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Innervation of the proximal human biliary tree

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Abstract

The autonomic nervous system plays a role in a variety of liver regenerative and metabolic functions, including modulating bile secretion and cholangiocyte and hepatobiliary progenitors of the canals of Hering. However, the nature and location of nerves which link to the proximal biliary tree have remained uncertain. We investigate the anatomic relationship of nerves to the proximal biliary tree including the putative stem/progenitor cell niche of the canal of Hering. Using double immunostaining (fluorescence, histochemistry) to highlight markers of cholangiocytes (biliary-type keratins), nerves (S100, neurofilament protein, PGP9.5, tyrosine hydroxylase), and stellate cells (CRBP-1), we examined sections from normal adult livers from autopsy or surgical resections. There is extensive contact between nerves and interlobular bile ducts, bile ductules, and canals of Hering (CoH). In multiple serial sections from 4 normal livers, biliary-nerve contacts were seen in all of these structures and were more common in the interlobular bile ducts (78/137; 57%) than in the ductules and CoH (95/294; 33%) ($p < 0.001$). Contacts appear to consist of nerves in juxtaposition to the biliary basement membrane, though crossing through basement membrane to interface directly with cholangiocytes is also present. These nerves are positive for tyrosine hydroxylase and are, thus, predominately adrenergic. Electron microscopy confirms nerves closely approximating ductules. Nerve fiber–hepatic stellate cell juxtaposition is observed but without stellate cell approximation to cholangiocytes. We present novel findings of biliary innervation, perhaps mediated in part, by direct cholangiocyte-nerve interactions. The implications of these findings are protean for studies of neuromodulation of biliary physiology and hepatic stem/progenitor cells.

Keywords Bile ducts · Nerves · Canals of Hering · Ductules · Stellate cells

Abbreviations

HSPC Hepatic stem/progenitor cells
PBS Proximal biliary structures
CoH Canal of Hering

NF Neurofilament protein
TH Tyrosine hydroxylase
HSC Hepatic stellate cell

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Introduction

Innervation of the liver is a relatively little studied subject but presents a puzzle. On the one hand, many liver metabolic and regenerative functions and processes are influenced by nerves of the autonomic nervous system; however, denervation of the organ at transplantation experimentally reveals little clinical change and variable morphologic changes. Nonetheless, it is difficult to solve this puzzle in the absence of detailed anatomic descriptions of the interactions of nerve fibers and elements of the liver. In this paper, we focus on innervation of the biliary tree given the demonstration that the autonomic nervous system mediates both bile secretion [1] and hepatobiliary stem/progenitor cell (HSPC) activation from the most proximal segments of the biliary tree, the canal of Hering (CoH) [2–4].

In terms of bile physiology, efferent hepatic innervations are known to play a role in the regulation of bile flow [5]. Experimental denervation studies have shown that innervation may play a role in the modulation of bile salt, cholesterol, and lipid output in response to somatostatin and feeding [6]. Adrenergic and cholinergic denervation of the liver promotes cell death and reduces proliferation of cholangiocytes in response to cholestasis. Furthermore, bile acids and autonomic innervation may interact to control cholangiocyte response to liver injury [7]. Several studies have shown that the cholinergic system regulates ductal bile secretion [8].

Regarding HSPC, it is understood that control of their alternating quiescence and activation is governed by cell-cell and cell-matrix interactions in the niche, the nature of which, however, remain largely unexplored [9]. It has been demonstrated that part of the web of control for HSPC is the autonomic nervous system, manipulation of which by pharmacologic intervention or severing of the vagus nerve leads to altered regulation of the oval cell (i.e., rodent HSPC) response [10–13]. Some of these experiments followed upon clinical observations in human livers that ductular reactions are diminished post-orthotopic liver transplantation, possibly due to

denervation at harvesting, and that hepatobiliary cells of the ductular reaction display muscarinic receptors for acetylcholine [11]. In the developing and regenerating mouse liver, intrahepatic bile duct formation precedes the development of portal innervation. In mice, biliary epithelial cells (BECs) reportedly secrete neural growth factor (NGF) and apparently guide the development of the intrahepatic neural network [14]. During human liver development, direct attachment of portal nerve fibers to the ductal plate, intrahepatic bile ducts, and peribiliary glands has been reported, but no direct nerve-cholangiocyte contact was seen [15, 16].

While these data convincingly demonstrate autonomic regulation of biliary functions, they do not explain their mechanisms. We hypothesize three possible structural mechanisms for autonomic regulation of the biliary tree, i.e., (1) direct nerve-cholangiocyte contact; (2) close approximation of nerves to cholangiocyte basement membrane, neurotransmitter depositing into the peri-biliary, extracellular milieu (as is seen in the digestive tract) [17]; (3) nerve interaction with intermediate effector cells, in particular the hepatic stellate cell (HSC) [18].

