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Morphometric study of hepatic ultrastructure in alcoholic hepatitis

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Summary. We undertook a morphometric analysis of hepatocellular organelles in an attempt to correlate their changes with the clinical stages of patients with alcoholic hepatitis. Although hepatic ultrastructural alterations did not correlate with disease severity, we found significant differences between patient and control groups in the measured parameters of non-organelle cytoplasm, mitochondria, SER, RER, glycogen, and lipid.

Key words: Morphometry - Ultrastructure - Alcoholic hepatitis

Introduction

A wealth of literature exists on the quantitative alterations of hepatic ultrastructure seen in experimental animals (Reith et al., 1976). Comparable work in the human liver is by contrast scanty. Although numerous ultrastructural studies have dealt with alcoholic liver disease (Porta et al., 1965; Klion and Schaffner, 1968; Grases et al., 1987), morphometric investigation of patients with alcoholic hepatitis in not available. The recent Veterans Administration Cooperative Study on Alcoholic Hepatitis provided us with the opportunity to examine the liver biopsied from patients with alcoholic hepatitis. We undertook the task of comparing the morphometric analysis of hepatic ultrastructure with the clinical stages of disease.

Materials and methods

Patient selection

Twenty-one randomly selected male patients from the Veterans Administration Study on Alcoholic Hepatitis on whom histology was available were stratified into three groups based on the severity of jaundice and degree of coagulopathy (Mendenhall et al., 1984a, b). Group I consisted of 5 patients with compensated liver disease and minimal jaundice (bilirubin less than or equal to 5 mg/dl); Group II consisted of 10 patients with liver decompensation with significant jaundice (bilirubin greater than 5mg/dl) but without severe coagulopathy (prothrombin time less than 4 seconds over control value); and Group III was comprised of 6 patients with both deep jaundice (bilirubin over 5mg/dl) and coagulopathy (prothrombin time prolonged more than 4 seconds). Liver histology was available on all 21 patients as part of their clinical evaluation. The diagnosis of alcoholic hepatitis was based on the criteria established by the International Association for the Study of the Liver (Nomenclature, 1976). The control group was selected from 5 non-alcoholic patients who had mild or no laboratory evidence of liver dysfunction and in whom liver biopsy was done to exclude metastatic or granulomatous disease. Informed consent was obtained from all patients who underwent liver biopsy.

Light microscopy

For light microscopy, a piece of the liver specimen was fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin-eosin, trichrome, reticulum, iron and orcein stains. Light microscopy tissue sections and stains were prepared at the Armed Forces Institute of Pathology and read independently by 3 pathologists.

Electron microscopy

Tissue preparation for electron microscopy was performed at the East Orange Veterans Administration Medical Center. A small fragment of liver was fixed in s-collidine buffered paraformaldehyde (ph 7.35) (Lynn et

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al., 1966) and post-fixed for 1 hr at 4 degree C in 1% osmium tetroxide buffered with 0.1M cacodylate at pH 7.35. After dehydration with graded ethanol and propylene oxide, the fragment was embedded in Spurrs' low viscosity medium. Thick (0.5 μm) sections were cut, stained with methylene blue and evaluated with light microscopy. Ultra thin sections (800-900 Angstrom) were obtained by sectioning on a Porter-Blum MT 2 Ultramicrotome with diamond knife. The sections were then double stained with uranyl acetate and lead citrate, and examined with a Hitachi HU-12 75 KV electron microscope.

Quantitative analysis

From each liver biopsy, six grids obtained from six blocks were prepared and provided for six electron micrographs (3000× magnification). Randomization was accomplished by positioning the microscope screen on a specific corner of the copper grid. Areas without a complete hepatocyte were excluded. Morphometric analysis was conducted to determine the volume density (VD), surface density (SD), and surface to volume ratio (S-V) ratio using the point counting method (Weibel et al., 1966, 1969). VD was measured as a percentage of hepatocyte volume or hepatocyte cytoplasm according to the formula VD=p/P where p is the number of points falling on the structure considered and P the number of points falling on the reference component. The test grid consisted of 195 points 1.5 cm apart drawn on a 8x10 in. plastic overlay. SD was estimated by counting the number of intersections with test lines on a grid system according to the formula SD=2I/LT where I is the number of intersections with the test lines with the element considered and LT is the total length of test lines of the grid system. The test grid used consisted of 11 parallel lines that spanned at 8x10 in. plastic overlay. S-V ratio was derived from a combination of point-counting volumetry with surface estimation by line intersection. The ratio was calculated according to the formula S/V=4N/ZP where N is the number of intersections of test lines with the structure considered, P is the number of test points (ends of test line) on the same structure, and Z the total length of test lines. The multipurpose test grid consisted of linear probes 1.5 cm long with 0.3 cm wide end points. These were arranged 7 in a line, with 11 rows, constructed on a 8x10 in. plastic overlay.

