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Structural Examination of Tryptase-, Chymase-, SP-, and VIP-Positive Mast Cells in the Human Common Bile Duct and Liver

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Human mast cells are divided into two subsets: mast cells with reactivity for tryptase but not chymase (MC_T) and reactive for tryptase and chymase (MC_{TC}). We investigated the MC_T and MC_{TC} subtypes in human livers containing metastases from gastrointestinal cancers and in the common bile duct in obstruction. We have found that there were increased numbers of mast cells (MC_T and MC_{TC}) in the surrounding liver parenchyma and in liver metastases originating from gastric, colorectal and pancreatic cancers. In the common bile duct with obstruction we detected greatly increased numbers of mast cells. Among the studied mast cell types MC_{TC} ones were the most numerous.

The ultrastructural appearance of tryptase- and chymase-positive mast cells showed three different types of granules, concerning the content of the reaction product (altered granules). These types were observed in the livers with metastases and in the inflamed common bile duct. In conclusion it can be stated that mast cells in human livers with metastases and in the inflamed common bile duct increased in numbers and showed signs of activation.

Key words: mast cells, immunocytochemistry, liver, holedoch.

Introduction

Mast cell heterogeneity is a key issue in mast cell biology [5], but its nature in humans is a subject of uncertainties.

Although the immunopathological role of mast cells has been acknowledged, these cells have aroused much controversy and confusion. One explanation for the contradictory opinions on mast cell function arises from their heterogeneity, which can express itself as differences in histochemical, biochemical, and functional characteristics.

The main criterion for mast cell differentiation in rodents is formalin resistance. In humans, mast cells are distinguished by their content of the mast-cell-specific proteases, chymase and tryptase [18, 22]. Tryptase and chymase are released from mast cells following IgE-mediated activation. In contrast to the findings in rat, where both mast cell serine proteinases are of chymotryptic specifity, only one human mast cell proteinase, chymase, displays this activity whereas the other enzyme, tryptase, is of tryptic specifity [3].

Human mast cells are divided into two subsets: mast cells with reactivity for tryptase but not chymase (MC_T) and reactive for tryptase and chymase (MC_{TC}). The human mast cell chymase belongs to the group of alpha chymases [4]. It is found predominantly in connective tissue mast cells and to a lesser extent in mucosal mast cells [4, 7]. Mast cell tryptase expression is largely confined to mucosal mast cells [4]. In humans, the differentiation of mast cells into mucosal or connective tissue phenotypes is less clear cut than in rodents [17]. Mast cells with variable granule morphologies immunolabelled for tryptase and chymase have been described in human lungs, skin and small intestine [4, 7].

Mast cells are intimately involved into the "cross-communication" between the nervous and immune systems [28, 30]. Mast cells have been shown to release some neuropeptides such as vasointestinal polypeptide (VIP) [8] and substance P (SP) [29, 32]. Biogenic amines secreted by these cells act together with the released biologically active peptides from endocrine cells at mucosal sites [11] thereby contributing to neurophysiological and neuropathological processes.

The role of tryptase- and chymase-positive mast cells for the fibrogenesis, epithelial cell proliferation, metaplasia and inflammatory cells recruitment is already described in other diseases [1, 2, 6, 31].

The biological role of mast cells in liver metastases and in cancer growth still remains speculative. Mast cells contain various bioactive substances such as histamine, heparin, etc. that appear to stimulate both mitogenesis and angiogenesis [6, 24 ,25], which may be detrimental for cancer patients. On the other hand, mast cell-derived tryptase stimulates fibroblasts [26], leading to fibrosis, which can form the stroma of metastasis or may limit tumor growth. Therefore, the study of the density of mast cell subpopulations (MC_{TC} and MC_{TC}) and granule morphology in "normal" livers and in liver metastases from gastric, colorectal and pancreatic cancers elucidated mast cell heterogeneity and functions in humans.

