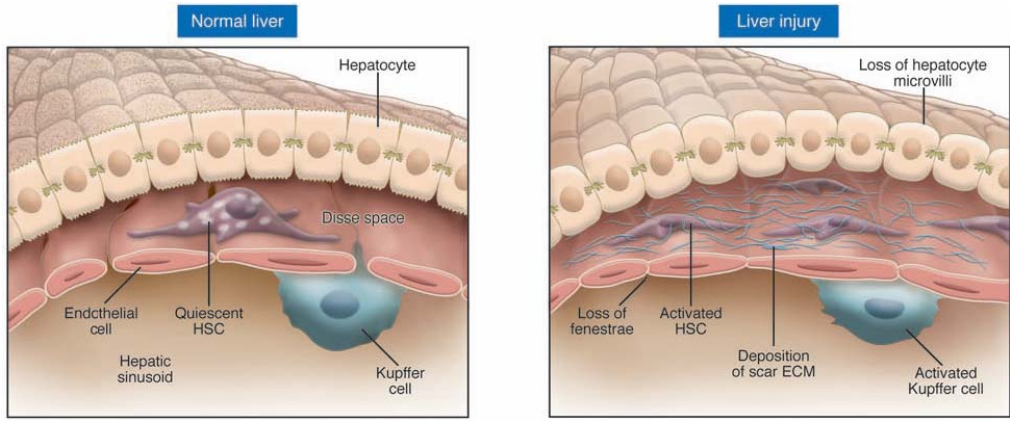

Figure 3. Sinusoidal changes during liver injury. Left: normal liver, and right: injured liver. Liver injury leads to activation of Kupffer cells, which reside in the sinusoidal space. Kupffer cell activation contributes to activation of hepatic stellate cells, located in the subendothelial space (Disse's space). Stellate cells deposit large amounts of fibril forming (scar) matrix. This deposition leads to loss of hepatocytic microvilli and sinusoidal fenestrae which results in deterioration of hepatic function. (Reproduced with kind permission from J Biol Chem 275: 2247-2250, 2000).

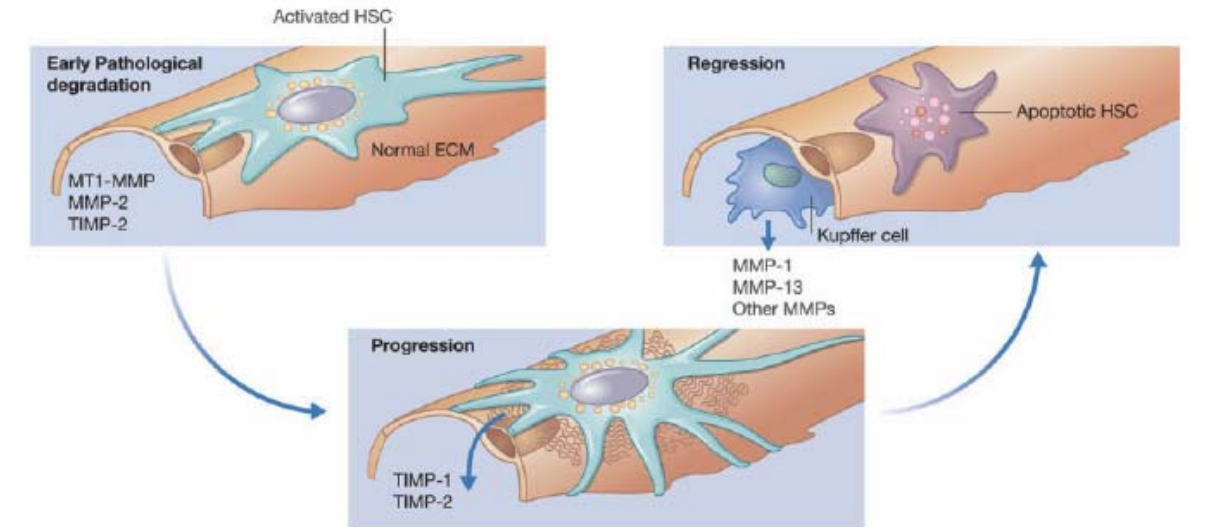

Figure 4. Sinusoidal endothelial cell layer (with one oval nucleus) covered by a perisinusoidally located hepatic stellate cell (HSC) in extracellular matrix. Emerging mechanisms of matrix degradation, fibrosis progression and fibrosis resolution in chronic liver disease. Left top: Activation of HSCs will be associated with pathological matrix degradation because of increased production of membrane type matrix metalloproteinase (MT1-MMP), matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinases-2 (TIMP2), eventually leading to replacement by interstitial collagen or scar matrix. Middle panel: During progression of fibrosis, sustained expression of TIMP-1 and -2 prevents matrix breakdown and apoptosis of activated HSCs. Right top: Fibrosis regression is associated with increased apoptosis of activated HSCs. Apoptosis and the following matrix regression requires decreased levels of TIMP-1 leading to a net increase in protease activity. This may correspond with MMP synthesis (likely MMP1 in man, and MMP13 in rodents). However, the producing cells (possibly Kupffer cells) have not been positively identified yet *in vivo*. (Reproduced with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc. Hepatology 43: S82-S88)

Activated fibroblasts, which transdifferentiate into **myofibroblasts** (MFs), play an essential role in hepatic fibrogenesis [6]. Based on location and immunohistochemical profile three MF subpopulations were described [3] [6]. These comprise 1) portal or septal MFs, present in the portal areas or in newly formed fibrous septa, 2) interface MFs, present at the interface between parenchyma and stroma of the portal areas or newly formed fibrous septa, and 3) the perisinusoidally located **hepatic stellate cells** (HSCs) (Figures 3 and 4). All types have fibrogenic potential, but many investigators regard HSCs as the principal fibrocompetent cell in the liver [2] [6]. Depending on the primary site of injury the resulting fibrosis may be restricted to the portal areas, as in most biliary diseases, or may be present in the hepatic parenchyma as seen in chronic hepatitis and cirrhosis.

Although incompletely understood, the activation or transdifferentiation of MFs/HSCs is a key event in liver tissue repair. This activation has been subdivided into two tightly associated successive steps: initiation and perpetuation [6], and may be followed by regression [2]. The activated or transdifferentiated state is characterized by increased proliferation, contractility and migration, as well as loss of vitamin-A containing lipid vacuoles and enhanced expression of α-SMA and desmin [2] [6]. Furthermore, HSCs produce opposing growth factors HGF and TGF-β as well as matrix

metalloproteinases (MMPs) and inhibitors of metalloproteinases (TIMP1 and TIMP2) and abundant amounts of extracellular matrix components including collagen, proteoglycans and glycosaminoglycans [1] [2] [6] (Figures 3 and 4).