Results

Liver histology. All three groups of patients displayed a spectrum of changes including the presence of fat, Mallory bodies, piecemeal necrosis, cholestasis, portal fibrosis, central perivenous sclerosis, and cirrhosis. HBsAg was not present on orcein stains. These alterations bore no correlation with the clinical severity of disease (Mendenhall et al., 1981).

Subcellular changes. The ultrastructural changes associated with alcoholic hepatitis have been reported in detail (Porta et al., 1965; Klion and Schaffner, 1968), and are not different in this study. Enlarged and disfigured mitochondria with distorted cristae, vesiculation and increase of smooth endoplasmic reticulum (SER), focal cytoplasmic degradation, steatosis, and Mallory bodies were seen in our series of patients.

Morphometric analysis. Among the three groups of patients, the VD, SD and S-V ratio of hepatocellular nuclei, mitochondria, microbodies, lipofuschin, rough endoplasmic reticulum (RER) and lipid did not differ significantly (see Tables 1, 2, 3).The VD of non-organelle cytoplasm, SER and glycogen was similarly unchanged. There was no correlation between the liver subcellular components and the mild, moderate and severe categories of patients with alcoholic hepatitis. To compare the control non-alcoholic patients with mild or no liver disease, the values of the three patient groups were combined. The VD of non-organelle cytoplasm, mitochondria and lipid was significantly greater than that of controls while glycogen was less than control. The SD of mitochondria, SER and RER exceeded the control levels. The S-V ratio of hepatocellular nuclei, lipofuschin and SER was higher than control values.

Table 1. VOLUME DENSITY OF HEPATOCELLULAR COMPONENTS

Component	Control (6)[1]	Group 1 (5)	Group 2 (10)	Group 3 (6)	Combined (21)
Nuclei	9.53 ± 2.51[2]	11.6 ± 2.3	11.7 ± 1.4	13.3 ± 1.0	12.2 ± 0.9
Non-organelle Cytoplasm	0.44 ± 0.28	9.7 ± 3.5	8.0 ± 2.3	10.3 ± 3.5	9.0 ± 1.6*
Mitochondria	15.76 ± 2.07	31.0 ± 1.9	24.7 ± 1.4	26.0 ± 3.3	26.6 ± 1.3*
Lipofuschin	1.36 ± 0.94	0.2 ± 0	0	1.5 ± 0.8	1.1 ± 0.6
Microbodies	1.64 ± 0.99	2.0 ± 0.3	2.7 ± 0.5	2.3 ± 0.8	2.4 ± 0.3
SER	0	5.0 ± 2.2	9.6 ± 2.0	6.3 ± 1.1	7.5 ± 1.2
Lipid	0	1.5 ± 1.2	7.1 ± 4.2	2.1 ± 1.2	4.4 ± 2.2
Glycogen	45.54 ± 4.10	10.3 ± 2.8	14.0 ± 4.6	8.4 ± 2.8	11.5 ± 2.4*
RER	5.70 ± 1.90	7.4 ± 1.4	6.9 ± 1.3	7.8 ± 1.1	7.3 ± 0.7

1. Number of patients.
2. Mean ± standard error of mean, percent of hepatocyte cytoplasm, except for cytoplasm where it is percent of hepatocyte volume.
* Significantly different from control, $P < 0.05$.

Table 2. SURFACE DENSITY OF HEPATOCELLULAR COMPONENTS

Component	Control (6)[1]	Group 1 (5)	Group 2 (10)	Group 3 (6)	Combined (21)
Nuclei	0.0 ± 2.25[2]	11.1 ± 0.9	12.0 ± 1.0	12.7 ± 0.3	12.0 ± 0.5
Mitochondria	92.67 ± 12.54	131.1 ± 14.1	143.8 ± 10.5	141.4 ± 13.2	140.1 ± 6.8*
Lipofuschin	6.67 ± 4.34	2.0 ± 0	0	4.0 ± 0	3.3 ± 0.7
Microbodies	6.67 ± 2.46	8.0 ± 1.3	15.9 ± 4.1	13.3 ± 2.4	14.0 ± 2.4
SER	0.67 ± 0.67	28.0 ± 17.5	42.3 ± 7.2	21.3 ± 6.1	32.4 ± 5.4*
RER	19.30 ± 5.41	39.0 ± 7.6	35.1 ± 7.7	52.2 ± 7.4	40.9 ± 4.7*
Lipid	0	4.4 ± 1.8	12.7 ± 10.5	3.2 ± 2.2	8.0 ± 4.7

