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The canine hepatic progenitor cell niche: Molecular characterisation in health and disease

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ABSTRACT

Hepatic progenitor cells (HPCs) are an adult stem cell compartment in the liver that contributes to liver regeneration when replication of mature hepatocytes is insufficient. In this study, laser microdissection was used to isolate HPC niches from the livers of healthy dogs and dogs with lobular dissecting hepatitis (LDH), in which HPCs are massively activated. Gene expression of HPC, hepatocyte and biliary markers was determined by quantitative reverse transcriptase PCR. Expression and localisation of selected markers were further studied at the protein level by immunohistochemistry and immunofluorescent double staining in samples of normal liver and liver from dogs with LDH, acute and chronic hepatitis, and extrahepatic cholestasis. Activated HPC niches had higher gene expression of the hepatic progenitor markers *OPN*, *FN14*, *CD29*, *CD44*, *CD133*, *LIF*, *LIFR* and *BMI1* compared to HPCs from normal liver. There was lower expression of albumin, but activated HPC niches were positive for the biliary markers SOX9, HNF1β and keratin 19 by immunohistochemistry and immunofluorescence. Laminin, activated stellate cells and macrophages are abundant extracellular matrix and cellular components of the canine HPC niche. This study demonstrates that the molecular and cellular characteristics of canine HPCs are similar to rodent and human HPCs, and that canine HPCs are distinctively activated in different types of liver disease.

Introduction

In dogs and other species, chronic liver disease frequently leads to progressive fibrosis, loss of regenerative capacity and reduced functional liver mass. Replication of mature hepatocytes can compensate for a substantial reduction in parenchymal mass (Fausto et al., 2006). However, the proliferative capacity of hepatocytes is insufficient in fulminant and chronic hepatopathies (Lunz et al., 2005). In these cases, hepatic progenitor cells (HPCs) become activated (Evarts et al., 1987; Katoonizadeh et al., 2006).

HPCs are a reserve population of adult stem cells located in the canals of Hering. Upon activation, HPCs can proliferate, migrate and differentiate into cholangiocytes or hepatocytes, depending on the type of injury and the microenvironment (Fausto, 2004; Duncan et al., 2009). Histologically, proliferating HPCs are often referred to as a 'ductular reaction' (DR), since they form structures resembling small ductules. HPCs differentiating into hepatocytes are described as 'intermediate hepatocytes'. These cells are present in close proximity to the DR and can be recognised by a larger cell size and lower ratio of nucleus to cytoplasm (Roskams et al., 2004). HPCs have potential as a therapeutic target in regenerative medicine; they could be used for cell transplantation or the resident progenitor pool could be stimulated with specific drugs (Sancho-Bru et al., 2009; Forbes and Newsome, 2012).

Most of the knowledge of HPC biology originates from experimental rodent studies, in which HPC responses are induced in specific liver injury models. In addition, the pattern of reaction of HPCs in response to liver pathology in human beings has been described extensively (Corcelle et al., 2006; Herrera et al., 2006; Yovchev et al., 2007, 2008; Zhang et al., 2008; Furuyama et al., 2011; Espanol-Suner et al., 2012). Numerous markers, such as keratins 7 and 19, epithelial cell adhesion molecule (EpCAM) and CD133, are expressed in rodent and human (activated) HPCs at the mRNA and/or protein level, and most of these are also expressed in biliary cells.

Adult stem cells reside in a specific stem cell niche. This niche is the immediate microenvironment of the cells and is composed of other cell types, signals and extracellular matrix components (Ohlstein et al., 2004). Dynamic cell signalling in this niche controls self-renewal and differentiation of stem cells (Fuchs et al., 2004). Activation of hepatic stellate cells and macrophages, which play a

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pivotal role in hepatitis, are involved in activation of the HPC compartment (Roskams, 2008; Viebahn et al., 2010; Boulter et al., 2012).

