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Copper/zinc and manganese superoxide dismutase immunoreactivity in hepatic iron overload diseases

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Summary. Iron overload to the liver induces hepatic injury, eventually ending up with liver fibrosis or cirrhosis. Pathogenic mechanisms involved in liver damage are only partially known, but there is evidence for an important role of iron-induced reactive oxygen species. We have, therefore, analyzed the immunohistochemical reactivity for two major free radical scavengers, copper/zinc and manganese superoxide dismutase (Cu/Zn- and Mn-SOD's) in three situations of hepatic iron overload, and compared enzyme patterns with grades of iron deposition, grades of fibrosis, and levels of microphotometrically measured type IV collagen immunoreactivity. Cu/Zn- and Mn-SOD reactivity was detectable in hepatocytes with a heavy and a low iron burden, but Cu/Zn-SOD staining was more intense than that of Mn-SOD in the three groups analysed. There was trend for microphotometrically measured type IV collagen levels to increase with the amount of iron, and increased collagen IV was correlated with higher grades of Cu/Zn-SOD, but not of Mn-SOD, reactivity. The findings suggest that the two SOD's may be differentially expressed in states of hepatic iron overload, and that low expression of the inducible radical scavenger, Mn-SOD, may play a role in chronic iron toxicity.

Key words: Hemochromatosis, Superoxide dismutase, Type IV collagen, Iron

Introduction

Chronic iron overload to the liver caused by genetic defect(s) of iron metabolism (genetic hemochromatosis) or due to other disorders (e.g., transfusional iron overload, alcoholic liver disease, hemoglobinopathies) induces liver injury, eventually ending up with hepatic fibrosis and cirrhosis (Deugnier et al., 1992). Hepatocellular damage and fibrosis seem to be directly related to the iron content of the hepatocytes and, therefore, the pattern of damage is similar irrespective of the aetiology (Sherlock and Dooley, 1993). There is experimental evidence for a direct profibrogenic effect of iron (Weintraub et al., 1985; Brissot et al., 1987; Park et al., 1987; Carthew et al., 1991). Early activation of hepatic genes in the iron-fed rat occurs in the absence of histologic changes (Pietrangelo et al., 1990), and recent findings indicate that selective localization of iron into liver parenchymal cells is required for the activation of collagen gene during long-term iron overload in rodents (Gualdi et al., 1994). On the other hand, mechanisms operational for hepatocellular damage are complex and seem to involve several pathways. Iron causes lipid peroxidation of membranes of organelles leading to functional defects of endoplasmic reticulum, mitochondria and lysosomes (Bacon and Britton, 1990; Myers et al., 1991), probably via a free radical-mediated process. In fact, iron can induce the production of free radicals in vitro (Halliwell and Gutteridge, 1985), and in particular nucleotide complexes of iron (Tien et al., 1981) and circulating low-molecular weight iron complexes (Gutteridge et al., 1985) are initiators of lipid peroxidation and promoters of hydroxyl radical formation.

Defense against oxidants in the liver is mediated by enzymatic and nonenzymatic systems (Tribble et al., 1987). Among free radical scavengers, superoxide dismutase (SOD), which catalyzes dismutation or destruction for most of superoxide anions, is considered as a key enzyme in protecting cells against oxidative injury (Gregory and Fridovich, 1973; Fridovich, 1975). SOD consists of two types, i.e. copper/zinc SOD (Cu/Zn-SOD), demonstrated throughout the cell, and manganese SOD (Mn-SOD), located mainly in the mitochondrial matrix (Slot et al., 1986; Bannister et al., 1987). Roles in vivo of these enzymes include prevention of injuries mediated by oxygen-derived free radicals, cytotoxic agents, and radiation damage (Yi, 1990; Koerner et al., 1991; Yamaguchi et al., 1994). The aim of the present retrospective study was to systematically analyze the patterns of Cu/Zn-SOD and Mn-SOD immunoreactivity in hepatic iron overload states and to correlate SOD immunoreactivity with type

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and severity of iron overload and with hepatic collagen type IV deposition.

Materials and methods

Patient groups

Biopsies from three groups of patients were used in this study: A) Patients with hereditary hemochromatosis (n=34; 10 female, 24 male; age range: 35 to 75 yrs); hemochromatosis in these patients was clinically and histologically confirmed; 17 patients were in the cirrhotic stage; B) Patients with hepatic iron overload of unknown reason (n=24; 5 female, 19 male; age range: 35 to 86 yrs); and C) Patients with hepatic iron overload due to either hyperhemolysis or exogenous iron administration (n=5; 3 female, 2 male; age range: 26 to 51 yrs). Biopsies of livers showing no relevant histologic change (staging biopsies) from 13 patients covering a similar age range served as controls.

Biopsies and light microscopic methods

Tissue consisted of needle biopsies exclusively. In the case of hemochromatosis, tissue was obtained before any therapy had been applied. Biopsies were fixed in 4% neutral buffered formalin for 2 to 4 hours and embedded in paraffin. 4 µm thick sections were processed for routine staining (hematoxylin-eosin, PAS, Van Gieson's, reticulin and chromotrope aniline stains). For the detection of stainable iron, Perls' iron stain was used (Perls, 1867).

Immunohistochemistry

Immunoreactive superoxide dismutases (SODs) were detected by use of a modified APAAP procedure. Sections were deparaffinized and rehydrated in Tris-NaCl buffer (0.1% Tris and 1% NaCl, pH 7.4) and preincubated with 3% serum albumin (BSA, Serva) in Tris buffer with 2% normal rabbit serum (Dako) for 30 min. Primary antibodies, i.e. mouse monoclonal antibody directed against human Cu/Zn-SOD (Sigma Immunohistochemicals) at a dilution of 1:200, and sheep polyclonal antibody directed against human Mn-SOD (The Binding Site, Birmingham, UK) at a dilution of 1:20, were applied on the sections and incubated for 1 hour. For Cu/Zn-SOD, monoclonal rabbit anti-mouse antibody at a dilution of 1:30 and monoclonal antibody APAAP complex at a dilution of 1:50 were used for incubating the sections for 45 min. For Mn-SOD, incubation with an alkaline phosphatase-conjugated donkey anti-sheep antibody (The Binding Site, Birmingham, UK) at a dilution of 1:50 and for 30 min was employed.

