Collagenisation of the Disse space in alcoholic liver disease

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SUMMARY Collagenisation of the space of Disse was systematically assessed to determine its relationship to the clinical and histological manifestations of chronic alcoholic liver disease. Ninety-four chronic alcoholics who had been submitted to biopsy were assessed by clinical manifestations of hepatic dysfunction and by a 17-parameter Combined Clinical and Laboratory Index (CCLI). Liver biopsies were scored for light (LM) and electron-microscopy (EM) abnormalities using a universal scoring system for both. Thirty-five patients with normal liver histology (LM) had an average collagen score of 0.6 ± 0.1 . Twelve cirrhotic patients and 29 with fatty liver, both groups with mild clinical manifestations, did not differ significantly. In 18 cirrhotic patients and five with fatty liver, both groups having severe clinical manifestations, the mean scores were 2.1 ± 0.8 ($P < 0.02$) and 2.5 ± 0.6 ($P < 0.01$) respectively. Collagenisation also correlated with CCLI ($P < 0.001$), serum bilirubin ($P < 0.001$), serum aspartate transferase (SGOT) ($P < 0.003$), and clinical evidence of portal hypertension and histological changes of necrosis, inflammation, and terminal hepatic vein sclerosis. These results suggest that collagenisation of the Disse space may be important in the pathogenesis of alcoholic liver disease.

The hepatic perisinusoidal space of Disse separates the endothelial lining cells of the hepatic sinusoids from the plasma membrane of the hepatocytes and constitutes the microenvironment from which exchange between the parenchymal cells and blood takes place. Normally, electron microscopy reveals either no collagen fibrils within the space, or occasional bundles of up to 10 fibrils (Popper *et al.*, 1961). In the early lesions of alcoholic liver disease these fibrils have been observed to be present in increased numbers to form bundles of up to 100 fibrils which cause considerable widening of Disse's space (Popper *et al.*, 1961; Schaffner and Popper, 1963; Phillips and Steiner, 1965). In more advanced alcoholic liver disease these thick collagen bundles form a continuous layer which, together with the formation of a basement membrane, may impede the interchange between the blood and hepatocytes and thus interfere with hepatic function (Schaffner and Popper, 1963). Furthermore, the widened space

of Disse, by decreasing the sinusoidal diameter, could increase the resistance to sinusoidal blood flow and thus raise portal pressure (Reynolds *et al.*, 1969; Boyer *et al.*, 1967; Kluge *et al.*, 1970; Sama *et al.*, 1971).

However, the relationship between ultrastructural changes in the space of Disse and the clinical manifestations of alcoholic liver disease have never been studied in a systematic way. We report here an attempt to assess the significance of such changes.

Methods

SUBJECTS

Ninety-four chronic alcoholics, with a daily alcohol consumption of more than 80 g for more than 10 years, were assessed clinically and biochemically for evidences of liver disease. The following laboratory tests were performed on each patient: serum aspartate transferase (SGOT), serum gamma glutamyl transpeptidase (GGT), serum alkaline phosphatase, serum bilirubin, serum albumin, prothrombin time, and haemogram. A liver biopsy was performed on each patient.

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Patients were divided according to the clinical, laboratory, and histological findings in the following way:

Normal

Thirty-five chronic alcoholic patients without clinical or laboratory evidence of liver disease and with normal liver histology on biopsy (LM);

With liver disease

This group of 59 patients was further subdivided into two categories:

Group A Forty-one patients with a minimal clinical manifestation of hepatomegaly and biochemically with an increase in SGOT of up to 100 U (normal 7–17 U) and SGGTP of up to 100 U (normal up to 27 U);

Group B Eighteen patients with marked clinical manifestations including spider naevi, palmar erythema, evidence of collateral circulation, hepatomegaly, jaundice and ascites; biochemical abnormalities included raised bilirubin levels, depressed serum albumin concentrations, and grossly raised liver enzyme levels. However, in all patients the prothrombin time was prolonged less than three seconds, permitting a liver biopsy.

The severity of the liver disease was assessed in each patient by a composite clinical and laboratory index (CCLI), based on the concept that the severity of the disease is reflected by the number of abnormal clinical and laboratory findings (Rankin *et al.*, 1978). These are added to give a final numerical score ranging from 0–27. The index is made up of 17 separate clinical and biochemical findings, and its validity has been discussed elsewhere (Orrego *et al.*, 1979).

TECHNIQUES

Transmission electron microscopy

Specimens studied by electron microscopy (EM) were fixed in a combination of 4% formaldehyde and 1% glutaraldehyde (MacDowell and Trump, 1976).

Specimens were cut on a Porter Blum MT2 with glass or diamond knives, stained with uranyl acetate and lead citrate and examined with a Philips 301 electron microscope. Sections approximately 0.5 μ thick were stained with toluidine blue for lobular localisation.

