Liver tryptase-positive mast cells and fibrosis in children with hepatic echinococcosis

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ABSTRACT

The hepatic echinococcosis in children is a serious surgical problem. The aim of this study is to investigate the participation of mast cells in liver inflammatory reactions triggered by echinococcal cysts. Liver biopsy samples were collected from the tissue surrounding the cysts from 16 sick children (11 boys and 5 girls) in the course of abdominal surgery and from 5 controls. Light and ultrastructural immunocytochemistry was performed using monoclonal antibody against tryptase. Light microscopical immunocytochemistry revealed abundance of tryptase-positive (MCT) mast cells in the capsules of the cysts (43.58 cells/mm²). There were also observed greatly increased numbers of mast cells in portal tracts surrounding the cyst, compared to those of control biopsies (26.49 vs. 1.78 cells/mm², p=0.0009, Mann-Whitney U test). Based on the ultrastructural appearance of tryptase-positive mast cell granules, morphological sings of activation of most of the mast cells were distinguished. In conclusion, we suggest that the accumulated and activated tryptase-positive mast cells in liver tissues surrounding the echinococcal cysts play a crucial role in modulation of the inflammatory liver response and could induce chronic inflammation and fibrogenesis, resulting in serious liver injury such as nonspecific reactive hepatitis.

KEY WORDS: Mast cells, Tryptase, Chymase, Echinococcosis

Hydatidosis or echinococcosis is an infection disease produced by the hydatid cysts, the larval stage of the tapeworms of the genus Echinococcus. Echinococcosis is manifested as disease of variable morbidity and mortality depending upon the species or the strain of Echinococcus involved. The activated onchosphere (embryo), which is liberated in the stomach and small intestine of the intermediate host or man, penetrates through the tissues and reaches an organ, where the development leading to the formation of a hydatid cyst begins. Hydatid disease occurs almost universally in the liver (about 75% of cases), however some other organs are also involved: lung 9%; muscle 5%; spleen and kidney about 2%; brain 1.5%; bones 1%, heart less 1% and other sites, 3.5%.[1]

In hepatic echinococcosis, the liver tissue reaction to the hydatid cysts consists in formation of fibrosis and marked inflammatory changes around the cysts. They may initiate reactive hepatic changes (nonspecific reactive hepatitis).[2] It has been shown earlier that mast cells are a source and a main inducer of FGFs and EGF (fibroblast and epithelial growth factors).[3] Mast cell tryptase promotes connective tissue formation[4] and epithelial cell proliferation.[5] It induces also neutrophil accumulation.[6,7]

Human mast cells contain mainly tryptase (MCₜ). [8] The ultrastructure of mast cell granules with reduced electron density has been widely recognized.[8–15] The ultrastructural characteristic of their granules may give information about the mast cells activation state.[8] Mast cell activation in interstitial cystitis[14] and in acute stress in mice heart[15] was investigated by electron microscopy and the reports definitely showed mast cell secretion.

The aim of the present study is to elucidate mast cells presence in the liver, around hydatid cysts in order to understand their participation in the inflammatory and fibrotic process there. The presence of inflammation and fibrosis around the hydatid cyst could aggravate the postoperative period. These changes should require radical resection (pericystectomy or liver resection), where it is necessary.
MATERIAL AND METHODS

Liver biopsy samples were collected from the tissue, surrounding the hydatid cysts from 16 children (11 boys and 5 girls) in the course of abdominal surgery. The children were between 2 and 15 years of age. In 12 children the cysts were solitary and in 4 – multiple. 3 cysts were complicated with ruptures. Control liver tissue was obtained from 5 young patients undergoing explorative laparotomy because of trauma. Liver tissue samples were processed for routine histology, stained with hematoxylin and eosin. The levels of fibrosis and inflammatory infiltrate in the portal tracts surrounding the hydatid cyst were evaluated semiquantitatively as weak (+) and intense (++). The weak inflammatory infiltrate comprises of scarce numbers of macrophages and lymphocytes, whereas the intense inflammatory infiltrate comprises of large numbers of neutrophils, macrophages and lymphocytes. Nine of the patients had weak, and 7 had intense fibrosis in the portal tracts, whereas the inflammatory infiltrate was weak in the liver of 9 and strong in 7 patients. Light and ultrastructural immunohistochemistry was performed on formalin-fixed paraffin-embedded and fresh samples as described before.[16] Informed consent for the operation was obtained from the parents of each patient. The liver tissue, attached to the capsule was extracted together with the hydatid cyst.