For type IV collagen (Col IV) staining, de-paraffinized sections were rehydrated in Tris-NaCl buffer as indicated above, incubated in protease solution (0.1% protease, Sigma, in Tris-NaCl buffer, pH 7.4) at 37 ºC for 10 min, then in 1% BSA (Serva) for 20 min at room temperature. The sections were rinsed three times in buffer. Mouse monoclonal antibody directed against human Col IV (Dako) at a dilution of 1:200 was applied, and sections were incubated overnight at 4 ºC. Monoclonal rabbit anti-mouse antibody (Dako) at 1:30 in Tris-NaCl buffer with 0.1% NaN_3 and 25% normal human serum, and monoclonal antibody of APAAP complex (Dako) at 1:50, were incubated on sections for 30 min, respectively.

For both, SODs and Col IV, alkaline phosphatase was developed in New Fuchsin substrate solution (0.01%; Dako Manual Book, 1986) for 20 minutes, and sections were counterstained with hematoxylin (Merck) and mounted with aquadex.

For immunohistochemical quantitation, all procedures were performed under strictly controlled conditions, and sections were processed in parallel using the same batches of antisera, substrate buffers, and washing solutions. Stained sections were kept in dark until use.

Histopathological assessment and grading of lesions

Granular deposits of stainable iron in hepatocytes were graded according to the proposition of Rowe (Rowe et al., 1977), in the modification of Searle and coworkers (Searle et al., 1987). This grading is based on the ease of observation and magnification required (eyepiece x objective), and works as follows: Grade 0, granules absent or barely discernible (x400); grade 1+, barely discernible (x250) or easily confirmed (x400); grade 2+, discrete granules resolved (x100); grade 3+, discrete granules resolved (x25); and grade 4+, masses visible (x 10, or naked eye).

Fibrosis and nodular change to the liver were assessed and graded according to a previously reported system (Knodell et al., 1981), with some modifications: Grade 0: no fibrosis; 1: mild, portal tract fibrosis only; 2: portal tract fibrosis plus incomplete fibrous septa; 3: complete septa, bridging portal-portal; 4: complete septa bridging portal-central, and/or focal complete cirrhosis; 5: diffuse complete and/or focal complete cirrhosis; and 6: diffuse complete cirrhosis.

Immunoreactivity of SODs was semiquantitatively assessed and graded according to the visible amount of reaction product in hepatocytes (Zimmermann et al., 1994). This approach had to be chosen because quantitation of reaction product (see below, for Col IV) was not possible in iron overload, the sensing channels of the CAS system not distinguishing SOD reaction product from densely packed hemosiderin granules. In SOD immunostains, immunoreactivity was separately graded for hepatocytes with lacking or low numbers of iron pigment granules (Hep L) and those with a high number of pigment granules (Hep H). Grading comprised: -, no reaction product; ±, questionable reaction product; +, slight staining; ++, moderate staining; and +++, strong staining. In addition to diffuse

Table 1. Hemochromatosis: histopathology and SOD immunohistochemistry.

CLINICAL DATA		HISTOPATHOLOGY GRADES			Cu/Zn-SOD					Mn-SOD		
Patient	Sex/Age	Rowe	Fibrosis	Cirrhosis	Hep L	Hep H	INV	NUC	BD	Hep L	Hep H	BD
1	F/36	4	0	0	+++	+++				++	++	+
2	M/43	4	4	1	+++	+++				-	-	+
3	M/43	4	5	1	+++	-		+	-	±	±	-
4	F/50	4	3	1	-	-		+	-	±	±	-
5	M/43	4	2	0	+++	+++		-	-	±	±	-
6	M/60	4	4	1		-		-	-	-	-	-
7	M/50	4	5	1	-	-		-	-	+	+	+
8	M/45	4	1	0	+++	+++		-	-	++	++	+
9	F/75	4	4	1	-			-	-	++	++	++
10	M/71	4	6	2	-			-	-	+++	+++	++
11	M/38	4	4	1	++	++		+	+	-	-	-
12	F/64	4	4	1	+++	++		+++	-	++	++	-
13	M/47	4	4	0	+++	+++		-	-	+	+	+
14	F/53	4	4	1	++	+		+	-	++	++	++
15	M/48	4	2	0	+++	+++		-	-	+++	+++	+
16	M/64	3	5	1	-	-		-	-	+	+	+
17	M/52	4	1	0	+++	+++		++	-	±	±	+
18	M/52	4	0	0	++	++		++	-	+	+	+
19	M/35	3	0	0	++	+++				++	++	+
20	M/51	3	3	0	+++	+++	-	-		±	±	+
21	F/49	4	0	0	+++	+++	-	+++		±	±	-
22	M/61	4	4	1	++	++	-	+		++	++	+
23	M/53	4	4	1	±	±	-	+	-	+	+	+
24	F/35	3	2	0	++	++	+++	+	-	±	±	-
25	M/56	4	4	1	-	-	-	-	-	+	+	+
26	M36	4	4	1	+++	+++	-	-	-	±	±	+
27	M/62	4	0	0	+++	+++	+++	+++	-	±	±	+
28	M/40	4	4	1	+++	+++	-	+	-	++	++	++
29	F/41	4	0	0	+++	+++				+	+	+
30	M/43	3	0	0	++	+++				±	±	-
31	M/61	3	2	0	-	-				+	+	+
32	F/54	4	2	0	+++	+++	-	-	-	±	+	+
33	M/67	4	6	2	+++	+++	+	+	-	±	±	+
34	M/51	4	2	0	+++	+++	-	++	-	++	++	+

Rowe: estimation of hepatocyte iron overload according to Rowe's grading; Hep L: grading of SOD immunoreactivity in hepatocytes with lacking or a low number of iron pigment granules; Hep H: grading of SOD immunoreactivity in hepatocytes exhibiting high numbers of iron pigment granules; INV: SOD immunoreactivity in intranuclear cytoplasmic invaginations; NUC: SOD immunoreactivity in hepatocyte nuclei not showing cytoplasmic invaginations; BD: SOD immunoreactivity in epithelial cells of bile ducts and ductules. Grading of fibrosis: see Materials and methods. Grading of cirrhosis: 0, no cirrhosis; 1, incomplete cirrhosis; 2, complete cirrhosis.

cytoplasmic SOD staining of hepatocytes, nuclear staining and staining of intranuclear cytoplasmic invaginations was assessed. Furthermore, sections were analyzed for SOD immunoreactivity in epithelial cells of small bile ducts and ductules.