A very early trigger in HSC activation results from edema. Edema leads to increased matrix stiffness which attributes to HSC activation [5] [6], TGF-β plays a pivotal role in initiation, promotion and progression of MF transdifferentiation [5]. Initially, TGF-β-1 is delivered by necrotic hepatocytes [11], platelets and inflammatory cells, including Kupffer cells [2] and is quickly released from matrix deposits [6]. Later on, during perpetuation, activated HSCs themselves become the major source of TGF-β-1, with autocrine and paracrine stimulating effects [2]. Moreover, HSC activation includes de novo expression of TGF-β receptors, thus further enhancing TGF-β effects [2]. Platelet derived growth factor (PDGF), mainly produced by Kupffer cells but also by hepatocytes, is the predominant mitogen for activated HSCs [6] [12]. Also oxidative stress and lipid peroxidation are important contributors to MF/HSC stimulation by formation of reactive oxygen species (ROS) [2] [6]. Well known inducers of oxidative stress in the liver include exogenous compounds like acetaminophen and 2-N acetyl aminofluorene, which are often used to reduce replicative capacity of hepatocytes in rodent animal models. Other activating factors include TNF-α, interferon-γ, IL-6, epidermal growth factor (EGF), insulin like growth factor (IGF), CD40, C-C chemokine ligand 21 (CCl21) [1]. Likewise, matrix fibrillar collagens (collagens I, III, V, all upregulated during fibrosis) can activate MFs by binding to HSC surface expressed discoidin domain receptor (DDR2) and integrins [6]. Interestingly, human HSCs express neuroendocrine markers (reelin, nestin, neurotrophins, synaptophysin, glial fibrillar acidic protein) and also receptors for neurotransmitters [1]; therefore they are likely also entangled in incompletely unraveled neural control mechanisms [14].

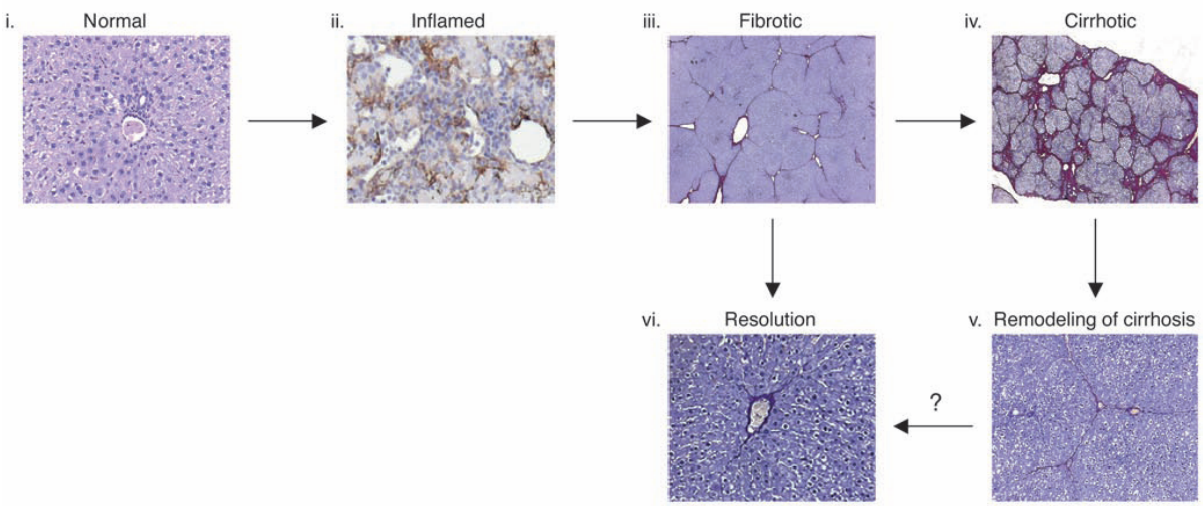

Figure 5. Lever cirrhosis as wound healing. Damage to the normal liver (i) results in hepatitis (ii) and activation of hepatic stellate cells to secrete collagen, culminating in the development of fibrosis (iii) and ultimately in cirrhosis (iv). Withdrawal of the injurious agent may allow remodeling of the fibrillar matrix, leaving attenuated cirrhosis (v). Spontaneous resolution of fibrosis after removal of injury results in a return to near normal architecture (vi). Whether "complete" resolution of cirrhosis can occur is currently unknown. (Reproduced with kind permission from B.M.J. 327: 143-147)

As described above, for the activation of hepatic MFs a plethora of factors are identified. However, concerning the equally important regression into quiescence or apoptosis of stellate cells/myofibroblasts, many questions remain open (Figure 4). Reversion of HSC activation is considered to attribute to fibrosis regression [2][4], (Figures 4 and 5*)*. Peroxisome proliferator activated receptor (PPAR)-γ was able to induce reversal of HSC activation [6]. Nerve growth factor, expressed by hepatocytes during injury, may drive HSC apoptosis [6]. Apoptosis of activated HSCs is successfully blocked by sustained TIMP1 expression [6], with activated NF-κB to preserve the activated state [6].

Hepatocellular proliferation is subdivided into three phases: priming, growth and growth inhibition. Priming is required to exert full effects of growth factor stimulation [6] [17]. The priming factors TNF-α and IL-6 [2] initiate, at least in part, hepatocellular proliferation by translocation of NF-κB and STAT3 to the nucleus [6]. In the rat PHx model (2/3 partial hepatectomy), early intracellular changes include translocation of Notch intracellular domain (NICD) and β-catenin to the nucleus [12]. After priming, growth is stimulated by three main growth factors: HGF, transforming growth factor-α (TGF-α) and epithelial growth factor (EGF). After liver tissue injury, increased uPA activity is observed within 5 minutes, leading to release of matrix bound HGF [12]. HGF and EGF receptors are activated within

30-60 minutes after PHx in rats [12]. Hepatocytes synthesize growth factors for stellate cells (e.g. PDGF) and endothelial cells, such as VEGF, fibroblast growth factor (FGF), angiopoetin, transforming growth factor α (TGFα). TGF-β inhibits hepatocellular and ductular proliferation [6]. However, due to timing and duration of its upregulation, it can not be the sole responsible factor for cessation of hepatocellular regeneration [12]. Likely activin, also belonging to the TGF-β family of cytokines, plays a major role in termination of hepatocellular proliferation [6] [12].

Progenitor cells are activated when hepatocellular proliferative capacity falls short. They ultimately differentiate into new hepatocytes or biliary epithelial cells [6] [12] [13] [17]. The progenitor cell compartment consists of resident progenitor cells in normal liver, and ductular reaction and intermediate hepatobiliary cells in the diseased liver. In the normal liver, resident progenitor cells are located in the finest branches of the biliary tree, the canals of Hering [15]. To date, no unique factor for identifying progenitor cells has been identified in man or dog. With some species specificity, the different subsets of progenitor cells transiently express combinations of markers, including stem cell markers, cholangiocytic, hepatocellular and neural markers, leading to extensive debate regarding the origin of these cells [12] [14] [17]. The regulation process of progenitor cell versus hepatocellular proliferation is incompletely unraveled (comprehensive review in [17]). Like in regeneration by mature hepatocytes, three phases are discerned: priming, growth/differentiation and growth inhibition [17].

