Relevance of Mast Cells and Hepatic Lobule Innervation to Liver Injury

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Abstract

Chronic liver diseases commonly result in liver fibrosis, and eventually liver cirrhosis. In the last decade, a new theory explaining liver fibrosis has been established. Accordingly, the development of liver fibrosis due to chronic liver diseases is thought to be mediated by inflammatory cells. They release fibrogenic mediators such as transforming growth factors (TGF)-beta, which are considered to be responsible for the activation and transformation of fat-storing cells. Recently, the involvement of mast cells and peripheral and autonomic nervous system in the fibrogenesis has been suggested. This study was aimed to establish the presence and distribution of mast cells and nerve fibers in the rat liver in the light of their implication in liver inflammatory and fibrotic disorders. Mast cells and afferent (sensory) fibers were detected immunohistochemically. An immunofluorescent method was applied to demonstrate tryptase and serotonin (SER) in the mast cells, while the primary sensory neuronal processes were identified by using antibodies against their marker calcitonin gene-related peptide (CGRP) and the proinflammatory mediator substance P (SP). The portal tracts and fibrous septa contained numerous mast cells, which exhibited strong immunoreactivity to tryptase and SER. SER-positive nerve fibers were also found. It is generally accepted that no nerve fibers are present in the hepatic lobules, but the current investigation clearly demonstrates availability of CGRP-, SP, and SER-immunoreactive nerve fibers there.

Our results indicate that in the rat liver portal tracts and hepatic lobules there are numerous mast cells, sensory and autonomic nerve fibers, which may be involved in liver injury by the inflammatory mediators they release.

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Keywords

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Rezumat

Hepatopatiile cronice conduc frecvent la fibroză, eventual ciroză hepatică. În ultimul deceniu a fost elaborată o nouă teorie despre fibroza hepatică. Conform acesteia, dezvoltarea fibrozei este mediată de celulele inflamatorii. Ele eliberează mediatori fibrogenetici precum factorii de transformare a creşterii-beta (TGF-beta), care sunt consideraţi responsabili pentru activarea şi transformarea celulelor de înmagazinare a grăsimii (fat-storing cells). De curând a fost sugerată implicarea mastocitelor şi a sistemului nervos periferic şi vegetativ în fibrogeneză. Acest studiu are scopul de a stabili prezenţa şi distribuţia mastocitelor fibrelor nervoase în ficatul de şobolan prin prisma implicării lor în inflamaţia şi fibroza hepatică. Mastocitele şi fibrele aferente (senzitive) au fost detectate prin imunohistochimie. A fost aplicată o metodă de imunofluorescenţă care detectează triptaza şi serotonina (SER) din mastocite, în timp ce procesele neuronale senzitive primare au fost identificate prin anticorpi contra markerilor acestora: peptidul calcitonin gene-related (CGRP) şi mediatorul proinflamator substanţa P (SP). Canalele porte şi septele fibroase au prezentat numeroase mastocite, care prezentau o puternică imunoreactivitate la triptază şi SER. Au fost găsite de asemenea fibre nervoase SER-pozitive. Se acceptă în general că nu există fibre nervoase în lobulii hepatici, dar acest studiu demonstrează existenţa fibrelor nervoase imunoreactive la CGRP, SP şi SER.

Aceste rezultate indică faptul că în ficatul de şobolan ramurile porte şi lobulii hepatici cuprind numeroase mastocite, fibre nervoase senzitive şi motorii, care pot fi implicate în leziunile hepatice, prin eliberarea de mediatori inflamatori.

Introduction

Chronic hepatocellular damage commonly results in liver fibrosis, and eventually liver cirrhosis. Over the past decade, a new theory regarding liver fibrosis has been established. Accordingly, the development of fibrosis due to chronic liver diseases is thought to be mediated by inflammatory cells. They release fibrogenic mediators such as the transforming growth factor (TGF)-beta (1), which is considered to be responsible for the activation and transformation of fat-storing (Ito) cells. In addition, the involvement of mast cells and liver innervation in the fibrogenesis has been recently suggested.