Microphotometrical quantitation of type IV collagen immunoreactivity

Col IV reaction product was measured by use of the two solid-state image sensing channels of the CAS 200 image analysis system (CAS Cell Analysis System, Inc., Becton Dickinson, Elmhurst, Illinois). The CAS 200 APAAP immunostain software program (Bacus and Grace, 1987; Bacus et al., 1988, 1990) employs two video cameras which are matched to the two components of stains used (first camera: at 260 nm; second camera: at 500 nm). The program, originally set up to measure her-2/Neu onco-gene product, and requiring cells with known amounts of DNA and oncogene product to calibrate the system, can also be used for conventional APAAP immunostained preparations by setting the calibration values manually. Thus, all measurements are done under the same modified conditions. The amount of reaction product was calculated as the summed-up optical density per mm^2 tissue surface.

Video images, seen via a x40 objective, result in an area of 0.01 mm^2 per field. Of each field measured, the total optical density of nuclear components was obtained from the first sensor, whereas the total optical density of immunostain reaction product was obtained from the second. At least 20 fields per sample were randomly chosen and measured. It was assumed that the nuclear DNA distribution of all specimens tested was constant.

Statistical methods

All results are expressed as means ± SD. Kruskal-

Wallis and U tests were employed. $p<0.05$ was considered to be statistically significant.

Results

Histopathological characterization of disease groups

The main histopathologic features observed in biopsies of the three disease groups are compiled in Tables 1-3. It is seen that among the 34 cases of hemochromatosis (Table 1), 28 biopsies (82.4%) showed severe iron overload (Rowe grade 4), whereas as 6 biopsies only were grade 3 (17.6%), and no biopsy showed a grade less than 3. Distribution of hemosiderin iron exhibited the features characteristic for hemochromatosis, i.e. predominance of iron pigment in the periportal acinar zone 1 (Searle et al., 1987; Fig. 1). In contrast, the 24 biopsies of patients with a hepatic iron overload of unknown reason showed lesser degrees of hemosiderosis (grade 1: 33.3%; grade 2: 58.3%; grade 3: 8,3%). No biopsy revealed a grade 4 hemosiderosis and, in contrast to hemochromatosis, granular iron deposits were found in Kupffer cells in addition to hepatocytes, and a distinct acinar gradient was not observed. The third patient group (hyperhemolysis or exogenous iron overload), showed grade 4 iron deposits in 3/5, grade 3 in 1/5, and grade 2 in 1/5, with hemosiderosis involving both, hepatocytes and Kupffer cells. Even though part of these biopsies had iron overload to a degree found in established hemochromatosis, the typical acinar distribution pattern was lacking.

17/34 cases (50%) of hemochromatosis were already in the cirrhotic stage, albeit only 2/17 with diffuse complete cirrhosis, whereas 2/24 cases (8.3%) in the second group showed cirrhosis, and no biopsy in the third group disclosed nodular change of the liver. Even though only half of cases with hemochromatosis were cirrhotic, fibrosis of variable degrees was noted in 27/34 cases (79.4%; Table 1). In contrast, only 6/24 (25%) cases of the second group showed fibrosis, in two cases associated with incomplete cirrhosis, and 3/5 biopsies of the third group exhibited low-degree fibrosis.

Qualitative distribution of Cu/Zn- and Mn-SOD immunoreactivity

In hepatocytes, immunoreactivity for both, Cu/Zn- and Mn-SOD was observed. For Cu/Zn-SOD, overall intensity of cytoplasmic staining varied considerably, but immunoreactivity was found in all biopsies of the three groups and in all normal central biopsies (Fig. 2a). In normal-looking hepatocytes, Mn-SOD staining was more frequently encountered and seemed to be focally more intense than that of Cu/Zn-SOD (Fig. 2a). In part of the biopsies, staining was noted within hepatocyte nuclei (Fig. 2a,b). and in intranuclear cytoplasmic invaginations (Fig. 2a). In contrast to normal-looking parenchyma, staining of hepatocytes in iron storage was generally much more pronounced for Cu/Zn-SOD than for Mn-SOD (Tables 1-3). In the group with hemochromatosis, Cu/Zn-SOD was +++ in 18/34 (52.9%) Hep L and in 18/34 (52.9%) Hep H, respectively, whereas Mn-SOD was +++ in 2/34 (5.9%) Hep L and 2/34 Hep H (Table 1). For the group with iron overload due to unknown reasons, Cu/Zn-SOD showed a grade +++ staining in 9/24 (37.5%) both for Hep L and Hep H, and Mn-SOD a grade +++ in only 1/24 (4.2%) both for Hep L and Hep H (Table 2). A similar picture turned out for the 5 cases of the third group (Table 3). Thus, strong cytoplasmic immunoreactivity in hepatocytes was detected both in parenchymal cells with low and high burdens of iron pigment granules. There were, however, biopsies with hemochromatosis where Cu/Zn-SOD staining was less expressed in periportal hepatocytes containing large amounts of iron pigment granules (Fig. 3). Immunostaining of nuclei and of intranuclear cytoplasmic invaginations only occurred for Cu/Zn-SOD