So far, only one factor selectively stimulated mitosis in mouse hepatic oval cells: TNF family member TWEAK (TNF-like weak inducer of apoptosis) [6]. Furthermore, progenitor cells share many priming factors with hepatocytes, e.g. TNF-α and IL-6. Moreover, IL-6 also acts as progenitor cell mitogen [6]. After priming, shared growth factors inducing proliferation and differentiation include HGF, TGF-α and acidic fibroblast growth factor (aFGF)/FGF1 [6] [17]. HSCs, proliferating simultaneously and in close contact with progenitor cells [6] [14] [17] are the main source of these growth factors (HGF, TGF-α and aFGF), suggesting paracrine regulation [17]. Interestingly, upregulation of FGF-receptors (FGFRs) differs in amount and subtype (FGFR-1 and -2) between hepatocytes, progenitor cells and stellate cells providing a possible explanation of different reaction patterns between progenitor cells and hepatocytes [6]. In contrast to its effect on hepatocytes, the stem cell factor (SCF)/c-kit growth factor receptor system is suggested to play a role in the initial activation of the progenitor cells [6] [17]. Reduction of progenitor cell activation is mediated by TGF-β. In addition to the surrounding HSCs, TGF-β is also synthesized by progenitor cells synthesize TGF-β during early phases of their differentiation [6]. Thus a negative feedback loop is supplied on excessive progenitor cell proliferation.

Moreover, intercellular interaction between HSCs and progenitor cells is partly mediated by cell-specific matrix proteoglycans such as heparan sulfate. These proteoglycans also mediate binding of growth factors to their receptors and regulate interactions between the progenitor cells and ECM (reviewed in [15]). Other factors exerting opposite effects on mature hepatocytes (growth inhibition) versus progenitor cells (stimulation) are IFN-γ and similarly functioning lymphotoxin-β (LT-β) [6] [17] Intercellular adhesion molecule-1 (ICAM-1) expression on progenitor cells indicates involvement of immune system cells in the microenvironment [6]. Also, the chemokine stromal cell-derived factor-1 (SDF-1) and its receptor CXCR4 were likely involved in activation and early expansion of the liver progenitor cell compartment in a rat liver model [6].

Matrix

The extracellular matrix (ECM) is composed of collagens (Table 1), structural glycoproteins (like laminin, fibronectin, tenascin, elastin) and proteoglycans divided into glycoaminoglycans (heparansulfate, hyaluronan) and core-proteins like decorin and syndecan) [5]. High turnover takes place during necro-inflammatory activity, tissue repair and fibrosis, with overt changes in amount, composition and distribution of ECM components (Figure 1). Changes in ECM composition can directly stimulate fibrogenesis, and during fibrosis a net deposition of ECM takes place [1]. ECM changes leading to stimulation of fibrogenesis comprise the presence of free collagen IV, fibrinogen and urokinase type plasminogen activator (uPA) which activate latent cytokines as TGF-β (bound to decorin) [6] [12]. What is more, the ECM functions as a binding place and reservoir for other important factors like HGF (bound to heparan sulfate) and MMPs, like MMP9 [6] [12].

Increased ECM deposition is the result of increased production combined with decreased breakdown [1]. In fibrotic dog livers, increased deposition of fibrillar collagens I and III was reported [6]. ECM components in scars are highly stable cross-linked molecules insensitive to most human proteases [2]. Only matrix metalloproteinases (MMPs), a group of calcium and zinc-dependent enzymes, can destruct ECM [2]. MMPs form five categories, with high substrate specificity for their "own" group of ECM components, and some species specific aspects (Table 1) [1] [2] [5]. To our knowledge, studies regarding MMPs in canine liver have not been conducted.

In tissue culture, rodent HSCs transiently express MMP3 (stromelysin) and -13 (collagenase 3), followed by MMP2 (gelatinase A), -9 (gelatinase B) and -14 (MT1-MMP) [8]. Of these MMP, only MMP2, 9 and 13 are reported to occur in rodent livers *in vivo* [1]. In man, during early fibrogenesis, MMP2 and -9 are upregulated facilitating breakdown of collagen IV from basement membranes, whereas MMP1 (degrading fibrillar collagens I, III, V) is downregulated [6]. HSCs are the main source of MMP production during liver fibrosis [6], although Kupffer cells and other inflammatory cells can also produce MMPs [4] [8]. Even hepatocellular MMP9 production is reported, *in vitro*, likely induced by TNF signaling [6]. MMP activation is balanced by binding to inhibitors known as tissue inhibitors of metalloproteinases (TIMPs), (Figure 2) [9]. In rat and man, fibrosis is characterized by upregulation in HSCs of TIMP1 and TIMP2, followed by increased synthesis of ECM fibrillar collagen I [6]. Thus, HSCs prevent breakdown of their freshly produced matrix, which leads to fibrosis. MMP/TIMP levels, resulting from local HGF/ TGF-β balance, seem to contribute to scar resolution or formation respectively [4] [6].

collagen type	collagen present in liver matrix	degraded by	MMP
fibrillar	I, III, V	interstitial collagenases	man: MMP1,-8,-13; rodents: MMP13
nonfibrillar	IV (reticulin), VI	gelatinases	MMP 1, 2 (man and rodents)

Table 1. Liver collagens and matrix metalloproteinases (MMP)

Few aspects of regression of fibrosis were unveiled. Indeed, it has yet to be established whether all types of fibrosis do regress in a similar way [1]. First of all, eradication of the primary insult is required, as ongoing necroinflammatory activity prevents HSC inactivation and induces hepatocellular apoptosis by TGF-β signaling [2]. In fibrosis regression, previously activated HSCs are either inactivated or removed by apoptosis [1] [2] [6]. Subsequently, a rapid decrease in TIMP1 expression in HSCs unlocks MMP activity (Figure 2). This results in increased collagenolytic activity and enables ECM breakdown [6]. In man, increased MMP2 expression is associated with HSC apoptosis [6]. Also, stimulation of Fas death receptors in activated HSCs and decreased expression of survival factors like TIMP-1 and NF-κB is reported to induce HSC apoptosis [1].

III Interplay of cells, matrix and signaling molecules

Liver regenerative attempt is a concerted action involving all resident liver cell types, the attracted incoming inflammatory cells as well as signaling molecules produced by these cells and the extracellular matrix. Three phases are distinguished: initiating, perpetuation and termination of the tissue repair response. Even in well controlled experimental settings, many questions regarding the regulation of this process, especially the initiation and termination phases, remain unanswered [12]. In patients the situation is likely even more complicated. The morphology of the liver reaction pattern appears similar between dog and man [6] [7]. Also at the molecular level, large similarities were observed between man and dog [6] [18].