One of the most important methodological problems that a study of this sort entails is the possibility of sampling errors. This factor, which has to be considered also in liver biopsies observed

under light microscopy (Scheuer, 1970), is greatly amplified in electron microscopic studies where the size of the zone observed is representative of a very small part of the total organ. Therefore, we adhered to certain criteria in an attempt to minimise this possibility.

The following framework was used in order to minimise the problem of sampling error and to ensure objectivity in grading the observed abnormalities:

1. The specimens were examined and classified by one of the authors (A.M.), who was blind to the clinical classification of the patient.

2. In all specimens thick sections stained with toluidine blue were observed for lobular localisation. It is recognized that this involves the problem of a certain degree of selection, and decreases the randomisation that we would have preferred. However, the 'blindness' of the pathologist with respect to clinical data ensured that these areas were not selected to fit a preconceived hypothesis. Furthermore, in the case of cirrhosis, the scores were compared only with other cirrhotics and not with other patient groups. The area for analysis was selected remote from portal triads and from scarred areas in abnormal livers. Once the area was selected, only the first four Disse spaces observed in the

Fig. 1 Grading of collagen fibrils in the Disse space (see Methods section for details).

selected specimen were photographed in their entirety, from magnifications ranging from 1000 to 25 000. At least 20 photographs were taken from each specimen. Irrespective of what might be seen in the fifth Disse space, only the findings in the first four observed on a random basis were graded

according to the devised scoring system.

3. A scoring system was devised to grade the degree of change observed in the space of Disse (Fig. 1). The items included in the system were the degree of collagenisation of the space, the presence or absence of basement membrane and/or the

Fig. 2 A. Fine structure of normal space of Disse with few collagen fibrils (arrows) between endothelial cell (E) and hepatocyte (H). $\times 20,800$ original magnification. B. Space of Disse widened by many (2+) fibre bundles (C arrows). The microvilli on the sinusoidal surface (S) of the hepatocyte are almost completely absent. $\times 26\,000$, original magnification.

presence of intercellular collagen. The maximum cumulative score obtainable in this scale is 5.5. Individual elements were graded as follows:

Collagenisation 0+, when minimal or non-existent; 1+ or 2+ (Fig. 2), when appearing in bundles widening the Disse space and flattening the microvilli of the hepatocytes, and graded according to the length of involvement of the space of Disse.

Formation of basal membranes As a continuous electron-dense material with a clearly defined structure separating the endothelial cells from the hepatocytes. When this finding was present it was assigned a score of 3+.

Collagen in intercellular space When present, 0.5% was added to the score.

When the above abnormalities were not diffuse, so that parts of the Disse space appeared abnormal, 0.5+ was subtracted from the score.

4. Reproducibility In order to assess the degree of reproducibility of the scoring of the sample, 10 micrographs were scored twice by the same pathologist using two different identification codes. The reproducibility was excellent, in that in each biopsy the first and second scores were identical.

Grading of light microscopy findings

Necrosis Severity was graded on a 1+ to 3+ scale: 1+, hepatocellular necrosis in zone 3 (Rappaport, 1973) or erosion of the limiting plate; 2+, more severe changes, widespread hepatocellular degeneration, some disorganisation of the liver cell plates, secondary collapse; 3+, diffuse necrosis, distortion of lobular organisation, confluent hepatocellular degeneration, and necrosis completely surrounding the hepatic acini.

Fibrosis (portal) 1+, expanded portal triads with increased connective tissue; 2+, fibrosis extending into the lobule, but not bridging portal triads with terminal hepatic venules; 3+, fibrosis bridging portal triads and terminal hepatic venules.

Inflammation 1+, zonal localisation; 2+, moderate, not confined to one zone of the acinus; 3+, diffuse.

Mallory bodies 1+, less than 10% of the hepatocytes contain Mallory bodies; 2+, more than 10% but less than 25% of hepatocytes contain Mallory bodies; 3+, more than 25% of liver cells contain Mallory bodies.

Fat 1+, less than 25% of the cells contain fat droplets; 2+, 25% to 50% of cells contain fat; 3+, over 50% of cells contain fat droplets.

Terminal hepatic vein (THV) sclerosis 1+, a rim of fibrous tissue around THV; 2+, extensive fibrosis around THV, lumen of vein still present; 3+, extensive fibrosis, lumen of vein obliterated. The total score was then divided by the number of THV observed in the liver biopsy and an average score was obtained.

Statistics Data are expressed as means $\pm$ standard error. When groups were compared, Student's *t* test was used. Analyses of correlations were performed using a special computer programme (SPSS). As values for some of the laboratory tests (serum enzymes) were not normally distributed, logarithmic conversion of these results was carried out before statistical calculations.