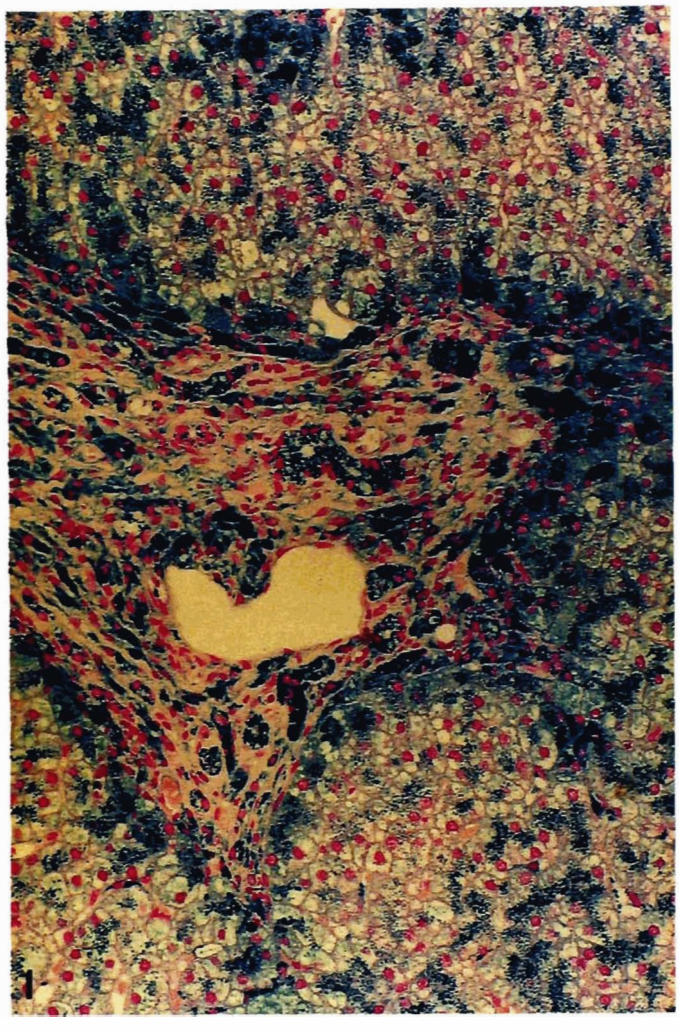


Fig. 1. Portal tract and periportal parenchyma of a liver biopsy in hemochromatosis. Note that hepatocytes close to the portal tract contain numerous blue granules representing iron pigment/hemosiderin. Perls iron stain. x 180

Table 2. Hepatic iron overload due to unknown reasons: histopathology and SOD immunohistochemistry.

CLINICAL DATA		HISTOPATHOLOGY GRADES			Cu/Zn-SOD					Mn-SOD		
Patient	Sex/Age	Rowe	Fibrosis	Cirrhosis	Hep L	Hep H	INV	NUC	BD	Hep L	Hep H	BD
1	M/65	2	2	0	-	-	-	-	-	+	+	+
2	M/41	2	1	0	++	++	-	+	-	+	+	+
3	M/78	2	0	0	+++	+++	-	+++	+	++	++	++
4	M/60	1	0	0	-	-	-	-	-	+	+	+
5	F/47	1	0	0	+++	+++	-	+++	-	±	±	-
6	M/53	1	0	0	+++	+++	-	-	-	+	+	-
7	M/42	2	0	0	+++	+++	-	-	-	+	+	+
8	M/35	2	0	0	-	-	-	-	-	-	-	-
9	M/46	2	0	0	++	++	-	-	-	++	++	+
10	M/77	2	0	0	+++	+++	-	++	-	+	+	+
11	M/40	3	0	0	+++	+++	-	+	-	++	++	+
12	M/41	2	0	0	±	±	-	+	-	++	++	+
13	M/50	2	5	1	+	+	-	++	-	+	+	+
14	F/71	2	0	0	++	++	-	+	-	+	+	+
15	M/86	2	0	0	±	±	-	+	-	+	+	+
16	M/46	1	0	0	+++	+++	-	-	-	+++	+++	++
17	M/48	2	0	0	+++	+++	++	-	-	++	++	+
18	M/46	2	0	0	±	±	-	-	-	±	±	-
19	M/53	2	0	0	+++	+++	-	-	-	±	±	-
20	M/38	3	0	0	-	-	-	+	-	-	-	-
21	M/54	1	2	0	+	+	-	-	-	+	+	+
22	M/58	1	0	0	+	-	-	++	-	±	±	+
23	M/61	1	5	0	-	-	+	-	-	-	-	+
24	F/59	1	4	1	-	-	-	-	-	-		

Rowe: estimation of hepatocyte iron overload according to Rowe's grading; Hep L: grading of SOD immunoreactivity in hepatocytes with lacking or a low number of iron pigment granules; Hep H: grading of SOD immunoreactivity in hepatocytes exhibiting high numbers of iron pigment granules; INV: SOD immunoreactivity in intranuclear cytoplasmic invaginations; NUC: SOD immunoreactivity in hepatocyte nuclei not showing cytoplasmic invaginations; BD: SOD immunoreactivity in epithelial cells of bile ducts and ductules. Grading of fibrosis: see Materials and methods. Grading of cirrhosis: 0, no cirrhosis; 1, incomplete cirrhosis; 2, complete cirrhosis.

Table 3. Hepatic iron overload due to hyperhemolysis or exogenous iron administration: histopathology and SOD immunohistochemistry.