Mast cells have an established role in a variety of pathological processes including immediate and delayed hypersensitivity reactions, inflammatory and fibrotic conditions of unknown etiology (2) and inflammatory bowel diseases (3). They have the capacity to release significant amounts of pro-inflammatory mediators, cytokines and growth factors (4). Furthermore, increasing evidence suggests that the secretory function of mast cells is regulated by the nervous system (5). Substance P (SP) is a mediator of the non-adrenergic non-cholinergic (NANC) part of the nervous system and is mainly present in the peripheral sensory nerves, particularly in small unmyelinated fibers. Besides its function as a neuromediator, SP is a non-immunologic secretagogue, which was reported to cause a degranulation of mast cells (5). This neuropeptide, in addition to classic adrenergic and cholinergic components, may play an important role in the regulation of the blood flow. On the other hand, mast cells, acting via histamine and nitric oxide, are responsible for increasing the blood flow in animal models, probably through the release of SP (6) and calcitonin gene-related peptide (CGRP) (7), which is considered to be a marker peptide for the primary sensory neurons.

Several studies on the distribution of nerve fibers in the normal liver (8-12) and in experimental hepatic injury (13) have been published. They suggest that both autonomic and sensory innervation play a significant role in the development of liver diseases (14). Patients with cirrhosis are known to have sympathetic hyperactivity that is manifested by increased nerve discharge rates, increased catecholamine levels and parasympathetic dysfunction (15, 16). These changes have been implicated in causing a hyperdynamic circulatory state and are frequently associated with an increased metabolic rate (17).

It is generally accepted that no sensory fibers are present in the hepatic lobules but in our previous studies we demonstrated CGRP-immunorective (IR) innervation of the hepatic lobules in the human liver (14, 18). SP and CGRP are involved in the regulation not only of the hepatic microcirculation, but in the control of the hepatocyte functions (19, 20, 21). In addition, numerous studies have demonstrated a close microanatomical association between mast cells and nerves in some tissues (22, 23).

As mast cells and hepatic lobule innervation are relevant to liver injury, this study aims to establish immunocytochemically the presence and distribution of mast cells and nerve fibers in the rat liver and to estimate their possible role in liver inflammatory and fibrotic disorders.

Material and methods

Twelve adult Sprague-Dawley rats of either sex weighting 250-300 g and provided by authorized suppliers were used in this study. The animals were deeply anesthetized with Ketanest (50 mg/kg i.p.) and perfused through the ascending aorta with 100 ml 1% heparine in cold 0.9% NaCl, followed by 500 ml Zamboni's fixative. Liver specimens were obtained and postfixed in the same fixative for 6 hours at 4 ^{o}C, and then immersed overnight at 4^{o}C in 0.01 M Tris-buffer , pH 7.4, with 20% sucrose for cryoprotection. The specimens were embedded in TissueTek OCT compound, frozen and sectioned at 20 µm in a cryostat at -20^{o}C. The sections were separated into five series. After rinsing in 0.1 M phosphate buffer saline (PBS), pH 7.4, each complete series of one-in-five sections was processed for indirect immunofluorescent technique of Coons (24). All processing was made on free-floating sections, which were incubated in a 1:1 000 dilution of polyclonal rabbit primary antibodies against SP or CGRP, polyclonal rabbit anti-SER antibody (Incstar Corporation, Stillwater MA, USA) in a dilution 1:5 000, or with polyclonal mouse anti-mast cell tryptase (DAKO, Glostrup, Denmark) in a dilution 1:50, at room temperature (RT) for 24 h. Afterwards, the specimens were incubated in a solution with goat anti-rabbit IgG conjugated to fluorescein isotiocyanate (FITC) or Texas Red (both Amersham, Buckinghamshire, UK), dilution 1:50 in PBS. Between the main steps, sections were rinsed in cold PBS.