In this study, we examine the structural relationship of nerves to the CoH, ductules, and interlobular bile ducts and find that they have extensive, though variable innervation, with nerve fibers stopping at the basement membrane or extending through the biliary basement membrane and, therefore, making direct contact with cholangiocytes. Because many contacts are identified in the CoH and bile ductules, we suggest that there is an element of nervous system control of the biliary tree by direct innervation.

Methods

Tissues and staining

Archival, formalin-fixed, and paraffin-embedded of 7 normal adult livers (clinical data summarized in Table 1) were

Table 1 S100-positive nerve contacts with the proximal intrahepatic biliary tree in 25 random small portal tracts

Case	Gender	Age	Source	Average # biliary profiles per portal tract*	Average # nerve-biliary contacts per portal tract	Total nerve-biliary contacts
1	Male	42	Autopsy**	10.5 ± 4.7	4.4 ± 1.9	110/262 (42%)
2	Male	51	Autopsy**	8.4 ± 3.5	2.8 ± 1.2	71/211 (36%)
3	Female	34	Resection***	10.9 ± 4.6	1.9 ± 1.8	47/243 (17%)
4	Female	63	Resection***	8.7 ± 5.3	2.6 ± 1.8	66/202 (33%)
5	Female	44	Resection***	9.0 ± 3.4	3.2 ± 1.7	78/216 (36%)
6	Male	64	Autopsy**	11.2 ± 2.6	3.1 ± 1.2	64/196 (33%)
7	Female	52	Autopsy**	9.7 ± 3.6	2.9 ± 1.4	92/223 (41%)

* “Biliary profiles” comprise cross sections of bile ducts, ductules, and canals of Hering

** Patients without clinical features of liver disease and with histologically normal liver

*** Liver tissue distant from resected cavernous hemangioma

sectioned for histochemical, immunohistochemical (IHS), and immunofluorescent (IF) staining. Cholangiocytes were immunostained with mouse monoclonal antibody cocktail AE1/AE3 which are directed against biliary-type keratins (including keratins 7 and 19) (DAKO, Carpinteria, CA), Schwann cells with rabbit polyclonal antibody to S100 protein (DAKO, Carpinteria, CA), and axons with monoclonal antibodies to neurofilament (NF) protein (DAKO) and PGP9.5 (Leica, UK). Immunostaining with antibodies for tyrosine hydroxylase (TH; Leica, UK) was used to identify adrenergic fibers. Serial sections of cases 1, 2, 6, and 7 were double-stained using the DAKO Envision kit (DAKO, Carpinteria, CA) according to manufacturer's instructions, visualizing biliary tree with biotinylated secondary antibodies and Schwann cell components of nerves with alkaline phosphatase conjugated secondary antibodies (chromogenic substrates, DAB and nitro blue tetrazolium or fast red, respectively). IF was performed with FITC-conjugated secondary antibodies for biliary structures and rhodamine for S100. Immunostaining of axons with NF was accomplished with biotinylated secondary antibodies (DAB) and counterstaining with Periodic Acid-Schiff after diastase digestion (PAS-D) according to standard protocols to highlight biliary basement membranes.

Immunostaining to highlight three-step connections of nerves to HSC to cholangiocytes was performed with double immunohistochemistry for cellular retinol binding protein-1 (CRBP-1; gift of A. Desmouliere), which stains all HSC (quiescent and activated) as well as many cholangiocytes [19, 20] and for S100 protein.

Quantification

Quantification of nerve-biliary relationships was made by evaluation of serial sections, 4 µm thick, of four normal livers (19 serial sections in case 1; 20 in each of cases 2, 6, 7), double immunostained for S100 and biliary-type keratins. Two small to medium-size portal tracts distant from the tissue edge were selected in each series. Total biliary profiles (complete ducts and “cuboidal strings” indicative of ductules and/or CoH [21]) were tabulated along with how many of these had apparent nerve contact on each level. Comparison of one level to the next confirmed that S100 structures were nerves rather than S100-positive dendritic cells, which do not form linked linear structures across many levels. Because bile ductules and CoH are not readily distinguishable in light microscopic sections, even with immunostaining, these were grouped together as proximal biliary structures (PBS). The same quantification was performed on single sections of the large pieces of the liver tissue available from all seven cases, with attention focused on randomly selected portal tracts containing bile ducts approximately 120 µm across in short cross-sectional profile or less (in order to confine evaluation to the PBS and first or second rank interlobular bile ducts) [22, 23].

