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Activation of Zinc-Dependent Hepato-Thymic Axis During Liver Regeneration in Hepatectomized Mice

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Zinc is an essential trace element for many biological functions including the processes of normal growth and functioning of the lymphatic system. In this study, we estimated the role of the thymus in liver regeneration following a 1/3 partial hepatectomy (pHx) as well as the role of zinc ions in these processes.
The data have shown that fast liver growth is followed by marked hypoplasia of the thymus and a difference in the proportion of the T-cell phenotype (increase of double negative $CD4^-CD8^-$ and single positive $CD4^+$ and CDB^+ cells, and decrease of double positive $CD4^+CD8^+$ cells). Simultaneously, a significant increase of tissue zind concentration was found both in liver and in thymus. The most pronounced increase was recorded 24 hours (liver) and 48 hours (thymus) after pHx. These results pointed to the possibility that the accumulation of zinc in both organs might be linked to the activity of growth factors which are activated by pHx. Furthermore, it is possible that activation of zinc-dependent thymic hormones or interleukins facilitates the zinc formation of T-lymphocytes with morphogenetic functions participating in the control of liver regeneration.

INTRODUCTION

The liver mass in adult mammals is in a dynamic equilibrium with the body mass. After surgical removal of liver tissue or a toxic injury, normal nonmitotic hepatocytes respond rapidly and proliferate to restore the appro-

priate organ size.[1,2] Several growth factors, derived from the hepatocytes themselves or coming from sources outside the liver, are involved in the triggering, progression and termination of hepatocyte replication.[3,4] Some of them are also related to the function of lymphatic tissue which might have a regulatory role on liver regeneration due to the hepatomodulatory properties of several lymphokines and monokines[5] as well as to the capacity of lymphatic cells to recognize the changes of self constituents after liver damage or after partial removal of hepatic tissue. It has been hypothesized that these processes lead to a breakdown of previously maintained tolerance to self antigens and activation of lymphocytes with morphogenetic capacities, capable of regulating the liver growth.[6] Our previous data emphasized the possible role of activated Kupffer cells in the regeneration of the liver,[7] and described the changes in the phenotype of spleen cells after partial hepatectomy in rats.[8] The consequences of *in vivo* depletion of CD4 and CD8 positive lymphocytes for on hepatic DNA, RNA and proteins[9] were also shown. Recently, the particular roles of the thymus and extrathymic T cells in the liver[10] were emphasized in the control of liver regeneration.

In the light of these findings, in this study we attempted: 1) to correlate the intensity of the changes found in the liver during regeneration with the changes in the thymus of hepatectomized mice, and 2) to elucidate the role of zinc ions in the liver and in the thymus during liver regeneration. Zinc is not only an essential trace element for the function of fundamental enzymes involved in nucleic acids and protein metabolism during the growth,[11] but it is also required for a normal function of the immune system.[11–13] Furthermore, the main thymic hormone, thymulin, also requires zinc ions for its full activity.[14]

EXPERIMENTAL

Induction and quantification of liver regeneration

Male, inbred BALB/c mice, aged 2–3 months, from our breeding colony, were subjected to 1/3 hepatectomy (pHx) under ether anaesthesia by aseptic extirpation of the median lobe. Mice in the control groups were subjected to the same surgical procedure, but without removal of the liver (sham Hx). To avoid diurnal variability, all operations were done between 8.00–9.00 a.m. The animals were sacrificed after 1, 2, 7 or 15 days, and the remaining liver lobes and thymus were aseptically removed for the evaluation of compensatory liver growth and zinc concentrations.