CLINICAL DATA		HISTOPATHOLOGY GRADES			Cu/Zn-SOD					Mn-SOD		
Patient	Sex/Age	Rowe	Fibrosis	Cirrhosis	Hep L	Hep H	INV	NUC	BD	Hep L	Hep H	BD
1	F/51	2	0	0	±	±	-	-	-	±	±	+
2	M/47	4	1	0	+++	+++	-	-	-	++	++	+
3	F/26	4	0	0	+++	+++	-	+++	-	++	++	+++
4	M/42	3	1	0	+++	+++	-	-	-	-	-	-
5	F/50	4	2	0	+++	++	-	+	-	++	++	+

Rowe: estimation of hepatocyte iron overload according to Rowe's grading; Hep L: grading of SOD immunoreactivity in hepatocytes with lacking or a low number of iron pigment granules; Hep H: grading of SOD immunoreactivity in hepatocytes exhibiting high numbers of iron pigment granules; INV: SOD immunoreactivity in intranuclear cytoplasmic invaginations; NUC: SOD immunoreactivity in hepatocyte nuclei not showing cytoplasmic invaginations; BD: SOD immunoreactivity in epithelial cells of bile ducts and ductules. Grading of fibrosis: see Materials and methods. Grading of cirrhosis: 0, no cirrhosis; 1, incomplete cirrhosis; 2, complete cirrhosis.

(Fig. 2a; Tables 1-3), and was grade +++ in 8.8% and 8.3% for the first and second disease group, respectively. Epithelial cells of small bile ducts and of ductules were Cu/Zn-SOD-positive in a minority of biopsies (2/63 overall), whereas Mn-SOD staining was visualized in 47/63 biopsies, being + in 40/47, ++ in 6/47, and +++ in 1/47 (Tables 1-3).

Qualitative distribution of type IV collagen immunoreactivity

In biopsies of non-fibrotic livers, perisinusoidal collagen IV immunoreactivity was either visible as a slight and focal reaction only, or was not detectable. In contrast, it was strong and clearly visible in situations of fibrosis or cirrhosis. Here, type IV collagen immunoreactivity was demonstrable in the form of a linear and dense product situated along the contours of sinusoids (Fig. 4a,b), sometimes with an apparent gradient of reaction density from peripheral to central parts of deformed acini or nodules (not shown). In addition, strong reactivity was found around intermediate-sized and small (in part proliferated) bile ducts and blood vessels located in portal tracts (Fig. 4c).

Immunohistochemical quantitation of type IV collagen

Microphotometrically quantified levels of type IV collagen (expressed as summed-up optical density per mm^2 tissue analyzed) and correlations of levels with other parameters are compiled in Table 4. Overall, no significant difference was observed between the three disease groups, group three however being represented with 5 cases only. There was a trend for type IV collagen levels to increase with the amount of stored iron (Rowe grade). Based on the qualitative findings of type IV collagen being more prominent in situations of fibrosis and cirrhosis it could have been expected that microphotometrically determined levels of type IV collagen should also be higher in these situations. As seen in Table 4, this was obviously not the case. The reason for this was most probably due to the fact that areas for measurement were randomly chosen, and that many measurement points in fibrotic and cirrhotic tissues were in fact occupied by fibrous tissue containing large amounts of interstitial collagens, but not type IV collagen, rather than by parenchyma containing sinusoids.

In Table 5, the relationship between levels of type IV collagen and grades of SOD immunoreactivity are shown, taken together for all disease groups. Microphotometrically measured type IV collagen was significantly correlated with higher grades of Cu/Zn-SOD immunoreactivity, both cytoplasmic and nuclear. Conversely, no significant relationship was found for Mn-SOD reactivity in the cytoplasm of hepatocytes.

Discussion

Hepatocellular damage and fibrosis or even cirrhosis occurring in hepatic iron overload states seem to be