Co-localization of SP and tryptase was demonstrated by the indirect immunoflourescence technique. After rinsing in 0.1 M PBS, pH 7.4, the sections were incubated in a solution containing polyclonal rabbit anti-SP and polyclonal mouse anti-tryptase antibody (DAKO, Glosrup, Denmark) in a dilution 1:1 000 and 1:100, respectively, at RT for 24 h. The second step was incubation in a solution with a goat anti-rabbit IgG conjugated to fluorescein isotiocyanate (FITC) (Amersham, dilution 1:50 in PBS) and biotinilated sheep anti-mouse Ig (1:100) for 3 h at RT. As a third step streptavidin Texas Red (Amersham), diluted 1:100 in 0.1 M PBS, pH 7.4 was applied. The incubation was followed by rinsing in 0.1 M PBS, pH 8.6, and the preparations were mounted and coverslipped with glycerol, diluted 3:1 in 0.1 M PBS, pH 8.6. All specimens were examined and photographed with a Leitz Laborlux S microscope, equipped with a 100 W super high pressure mercury lamp and appropriate filter sets for FITC and Texas Red .

Negative controls included incubating some sections in the absence of the primary antibody or replacement with a non-immune normal serum using the same concentration, as well as antigen-antibody preabsorption experiments with the native antigen (10 mg/ml) at 4°C for 48 h. In all cases, this resulted in the total absence of staining.

Results

Ten randomly taken sections from each series of rat liver parenchyma were investigated in order to determine the presence and localization of neuronal fibers and mast cells. In the normal livers, the intrahepatic structures (portal tracts and liver lobule with hepatic cords, sinusoids and central vein) were observed. The larger intrahepatic branches of hepatic artery, portal vein and bile duct were also observed in the connective tissue near the hilum of the liver lobule. Neuronal fibers were demonstrated immunocytochemically with dark field microscopy using antibodies against three of their neurotransmitters – SP, CGRP and SER. Immunoreactivity to SP and CGRP in neuronal fibers and terminal-like arborizations was present abundantly in the portal tracts. Many immunolabeled axonal varicosities were observed in close contact with hepatic arteries and portal veins. A striking accumulation of CGRP-positive neuronal terminals was found in the portal vein where they crossed the vessel's wall and ended below the endothelial cells (Fig.1). Some varicose neuronal branches could be followed further inside hepatic lobules along sinusoids (Fig.1). However, when compared with CGRP-IR neuronal processes, SP-IR nerves had more widespread distribution in the lobules near the sinusoidal cells (Fig.2). In cross sections of portal tracts SP- and CGRP-IR fibers and terminal-like arborizations were mainly found within the artery and bile duct walls. The nerves were denser around the branches of the hepatic artery

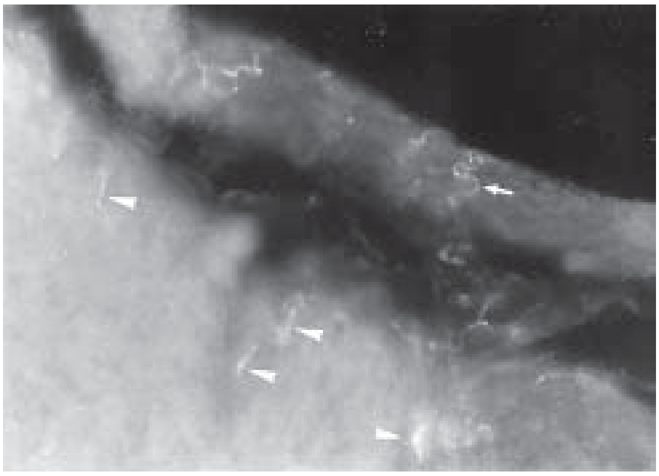

Fig.1 Dark field microphotograph of a larger intrahepatic branch of portal vein in the connective tissue near the hepatic lobule. CGRP-IR fibers and terminal-like arborizations within the vessel wall (arrow). Some labelled varicose neuronal branches can be followed further into the hepatic lobule (arrowheads). x 250.

as compared with those found around the portal vein branches. Some of the fibers were present underneath the endothelial layers of the interlobular vessels. Positivity for SP and CGRP occurred more frequently than positivity for SER. The number of SER-labeled neuronal fibers was smaller in portal tracts and septa compared to the number of SP- and CGRP-stained varicosities, and they were found in extremely low numbers in perisinusoidal spaces (Fig.3). In cross sections of the portal tract immunostaining with tryptase (Fig.4) and SER (Figs.5, 6) demonstrated numerous mast cells in close contact with bile duct and parenchymal cells. When single incubation of hepatic specimens with anti-SP antibody was applied, a significant number of positive cells were observed in the portal tracts (Fig.7). Double labeling of the sections with anti-tryptase (Fig.8a) and anti-SP antibodies (Fig.8b) revealed that all of the SP-containing cells are tryptase-positive, and thus they were identified as mast cells. Mast cells containing both SP and tryptase were found dispersed throughout the lobule and did not have a specific location.