Liver regeneration was monitored by estimation of the total quantity of hepatic DNA, RNA and proteins found in 1/3 of the regenerated liver (obtained from pHx mice) or in 1/3 of the intact liver (obtained from sham operated mice), expressed as wet weight in mg/100 g of body weight of animals. DNA, RNA and proteins were determined by standard methods.[15–17]

Determination of the phenotypic profile of thymocytes in hepatectomized mice

In additional experiments, mice were sacrificed by cervical dislocation and their thymuses were aseptically removed on days 1, 2, 7 or 15 after the pHx or sham operation. Single thymocyte suspensions were prepared in RPMI 1640 by tapping the thymuses on a 100 wire mesh gauze. Red blood cells were lysed by Tris-buffered ammonium chloride for 5 min. After washing, the suspension of cells was filtered through fine nylon mesh, resuspended in complete media and adjusted to the desired final concentration for flow cytometric (FACS) analyses. The expression of membrane markers on thymocytes was assessed by two-colour flow cytometry in a direct immunofluorescence assay. The monoclonal antibodies (mAbs) used were fluorescein isothiocyanate (FITC)-conjugated anti-CD4 and phycoerythrin (PE)-conjugated anti-CD8 mAbs (Becton Dickinson, Mountain View, CA). Data were analyzed by a FACScan (Becton Dickinson 440), using FACScan Research Software. Propiduim iodide (1 μg/ml)-stained dead cells were excluded by electronic gating. Relative fluorescence intensities were expressed in a log scale, with 2 x 10^4 cells analyzed.

Sample preparations for mineralization and determination of zinc in the liver and thymus

Samples of thymus (20–50 mg) or liver (150–300 mg) were prepared according to the modified method of Mascia *et al.*[18] They were dried at 105 °C for 5 hrs, 1 mL of conc. HNO_3 was added, and the mixture was heated at 60 °C for 1 hr on a hot plate. 1 mL of conc. HNO_3 was then added and the mixture was left at ambient temperature for 24 hours. The samples were then heated in a fire-box at 100 °C and then burned to ashes. Ashes were solubilized with 2 successive portions of 7.5 mL of 20% HNO_3 and diluted to 5 mL with deionized water so as to bring the zinc concentration to the optimal analytic range. For controls, parallel mineralization of an analogous sample containing a known amount of zinc, was carried out. The determination of zinc in samples was achieved by ICP spectrometry on a Pye Unicam 7000 spectrometer. Values for zinc found in the tissue were expressed as concentrations (μg/mg) or as the total quantity of zinc in thymus or liver, expressed as mg/100 g of the animal body weight.

Statistical analyses

Results were analyzed using the Sigma Plot Scientific Graphing system, version 4.03. Statistical analyses were performed by two-tailed Student's *t*-tests for unpaired samples. A p value < 0.05 was considered significant. The results are presented as mean +/– SD.

RESULTS

Characteristics of changes in the liver after 1/3 hepatectomy

Removal of 1/3 of the liver in adult BALB/c mice was followed by fast regeneration, characterized by a progressive increase of DNA, RNA and proteins in the remaining hepatic tissue (Figure 1). On the 15th p.o. day the

DNA content in pHx mice was 47% higher than that found in sham operated mice, indicating the persistence of hyperplasia in regenerating liver. Data expressed as RNA/DNA and protein/DNA (not shown) confirmed the presence of an early hypertrophic phase in regenerative response.