Three-dimensional reconstruction

Serial sections of one portal tract from a double-immunostained liver tissue section were photographed at × 40 with Sony Digital Imaging System. TIFF files were obtained in Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA) without compression, for maximum color gamut and minimizing of imaging artifacts. Images were then converted into Joint Photographic Experts Group (JPEG) files with maximum quality compression on Macintosh OSX 10.4.1 and transferred to a computer with Amira 3.1 (Indeed-Visual Concepts GmbH, Berlin, Germany) image processing software. Sections were aligned using the Amira software, using a least squares method of comparing grayscale data in a pixilated grid between two images. The image alignment correlation was kept above 90%. The histologic structures were then detected within the slide by 3D-Doctor's edge detection tool (Able Software Corporation, Lexington, MA). The three-dimensional model was exported from Amira to Maya Unlimited 6.0 (Alias-Wavefront Corporation, Toronto, Canada). All S100-positive structures which appeared in three or fewer levels were considered likely to be S100-positive dendritic cells and deleted from the composite figure.

Electron microscopy

Surgical biopsies of normal liver performed during abdominal surgery from four patients were perfusion-fixed with 1.5% glutaraldehyde and processed for routine electron microscopy with embedding in Epon resin. Clear perfusion-fixed areas around small portal tracts were selected on 1-µm-thick sections stained with toluidine blue. Serial thin sections of these were prepared. Grids were double-contrasted with uranyl acetate and lead citrate, and observed under a Philips Tecnai electron microscope (SERCOMI, University Bordeaux 2, France).

Results

Immunostaining for S100, NF, and PGP9.5 identified variable numbers of nerve fibers in different portal tracts and in specimens from seven different patients. Numbers of nerve contacts in the examined portal tracts were likewise variable. In cases 1, 2, 6, and 7, in which serial levels were examined, nerve contacts (defined by S100 protein immunostaining) were seen in both small interlobular bile ducts and the PBS, the former (78 biliary-nerve contacts/137 bile ducts; 57%) more commonly than the latter (95 biliary-nerve contacts/294 ductules/CoH profiles; 33%) /137; 57%) ($p < 0.001$). The data from examination of single sections is summarized in Table 1. Of particular note, the number of nerves per portal tract was highly variable between specimens as was, therefore,

the number of nerve-biliary duct contacts. Comparison of sequential levels of tissue stained for PGP9.5 and TH revealed that the majority (greater than approximately 80%) of nerve fibers were adrenergic, and that there was clear nerve-bile duct/ductule contact.

Figure 1 demonstrates common features of nerve-bile duct relationships. Nerves focally enter into small bile duct structures (Fig. 1a, e), but more frequently appear to touch and/or stop at the basement membrane (Fig. 1b–d, f). All proximal segments of the biliary tree, from small interlobular ducts (Fig. 1a–c, e, f) to bile

ductules (Fig. 1c, d) to CoH (Fig. 1d) are involved. Confirmation that nerves can cross the biliary basement membrane is demonstrated in Fig. 1e, where a NF-positive axon crosses through a PAS-D-positive basement membrane. Figure 2 shows close contact of a PGP9.5-positive nerve fiber (Fig. 2a) with bile ductules at the portal tract-parenchymal interface and a TH-positive nerve fiber in direct contact with an interlobular bile duct (Fig. 2b). Three-dimensional imaging confirmed an impression obtained from microscopy of individual sections, namely that nerves often travel longitudinally along bile

Fig. 1 Interactions between nerves and proximal branches of the biliary tree. **a** Serial sections stained for biliary-type keratins (AE1/AE3, blue) and S100 protein (brown). Level 1 has a cross section of a nerve that then appears to turn to the right (2) and then enter the bile duct (3). The route of the nerve within the bile duct is either erratic across the plane of tissue sectioning or it is branching, resulting in the three cross-sectional brown dots (highlighted by arrows when keratin staining is dark) in levels 2, 4, and 5, $\times 400$. **b** Immunofluorescent staining (S100, red; biliary keratins, green) show the frequent appearance of nerves traveling along biliary structures. At the arrowhead, a red nerve enters the bile duct to overlap with the green cholangiocytes, $\times 200$. **c** Immunostaining for S100 (brown) and biliary keratin (red) shows a nerve encountering a proximal

biliary structure (either CoH or ductule; open arrowhead) and a small bile duct, $\times 400$. **d** Immunostaining as in (c) shows nerves encountering a canal of Hering sampled longitudinally (open arrowhead) and in cross section (solid arrowhead), $\times 400$. **e** PGP9.5 immunostaining highlights a nerve ending (white arrow) in direct contact with a cholangiocyte of a bile ductule. Counterstain is Periodic Acid-Schiff after diastase digestion (PAS-D), highlighting the magenta-colored basement membrane of the duct, through which the nerve fiber passes, $\times 600$. **f** Immunostaining for S100 (brown) and biliary keratin (blue) showing a nerve which approaches a bile duct and appears to stop at the basement membrane (analysis of serial sections shows this to be the end of the nerve), $\times 400$