The individual characteristics and actions of the resident liver cell types, signaling molecules produced by these cells and the extracellular matrix have been described above. An outline of the major order of these events, as concerted actions and in chronological order, occurring after primary liver injury follows now. The primary event always is cellular degeneration or death, whether necrosis or apoptosis, of **hepatocytes** or **cholangiocytes**. Subsequently, reactive oxygen species (ROS) and fibrogenic mediators are released, and **lymphocytes** and **neutrophils** are recruited [1]. Formation of ROS is the result of oxidative stress and lipid peroxidation and includes intermediate reactive metabolites, free radicals and nitric oxides. These are generated by damaged hepatocytes, attracted neutrophils and activated Kupffer cells [2]. ROS lead to HSC stimulation [6].

Activated **Kupffer cells** secrete elevated levels of TGF-β, TNFα, epidermal growth factor (EGF) and insulin like growth factor (IGF), IL-1, IL-6, ROS, prostaglandins D_2, E_2 and $F_{2\alpha}$ and thromboxane A_2 [1] [6]. Subsequently, TNFα and IL-6 can prime proliferation of both hepatocytes as well as progenitor cells [6] [17]. **Myofibroblasts (MFs),** including **hepatic stellate cells (HSCs)** are activated by Kupffer cell products ROS, TGF-β-1, TNF-α, IL-6 and IGF. MFs are not only activated by Kupffer cells, but also by T-lymphocytes, especially the Th2 subset (by IL-6, interferon-γ (IFN-γ), CD40, C-C chemokine ligand 21(CCL21)) and by degenerated hepatocytes (by ROS, TGF-β-1, TNF-α, EGF and IGF). Also, apoptosis of hepatocytes stimulates fibrogenic actions of MFs/HSCs [6]. Finally, changes in the composition of **ECM** can directly stimulate fibrogenesis by MFs/HSCs. Collagen type IV, fibrinogen and urokinase type plasminogen activator activate latent TGF-β [6] which activates MFs/HSCs. Activated MFs/HSCs have contractile, proinflammatory and fibrogenic properties. They migrate and accumulate at the sites of tissue repair, produce HGF and TGF-β, secrete large amounts of fibril

forming ECM and regulate ECM degradation [1] [2] [6]. These accumulating events result in fibrosis, as depicted in Figure 3.

Hepatocytes are primed for proliferation by Kupffer cell derived TNF-α and IL-6 [12]. Hepatocellular proliferation itself is stimulated by HGF (from MFs, endothelial cells and plateles), TGF-α (from hepatocytes and matrix stores) and EGF (from Kupffer cells) [17]. Hepatocytes and **endothelial cells** mutually support proliferation: hepatocellular VEGF induces endothelial cells to synthesize HGF [6]. Endothelial cell mitogenic factors produced by hepatocytes include FGF1 and 2, angiopoetins 1 and 2 and TGFα. Thus, neovascularization of newly proliferated hepatocytes seems to be secured [12]. Activated MFs/HSCs are likely to play a role in termination of hepatocellular proliferation by production of TGF-β and activin [6] [12].

Progenitor cells are activated when hepatocellular proliferative capacity falls short [6] [12]. HSCs, proliferating simultaneously and in close contact with progenitor cells [6] [14] are the main source of stimulating growth factors HGF, TGF-α and aFGF, suggesting paracrine regulation [6] [12]. Moreover, intercellular interaction between HSCs and progenitor cells is partly mediated by cell-specific matrix proteoglycans such as heparan sulfate. These proteoglycans also mediate binding of growth factors to their receptors and regulate interactions between the progenitor cells and ECM (reviewed in [14]). In addition to the surrounding HSCs, also progenitor cells synthesize TGF-β during early phases of their differentiation, probably supplying a negative feedback loop on further proliferation [6]. Also IFN-γ (produced by activated CD 4+ T cells, NK cells, hepatocytes) leads to priming and maintaining the progenitor cell response [6]. Additionally, Kupffer cells attribute to progenitor cell proliferation by synthesis and excretion of stimulating lymphotoxin-β (LT-β) [12].

Diagnosis of liver pathology

Histological examination is considered essential for a good diagnosis of liver disease [6]. Liver biopsies secure assessment of necroinflammatory type, pattern and extent, a possible cause and presence, pattern and extent of fibrosis and regeneration [7]. In biomedical research, combination of histology and molecular studies yields more information than either of these methods separately. Where ideally, one biopsy should be suitable for use of all chosen techniques, in reality multiple biopsies and associated preservation methods are required to combine all surveys. Unfortunately, taking liver biopsies is an invasive technique, and sampling error can occur, especially in small biopsies [1] [6]. Therefore, there is an increased call for non-invasive techniques, especially to differentiate intermediate grades of fibrosis [1]. Several ideas were suggested, such as the use of biomarkers or surrogate markers for liver fibrosis (serum levels of hyaluronic acid, TIMP1), [6] and the use of imaging techniques as ultrasonography [1] [3] [6]. However, to date no method has yet replaced the golden standard of histological evaluation [1] [3] [6]. In our opinion this is not surprising, as "classical" pathological evaluation by a well-trained pathologist will provide essential microscopical spatial insights not to be delivered by any other method. The specific microarchitecture of the liver is critical for its function. Nowadays, immense possibilities unfold to investigate canine liver pathology, e.g. by genome wide screening, using dog-specific micro-arrays [6]. Elegant combinations of spatial, morphological evaluation with molecular techniques by *in situ* hybridization and laser micro-dissection promise improved, detailed insight into canine liver disease. Developments in this field will be beneficial to translational medicine.

Future directions

Research into the reversal of hepatic fibrosis now concentrates on two areas: eradication of the primary insult, and secondly a direct attack on the pathways of fibrogenesis [6]. Compiling evidence challenged the old dogma of hepatic fibrosis being static and irreversible [6]. Documented cases of spontaneous resolution of fibrosis in animal models appeared simultaneously with new antiviral trials in man. Surprisingly, successful viral eradication appeared to associate with fibrosis regression in human patients [6] [8] [10] (Figure 5). However, to date no antifibrotic therapy is clinically available. In case of development of hepatic decompensation in cirrhosis, liver transplantation remains the only option in man [6].