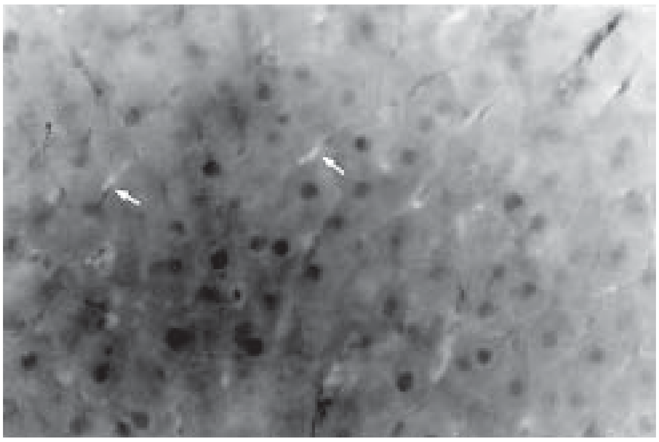

Fig.2 Photomicrograph of varicose SP-IR neuronal processes (bright white) near sinusoids of normal liver. This location suggests a close apposition to the Ito cells. When compared with CGRP-IR neuronal processes, SP-IR nerves have more widespread distribution in the lobules. x 250.

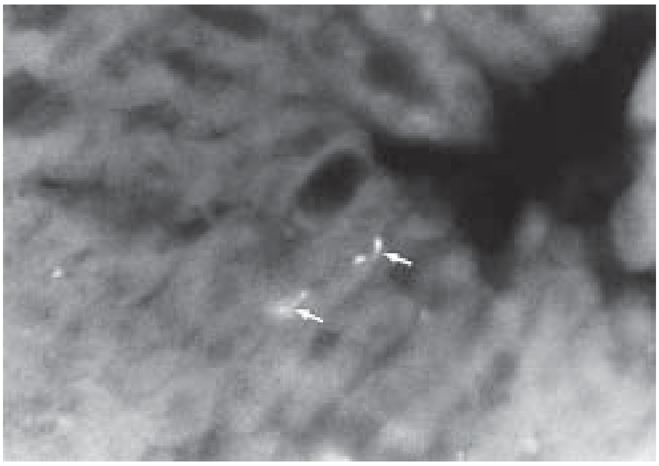

Fig.3 The number of serotonin (SER)- IR neuronal fibers is smaller in portal tracts and septa, and extremely low in perisinusoidal spaces (arrows). x 250.

Discussion

The present study shows that in the normal liver of the rat SP-, CGRP-, and SER-IR nerves are not restricted to the

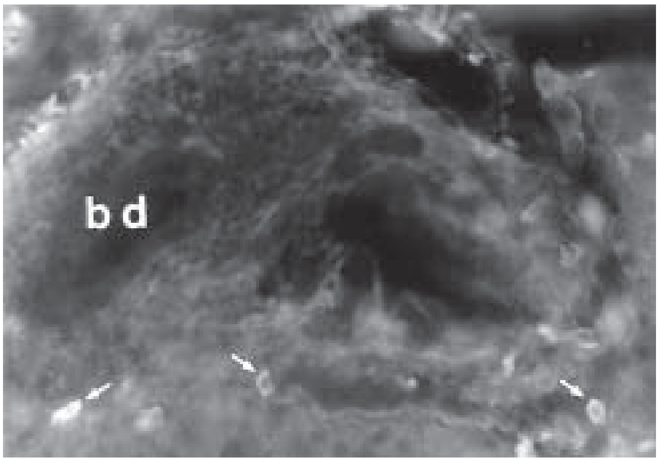

Fig.4 Cross section of portal tract with numerous tryptase-positive mast cells (arrows) in close contact with bile duct (BD) and parenchymal cells. x 250.