All specimens (size, approx 10 x 8 x 5 mm) were immersion-fixed, and then cryostat sections were prepared for light and ultrastructural immunocytochemistry. The remainder of the tissue was embedded in paraffin for routine histology.

Routine histology
Paraffin sections (5 mm thick) were stained with haematoxylin and eosin and were used to determine the stage of inflammation and fibrosis.

Immunoochemicals
The antibody used was mouse anti-human mast cell tryptase clone AA1 (M7052) (Dako, Glostrup, Denmark) in a dilution 1:50. The detection system was immunostaining kit StrAviGen MultiLink kit, concentrated (LP000-UL; Biogenex, San Ramon, USA). The chromogen used was 3,3’-diaminobenzidine (DAB) (Sigma, St. Louis, MO, USA).

Light microscopical immunocytochemistry
Cryostat sections from liver tissue surrounding the cysts (5-10 mm thick) were used. Quenching of endogenous peroxidase activity was made in 3% hydrogen peroxide in methanol for 30 min, followed by rinsing with 0.1 M phosphate buffered saline (PBS), pH 7.4, for 15 min. Then the sections were incubated in a solution of the primary antibody for 24 h, at room temp. Afterwards, they were reacted with biotinylated anti-mouse antibody for 4 h, and then with peroxidase-conjugated streptavidin, for 4 h, at room temp. Peroxidase activity was localized by using a mixture of 3 mg DAB, in 15 ml 0.05 M Tris-HCl buffer, pH 7.5, and 36 ml 1% hydrogen peroxide for 10–20 min. The reaction was stopped by rinsing in distilled water, before mounting.

Electron microscopical immunocytochemistry
The method has been described earlier.[16] Specimens were immersion-fixed in 0.05 M cacodylate buffer, pH 7.2, containing 2% paraformaldehyde, and 0.2% glutaraldehyde for 2 days at 4°C. After fixation, tissue blocks were incubated in 30% surose in distilled water at 4°C for 24 h. Cryostat sections (20-30 mm thick) were prepared and before the immunocytochemical procedures sections were thawed in 0.05 M cacodylate buffer, pH 7.2, overnight. Quenching of the endogenous peroxidase activity was done as described above. After that, the free-floating sections were incubated with the primary antibody in a dilution of 1: 50 for 24 h at room temp, rinsed in 0.1 M PBS, pH 7.4, incubated with biotinylated anti-mouse antibody in a dilution 1: 20 for 4 h, and then rinsed in 0.1 M PBS, pH 7.4 for 15 min. Later sections were reacted with peroxidase-conjugated streptavidin in a dilution 1: 20 for 4 h, rinsed in 0.1 M PBS, pH 7.4, and then in 0.05 M Tris-HCl buffer, pH 7.5, for 10 min. Peroxidase activity was localized as described above by using DAB-solution, and rinsed in 0.1 M PBS, pH 7.4. Then, sections were postfixed in PBS containing 2% osmium tetroxide for 30 min at 2°C, followed by rinsing in 0.1 M PBS, pH 7.4. Finally, sections were dehydrated in graded concentrations of ethanol and propylene oxide, and flat-embedded with Durcupan, between cellophane sheets. Ultrathin sections were cut of portal tracts surrounding hydatid cysts, and some were cut from the fibrous capsule, where mast cells were accumulated. Ultrathin sections were counterstained with uranyl acetate only and examined and photographed with an EM 109 electron microscope (Zeiss-Opton, Jena, Germany) at 50 kV. All the procedures were carried out immediately after the biopsies were taken.

Negative controls
Control sections were incubated with non-immune sera instead of the primary antibodies.

Quantitative measurements
Mast cells were quantified in sections immunostained for tryptase. The number of mast cells was counted at magnification x 160, as the field of vision at this magnifica-
tion has a size of 0.47 mm² and covers only connective tissue from the capsule or from enlarged and fibrous portal tract. Total numbers of MC₄ in the capsule, were determined in 5 adjacent measuring fields of 0.47 mm² (magnification, x 160) and the mean number of mast cells per mm² was calculated. Total numbers of MC₄ in the portal tracts were determined in 5 randomly chosen portal tracts at magnification x 160, and the mean number of mast cells per 1 mm² was calculated.