Results

Of the 41 patients classified clinically as mild (group A), 29 had fatty liver and 12 cirrhosis with and without hepatitis on histology. Of the 18 patients classified clinically as severe (group B), 13 had cirrhosis with and without hepatitis and five had fatty liver on histology (Table 1).

The mean scores for the changes in the space of Disse of patients in group A, irrespective of whether they had fatty liver or cirrhosis on biopsy, were not significantly different from those of the normal group (Table 1). In contrast, the 13 patients in clinical group B with cirrhosis with or without hepatitis on biopsy had a mean EM collagen score that was significantly different not only from normal patients and group A patients with fatty liver

Table 1 Histological and clinical severity of liver disease and Disse space collagen score

Histological diagnosis	Patients (no.)	Clinical group	Average score*	Difference
Normal	35		0.6 $\pm$ 0.1	
Cirrhosis with/without hepatitis	12	A (mild)	0.5 $\pm$ 0.3	P < 0.02
Cirrhosis with/without hepatitis	13	B (severe)	2.1 $\pm$ 0.8	
Fatty liver	29	A (mild)	0.7 $\pm$ 0.2	P < 0.01
Fatty liver	5	B (severe)	2.5 $\pm$ 0.6	

*Score indicates the electron microscopic rating of extent of collagen accumulation in Disse's space, on a scale of 0-5.5+, as explained in Methods section.

Table 2 Collagen in Disse space and clinical evidences of portal hypertension (ascites and/or splenomegaly and/or collateral circulation)

Clinical evidence of portal hypertension	Patients (no.)	Score of collagen in Disse space		P
		0-2.4 (%)	2.5 and over (%)	
Absent	41	97.6	2.4	<0.0001
Present	18	16.7	83.3	<0.0005

($P < 0.01$), but also from the group A patients with cirrhosis ($P < 0.02$). The patients in clinical group B who had only fatty liver on histology, but who presented moderate ascites, had a mean EM collagen score that was significantly different from the 29 patients with fatty liver in clinical group A (Table 1). The 94 patients were divided into two groups on the basis of the degree of collagen found in Disse's space. Those with collagen scores of 0-2.4 ($N=76$) had a mean CCLI score of 2.2 ± 0.4 , while those with collagen scores of 2.5 or more ($N=18$), had a mean CCLI of 10.0 ± 4.2 . The difference in collagen scores was highly significant ($P < 0.001$). Furthermore, there was a significant positive correlation between the EM collagen score and the CCLI when compared in all 94 patients in the series (Fig. 3).

Finally, the relation between the EM collagen score and the clinical manifestations of portal hypertension was assessed by comparing the groups with high and low EM collagen scores, divided again at the 2.5 mark, with respect to the presence or absence of ascites and/or splenomegaly and/or evidence of collaterals. There was no clinical evidence of portal hypertension in over 97.6% of patients with no, or minimal, collagen deposition, whereas 83.3% of patients with high EM collagen scores had evidence of portal hypertension, this association being statistically highly significant (Table 2).

Collagen in Disse's space was found to be correlated positively with the serum bilirubin ($P < 0.001$) and SGOT ($P < 0.003$) levels, but not with alkaline phosphatase or albumin. Among the histological findings on light microscopy, necrosis ($P < 0.002$), inflammation ($P < 0.003$) and terminal hepatic vein sclerosis ($P < 0.002$) correlated positively with Disse space collagen. On the other hand, no

correlation was found with fibrosis as detected on light microscopy, with fat accumulation or with the presence of Mallory bodies (Table 3).

Discussion

The results in this study have shown highly significant correlations between the degree of collagenisation and capillarisation of the space of Disse and the severity of clinical manifestations and biochemical

Fig. 3 Correlation of the combined clinical and laboratory index (CCLI) with the grading of collagen in the Disse space in 94 chronic alcoholics with and without liver disease.

Table 3 Correlation between collagen in the Disse space and laboratory and histological abnormalities in 94 chronic alcoholics

Laboratory abnormalities	P	r	Histological abnormalities	P	r
Bilirubin	<0.001	0.34	Necrosis	<0.002	0.31
SGOT	<0.003	0.31	Inflammation	<0.003	0.30
Albumin	NS	0.18	Terminal hepatic vein sclerosis	<0.002	0.35
Alkaline phosphatase	NS	0.23	Mallory bodies	NS	0.19
			Fibrosis	NS	0.03
			Fat	NS	0.08

changes associated with hepatic dysfunction in alcoholic liver disease. Before discussing the importance of these findings it is necessary to examine the significance of the data itself.