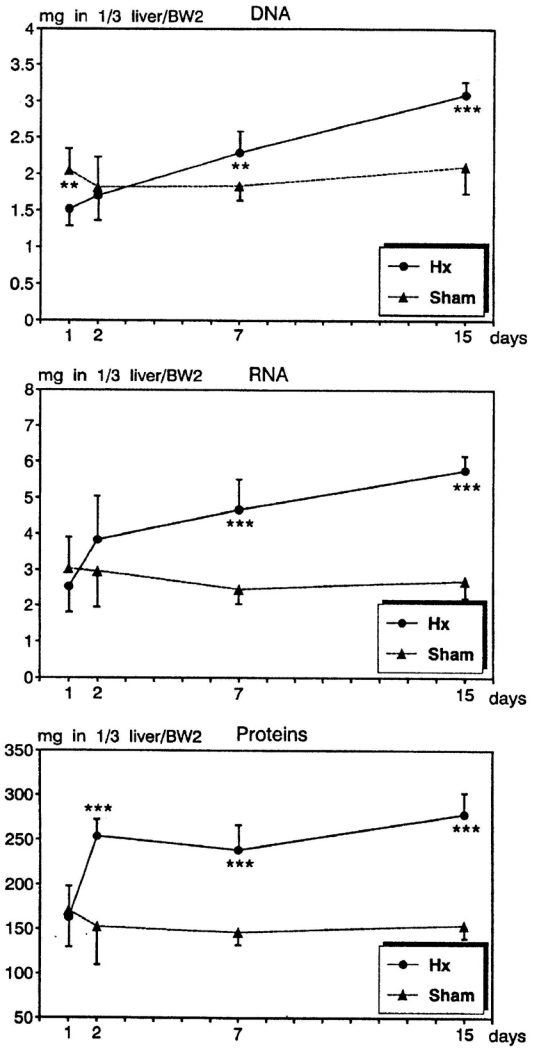

Figure 1. Dynamics of hepatic DNA, RNA and proteins in the liver of partially hepatectomized (Hx) or sham operated mice. Values are presented as mg in 1/3 of liver, expressed on 100 g of total body weight (BW_2). Data are expressed as mean +/– SD with 5–6 mice per group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Kinetic changes of cellularity and T-cell subsets in the thymus after partial hepatectomy

During liver regeneration after partial hepatectomy, a transient hypoplasia of thymus was noticed both in the pHx and in sham operated mice. However, atrophic changes in pHx animals at 48 hrs after pHx were greater than those provoked by operative stress in sham treated animals (Figure 2). Analyses of the expression of CD4 and CD8 antigens in the thymus (Table I.) revealed that, during liver regeneration, the proportion of double negative $CD4^-CD8^-$ cells increased up to the 7^{th} postoperative day (from 2.2 to

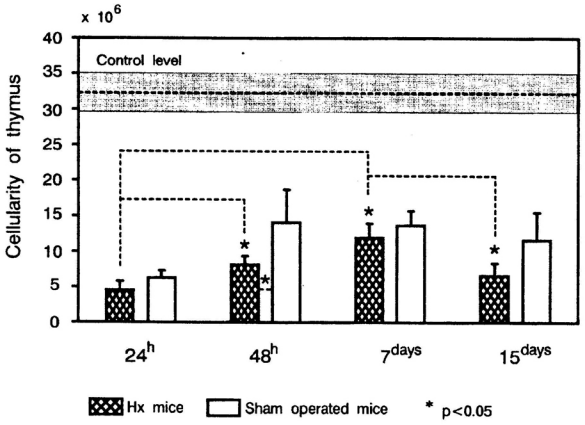

Figure 2. Changes of thymic cellularity after partial (pHx) and sham (sham) hepatectomy. Data are expressed as mean +/– SD with 3 mice per group.

TABLE I

Percentages of lymphocyte subsets of thymus in hepatectomized mice

Thymocyte subsets	Intact mice	Time after the operation					
		24 h		48 h		7 days	
		Hx	Sham	Hx	Sham	Hx	Sham
$CD4^-CD8^-$	2.2	2.37	1.93	2.43	1.56	5.89	4.11
$CD4^+CD8^+$	81.6	79.21	80.03	75.79	78.77	77.47	80.46
$CD4^+CD8^-$	10.9	12.34	10.97	14.26	11.93	9.84	8.47
$CD4^-CD8^+$	5.3	6.08	7.07	7.52	7.74	6.8	6.96

Results are presented as mean percentages of cells, found by flow cytometric analysis in pooled suspension of thymocytes obtained from 3 partially hepatectomized (pHx), sham operated (sham Hx) or intact mice.