A great amount of mast cells was already noticed in the common bile duct in secondary cholangitis [15]. It will be interesting to disclose the structural morphology and the proportions of the different subsets of these cells and also to discuss their role in physiologic and pathologic conditions. It is also interesting to study the structural morphology and the density of mast cell subpopulations (MC_T and MC_{TC}) in livers containing metastases from gastric, colorectal and pancreatic cancers.

Materials and Methods

Liver metastases. Liver tissue was collected from 30 patients with colorectal (n=15), gastric (n=8), pancreatic (n=7) cancers. The "normal" liver tissue was obtained from 5 patients operated with diagnostic purpose (explorative laparotomy). Controls had no previous history of liver diseases [13].

Extrahepatic bile duct. Surgically resected specimens from the lower part of the common bile duct (at 2 cm distance from the papilla of Vater) were collected from 50 patients (19 men and 31 women), aged between 31 and 81 years. Twenty-six patients (9 men and 17 women) had calcium-bilirubinate stones in the common bile duct, 3 had pancreatic cancers (two men and one woman) and two patients had stenosis (one male and one female). All these lesions caused acute obstruction and extrahepatic jaundice. The biopsy specimens of these patients showed secondary chronic exacerbated cholangitis. Eighteen patients (7 men and 11 women) had previous attacks of calculous obstruction and one woman had stenosis, but at the time of operation showed only secondary chronic sclerotic cholangitis. Biopsy samples were collected after resection of the common bile duct for

drainage and stone extraction. As controls, we obtained 5 specimens from patients (3 men and 2 women) who had died of myocardial infarction and had no pathology of the biliary pathways. Informed consent had been previously obtained from each patient [14].

Cryostat sections cut from one sample were prepared for light and ultrastructural immunocytochemistry. The remainder of the tissue was embedded in paraffin wax for routine histology. The methods were described earlier [13, 14, 15].

Immunochemicals. The antibodies used were mouse anti-human mast cell tryptase [clone AA1 (M7052)], mouse anti-human serotonin (N1530 from Dako, Glostrup, Denmark), mouse anti-human mast cell chymase [clone CC1 (MCA1930)], rabbit anti-human VIP (PEPA41 from Serotec, Oxford, UK), and rabbit anti-human substance P (PEPA40 from Serotec). The DAKO (Glostrup, Denmark) immunostaining detection system kit, the LSAB 2 System and HRP (K0675), was used together with either 3,3'-diaminobenzidine (DAB; from Sigma, St. Louis MO, USA) or 3-amino-9-ethylcarbazole (AEC; from Dako) as the chromogen.

Results and Discussion

Mast cells in human livers with metastases

We have found an increased numbers of mast cells (MC_T and MC_{TC}) in the liver parenchyma, situated around metastases, originating from gastric, colorectal and pancreatic cancers, as compared to controls (2.9 v.s. 0.6 cells/mm^2, p=0.003 for MC_T and 8.6 v.s. 0.7 cells/mm^2, p=0.004 for MC_{TC}). Mast cells were largely located in portal tracts surrounding metastases (13). In the sinusoids there could rarely be observed mast cells. Therefore, we can state that liver sinusoidal mast cells do not increase in parenchyma surrounding metastases from gastric, colorectal and pancreatic cancers [13]. The number of mast cells was great in the stroma of metastasis (6.9 ± 4.8 cells/mm^2 for MC_T и 22.8 ± 14.7 cells/mm^2 for MC_{TC}). Therefore, these cells actively participate in the metastatic process [13].

The current study indicates that human liver mast cells do not react with VIP and SP in "normal" liver or in the presence of metastases.

Two theories have been advocated in literature about intratumoral mast cells. The first is that mast cells may play a role in tumor growth and angiogenesis forming tumor stroma [16, 25]. The second is that mast cells exert cytotoxicity on tumor cells by immunologic mechanisms [12]. In the current work, a relatively large number of mast cells was present in the stroma of metastases and around them, suggesting that the connective tissue formation in liver malignancies may be associated at least in part with the presence of mast cells.