Interestingly, as regression of cirrhosis seemed to be related to the extent of collagen cross-linking [10], the need of determining different degrees and characteristics of fibrosis and cirrhosis has arisen [6]. Many targets of therapeutic intervention are conceivable, varying from pharmacological treatment to gene therapeutical approaches [4] [6] [8] [11]. In this light, further identification of injurious agents will remain a challenge in veterinary hepatology. Furthermore, the increased appreciation of spontaneously diseased dogs, mimicking human patients in a better way than artificially induced rodent models, may have mutual advances for veterinary and human patients [6] [13] [18] [19]. Dogs

may be able to bridge the existent gap between rodents and man. Thus, newly designed anti-fibrotic therapies may be reliably tested in a clinical situation in canine liver disease and may become available even sooner in veterinary and human medicine if the two research fields combine their efforts.

Discussion

Though liver disease is certainly not restricted to companion animals, the main proportion of veterinary hepatology patients submitted for therapy consist of dogs, and fewer cats. In contrast to husbandry, for companion animals not only economical but also social-emotional arguments are of importance and thus these animals are more easily submitted for therapy. Important and interesting liver diseases in other domestic animals include e.g. pyrrolizidine alkaloid poisoning from *Senecio spp.* (common groundsel/ tansy ragwort) in horses, hepatitis dietica (Selenium/ vit.E deficiency) in pigs, Rift valley fever, hydatid liver disease (*Echinococcus* sp.) and fasciolasis/ distomiasis (liver flukes) in ruminants or Leptospirosis in many animal species [6]. A large proportion of canine liver disease consists of hepatitis, generally of unknown origin (idiopathic), whereas in cats, acute hepatitis occurs relatively more often (see Introduction). Meanwhile, the importance of other liver diseases such as portosystemic shunting, primary portal vein hypoplasia or tumors should not be underestimated.

Hepatitis is diagnosed histologically on 3 µm sections stained with haematoxylin and eosin (HE). The standard criteria for hepatitis were followed [6] [7]. In veterinary hepatology this always includes hepatocellular cell death: necrosis and/or apoptosis, and an inflammatory infiltrate varying in type and extent [16]. Furthermore, acute hepatitis (AH) sometimes features regeneration, whereas chronic hepatitis (CH) always implies regeneration and fibrosis. Fibrosis, a detectable deposit of extracellular matrix [6], can develop into cirrhosis, which has a poor prognosis. Lobular dissecting hepatitis (LDH) is a special form of cirrhosis typically occurring in younger dogs [16] [20]. LDH has an even fiercer clinical course which rapidly progresses to a fatal outcome. To date, it is unknown which factors determine the LDH reaction pattern versus "normal" cirrhosis.

To overcome insults like hepatitis, the liver has an almost mythically large, though not infinite, regenerative capacity [12]. However, when liver regeneration is charged too heavily, clinical symptoms develop. Dogs and cats with hepatitis present with apathy, anorexia, vomiting and/or jaundice, and in cases of chronic hepatitis often with signs of liver decompensation with ascites and hepatic encephalopathy. The presence of liver disease can be confirmed by finding high levels of serum alkaline phosphatase (AP), alanine aminotransferase (ALT) and/or bile acids. Unfortunately, therapeutical options are limited. Based on a better understanding of the underlying reaction pattern of tissue repair in the liver we hope to be able to develop new therapies. Moreover, the liver is a very interesting model organ for tissue repair in general. In contrast to most other organs, disease progress and associated tissue repair attempts can be monitored relatively easily by needle biopsies. The liver is easily accessible by its location and its size. Furthermore, due to its functional organization and large regenerative capacity performing needle biopsies does not inflict any organ dysfunction nor is it life-threatening.

In order to benefit from our large, 25 year spanning paraffin archive (UU, Veterinary Pathobiology) we studied mainly formalin fixed paraffin embedded liver specimens [6]. This enabled us to pick representative samples of any disease group, and also to form groups of interesting disease entities which are less often diagnosed, such as lobular dissecting hepatitis, without waiting for years to collect a sufficient amount of samples. Moreover, we were primarily interested in the complex interactions of cells, matrix and signaling factors as they occur in real-life patients submitted to the clinic, and the large majority of our archival samples, stored in paraffin blocks, is obtained from similar patients. Besides, morphology of formalin fixed, paraffin embedded tissue is excellent [6].

A disadvantage of using this archival material is the sampling variability regarding time between decease of the animal and sampling, variation in cooling of the body after death until sampling and duration of formalin fixation. However, these circumstances realistically covered the medical approach of the patient population of interest. On the other hand, immunohistochemistry on frozen samples would not be hampered by formalin-fixation artifacts and time-consuming epitope-unmasking strategies. Nevertheless, the very limited availability of frozen patient archival material and its lower morphological quality were the basis to choose almost exclusively for formalin-fixed samples [6].

Identification of cell types

Liver tissue is composed of many cell types which coordinately safeguard hepatic functions. In this study, several key cell types regarding fibrosis and regeneration were identified immunohistochemically: hepatocytes, cholangiocytes, fibroblasts including hepatic stellate cells and

progenitor cells [6]. Staining results were always related to normal liver tissue, and canine progenitor cells were compared to their human counterparts. Immunohistochemical results are summarized in Table 2.

Hepatocytes

Hepatocytes in dogs and cats were uniquely identified by human hepatocyte marker (Hepar1) in the cytoplasm (Figure 6) and Multidrug Resistance binding Protein-2/ ATP Binding Cassette C2 (MRP2) staining in the bile canaliculicar membrane (Figure 7) [6]. Also, Breast Cancer Related Protein/ ATP binding cassette G2 (BCRP) was positive in hepatocytes [6], as in man, but in contrast to rat [20].

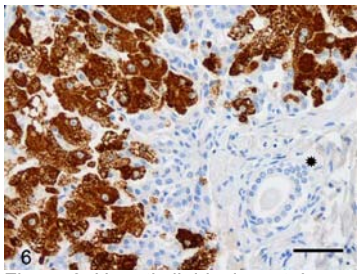

Figure 6. Hepatic lipidosis, cat. Large, mature hepatocytes (left, and right top) show intense brown granular cytoplasmic positivity, while the smaller epithelial cells in ductular reaction (center) are negative. Portal area: asterisk. Paraffin section, Hepar1 immunolabeling, Mayer's hematoxylin counterstain. Bar = 50 micron.

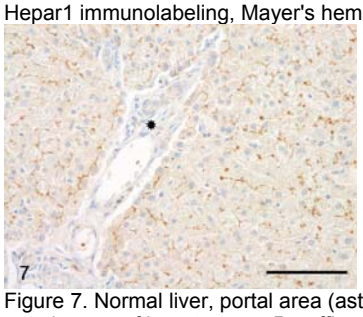

Figure 7. Normal liver, portal area (asterisk) and parenchyma, dog. Strong signal (brown) is present along the canalicular membranes of hepatocytes. Paraffin section, MRP-2 immunostaining, Mayer's hematoxylin counterstain. Bar = 100 micron.