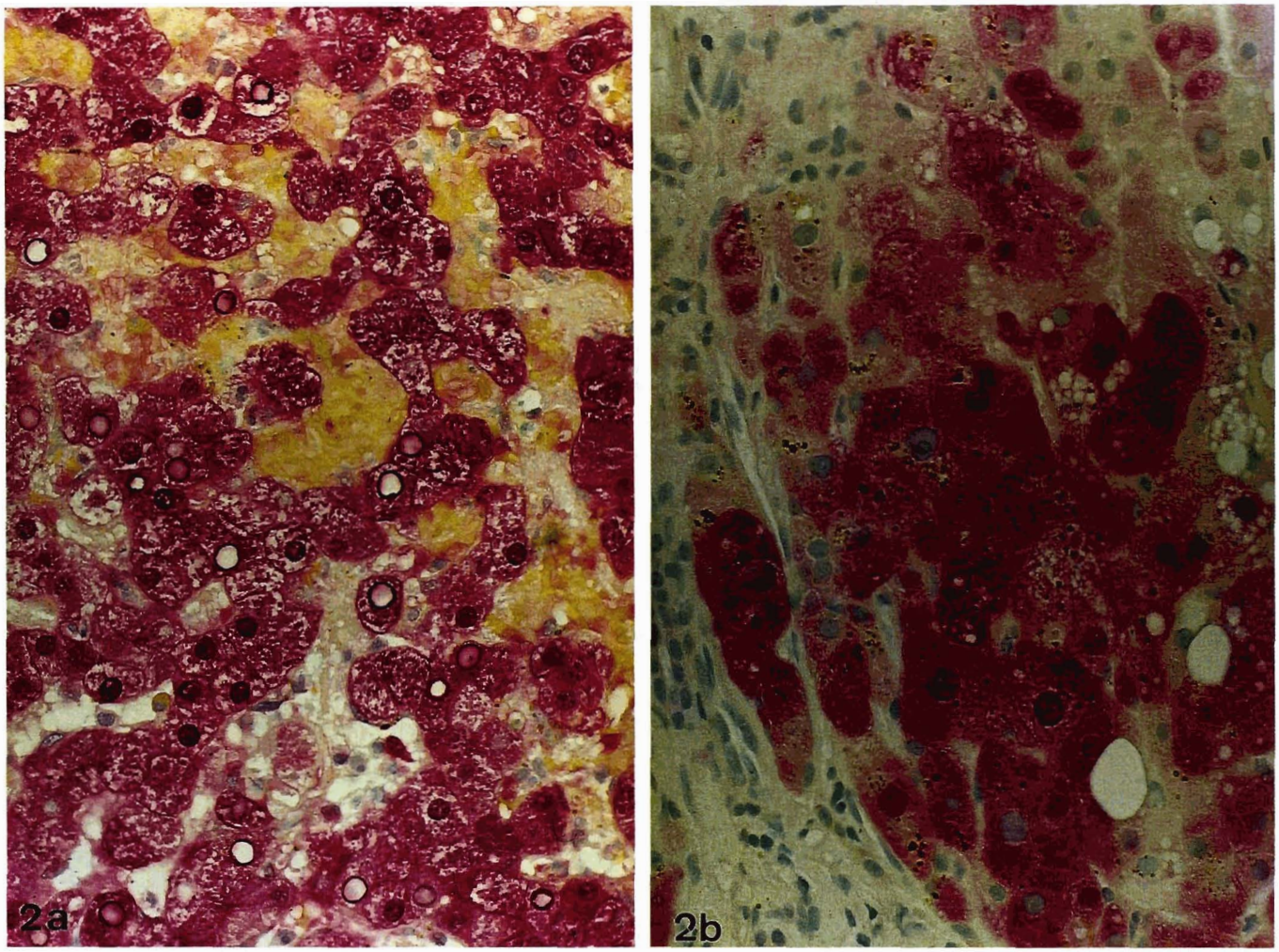


Fig. 2. a. Liver biopsy, hemochromatosis, Cu/Zn-SOD immunostain. In this area of hepatic parenchyma, the majority of hepatocytes disclose a granular red reaction product in the cytoplasm. Immunostaining is also visualized in some hepatocyte nuclei and in intranuclear cytoplasmic invaginations. **b.** Liver biopsy, hemochromatosis, Cu/Zn-SOD immunostain. in contrast to a, staining for the enzyme is more heterogeneous, in that only part of the hepatocytes exhibit strong cytoplasmic positivity. One hepatocyte lacking immunoreactivity in the cytoplasm expresses SOD in its nucleus (right hand of figure). Cu/Zn-SOD immunostain; APAAP method. a, x 360; b, x 580

Table 4. Immunohistochemical quantitation of type IV collagen: correlation with disease group, degree of iron storage and cirrhosis.

PARAMETER	n	TYPE IV COLLAGEN*	
*Disease group***			
Group 1	34	5.7±1.2	p:0.11
Group 2	24	3.2±1.4	
Group 3	5	8.8±3.0	
Iron storage (Rowe grade)			
Grade 1	8	2.3±2.3	p:0.24
Grade 2	15	2.9±1.7	
Grade 3	9	5.9±2.2	
Grade 4	31	6.4±1.2	
Cirrhosis			
No cirrhosis	44	4.7±1.0	p:0.91
Incomplete cirrhosis	17	6.1±1.7	
Complete cirrhosis	2	2.2±4.8	

*: immunohistochemical quantitation of type IV collagen is indicated as summed-up optical density per mm^2 tissue surface. **: group 1, hemochromatosis; group 2, iron overload due to unknown reasons; group 3, iron overload due to hyperhemolysis or exogenous iron administration. For iron storage and cirrhosis, values of all cases for the three disease groups are cumulated. n: number of biopsies. p: p according to Kruskal-Wallis test (see Materials and methods).

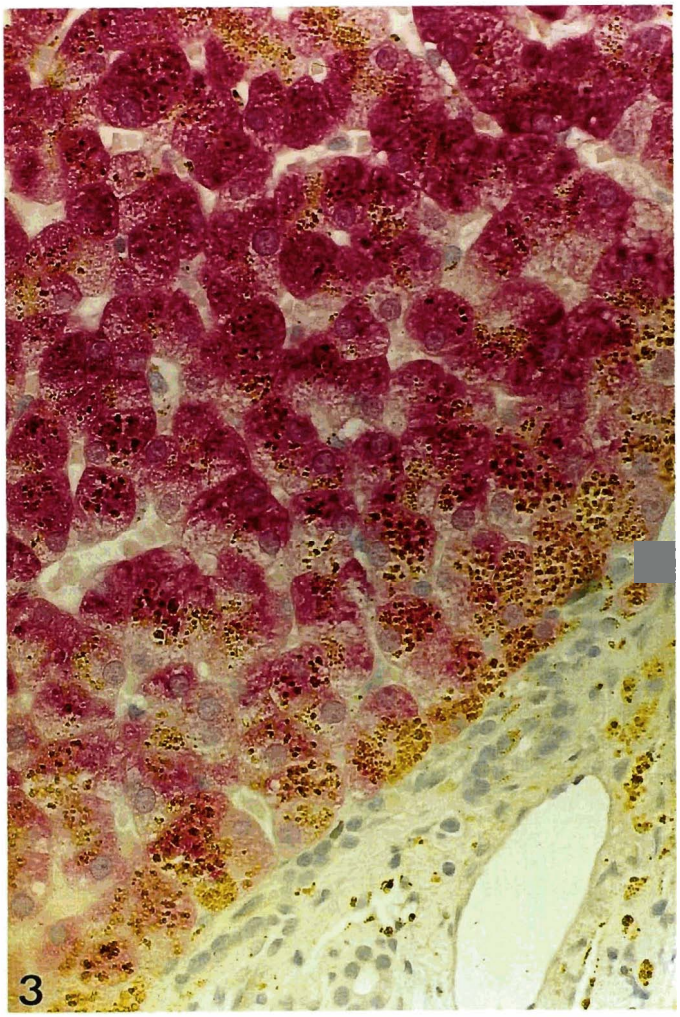


Table 5. Immunohistochemical quantitation of type IV collagen: correlation with grading of SOD immunoreactivity.