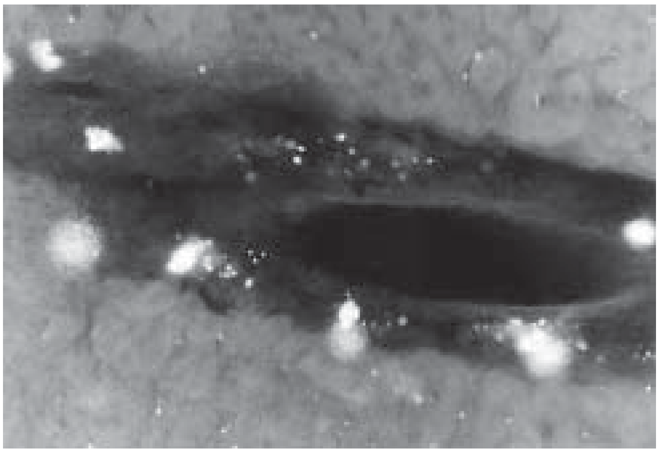

Fig.5 Dark field photograph of SER-labelled mast cells (bright white) in the portal tract. Many of them are in contact with hepatocytes. x 250.

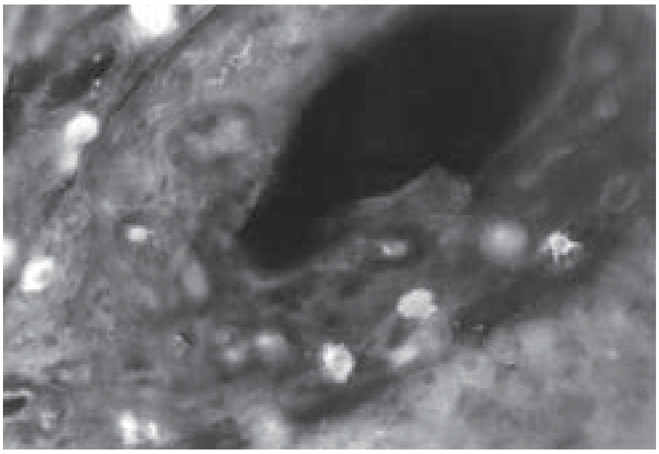

Fig.6 Microphotograph showing numerous SER-IR mast cells (bright white) in the portal tract. x 400.

portal tracts, but part of them also run into the hepatic lobules. Innervation of the liver plays a significant role in its physiology (9, 25) and pathology (26). The exact function of neuropeptide-containing fibers is not completely elucidated yet, but it is clear that glucose and lipid metabolisms are regulated by neuronal projections into the

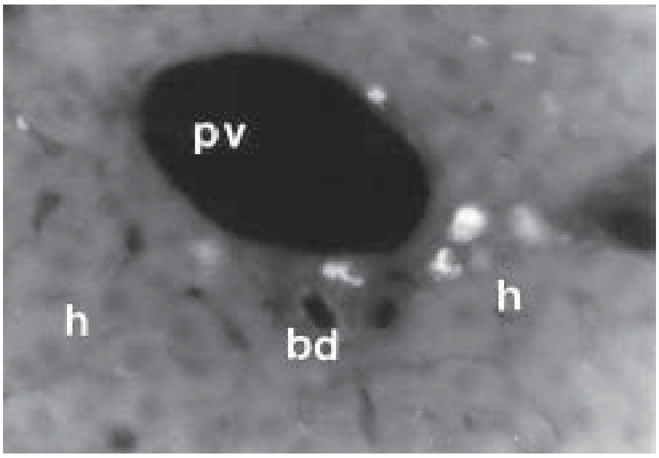

Fig.7 Single labelling of SP-IR mast cells (bright white) in the portal tract, in close contact with the interlobular vessels and parenchymal cells. PV portal vein, BD bile duct, H hepatocyte. x 250.