Cholangiocytes

Cholangiocytes were positive for cytokeratin-7 (CK7) (Figure 9), but not uniquely as the progenitor cell compartment also stained CK7 positive (Figure 8) [6]. Therefore, identification of cholangiocytes always includes combination of cellular morphology (form, size) and location.

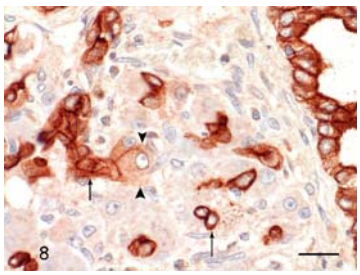

Figure 8. Chronic hepatitis, dog. Strong cytoplasmic staining of bile duct (right top) in portal area. Ductular reaction in the interface location consists of small, strong and diffusely positive cells (arrows) and larger intermediate cells (between arrowheads) with a prominent submembranous staining (uppermost arrowhead) and a less prominent cytoplasmic positivity. Paraffin section, CK7 immunolabeling, Mayer's hematoxylin counterstain. Bar = 20 micron.

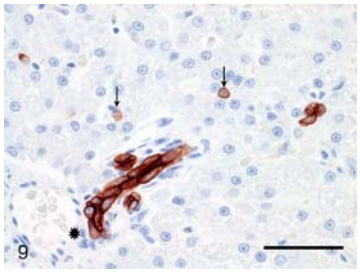

Figure 9. Normal liver, portal area (asterisk) and periportal parenchyma, dog. Strong cytoplasmic staining is present in bile duct epithelial cells in the portal area and in isolated small oval-shaped cells in the periportal parenchyma (arrows). Paraffin section, CK7 immunolabeling, Mayer's hematoxylin counterstain. Bar = 50 micron.

Myofibroblasts

Activated fibroblasts which develop myofibroblastic characteristics play an essential role in hepatic fibrogenesis. They comprise three subpopulations: 1) portal or septal myofibroblasts, 2) interface myofibroblasts and 3) the perisinusoidally located hepatic stellate cells (HSCs) [3]. We identified markers for myofibroblasts (MFs) in the normal canine liver and we studied staining characteristics of activated MFs and HSCs in chronic hepatitis in the dog [6]. Antibodies to alpha-smooth muscle actin (α-SMA) (Figure 10), muscle-specific actin clone HHF35 (HHF35) (Figure 11) and to a lesser extent desmin were useful markers for the canine MF in normal canine liver, in formalin fixed, paraffin embedded samples [6]. Furthermore, vimentin proved less useful for this purpose, due to the generally negative staining of HSCs, in contrast to other MFs. Finally, neural crest markers synaptophysin, glial fibrillar acidic protein (GFAP) and neural cell adhesion marker (NCAM) did not seem suitable for marking of canine HSCs under the studied conditions (formalin fixated, paraffin embedded material). The positivity of HSCs for α-SMA and HHF35 in the normal canine liver may eventually reflect a more active regulation of hepatic sinusoidal flow by these HSCs compared to other species. Possibly, the vanishing of this active α-SMA vascular tone in shock allows the stasis of a relative large blood volume in shock. This may explain the fact that in the dog, the liver is a so-called "shock" organ [6].

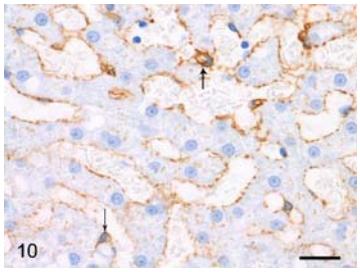

Figure 10. Normal liver, dog. Perisinusoidal hepatic stellate cells display a thin irregular positive band lining the sinusoids. Stellate cells with small cytoplasmic vacuoles stain positive (arrows). Paraffin section, alpha-SMA immunolabeling, Mayer's hematoxylin counterstain. Bar = 20 micron.

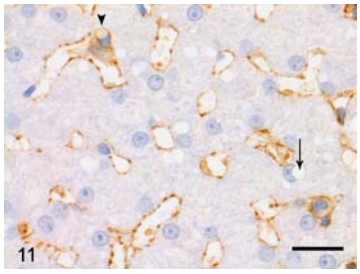

Figure 11. Normal liver, dog. Hepatic stellate cells (HSCs) stain positive, producing a thin irregular lining of the sinusoids. A HSC with small cytoplasmic vacuoles shows a positive reaction (arrowhead), while a HSC with a single, large cytoplasmic vacuole and a dislocated nucleus remains negative (arrow, head is placed in vacuole). Paraffin section, HHF-35 immunolabeling, Mayer's hematoxylin counterstain. Bar = 20 micron.

	differentiated		progenitor			MF	
antibody	Hep	chol	N liver	DR	intermed.	quiescent	active
Hepar1	+	-	-	-	-	-	-
MRP2	+	-	-	-	-	-	nt
BCRP *	+	+	+	+	+	-	-
CK7	-	+	+	+	+	-	-
α-SMA	-	-	-	-	-	+	+
HHF35	-	-	-	-	-	+	nt
Desmin	-	-	-	-	-	+	nt
Vimentin	-	-	-	-	-	+/-	nt
Syn.ph.	-	-	-	nt	nt	-	-*
GFAP	-	-	-	nt	nt	-	-*
NCAM	-	-	-	nt	nt	-	-*

Table 2. Immunohistochemical results in the dog, per cell type. (Formalin-fixed tissue, except *: frozen tissue) + positive; - negative; nt = not tested [6].
Progenitor = progenitor cell compartment; MF = myofibroblasts; Hep = hepatocyte; chol = cholangiocyte; N liver = normal liver; DR = ductular reaction; intermed = intermediate hepatobiliary cell; Hepar1 = human hepatocyte marker1; MRP2 = multidrug resistance protein2/ ATP binding cassette C2; BCRP = breast cancer resistance protein/ ATP binding cassette G2; CK7 = cytokeratin-7; α-SMA = alpha smooth muscle actin; HHF35 = muscle specific actin clone HHF35; syn.ph .= synaptophysin;

GFAP = glial fibrillar acidic protein; NCAM = neural cell adhesion marker [6].

In canine livers with chronic hepatitis associated with fibrosis we found that activated HSCs and other MFs proved to contribute to fibrosis, which is in agreement with findings in rat and man [3]. In fibrotic livers HSCs and other MFs showed dramatically increased α-SMA expression. Thus, activation of MF/HSCs was not indicated by the presence of α-SMA expression as such, but by the considerable increase in α-SMA production in diseased livers. Also, α-SMA expression increased with the stage of fibrosis. In the dog morphological changes or functional changes such as increased cell size, loss of lipid vacuoles and enhanced production of TGF-β and other substances assist to discriminate between a quiescent and activated phenotype of hepatic MFs.