The ultrastructural immunocytochemical localization of tryptase and chymase revealed 3 distribution patterns of the antigen in mast cell granules: granules with darkly precipitated reaction product, electron lucent granules without reaction product and electron lucent granules with sparse reaction product (altered granules). Mast cell granules with MC_{TC}-phenotype had larger diameters (over 0.203 μm) as compared to granules positive only for tryptase (about 0.13 μm) [(7]. The mast cells, described by us in human liver had granules with larger diameters about 0.45 μm. Morphologically, they looked like mast cells with MC_{TC}-phenotype.On the basis of localization of tryptase (Fig. 1) and chymase (Fig. 2) and the mean diameters of granules, these mast cells resembled tryptase/chymase-positive (MC_{TC}) as described by G r a i g et al. [7] in human lung, intestine and skin.

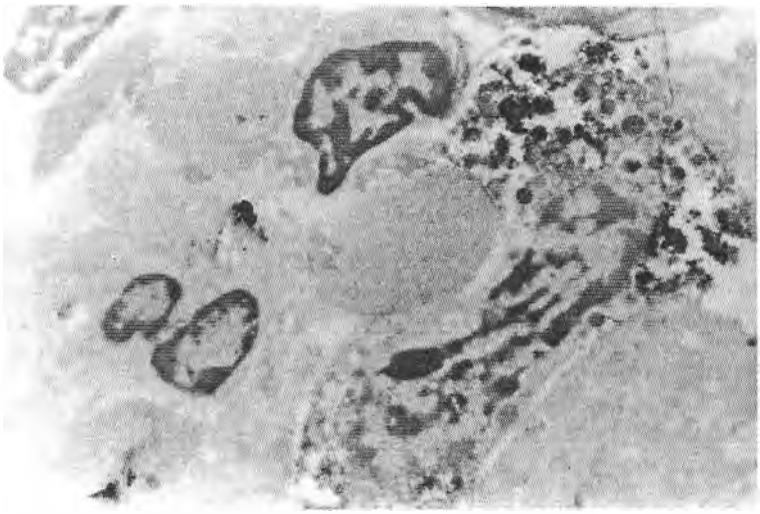

Fig. 1. Tryptase-positive mast cell with many electron dense granules in the portal tract of a liver with metastasis from gastric cancer (× 4400)

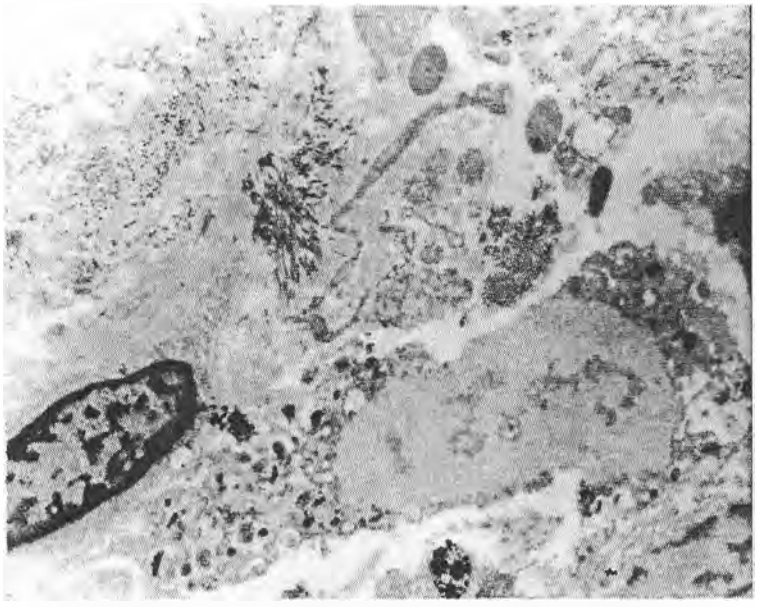

Fig. 2. Chymase-positive mast cell in the liver with metastasis from gastric cancer. Most of the granules are altered with electron-lucent matrices, having sparse amount of reaction product (× 7000)

The "altered granules" with discrete and focally located DAB reaction product observed by us, resembled to these described earlier [4, 19]. The immunoreactivity of mainly chymase and less for tryptase was largely lost in these "altered granules". This can be explained with the degranulation process of mast cells [4]. Chymase and tryptase labelling densities in our study were related to electron dense portions of granules including particulate and lattice patterns, as reported for heparin and histamine [9].