Fig. 2 **a** PGP9.5-positive nerve fibers (brown) in direct contact with a bile ductule at the portal-parenchymal interface, DAB $\times$ 600. **b** A TH-positive nerve fiber (brown) in close contact with the basement membrane of a bile duct (black arrow), DAB and PAS-D $\times$ 400. A TH-positive nerve fiber in close contact with an interlobular bile duct (black arrow), probably originating from the larger TH-ergic portal nerve (white arrow) seen in the upper right (white arrow), DAB $\times$ 400. **c** TH-positive nerve fiber (brown) in close contact with the basement membrane of a bile ductule (black arrow), DAB and PAS-D $\times$ 400

ducts, sometimes yielding several contacts along its route, but also sometimes crossing between adjacent bile ducts (Fig. 3). Electron microscopy also demonstrated close proximity of nerve to basement membrane of a small duct or ductule (Fig. 4); we have not yet found a sample that shows the nature and structure of intercellular interactions when nerves cross the basement membrane.

In sections of histologically normal liver double-stained for CRBP-1 and S100, very rare juxtapositions of an S100-positive nerve fiber with a CRBP-1-positive HSC were observed; these were not the norm (Fig. 5).

Fig. 3 Three-dimensional reconstruction of scanned images of a single portal tract with bile ducts and ductules (blue) and nerves (yellow orange). Shadowed brown structures toward the top and right show original S100 protein-positive structures before manual deletion of non-contiguous cell profiles (i.e., dendritic cells). This segment demonstrates that nerves both travel along biliary structures and cross between them

Discussion

The liver has abundant portal and parenchymal innervation [16, 24–28]. Both sympathetic and parasympathetic nervous systems have afferent and efferent intrahepatic connections utilizing a variety of neurotransmitters, including aminergic, cholinergic, peptidergic, and nitrergic compounds. The target cells of these nerves are varied, including endothelium, vascular smooth muscle, HSC, and HSPC [29] but direct innervation of cholangiocytes in the proximal branches of the biliary tree has not, to our knowledge, been previously described. Our data add the possibility of a widespread interface between nerves and the smallest branches of the biliary tree, including interlobular ducts, ductules, and CoH.

There is a significant difference between the number of contacts between nerves and interlobular bile ducts compared with those with ductules and CoH, but this may reflect size of structures and thus the possibility of identifying an interface on any particular level. The variability in numbers of contacts between the livers examined in this study, however, may reflect that prospective tissue sampling was not systematically performed, presence of nearby lesions (in the resection specimens), age, or may simply reflect the “normal” range. This variability suggests that a “reference standard” for the normal number of such interfaces may not be obtainable, complicating the studies of innervation in disease.

Our data indicate two forms of juxtaposition of nerves and biliary components. Some nerves directly abut the basement membrane beneath the cholangiocytes, but others cross the basement membrane into the bile duct proper. The

Fig. 4 Nerve with 4 axon sections (*), surrounded by Schwann cell (SC) processes is in close contact with a cholangiocyte of a small bile duct or ductule; the basement membrane of bile duct (arrow heads) is still present in between. Co, collagen fibers in portal tract

submembranous juxtaposition appears similar to the lamina propria terminus of some gastrointestinal innervation, with diffusion of neurotransmitters through the extracellular milieu [18]. Nerves crossing the basement membrane to interface directly with cholangiocytes are still more provocative. Electron microscopic analysis of these connections is, of course, vital. We have begun such analysis and can confirm

close approximation between a nerve and the basement membrane of a duct or ductule. A third possible structural interaction between nerves and cholangiocytes might be the serial linking of these structures by an intermediate cell, a likely candidate for which is the HSC. It is known that HSC are sometimes innervated [18], and activated HSC have been noted in animal models in close association with oval cells in chemical injury [30]. Although we observed some HSC in close approximation with nerve fibers, we are unable to identify a network-like connection with cholangiocytes as the third structure. While this absence may reflect limitations of sampling, clearly the other forms of nerve-cholangiocyte structural juxtapositions are far more common, since these were readily identified in all specimens.