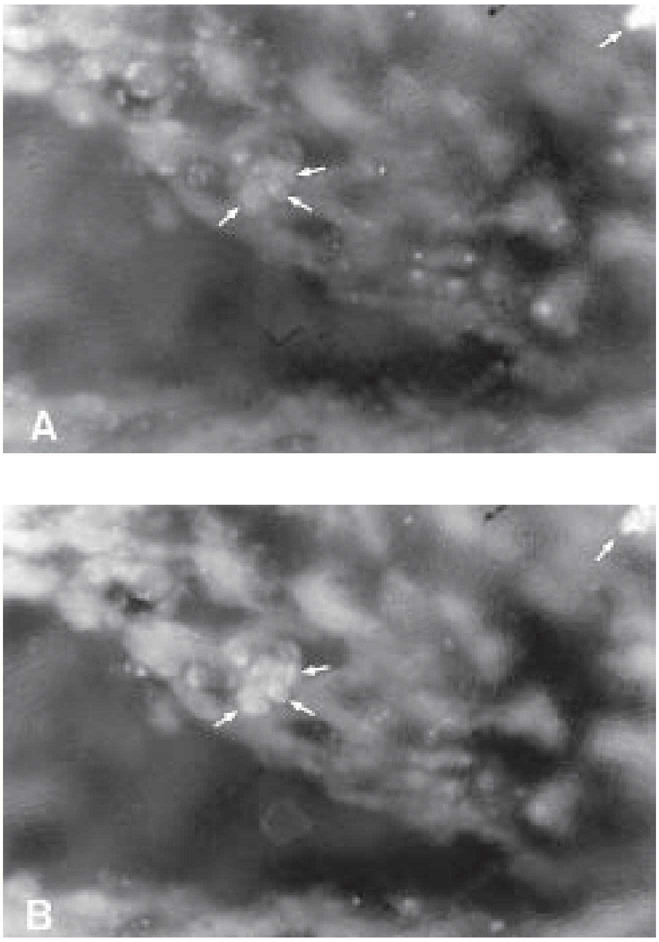

Fig.8 Double labelling of mast cells with tryptase (a) and SP (b). Some of the mast cells are SP-positive (arrows). x 250.

liver parenchyma. This neuronal control could be exerted directly upon parenchymal and non-parenchymal liver cells by release and diffusion of neurotransmitters, or indirectly as a result of alterations in the blood flow (27). This suggestion is well illustrated by the present findings of neuronal processes in the blood vessel walls. A direct sensory innervation of the parenchymal and sinusoidal cells in the rat liver is also clearly demonstrated. Our data support the hypothesis that neuronal fibers might affect inflammatory and immunological processes in the sinusoids (28, 29).

CGRP and SP are present in the sensory-motor nerves (30). A widespread distribution of CGRP- and SP-IR nerves was observed in the normal rat liver and it was reported that they might play some role in the control of intrahepatic blood vessel tone (31). CGRP is capable of causing some relaxation of the rat hepatic artery (32). Availability of CGRP- and SP-positive nerve terminals near the sinusoidal wall demonstrated in our study suggests a close apposition to the Ito cells and leads to the idea that in the liver tissue peptidergic neuronal fibers affect Ito cells in their transformation into the myofibroblastic phenotype. It is known that SP stimulates the production of interleukin-1 in activated macrophages (33), which is one of the stimulators of Ito cell transformation (34). These transformed cells play a central role in the regulation of portal hypertension by enhanced contractility, leading to their bulging into sinusoidal lumina (35, 36).

In the human liver, CGRP-positive neuronal fibers had been found only in portal tracts, mainly in the fibromuscular layer of the biliary tree, surrounding the portal vein, and in the stromal interlobular compartment of the portal tracts. According to many investigators, these fibers are not present in hepatic parenchymal lobule (37). In the present investigation, a close apposition of GCRP-immunoreactive nerve processes to hepatocytes and sinusoidal cells in the lobules is clearly demonstrated. Specific CGRP receptors linked to cAMP accumulation have been evidenced in the rat liver (38, 39) and it has been established that CGRP inhibits insulin-stimulated glycogen synthesis in isolated rat hepatocytes (40). Experiments in capsaicin-pretreated rats and cultured Ito cells (41, 42) suggest that local release of sensory neuropeptides such as SP and CGRP may be involved in the genesis of liver fibrosis (43). In rats, CGRP stimulates production of glycosaminoglycans by Ito cells (41). On the other hand, SP stimulates collagen type I production via NK-1 receptors but not that of glycosaminoglycans (42). Therefore, these two neuropeptides, which we found in neuronal processes innervating rat liver parenchyma, probably act in a coordinated manner to stimulate, via specific receptor-mediated mechanisms, the production of the two main components of the extracellular matrix. These data support the hypothesis that local release of sensory neuropeptides may contribute to the development of fibrotic tissue during cirrhosis (44).