In conclusion, our immunocytochemical ultrastructural study suggested that most of chymase- and tryptase-positive mast cells showed granule diversity with many "altered" granules, considered as a state of activation.

Mast cells in the human common bile duct with obstruction

In the common bile duct with biliary obstruction we detected greatly increased numbers of mast cells [14, 15]. Among the studied mast cell types MC_{TC}-positive ones were the most numerous (mean value of 62.9 ± 14.6 cells/mm^2, $p<0.0001$, Wilcoxon Signed Rank test. VIP-positive mast cells were less in number (26.7 ± 6.6 cells/mm^2), followed by SP-positive mast cells (24.7 ± 6.3 cells/mm^2), and then by mast cells producing only tryptase MC_T (11.3 ± 7.8 cells/mm^2). In addition, some SP-, VIP-, and S-100-positive nerve fibers were observed around glands beneath surface epithelium and in muscle layer. Tryptase-positive mast cells could be found near the nerve cell bodies of multipolar neurons of the ganglionated myenteric plexus. SER-positive nerve fibers and endocrine cells were also found in hyperplastic glands.

Ultrastructural analysis: **Tryptase**-positive mast cell granules were small and large, electron-dense, particulate or with scrolls (Fig. 3). **Chymase**-positive mast cell granules were large as lattice and grating configurations and particulate structure were characteristic for them. The reaction product in both types of cells was precipitated in three different patterns: in large amount over electron-dense granules; or it was sparse over electron-dense regions of the granules (altered granules); or there were observed electron lucent granules without reaction product. We interpreted this as a sign of activation. VIP-positive mast cells contained electron-dense or particulate granules (Fig. 4). SP-positive mast cell granules were mainly of particulate/beaded pattern (Fig. 5) [14, 15].

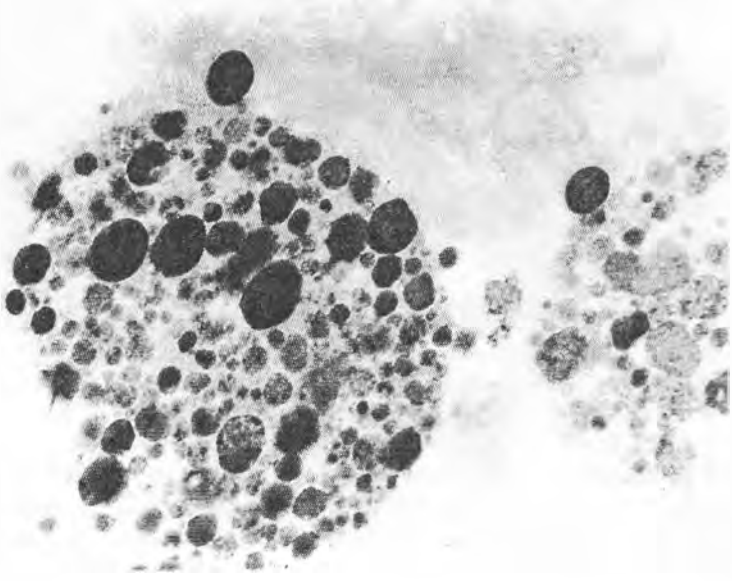

Fig. 3. Tryptase-positive mast cell in the common bile duct of a patient with chronic cholangitis (× 20 000)