HPCs have been identified in the canine liver (Yoshioka et al., 2004; Mekonnen et al., 2007; Arends et al., 2009a and b; Schotanus et al., 2009; Ijzer et al., 2010), but HPCs and the HPC niche have not been characterised in detail. The aim of the present study was to characterise canine HPCs and the HPC niche using a large marker set selected from studies in rodents and human beings. The markers were validated by gene expression analysis in activated and quiescent HPCs. Samples of canine liver were collected by laser microdissection (LMD) from cryosections of cases of lobular dissecting hepatitis (LDH), representing an activated niche characterised by a pronounced ductular reaction, and normal liver, representing a quiescent niche (van den Ingh and Rothuizen, 1994; Schotanus et al., 2009; Spee et al., 2010). Selected markers with increased mRNA expression in the activated HPC niche were investigated at the protein level using immunohistochemical and immunofluorescent double staining on sections of normal liver, along with liver from cases of LDH, acute hepatitis (AH), chronic hepatitis (CH) and extrahepatic cholestasis (EHC), the latter representative of biliary injury.

Materials and methods

Liver samples

Samples of liver were obtained from healthy dogs ($n = 7$) used in non-liver related research projects (surplus material, University 3R policy) and at postmortem examination from dogs presented to the University Clinic for Companion Animals of Utrecht University with LDH ($n = 9$), AH ($n = 5$), CH ($n = 5$) and EHC ($n = 5$). Diagnoses were confirmed histologically according to World Small Animal Veterinary Association standards (Rothuizen et al., 2006). For immunohistochemistry and immunofluorescence, tissue samples were fixed in 10% neutral buffered formalin for 24 h, dehydrated in ethanol, embedded in paraffin wax and sectioned at 4 μm. For LMD, samples were frozen in liquid nitrogen and 10 μm thick cryosections were cut in an RNAse free environment and stored at –70 °C until further processing.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded samples of liver from normal dogs and dogs with LDH, AH, CH and EHC ($n = 4$–5). For each antibody, the antigen retrieval method, antibody dilution and incubation times are summarised in Table 1. Sections were stained for keratin 19 (K19), CD29, CD44, B cell-specific Moloney murine leukaemia virus integration site 1 (BMI1), hepatocyte nuclear factor 4α (HNF4α), sex determining region Y box 9 (SOX9) and HNF1β (van Steenbeek et al., 2013). For CD44 staining, an additional rabbit anti-rat secondary antibody labelling was performed for 45 min at room temperature (dilution 1:2,000). For LMD, rapid immunohistochemistry for keratin 7 (K7) was performed on cryosections (Spee et al., 2010). For immunohistochemistry on frozen sections, an antibody against K7 was used instead of K19 (Table 1).

Double immunofluorescence

Immunofluorescent double staining for pan-cytokeratin (panCK)-α smooth muscle actin (αSMA), panCK-MAC387 and K19-laminin were performed in a parallel approach on paraffin-embedded liver sections. On the basis of immunohistochemical staining, 2–7 representative samples per disease were selected for immunofluorescence. After antigen retrieval, primary antibodies were incubated overnight at 4 °C, secondary antibodies were incubated at room temperature for 60 min and nuclei were counterstained with To-Pro-3 or 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies) at room temperature for 10 min. For negative controls, the primary antibody was omitted. Slides were analysed using a Leica DMRE fluorescent microscope with Photometrics Coolsnap CCD digital photo camera and CellB software (AnalySIS, Olympus). Cell counts were performed for panCK and MAC387 and expressed relative to total cell number (To-Pro-3 positive). For each sample, five fields were counted at 200× magnification using ImageJ 1.44 software. Statistical analysis was performed using Mann–Whitney *U* post hoc tests to assess differences in cell counts among diseases. $P < 0.05$ was considered to be significant.

Laser microdissection of keratin 7 positive cells

K7 positive cells were dissected together with neighbouring cells and stroma (total of $2–3.5 \times 10^6$ μm^2 tissue per sample), representing their histologically defined niche. LMD was performed with a Nikon Eclipse TE300 inverted microscope connected to a Sony 3-CCD Microscope Colour Video Camera using MMI CellTools software (Molecular Machines & Industries). Dissected cell niches were collected with adhesive lid tubes (MMI), lysed in 50 μL Extraction Buffer (PicoPure RNA isolation kit, Molecular Devices, MDS Analytical Technologies) and stored at –70 °C until further use.