The implications of these findings are revealing of possible novel pathways of modulation of epithelial components of the liver (and possibly other organs) by the nervous system. Cholangiocytes not only have muscarinic [10, 11], dopaminergic [10, 18], and serotonin receptors [31], but also produce at least one neurotransmitter themselves, namely serotonin [31]. Our findings suggest that there may not only be autocrine regulatory loops involving these receptor-ligand pairings, but cholangiocyte-nerve regulatory loops as well, involving efferent and/or afferent neural networks. Specifically, correlating to observations of muscarinic receptors on cholangiocytes, we found TH to be present in the majority of periductal/ductular nerves, indicating adrenergic innervation.

Liver transplantation offers a unique insight in the dynamics of liver innervation as the procedure includes denervation

Fig. 5 Direct contact of a S100-positive nerve fiber (white arrows) with a CRBP-1 hepatic stellate cell (HSC, black arrow) in histologically normal liver double-stained for CRBP-1 and S100. The HSC contains tiny CRBP-1-negative lipid droplets, DAB × 600

of the liver. Two early studies followed the morphologic changes in liver innervation in early and as late as 4-year post-transplant liver biopsies [32, 33]. Different markers were applied that also included antibodies used in the current study, e.g., S100 and PGP9.5. These studies elegantly documented the rapid loss of nerve fibers both in the portal tracts and liver parenchyma between the first week and 6-week post-transplantation, regardless other conditions such as rejection. No nerve fibers were observed in a long period hereafter, varying between 8 months and 1 year. Reappearance of nerves after this period was observed in only 30% of cases, mainly in the portal tracts. Boon et al. [32] hypothesized that the post-transplant degeneration mainly affects the long post-ganglionic sympathetic fibers. This concept is corroborated by the findings of Kjaer et al. who found a rapid and significant decrease of epinephrine and norepinephrine concentrations without recovery to normal control values even in biopsies of 4-year post-transplant [34]. For unknown reasons, in larger septal portal tracts and hepatic hilum of transplanted livers persistence of hypertrophic nerve bundles were observed without signs of outgrowth beyond these large septal portal tracts [32]. These structures probably represent traumatic amputational biliary neuromas similar to their extrahepatic counterparts that have been described in several papers and assumed to result from failure of axonal regeneration (reviewed in Colle I et al [35]). These amputational neuromas may be associated with biliary strictures after transplantation [36]. The physiologic consequences of denervation have been reviewed by Kandilis et al. [28].

In terms of HSPC regulation by the autonomic nervous system, in rat liver the hepatic autonomic nerves are suggested to play a role in the proliferation of HSPC during liver regeneration [13]. One report suggested that innervation may play a role in stem cell regulation in hematopoiesis [37]. That study indicated that stem cell activity is modulated by the sympathetic nervous system through an intermediate cell, namely the osteoblast. We have not been able to confirm a similar networking between nerves, HSC, and HSPC in the human liver, suggesting that even if such a network is present, it is a minor mechanism of nerve-HSPC interaction in the human intrabiliary stem cell niche.

Many questions are raised by these findings, including the following: Is there direct central nervous system monitoring or control of biliary flow and/or composition? If hepatic regeneration through HSPC activation in ductular reactions is modulated directly by the autonomic nervous system, can survival in fulminant hepatic failure be influenced by medically manipulating that system? If ductular reactions are a common antecedent, if not direct precursor, to some hepatic scarring could such manipulation also play a role in slowing disease progression? Does the liver have a neural network independent of central control like the enteric nervous system?

On a larger scale, this work raises the possibility of more widespread roles for nervous system regulation of epithelia throughout the body. Bohorquez et al. have identified such nerve-epithelial contacts with enteroendocrine cells of the gut, in which a process from the nerve enters the epithelial cytoplasm, that the authors term a neuropod [38]. Nerves approximating to basement membrane under collecting duct epithelial of the kidney are also known [39]. Innervation of epithelia of the cornea [40] and salivary gland have been described [41]. In the liver, whether nerve-epithelial contacts are mediated by gap junctions, synaptic junctions, or tight junctions or by combinations of these, for example, requires further study.

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Author contributions AZ: experiments and procedures, data analysis, JR: experiments and procedures, HJF: experiments and procedures, data analysis, manuscript preparation, JQ: experiments and procedures, ASG: study design and concept, manuscript preparation, JO: experiments and procedures, AK: experiments and procedures, CB: study design and concept, experiments and procedures, PB-S: study design and concept, data analysis, manuscript preparation, DGT: study design and concept, experiments and procedures, data analysis, manuscript preparation, NDT: study design and concept, experiments and procedures, data analysis, manuscript preparation

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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