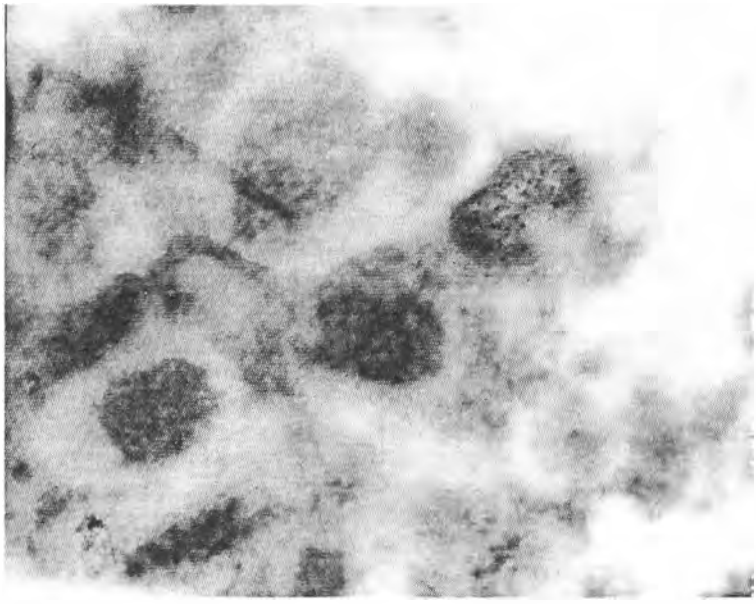

Fig. 4. VIP-positive mast cell granules in the common bile duct of a patient with chronic cholangitis (× 20 000)

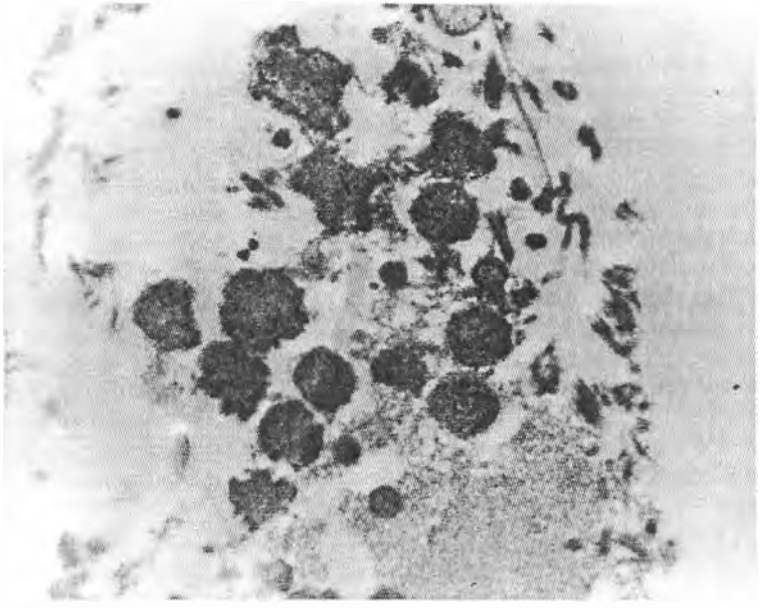

Fig. 5. SP-positive mast cell in the common bile duct of a patient with chronic cholangitis (× 20 000)

VIP-, SP- and chymase-labelled granules are commensurable (with mean values and standard deviations of 0.385±0.14; 0.357±0.8 and 0.360±0.10, respectively, $p>0.05$, t-test). Tryptase labelled granules were two types: large ones with mean value of the diameter

of 0.256±0.04 μm, and small granules with mean value of the diameter 0.109±0.04 um. Both types tryptase-positive granules appeared to be significantly smaller than all the other studied granules ($p<0.0001$, t-test).

Similar prevalence of tryptase-positive mast cells has been reported in human liver diseases [23], at mucosal sites [4] and in lung [7]. Chymase-positive mast cells have been found predominantly in collagen rich connective tissues and to a lesser extent at mucosal surfaces [4].