**Statistical analysis**

Immunocytochemical and clinical data were analyzed using the StatView™ package for Windows, v.4.53 (Abacus Concepts, Berkeley CA, USA). Basic descriptive statistics were used to calculate mean values and standard deviations (SD). Because of the non-normal distribution of the variables the nonparametric Mann-Whitney U test was used to evaluate the significance of the differences in the number of MC₄ between the controls’ and patients’ samples (independent groups). When p<0.05 differences were considered to be statistically significant.

**RESULTS**

**Routine histology**

Most of the hydatid cysts in our cases had laminated membrane with protoscolices [Figure 1a]. Massive fibrosis surrounded the echinococcal cyst [Figure 1b]. Portal tracts around it were with extensive fibrosis [Figure 1c], forming connective tissue septa in the periporal zone. Perisinusoidal fibrosis in this zone could be observed also. The inflammatory infiltration in fibrous portal tracts [Figure 1c] around the cyst and in the fibrous capsule consisted in monocytes, lymphocytes and eosinophils.

**Light microscopical immunocytochemistry**

In control livers MC₄ were less in number as compared to livers containing hydatid cysts. Single tryptase [Figure 1d]: A portal tract from a control liver with one tryptase-positive mast cell (arrow). Magnifications, a x 250, b x 100, c x 200, d x 300

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**Figure 1a:** A laminated (L) membrane and protoscolices (P) in a hydatid cyst. **Figure 1b:** Fibrous capsule around liver hydatid cyst with inflammatory infiltrate. **Figure 1c:** Enlarged and fibrous portal tract infiltrated with many inflammatory cells, situated near the hydatid cyst. **Figure 1d:** A portal tract from a control liver with one tryptase-positive mast cell (arrow). Magnifications, a x 250, b x 100, c x 200, d x 300
Multiple tryptase-positive mast cells in the fibrous capsule around the cyst. 

Livers with hydatid cysts

Tryptase (Figures 2a)–positive mast cells were multiple in the fibrous capsule around the cyst. In the neighboring portal tracts tryptase-positive mast cells (Figure 2b) were more than those in control livers (Figure 1d).

Mast cells had oval or elongated forms. There could be observed mast cells with granules tightly gathered around the nucleus (Figures 2c). Some mast cells could be seen in the sinusoids around the portal tracts (Figure 3).

Statistical analysis

In controls MC\(_T\) varied from 1 to 3 cells per \(\text{mm}^2\) of portal tract (mean values of 1.78 ± 0.19 cells/\(\text{mm}^2\)).

In sections of livers with hydatid cysts numerous MC\(_T\) (mean value of 43.58 ± 2.67 cells/\(\text{mm}^2\)) could be observed in the fibrous tissue surrounding the cysts. In the fibrous portal tracts their number was also high (mean values of 26.49 ± 5.37 cells/\(\text{mm}^2\)). A statistically significant increase of the number of MC\(_T\) was found in the fibrous portal tracts of the patients with echinococcosis compared to the portal tracts of control patients (\(p = 0.0009\), Mann-Whitney U test) (Figure 4). Also the numbers of MC\(_T\) per \(\text{mm}^2\) in portal tracts with strong fibrosis and intense inflammatory infiltrate appeared to be significantly higher than that with weaker fibrosis and scarce inflammatory infiltrate (30.31 ± 5.79 vs. 23.52 ± 2.48 cells/\(\text{mm}^2\) for fibrosis, \(p = 0.004\); 29.44 ± 5.30 vs. 22.70 ± 2.14 cells/\(\text{mm}^2\) for inflammatory infiltrate, \(p = 0.0008\), Mann-
Electron microscopic immunocytochemistry

Tryptase-positive mast cells had mainly two groups of granules concerning their size. The bigger granules had mean diameter of 0.586 mm ± 0.11 mm and the smaller granules - 0.283 mm ± 0.07 mm. Granules with scrolls and particulate and beaded pattern were observed [Figures 6 a,b].

There were observed three main types of granules concerning their granular content: electron-dense granules with dark precipitated reaction product; electron-lucent granules without reaction product; and electron-lucent granules with sparse reaction product, considered to be activated granules [Figures 6 a,b].