5.89%). At the same time, the proportion of double positive $CD4^+CD8^+$ cells dropped to the lowest level (from 81.6 to 75.79%). Furthermore, at the time of prominent thymic atrophy, the proportion of single-positive $CD4^+$ became higher (12.34 and 14.26% at 24 and 48 hour intervals, respectively) and the proportion of $CD8^+$ cells slightly increased (from 5.3 to 7.74%).

Changes in the tissue zinc concentration in the liver and thymus during liver regeneration

The data described above suggest that processes occurring in the liver and thymus after pHx are closely interrelated. Since each of them might be under the influence of zinc, in further experiments the tissue kinetics of zinc in both organs was evaluated. The results showed that, during liver regeneration, the specific accumulation of zinc occurs in both organs. Zinc content in the liver was maximal 24 hrs after pHx. This increase preceded the accumulation of zinc in the thymus which had a peak 48 hrs after pHx (Figure 3). The latter peak of thymic zinc correlated very well with the described changes in the cellularity and phenotype of thymic T-subsets (Figure 2 and Table I). The early peak of zinc in the regenerating liver probably suggests that some of the growth factors that arose after partial Hx are able to stimulate zinc uptake, first in the liver, and later in the thymus. On the 15^{th} post-Hx day, the concentration of zinc in both organs was still increased in comparison with the sham operated and intact controls (Figure 3).

DISCUSSION

Liver regeneration that occurs after partial hepatectomy is a well defined process[1–4] which is accompanied with a unique modification of immune functions.[8–10] Thus, it is well established that partial pHx induces activation of NK and autoreactive T-cells,[19] antigen specific and unspecific suppressor T-cells,[20] as well as modification of T-lymphocyte subsets in peripheral blood and spleen[8] followed by specific activation of unique, intermediate T-cell receptor positive cells in the liver.[10] Our data, showing a greater expression of class II major histocompatibility complex (MHC) antigens on Kupffer cells and other structures in the regenerating liver[7] as well as changes of the phenotypic profile of cells in spleen after Hx, support these findings.[8,9]

The data presented in this work give an additional support to the hypothesis that thymus plays a notable role in these events. Similarly, like after 2/3 hepatectomy,[10] we found that 1/3 partial Hx induces a marked hypoplasia of thymus, followed by an increased proportion of double negative $CD4^-CD8^-$ cells, decreased levels of double positive $CD4^+CD8^+$ cells and increased levels of single-positive $CD4^+$ and $CD8^+$ cells at the time of the prominent atrophic phase (Table I). Since other subsets are all generated

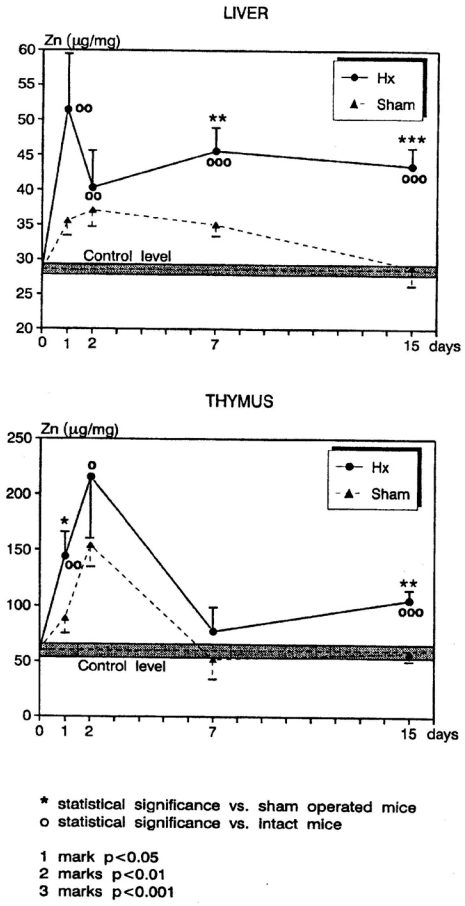

Figure 3. Changes of hepatic and thymic content of zinc in hepatectomized and sham operated mice. Data are expressed as mean +/– SD with 5–6 mice per group.