Numerous mast cells can be detected in the connective tissue of the portal tracts and only single ones were in close contact with parenchymal cells. The mechanism by which SP activates mast cells remains unclear. Evidence was produced that mast cells release histamine in response to a specific activation of neurokinin receptors (45, 46). On the other hand, it was suggested that SP activated mast cells throuth a nonreceptor mediated interaction (47), possibly through an interaction with cell surface sialic acid residues (48) or other NK1 and NK2 receptor-independent mechanisms.

Although application of SP in pharmacologic doses elicits histamine release from the mast cells, this has no effect on the blood flow. These findings are more consistent with SP playing a role as a cofactor rather than acting as a key mediator that initiates mast cells degranulation and a hyperemic response (47). SP and CGRP have been found to have synergistic effects on mast cell exocytosis (49). In addition, differential effects on mast cells and vascular tone could account for the present findings. The increase in histamine release via SP- and CGRP-IR neuronal fiber stimulation, shows that mast cells, which are numerous in the portal tracts, some of them being in close apposition to the hepatic parenchyma, are capable of responding to neuronal signals.

Recently, SP-containing human mast cells have been demonstrated for the first time in the skin (50). The finding that the amount of SP in mast cells in dermatitis is larger than that in normal skin suggests that SP is synthesized within mast cell granules as a newly generated mediator under environmental stimulation, such as inflammation (50). Using a double labelling for tryptase and SP in this study, we for the first time demonstrate SP-containing mast cells in the rat liver.

Serotonin (or 5-hydroxytriptamine (5-HT)) is a small molecule of the indoleamine family, which has been identified as a neurochemical messenger (51). It has a wide distribution across animal species and is present in several tissues (52). Physiological investigations performed in the cat have shown that SER has some effects on nociception and cardiovascular parameters mediated via 5-HT1a receptors (53, 54). It is shown that SER may increase the discharge of vagal afferent fibers from the stomach and proximal intestine in ferrets (55), and stimulate vagal nodose ganglia activities in rats (56). In the current study, we demonstrate numerous SER-containing mast cells localized in the liver portal tracts and in the vicinity of the hepatocytes. It is likely that SER released from these mast cells and SER-IR neuronal fibers may stimulate vagal and spinal afferent nerve endings innervating lobules and thus activating hepatocytes. This conclusion is in consent with the previously reported role of SER in the upper gastrointestinal tract (57).

Taken together, all these facts suggest that activation of sensory nerves in the liver might be involved in the histamine, SP and SER release from mast cells. In view of the significant role of SP and SER in the inflammation, this study provides further evidence for the important role of mast cells in the pathogenesis of inflammatory and fibrotic liver diseases, and a new insight into the neuroimmune interaction that is likely to occur during these processes.

The present data help to elucidate the role of neuropeptides and mast cell-nerve interaction in the inflammatory- and fibrotic-type response of the liver triggered by different factors.

Although a lot of unresolved questions remain, we may conclude that mast cells and innervation of the liver may play an important role in the pathogenesis of circulatory and metabolic changes by stimulating sinusoidal vasoconstriction, Ito cell transformation and liver fibrogenesis. Moreover, it may also influence the course of the liver disease. A close relationship has been demonstrated between some neuromediators like catecholamines and survival, so plasma levels of noradrenalin might serve as an independent prognostic factor in cirrhosis (58, 59). Alterations in the density and distribution of neural elements have been described in different liver diseases including cirrhosis (60). A better understanding of the role of mast cells, liver innervation and their alterations under pathological conditions might contribute to the development of specific pharmacological agents for the treatment of chronic liver disease.

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