RNA isolation and amplification

Total RNA was extracted from LMD samples using the PicoPure RNA isolation kit (MDS Analytical Technologies) with an on-column DNase treatment (0.1 U/μL; Qiagen). RNA integrity was moderate to good and comparable between samples (Bioanalyzer 2100, Agilent Technologies). RNA was stored at –70 °C. RNA was reverse transcribed to cDNA and amplified using the WT-Ovation RNA amplification system (NuGEN Technologies) (Spee et al., 2010).

Quantitative reverse transcriptase PCR and relative expression analysis

For gene expression analysis, a SYBR Green-based quantitative PCR (qPCR) was performed using a Bio-Rad My-IQ detection system (van Steenbeek et al., 2013). Primer details and PCR conditions are listed in Table 2. Gene products measured were *keratin 7 (KRT7)*, *keratin 19 (KRT19)*, *osteopontin (OPN)*, *tumour necrosis factor receptor superfamily member 12A (FN14)*, *integrin β1 (CD29)*, *CD44*, *prominin1 (CD133)*, *leukaemia inhibitory factor (LIF)*, *LIF receptor (LIFR)*, *BMI1*, *α fetoprotein (AFP)*, *hepatocyte nuclear factor 4α (HNF4α)*, *albumin (ALB)*, *SOX9*, *HNF1β* and *neural cell adhesion molecule (NCAM)*. Sequencing reactions confirmed the specificity of the amplified products. Accurate normalisation was secured by using four reference genes (*B2M, HPRT, RPS5* and *RPS19*) selected upon stability determination. Relative expression of each gene product ($^{\Delta}$Cq method) was used to compare normal liver with liver from cases of LDH. Expression levels that were undetectable were arbitrarily set to a Cq value of 45. SPSS20 (Benelux) was used for statistical analysis. The non-parametric Mann–Whitney *U* test was used to compare gene expression levels from normal and diseased HPC niches. *P* values <0.05 were considered to be significant.

Table 1
Antibodies used in this study.

Antibody target	Antibody source/type	Clone	Company	Application	Dilution	Incubation	Antigen retrieval
K7	Mu/Mo	OV-TL 12/30	Dako	IHC/LMDC	1:50	1 h RT	–
K19	Mu/Mo	K4.62	Sigma	IHC	1:100	O/N 4 °C	Proteinase K RT
CD29	Mu/Mo	18/CD29	BD Biosciences	IHC	1:100	O/N 4 °C	Citrate 98 °C
CD44	Rat		Hubrecht Institute	IHC	1:200	O/N 4 °C	Citrate 98 °C
BMI1	Mu/Mo	F6	Millipore	IHC	1:150	O/N 4 °C	TE 98 °C
HNF4α	Rb/Po		Santa Cruz	IHC	1:300	O/N 4 °C	TE 98 °C
SOX9	Rb/Po		LS Biosciences	IHC	1:250	O/N 4 °C	Citrate 98 °C
HNF1β	Rb/Po		Sigma	IHC	1:400	O/N 4 °C	Citrate 98 °C
PanCK	Rb/Po		Dako	IF	1:400	O/N 4 °C	Proteinase K RT
αSMA	Mu/Mo	1A4	BioGenex	IF	1:200	O/N 4 °C	Citrate 98 °C
MAC387	Mu/Mo	MAC387	Abcam	IF	1:500	O/N 4 °C	Proteinase K RT
Laminin	Rb/Po		Abcam	IF	1:100	O/N 4 °C	Proteinase K RT

Mu Mo, mouse monoclonal; Rb Po, rabbit polyclonal; IHC, immunohistochemistry; IF, immunofluorescence; LMD, laser microdissection cryosections; RT, room temperature; O/N, overnight; TE, Tris EDTA.