It is already known that mast cells play a major role among the immune effector cells involved in neuroimmune communication [30]. Previous studies revealed an intimate association between mast cells and neurons in peripheral nervous system [28]. In our study mast cells were detected near the nerve cell bodies of multipolar neurons comprising the ganglionated myenteric plexus. Because of this proximity mast cells appear to act as bidirectional information carriers between the neurons and immune system. It has been reported that nerve endings are remodeled at the acute phase of inflammation due to the degeneration and during recovery stage due to regeneration [27]. Nerve growth factor (NGF) and SP released from peripheral sensory nerves are the cause for mast cell degranulation. The liberated by the mast cells histamine, in turn, activates peripheral sensory nerves [30]. On the other hand, SP may also trigger immune responses (in neurogenic inflammation) via binding to neuropeptide receptors located on other immune cells, such as T-cells, B-cells and macrophages, thus generating immune responses [20, 30, 32]. Therefore, our finding of mast cells near nerve fibers and nerve plexuses implies their role in neuroimmune interactions during chronic inflammation. We found ganglionated myenteric plexus and many S-100- and NSE-positive nerves in the vicinity of mucous glands in the lower part of d. choledochus situated near the sphincter of Oddi. We also observed many serotonin-positive endocrine cells from the EC type with small discoid granules in that region of choledochus. Thus, it can be supposed that lower part of the large bile duct, participates in the regulation of motility and mucous and hormonal secretion of the biliary pathway.

In two previous reports it was shown that skin mast cells in atopic dermatitis [32] and intestinal mast cells in ulcerative colitis [29] are SP-positive. In the current study, we also found SP-positive mast cells and nerve fibers in the inflammatory infiltrate in secondary cholangitis caused by bile obstruction. This suggest that prolonged chronic inflammation caused by biliary toxins stimulates mast cells to synthesize SP within their granules that in turn may stimulate sensory neurons to release more SP, resulting in prolonged inflammation. So, SP in chronic inflammation could be released from both peptidergic nerves and SP-positive mast cells. It stimulates inflammatory cells involved in chronic inflammation [20, 32], as well as mast cells themselves, which may explain the significantly increased population of activated mast cells observed in the choledochal wall.

The great number of VIP-positive mast cells in secondary cholangitis could be explained with the need of smooth muscle relaxation in the obstructed bile duct [10, 15] and of increased mucus secretion from hyperplastic glands in choledochus as a result of biliary intoxication [15, 21].

Since the large bile duct in chronic cholangitis has a thin mucous layer and a thick connective tissue layer (caused by chronic inflammation) the mast cells belong to various subsets. It is known that human mast cells at mucosal sites have more scroll granules (tryptase-positive) and connective tissue mast cells possess "lattice" or "grating-like" configuration in their granules (chymase-positive) [4].

In our study, the large bile duct tryptase-positive granules were electron-dense, particulate or with scrolls. Similar granular morphology was reported also for tryptase-positive mast cells in the human lung [4, 7]. Choledochal chymase-positive mast cell granules were mainly of the particulate and beaded pattern. The ultrastructural immunocytochem-

ical localization of tryptase and chymase revealed 3 distribution patterns of the antigen in mast cell granules: granules with darkly precipitated reaction product, electron lucent granules without reaction product and electron lucent granules with sparse reaction product (altered granules). On the basis of localization of tryptase and chymase and the diameters of granules (large and small in size), these mast cells resembled tryptase/chymase-positive as described earlier [7] in human lung, intestine and skin. The observed "altered granules" resembled those reported earlier by B e i l and P a m m e r [4]. The decrease or lack of immunoreactivity mainly for chymase and less for tryptase in the observed "altered granules" can be explained with the degranulation process of mast cells [4].

VIP- and SP-positive granules were electron-dense and particulate. Their diameters and appearance were like chymase-positive granules. Further investigations with co-localization are necessary to determine whether SP- and VIP-positive mast cells could be also chymase-positive.

Therefore the presence of such variety of mast cells, nerves, ganglionated plexus and endocrine cells in the lower part of the human large bile duct (at a 2 cm distance from the sphincter of Oddi) suggest the importance of this segment in the regulation of the process of motility, evacuation of bile in the duodenum, hormonal secretion, and some other physiological functions in human.

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