Progenitor cells

When hepatocytes are not severely injured, like in the classical rodent partial hepatectomy, liver regeneration starts with a wave of mitoses of hepatocytes [12]. However, when hepatocellular replication is impaired, as occurs in acute fulminant hepatitis or severe liver fibrosis, the hepatic progenitor compartment becomes of vital importance in liver regeneration [15] [17]. The progenitor cell compartment of dogs and cats was investigated (immuno)histochemically [6]. In dogs and cats the normal liver showed a similar morphology and immunohistochemical reaction of the progenitor cell compartment as in man. Also a comparable ductular reaction with respect to amount, location and immunohistochemistry was observed in canine and human acute and chronic hepatitis. Cytokeratin-7 (CK7) was a good marker for canine and feline progenitor cells, though cholangiocytes were positive as well (Figure 8 and Figure 12). The presence of feline intermediate hepatobiliary cells was confirmed by CK7 staining in various liver diseases [6]. In the dog intermediate hepatobiliary cells were identified in acute and chronic hepatitis with CK7 (Figure 8) [6]. Between man and dog a similar increase in size of the progenitor cells was observed when differentiating towards the hepatocellular lineage, combined with a gradual decrease in CK7 expression of intermediate cells (Figure 8). MRP2 positivity of intermediate cells was suspected in acute and chronic hepatitis [6]. Hepatocellular differentiation of intermediate cells could not be confirmed by staining for Hepar1 (Figure 12) or MRP2 in both cats and dogs. BCRP antibody yielded identical results in both human and canine progenitor cells and hepatocytes [6]. The classical rodent "oval cell" proliferation is very easily provoked and is often dispersed throughout the lobules. Compared to rodent liver, the parenchymal ductular reaction in the dog and cat was less extensively present. This pattern more closely resembled the human progenitor cell compartment, where it appears less common (though still often) and less extensive than the rodent "oval cell" proliferation.

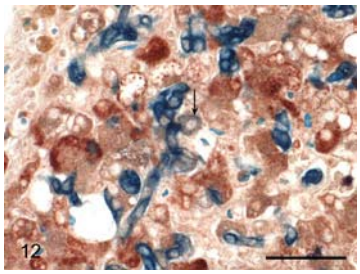

Figure 12. Chronic hepatitis, dog. CK7 positive ductular reaction (blue) closely associates with hepatocytes (brown) in liver cell plates. Incidentally, in an intermediate sized cell overlap of the two signals (arrow) might be present. Paraffin section double stained with anti-Hepar1 antibody (brown) and anti-CK7 (blue), Mayer's hematoxylin counterstain. Bar = 50 micron.

These findings underline the similarities between canine and human liver reaction patterns, and thus offer mutual advantage of research in human and canine spontaneous liver diseases. Interestingly, feline hepatic lipidosis may be a spontaneous animal model for human fatty liver disease (non-alcoholic steato-hepatitis) where oxidative stress leads by hepatocellular replicative senescence to similarly extensive progenitor cell proliferation in the liver [6] [21].

HGF/TGF-β balance

Hepatocyte growth factor (HGF) and transforming growth factor β (TGF-β) are two major factors determining the outcome of hepatic tissue repair triggering hepatocellular replication and fibrosis, respectively [1] [12]. We investigated the immunohistochemical localization of HGF, HGF receptor (c-Met) and TGF-β-receptor1 (TGF-β-R1) in spontaneous canine hepatitis, and compared it to previously determined associated mRNA and protein expression levels in comparable canine patient groups [6]. Unfortunately, reliable immunohistochemical staining results for TGF-β could not be obtained. Formalin fixed, paraffin embedded archival liver specimens of dogs with acute hepatitis (AH), chronic hepatitis/ cirrhosis (CH), and lobular dissecting hepatitis (LDH) were used and compared with normal liver tissue. In our canine patient material, absolute HGF availability did not seem the limiting factor in

hepatic regeneration. In AH, upregulation of HGF expression by hepatic stellate cells was restricted to locations adjacent to necrotic areas. Most likely, TGF-β derived from damaged hepatocytes and Kupffer cells at the primary site of injury played a paracrine role in this upregulation. All reactive ductules (proliferating progenitor cells) showed similar mitogenic stimulation as evidenced by their HGF/c-Met positivity. Likely, septal reactive ductules were restrained from further differentiation by TGF-β signaling from encompassing TGF-β-R1 positive mesenchymal cells. Finally, immunohistochemical results regarding HGF, c-Met or TGF-β-R1 reactivity of LDH closely mimicked the findings in interface locations of CH. The structure of the newly formed connective tissue in LDH also mimicked the loose structure of the interface location in CH, where the inflammatory process usually is more active than in cell-poor fibrotic septa. The diffuse presence of this active phenotype in LDH could possibly explain the rapidly progressing fatal course of LDH compared to CH.

Extracellular matrix

During fibrosis, the extracellular matrix (ECM) is continuously remodeled and increases in volume due to the production of various proteins, including shifts in the local balance of matrix metalloproteinases (MMPs) and tissue inhibitors of MMP (TIMPs, see Introduction) [2]. We studied the distribution of the ECM protein tenascin-C (TN-C) which is highly upregulated in tissue repair processes, but no data regarding the dog were present to date [6]. Its correlation with the necro-inflammatory activity and expression of α-SMA, CK7 and CD3+ T-lymphocytes in canine chronic hepatitis is reported. Patient groups included LDH and CH and were compared to livers from normal dogs. In normal liver, TN-C was localized in Disse's space and surrounded bile ducts and blood vessels. In the diseased livers, TN-C proved to be upregulated, which is in line with other species. The distribution patterns differed between the CH and LDH cases. In CH, TN-C was present at the periphery of the regenerating nodules and was conspicuous in the bridging fibrous bands. In LDH, TN-C was diffusely distributed along the reticular fibers that crossed over between single cells or groups of hepatocytes.

The presence and interpretation of α-SMA and CK7 results were already discussed in relation to the identification of cell types. The distribution pattern of CD3+ lymphocytes was inversely proportional to that of TN-C. This is consistent with other studies, where the mutually opposing effects of TN-C expression on T-cell activation were described [6]. These results underline the active role of TN-C specifically, and ECM proteins in general in the tissue repair response. In our study there was no difference in TN-C, CK7, and CD3 expression between the CH and LDH groups. These results showed that TN-C expression strongly correlated with increased fibrotic stage, inflammatory activity, and expression of CK7 and α-SMA [6].

Interestingly, the TN-C distribution in LDH and CH matched the active interface phenotype which was also accentuated by the presence of activated α-SMA positive MFs, and HGF, c-Met and TGF-B-R1 reactivity. As already suggested, the widespread presence of this active phenotype may account for the fierce clinical course of LDH. Possibly, TN-C staining combined with α-SMA could be useful to asses the type or activity of the fibrosing process, and HSC activation, respectively. At sites (as seen in CH) with enhanced ECM turnover, the disease process is active, with generally a poorer prognosis when widely present (as seen in LDH). However, we expect that this activity also includes potential reversibility of fibrosis [4] [9] [10], thus an indication of fibrosis activity may predict therapeutical accessibility and success rate.