1. Number of patients
2. Mean ± standard error of mean, μ^2/μ^3 of hepatocyte cytoplasm
* Significantly different from control, $P < 0.05$

Table 3. SURFACE-VOLUME RATIO

Component	Control (6)[1]	Group 1 (5)	Group 2 (10)	Group 3 (6)	Combined (21)
Nuclei	1.04 ± 0.23[2]	5.0 ± 2.3	9.6 ± 1.5	11.2 ± 2.0	9.0 ± 1.2*
Mitochondria	3.92 ± 0.40	7.7 ± 4.8	3.2 ± 0.1	12.4 ± 0.6	6.9 ± 2.1
Lipofuschin	0.83 ± 0.54	2.0 ± 0	0	14.0 ± 6.0	10.0 ± 5.3*
Microbodies	1.67 ± 0.80	1.9 ± 0.1	15.5 ± 7.4	4.6 ± 2.4	10.1 ± 4.2
SER	0.33 ± 33	3.9 ± 1.4	4.6 ± 0.4	2.8 ± 0.50	3.8 ± 0.40*
RER	3.11 ± 1.84	3.8 ± 0.2	3.3 ± 0.5	4.2 ± 0.60	3.7 ± 0.30
Lipid	0	1.8 ± 1.0	1.1 ± 0.4	0.50 ± 0	1.3 ± 0.40

1. Number of patients
2. Mean ± standard error of mean
* Significantly different from control, $P < 0.05$

Discussion

Our control values for hepatic organelles differ markedly from those previously reported. Our VD is approximately ten fold, and the SD approximately a hundre fold higher than those determined for normal human liver (Rohr et al., 1976). The discrepancy may be explained, in part, by a difference in the number of points counted (350 versus, 3,630), magnification of electron micrographs (3000 versus 37,000) and the levels of magnification taken for correlation. Rohr et al. (1976) include determination by light microscopy for reference whereas we base ours on ultrastructure alone. Another source of error is that we did not have a standard period of primary fixation of the liver specimens which were obtained from six centers. Nevertheless, we have data that are internally consistent within the control and patient groups, and this provides a basis for comparison.

It is widely held that liver histology does not predict clinical severity in patients with alcoholic liver disease (Rankin et al., 1978). Our study demonstrates that hepatic ultrastructure also does not correlate with clinical manifestations in patients with alcoholic hepatitis. The lack of correspondence between subcellular alterations and the severity of alcoholic liver disease has been previously suspected (Horvath et al., 1973). However, it may be argued that our patient groups had severe disease and were already in the late stage of evolution, and the morphologic quantitation would be more appropriate if comparison was made with a mild and early phase of alcoholic liver injury. Although intergroup values did not differ, significant changes occurred between the controls and patients in terms of hepatocellular non-organelle cytoplasm, mitochondria, SER, RER, lipid and glycogen. These differences have been reported on a qualitative basis (Porta et al., 1965; Klion and Schaffner, 1968).

The increase of non-organelle cytoplasmic volume accounts, in part, for the enlargment of the hepatocyte that marks the early phase of alcoholic injury. Prior to the appearance of fat, the increase in cell size is due to the accumulation of water and protein (Orrego et al., 1981). The cellular hypertrophy narrows the sinusoidal space resulting in portal hypertension and collagenization of the space of Disse (Blendis et al., 1982). This progression of events forms a major element in the pathogenesis of alcoholic liver disease. Large hepatocytes also are encountered during the cirrhotic stage (Kaneda and Takahashi, 1970). Our study confirms that the enlargement of the liver cells is due to the increased cytoplasmic but not nuclear volume.

We found that the mitochondria is consistently enlarged in the mild, moderate and severe stages of alcoholic hepatitis, representing a characteristic feature of alcoholic liver ultrastructure. Its appearance has been linked to the amount of ethanol consumption, malnutrition, copper deficiency and hypervitaminosis A (Uchida et al., 1984). Our recent study reveals that the presence of megamitochondria is associated with a more benign course of alcoholic hepatitis (Chedid et al.,

1986). At the morphometric level, no differences can be discerned in the mitochondria among patients with varying clinical manifestations. Both mitochondrial VD and SD in patients are approximately one and a half times those of the controls, but the S-V ratio for the two groups remains similar. This suggests that although the mitochondrias are enlarged, their mean width does not increase (Weibel et al., 1969).

Both patient and control hepatocytes display an amount of RER that exceeds the SER. Some studies suggest the reverse is true for the human liver (Jezequel et al., 1974). We cannot account for the discrepancy except that our controls were not healthy individuals. However, the increase of SER is attributable to the ingestion of ethanol (Rubin et al., 1968). That RER appears consistently greater than control level has not been reported. The amount of RER is said to be normal in most cases of alcoholic liver disease (Hovrath et al., 1973).

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