SUPEROXIDE DISMUTASE (SOD)	n	TYPE IV COLLAGEN	
Cu/Zn-SOD, cytoplasmic			
0	14	2.0±1.8	p<0.05
±	5	1.0±3.0	(+++ vs.
+	3	3.7±3.8	0 and ±;
++	8	5.8±2.3	++ vs. 0
+++	33	6.8±1.2	and +)
Cu/Zn-SOD, nuclear			
0	35	4.1±1.1	p<0.05
+	16	4.4±1.6	(+++ vs.
++	6	2.9±2.6	0, + and
+++	6	13.6±2.6	++)
Mn-SOD, cytoplasmic			
0	8	4.0±2.4	NS
±	17	4.5±1.6	
+	17	3.6±1.6	
++	18	7.7±1.6	
+++	3	1.8±3.9	

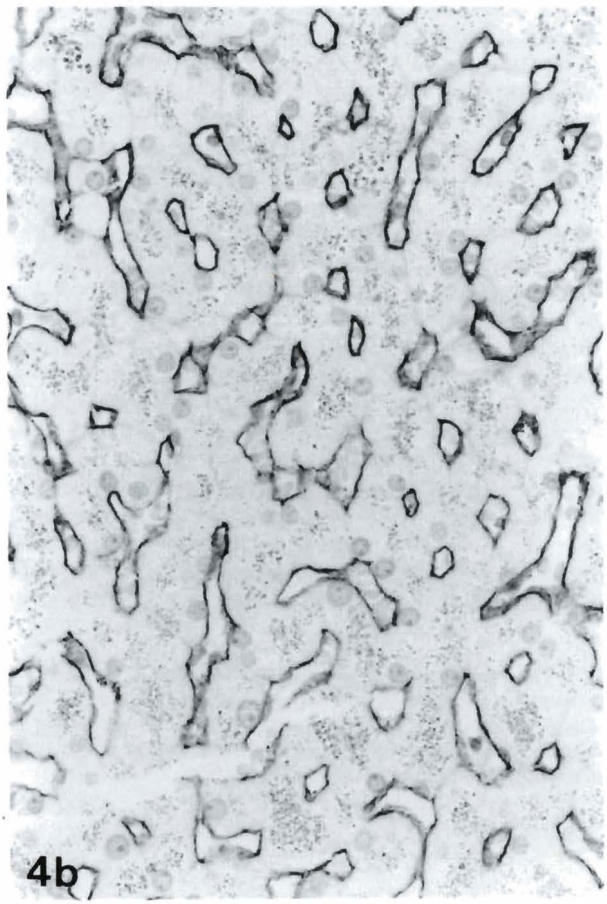
n: number of biopsies; Cu/Zn-SOD: copper/zinc superoxide dismutase; Mn-SOD: manganese superoxide dismutase; NS: not significant. Immunohistochemical quantitation of type IV collagen is indicated as summed-up optical density per mm^2 tissue surface. All cases of the three disease groups ar cumulated. p is based on the U test (see Materials and methods).

directly related to the iron content of hepatocytes, but the pathogenic mechanisms involved are only partially known (MacDonald, 1964; Nichols and Bacon, 1989; Deugnier et al., 1992; Sherlock and Dooley, 1993; Gualdi et al., 1994). There is evidence to suggest that one of the factors of iron toxicity is related to the induction of excess reactive oxygen species (ROS), leading to formation of lipid peroxides and, subsequently, to membrane and organelle damage (Tien et al., 1981; Halliwell and Gutteridge et al., 1985; Bacon et al., 1986; Cederbaum, 1989; Bacon and Britton, 1990; Houglum et al., 1990; Davis and Petry, 1994; Itoh et al., 1994; Lamb and Leake, 1994; Sadrzadeh et al., 1994). Oxidative stress induced by iron appears to ensue rapidly, and is detectable in rats already six hours after iron supplementation (Galleano and Puntarulo, 1994). Iron bound in the form of hemosiderin or prehemosiderin (Ward et al., 1994), storage forms excessively accumulated in hemochromatosis and other situations of severe hepatic iron overload, may at least in part protect the cells from the damaging effects of hydroxyl radicals (O'Connel et al., 1986). On the other hand, superoxide-generating systems appear to have an effect on the intracellular availability of iron, in that superoxide can release iron from ferritin stores, and ferritins with

Fig. 3. Liver biopsy from a patient with hereditary hemochromatosis. Part of the hepatocytes show a strong reaction product for Cu/Zn-SOD in the cytoplasm. Note that periportal hepatocytes containing large numbers of iron pigment granules exhibit much weaker SOD staining. Cu/Zn-SOD immunostain, APAAP method. x 360

a high rate of superoxide-dependent iron release are in tissues known to be susceptible to iron damage (Harris et al., 1994). That free iron may be the determinant of cellular injury is further supported by the observation that the iron chelator, desferroxamine, has antioxidant effects (Davis and Petry, 1994). In chronic hepatic iron overload, cellular damage is then frequently associated with fibrosis or cirrhosis, and the hepatic disorder of circulation occurring in these states may then further be influenced by accumulation of iron, because iron has been shown to exert an influence on the synthesis of one of the major vasoactive agents, nitric oxide (Mikoyan et al., 1994; Weiss et al., 1994).