DISCUSSION

To the best of our knowledge, the present study is the first that examines tryptase-positive mast cells in the liver tissue, surrounding hydatid cysts in children. Liver changes such as moderate portal inflammation and fibrosis have been described in various conditions leading to nonspecific reactive hepatitis. In some cases periductal fibrosis and slight cholestasis could be detected. The presence of intense inflammatory infiltration in

**Figure 4:** Numbers of MCT (MC T) per mm² in samples of controls and patients with echinococcosis. (*p = 0.0009, Mann-Whitney U test, MCT in portal tracts of patients vs. MCT in portal tracts of controls)

**Figure 5:** Numbers of MCT (MC T) per mm² in portal tracts of patients with echinococcosis according to the levels of fibrosis and inflammatory infiltrate (Inflam. Inf.) (*p = 0.004, Mann-Whitney U test for MCT of patients with weak (+) vs. intense (++) fibrosis; **p = 0.0008, Mann-Whitney U test for MCT of patients with weak (+) vs. intense (++) inflammatory infiltrate)

Whitney U test) [Figure 5].
liver parenchyma surrounding the hydatid cysts could cause hepatocyte derangements and stimulation of fibrogenesis. Mast cells could participate in the perpetuation of inflammation, the development of fibrosis and aggravation of the pathologic process in the liver. Mast cell occurrence has been investigated mainly in livers with cirrhosis [18] or in hepatobiliary disorders [19] however no such studies of the liver have been performed in echinococcosis in children.

Our study revealed the presence of greatly increased number of mast cells (tryptase-positive) in fibrous tissue surrounding the echinococccal cysts and in portal tracts in the neighboring liver tissue. That suggests their importance in inflammatory and fibrotic reaction in the liver with echinococcosis. In our study mast cells were largely located in portal tracts and less in the perportal sinuoids. In the literature increased numbers of mast cells were found only in the sinusoids-like vessels in human hepatocellular carcinoma[20].

Reports on the ultrastructural aspects of mast cells focused mostly on the morphology of cytoplasmic granules. MCs had granules with grating and lattice substructures, or granules containing discrete scrolls and mainly smaller diameters [21]. In chronically inflamed tissue, a process termed piccemeal degranulation is characteristic for mast cells. It is associated with reduction in granule numbers and in their electron-dense content. [22, 23] The presence of mast cell granules with reduced electron density has been widely recognized. [8, 9, 10, 11, 12, 13, 22, 23, 24, 25] The acute process of mast cell degranulation was studied after stimulation with various substances such as platelet-activating factor (PAF) or calcium ionophore A-23187, compound 48/80 etc. [12, 26] Ultrastructurally it was demonstrated by swollen granules with lost electron density, fusion of granule membranes and formation of degranulation channels. [22]

According to the ultrastructural findings we identified three types of tryptase-positive granules, two of which had reduced electron-dense matrices. These were the electron-lucent granules with sparse reaction product and the electron-lucent granules without reaction product (activated granules). The presence of “activated granules” (called “altered”), containing electron-dense remnants and wide electron-lucent halo was described earlier in human lung tissue surrounding tumors. [30] Therefore, the “activated granules” in our study were considered to be a result of mast cell activation in the chronically inflamed liver tissue in echinococcosis.

The observed association between the presence of great number of activated mast cells and the intense inflammatory infiltrate in the fibrous tissue surrounding the hydatid cysts can be explained with the ability of mast cells to induce neutrophil, monocyte and eosinophil recruitment. [27, 28] It has also been shown that human mast cells stimulate endothelial cells to express leukocyte adhesion molecules, such as VCAM-1 and E-selectin [29] and ICAM-1 and ICAM-3. [30] Thus mast cells contribute to leukocyte extravasation in the liver in echinococcosis. Therefore, we can conclude that the presence of great number of mast cells could be necessary for modulation of the inflammatory response. [7]

Another main function of the tryptase-positive mast cells is the promotion of fibroblast proliferation. [31] In our study we detected increased number of tryptase-positive mast cells situated in the area of active fibroplasia around the cysts and in neighboring portal tracts. Similar data have been reported for the wall of intrahepatic bile ducts in cholestatic hepatitis [32] and for liver fibrosis. [33] Thus, our finding suggest that the accumulation of mast cells positive for tryptase around liver echinococcosis plays a role in active fibrogenesis seen there.

In conclusion, we may state that by their ultrastructural appearance mast cells in echinococcosis showed morphological signs of activation. We also suppose that the accumulation of mast cells around echinococccal cysts in liver could induce chronic inflammation and fibrogenesis, and probably leads to serious liver injury such as nonspecific reactive hepatitis. Therefore, surgeons must have in mind that liver echinococcosis may trigger serious liver chronic inflammatory changes, when they choose the right type of treatment and the precise moment of operation. All these changes discharged the performance of radical surgical methods for hydatidosis in childhood.

REFERENCES