stepwise from the $CD4^{-}CDB^{-}$ cells, it is likely that these data reflect the activation of self-regulating clones of lymphocytes with acceleration of programmed cell death in the double positive $CD4^{+}CD8^{+}$ stage. Reciprocal inactivation of the intrathymic T cells during the activation of extrathymic T cells in the liver was observed also in mice treated by heat killed *Escherichia coli*.[21]

These results clearly point to a hepato-thymic interaction during the liver regeneration. Additional data showed that these events are followed by an early accumulation of zinc in liver as well as in thymus but with a 24 hr delay. As it is well known, zinc is essential for the function of more than 200 metalloenzymes that play key roles in the metabolism.[22] Among them

are also DNA and RNA polymerase, reverse transcriptase and deoxythymidine kinase,[23] which are activated during the nucleic acid and protein syntheses and the degradation in liver regeneration. Zinc restriction therefore, results in an early impairment of DNA snythesis[24] and wound healing,[25] as well as in an impairment of cell replication in all tissues with a high replication rate. Very sensitive to the zinc supply, in this sense, is also the lymphatic tissue, and recent evidence indicates that zinc ions are required not only for a normal development and function of several lymphatic cells[11–13] but also for the activity of thymic epithelial cells (TEC) which are the main producers of the thymic hormone thymulin,[14] as well as other hormones that influence maturation of T-lymphocytes.[12]

Our data give some new evidence of the activation of the zinc-dependent hepato-thymic axis after partial Hx. Accumulation of zinc in the liver preceded that in the thymus by one day, suggesting that there is a link between some zinc-dependent event in the regenerating liver and in the thymus. This link might be responsible for the activation of self regulating T-cells and/or humoral mediators with growth regulating properties.

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SAŽETAK

Aktivacija o cinku ovisne hepato-timusne osovine tijekom regeneracije jetre u hepatektomiranih miševa

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Poznato je da cink sudjeluje kao kofaktor u brojnim metaloenzimima koji su bitni za različite stanične funkcije. Među njima su i procesi koji kontroliraju fiziološki rast i aktivnost limfatičkog tkiva.

U ovom radu nastojali smo utvrditi da li tijekom regeneracije jetre, koja nastaje nakon djelomičnog odstranjenja jetrenog tkiva, dolazi do promjena u staničnosti timusa i fenotipskoj pripadnosti njegovih T-staničnih subpopulacija, te da li ti procesi mijenjaju tkivne koncentracije cinkovih iona u jetri i timusu. Kontrolne skupine sačinjavali su lažno operirani miševi, a promjene su bile analizirane tijekom 15 dana nakon parcijalne hepatektomije (pHx), odnosno lažne operacije. Utvrdili smo da pHx izaziva hipoplaziju timusa i promjene u proporciji T-staničnih subpopulacija (porast dvostruko negativnih $CD4^-CD8^-$ i jednostruko pozitivnih $CD4^+$ i $CD8^+$ t-limfocita, a smanjenje dvostruko pozitivnih $CD4^+CD8^+$ stanica). Procesi rasta u jetri i promjene u timusu bili su popraćeni nakupljanjem cinka u oba organa. Pritom je nakupljanje cinka u jetri bilo najizraženije 24 sata nakon pHx, dok je nakupljanje cinka u timusu uslijedilo s pomakom od jednog dana (48 sati nakon pHx).

Zaključeno je da bi akumulacija cinka u jetri i timusu mogla biti inducirana djelovanjem određenih faktora rasta, koji se oslobađaju tijekom regeneracije jetre, a koji potom aktiviraju u timusu ovisne procese o cinku, što rezultira stvaranjem hepatoregulacijskih limfocita ili humoralnih tvari, potrebnih za regulaciju rasta preostale jetre.