Information obtained from microscopic examination suffers from the handicap of being 'soft', in that it depends entirely on the criteria of the observer as compared with the instrumental result of a biochemist. Nevertheless, within the limits of such an investigation, by laying down as rigid criteria as possible for scoring, by adhering to them throughout the study, and by using the same observer, one can minimise this source of error as seen by the excellent reproducibility found when the same samples were scored again for EM collagen. In addition, such an investigation also encounters the problem of sampling, which is exaggerated in an EM study by the smallness of the sample examined. This is minimised in diffuse liver disease by the similarity of the changes throughout the liver. It is maximum in the cirrhotic liver because of the differences that occur depending on whether the sample is in the middle or edge of a regeneration nodule or whether it is close to a portal tract or central vein. Again, an attempt was made to minimise the sampling problem by first attempting to analyse similar sites in the lobule in each specimen, secondly by grading only the first four spaces of Disse observed at random, and thirdly by the 'blindness' of the observer to the clinical conditions of the patients.

With these reservations in mind, it would be extremely unlikely that by chance all the samples from cirrhotics in clinical group A would be chosen from areas with minimum EM lesions, while all those from group B would be chosen from areas with marked EM lesions.

On the other side of the equation is the problem of clinically assessing hepatic function. In this study we have used two methods. One consisted of dividing our patients into two groups, one mild with only hepatomegaly as commonly found in fatty liver, and the other moderate or severe with many of the other clinical and biochemical manifestations such as spider naevi, palmar erythema, ascites and jaundice, plus abnormal bilirubin, albumin, and enzyme levels. The second method was a combination of both clinical and biochemical abnormalities, which gives a 17-factor index. In a previous study this combined clinical and laboratory index was found to be a good monitor of acute changes in hepatic function and to be statistically better than any single parameter used in the index (Rankin *et al.*, 1978).

It is of interest, therefore, that assessing the patients by both methods produced the same result—namely, that a deterioration in function was asso-

ciated with increased changes in Disse's space. Previous studies have drawn attention to these changes and have suggested that they are early indications of alcoholic liver disease (Popper *et al.*, 1961; Schaffner and Popper, 1963; Phillips and Staines, 1965; Reynolds *et al.*, 1969; Edmondson *et al.*, 1967). By studying a large group of patients systematically we have been able to show that, on the contrary, in patients who show no clinical, biochemical, or histological light-microscopy evidence of liver damage after drinking heavily for more than 10 years, the so-called 'normal' group, there were no EM abnormalities in the Disse space. Furthermore, significant collagenisation occurred only with gross clinical and biochemical evidence of liver dysfunction. A good example of this are the five patients with fatty liver who presented clinically with moderate ascites. Collagenisation of the Disse space was as marked in these patients as in the cirrhotics with evidence of portal hypertension. Ascites in association with fatty liver is uncommon, but it is well described (Rankin *et al.*, 1978), although the mechanism has not been clarified. On the other hand, cirrhotics in group A with minimal clinical abnormalities had the same lack of collagen in the Disse space in the zone observed as did alcoholics with biopsies showing normal livers. Also, the positive correlation found between collagen in the Disse space and serum bilirubin and SGOT levels, and with necrosis and inflammation on histology, points to an association of this lesion with activity of the liver disease.

The low correlation coefficients (r) found in all of these correlations is consistent with the possibility that both changes in severity of liver disease and in histology reflect a multifactorial pathogenesis, of which the abnormalities in the space of Disse are only one factor.

What is the significance of these observations? The Disse space is a highly specialised lymphatic vascular bed characterised by an absence of a basement membrane, and by fenestrations in the endothelial lining through which the microvilli of the plasma membrane of the hepatocyte can project into the lumen (Layden *et al.*, 1975; Jones and Schmucker, 1977). Thus, in this micro-environment the absorptive surface of the hepatocyte is uniquely exposed to the circulation, which facilitates the transfer of plasma constituents and of oxygen into the hepatocyte. With collagenisation of the space and the interposition of a basement membrane it is to be expected that this process would be hindered and hepatic function impaired (Schaffner and Popper, 1963; Phillips and Steiner, 1965; Tanikawa, 1975). For example, it has been shown that hypoxia in chronically alcohol-treated rats can lead to liver cell

necrosis (Israel *et al.*, 1977). Thus, interference with oxygen diffusion from the sinusoids into the liver cells could additionally contribute to liver cell necrosis in the alcoholic.

Furthermore, a diminution of the sinusoidal diameter caused by widening of the Disse space could result in an increase in portal pressure (Sama *et al.*, 1971). It has been postulated that this mechanism is responsible for the portal hypertension found in idiopathic portal hypertension (Boyer *et al.*, 1967; Kluge *et al.*, 1970; Sama *et al.*, 1971). Alternatively, these EM changes may simply be a consequence of prolonged hepatic dysfunction and increased sinusoidal pressure due to, for example, an increase in the size of the hepatocytes. Whatever the mechanism, the findings in this study would seem to be important in the understanding of the pathogenesis of hepatic dysfunction and portal hypertension in alcoholic liver disease.

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