Fixation

During the course of testing of several antibodies a significant difference in staining characteristics occurred between identically fixed needle biopsies and larger tissue sections (unfortunately these kind of important results are hard to publish). We hypothesized that in the relatively small biopsies strong formalin-induced epitope masking could occur, resulting from overfixation. As combined analysis of molecular data and (immuno)histochemistry is much more powerful than application of either single survey, we tried to establish a fixation protocol for combined histological and molecular studies of liver biopsies [6]. To optimize the use of a single liver biopsy and to minimize the number of biopsies per animal we evaluated different fixation- and RNA isolation methods available in our laboratory. Moreover, a simplified protocol could reduce sampling variability between institutions and enable first-line clinics to participate in studies, which potentially increases the availability of sampled cases.

Evaluated preservatives for histology included formalin with different fixation times, Boonfix, RNA-later and snap-freezing. Boonfix is a fixative of patented, thus unknown, composition, but free of formalin and based on ethyl alcohol and polyethylene glycol. It is supposed to be good for (immuno)histochemical and molecular studies. RNA-later, also of patented, unknown, composition, is designed for optimal molecular studies. Snap-freezing is used for molecular studies and can also be applied for immunohistochemical studies, but mostly high morphological quality can not be obtained.

Finally, Boonfix, RNA-later, snap-freezing and Beta-mercaptoethanol supplemented RLT (B-RLT) preservation was used for molecular studies. Molecular analysis evaluated RNA yields and the RNA quality, measured by RIN values. Histological evaluation was based on HE, reticulin (fibrosis), rubeanic acid and rhodanine (copper) staining, and finally on immunohistochemistry for CK-7 (progenitor cells and cholangiocytes), MRP-2 (bile canaliculi) and hepar1 antibody (hepatocytes) [6].

Not one fixation method proved reliable for all purposes. Standard histological evaluation required formalin or Boonfix fixation. Reliable evaluation of copper storage by rhodanine or rubeanic acid was only possible in formalin fixed wedge or needle biopsies. Reduction of formalin fixation time to 1-4 hours improved immunohistochemical reactivity in liver biopsies. Although Boonfix could be used for immunohistochemical evaluation in liver biopsies, formalin fixation (1-4 hrs) still proved superior. RNA-later was not suitable for any tested histomorphological application. Optimal RNA-quality, assessed by RIN values, was obtained after the Menghini/ NaCl 0,9% biopsy technique technique followed by RNA-later preservation and RNAeasy isolation [6].

Conclusively, we found that at least two biopsies are needed. One for histology (formalin 1-4 hrs) and one for molecular assays: RNA-later. Since these two biopsies can be dispersed in non-toxic liquid preservatives, this combination easily provides researchers with material for high-throughput expression analysis. Moreover, biopsies can simply be transported from the clinics to the research facilities. Arrived there, the RNA in RNA-later fixed samples will remain intact if immediately stored at minus 70˚C. Also, the paraffin embedding after formalin fixation of the histological samples guarantees good preservation of tissue morphology and consistent, good results in immunohistochemistry [6].

Future continuation

A logical next step will be the further identification of relations between cells and their microenvironment: the cellular niche consisting of the surrounding cells, matrix and signaling factors. The cellular niche likely plays a decisive role in the fate of cells [17]. Of special interest in liver fibrosis and regeneration are myofibroblasts/ hepatic stellate cells and the stem cells/ liver progenitor cells respectively. Also, the active role of the matrix in storage and release of growth factors and the active role of ECM proteins, like tenascin, will deserve attention. Especially development of markers to asses the potential of scar regression in patients justifies great interest. Scar regression potentially means clinical improvement in currently untreatable diseases like liver cirrhosis.

Apart from removal of the primary injurious agent, suggested options to induce scar regression include modulation of HSC activity, disruption of TGF-β synthesis, administration of growth factors (e.g. HGF and insulin like growth factor), and degradation of ECM, up to gene therapy [1] [8] [11]. Earlier studies described several potential antifibrotic targets: HSCs, TGF-β, TGF-β-R1 and TN-C [6]. Moreover, stimulation of hepatic regeneration may be helpful, by enhancing replicative activity of hepatocytes and/or progenitor cells, while simultaneously avoiding tumor development. Keys to address this subject may be found in temporarily attenuation of the progenitor cell niche. Earlier studies contain the first description of the presence of the liver progenitor cell compartment in the dog and the cat [6]. In addition, the niche of progenitor cells and MF/ HSCs was described in relation to the key players in the tissue repair response (HGF, c-Met, TGF-β-R1), and the location and identification of closely located cells and ECM protein TN-C.

For further research, suitable molecular/ microbiochemical methods include *in situ* hybridization for localization of mRNA and laser micro dissection of microscopically selected sites followed by Q-PCR to assess mRNA levels of various cell types. These methods require another fixation than formalin fixation [6]. Likely, new patients will have to be sampled for these purposes, and due to time pressure study groups will be of smaller size. However, the combination of modern molecular techniques with histology will yield such an amount of information, that somewhat smaller group size within one specific disease will not be too big a disadvantage. Besides, formalin fixed paraffin embedded specimens remain of utmost importance to establish a primary histological diagnosis.

Translational medicine

Further studies emphasized similarities in liver diseases and liver reaction patterns between man and animals, particularly dogs and cats [6]. Earlier found parallels presented in this review include the presence of the CK7 positive liver progenitor cell compartment in dogs and cats with an analogous distribution and reaction pattern as in man, when compared to rodents [6]. Moreover, also BCRP immunohistochemistry of hepatocytes and progenitor cells mimicked that in man, better than rodents. We expect that far more immunohistochemical markers will prove to react similarly in the dog as they do in man. Frozen tissue sections will unveil immunohistochemical reactivity more easily than formalin fixed samples, as we experienced with CK7 and BCRP staining.

Spontaneous hepatologic canine patients may have large potential as useful animal model to human liver disease. First, the dog is one of the very few species, apart from man, in which liver diseases are therapeutically treated. Thus, dogs harbor and demonstrate similar complex interaction patterns as occur in natural liver disease in man and much less artificial than in artificially induced rodent models. Second, spontaneous liver pathology in dogs occurs regularly and frequently. Third, dogs submitted to a veterinary clinic usually are pet animals and therefore share many environmental factors with man. Finally, dogs have a relatively long life-span, granting time to the before mentioned environmental factors to inflict disease. Interestingly, dogs were recently advocated as a model animal in translational research [13]. With the drive to cure privately owned pet animals, the existing gap between rodent animal models and clinical practice in man [9] could be bridged by veterinary medicine. This offers multiple promising mutual clinical benefits for both dog and man.

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