In the liver, effects of reactive oxygen species can be



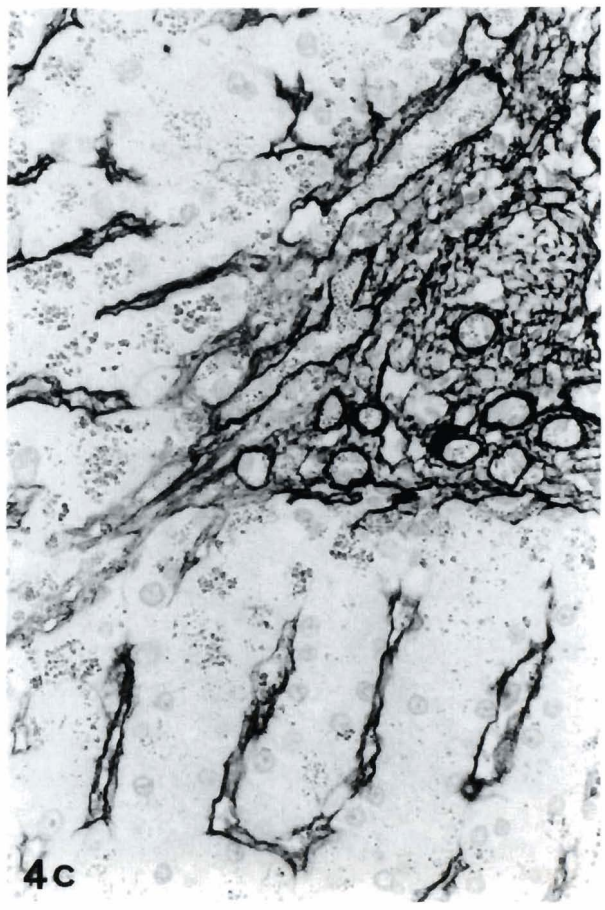


Fig. 4. a. Hemochromatosis in the stage of incipient septal fibrosis. The portal tract (top of figure) is increased in size, and an incomplete fibrous septum is seen (middle part of the figure). This section is immunostained for type IV collagen. Strong immunoreactivity is detectable along the sinusoids, indicating capillarization. **b.** Type IV collagen immunostaining of hepatic parenchyma in hemochromatosis at higher magnification. The dark contours represent strong reaction product located along Disse's space of most sinusoids. **c.** Portal tract and periportal hepatic parenchyma in hemochromatosis, staining for type IV collagen. Hepatocytes in the periportal zone contain dark iron pigment granules. Note that, in addition to the perisinusoidal space, strong immunoreactivity for type IV collagen is also visualized around small bile ducts and vessels in the portal tract. Type IV collagen immunostain, APAAP method. a, x 90; b, c, x 180

counteracted by antioxidant protective mechanisms comprising enzymatic and non-enzymatic defense systems (Tribble et al., 1987). Among free radical scavengers, superoxide dismutase (SOD; E.C. 1.15.1.1.), which catalyzes dismutation or destruction of most superoxide anions, is considered to be a key enzyme in protecting cells against oxidative injury (Gregory and Fridovich, 1973). In the present study, immunoreactivity for both Cu/Zn and Mn-SOD was noted in hepatocyte cytoplasm of the majority of biopsies with histologically verified hepatic iron overload, and of all control biopsies, but Cu/Zn-SOD staining was more intense than that for Mn-SOD. Presence of both species of SOD in liver cells has previously been demonstrated and has been shown to be developmentally regulated: hepatocytes in the developing rat liver expressed Cu/Zn-SOD at 14 days and Mn-SOD at 17 days of gestation (Munim et al., 1992). In hepatocytes of the present study disclosing SOD immunoreactivity, staining was reproducible, and therefore in line with previous reports showing that these enzymes are detectable by use of immunohistochemistry (Slot et al., 1986; Chan et al., 1988; Dobashi et al., 1989; Wakai et al., 1994). The fact that Mn-SOD immunoreactivity was less prominent than that of Cu/Zn-SOD may either be due to technical reasons or to the phenomenon that Mn-SOD is inducible by several factors, such as hyperoxia, tumor necrosis factor, interleukin-1, lipopolysaccharides and active phorbol esters (Wong and Goeddel, 1988; Mokuno et al., 1994), its expression therefore being susceptible to regulation, whereas Cu/Zn-SOD, which is primarily a cytosolic protein in human cells (Crapo et al., 1992) seems to show less variable expression. Cu/Zn-SOD, however, but not Mn-SOD, was also expressed in hepatocyte nuclei in a large proportion of cases. Qualitatively, there was no difference in overall SOD staining for the three disease groups analyzed, but staining intensity, in particular for Cu/Zn-SOD, considerably varied among the hepatocyte population. We were, therefore, interested to analyze whether hepatocytes exhibiting numerous iron pigment granules had a similar SOD immunoreactivity as those with few or no visible pigment granules, and whether this would be the reason for staining heterogeneity. It turned out that strong immunoreactivity for both, Cu/Zn and Mn-SOD was detectable in hepatocytes with a heavy and low pigment burden, and in all three disease groups, even though a strong reaction for Mn-SOD was observed in one biopsy only. It thus appears that, with the exception of periportal hepatocytes in some biopsies, Cu/Zn-SOD may not be differentially expressed in hepatocytes with respect to cellular iron pigment burden, whereas Mn-SOD has a lower level of immunohistochemical expression. The latter finding is of interest insofar as the inducible Mn-SOD has been proposed to be the major antioxidant SOD species (Dobashi et al., 1989; Ono et al., 1991; Yoneda et al., 1992). Furthermore, cellular injury can be inhibited by a synthetic manganese-based SOD mimetic (Black et al., 1994). One may therefore suggest that the lower levels of Mn-SOD reactivity in iron overload cells, in comparison with controls, may play a pathogenic role in iron toxicity.

In a further step, we determined the immunohistochemical expression of type IV collagen in the three groups of hepatic iron overload by use of quantitative microphotometry. Type IV collagen is a major constituent of the extracellular matrix, in particular basement membranes (Timpl et al., 1981). In normal human livers, only small amounts of type IV collagen-containing extracellular matrix are detectable in the perisinusoidal space, whereas most types of chronic liver disease are, irrespective of their etiology, associated with an increase in hepatic extracellular matrix (Hahn et al., 1980; Milani et al., 1990; Schuppan, 1990; Griffiths et al., 1991; Gressner, 1992; Gressner and Ramadori, 1992). This phenomenon is accompanied by sinusoidal capillarization (Schaffner and Popper, 1963), a determinant of the disordered intrahepatic circulation in fibrosing liver disease. We have previously demonstrated that deposition of type IV collagen can be quantified by microphotometry on immunostained sections (Zhao et al., 1994). In biopsies of iron-loaded liver with or without fibrosis or cirrhosis, immunoreactive type IV collagen was visualized in Disse's space in considerable amounts, in contrast to control biopsies, indicating that capillarization takes place in these situations. However, when quantifying the reaction product, no significant difference was observed between the three disease groups, and between biopsies with or without fibrosis. We assume that the reason for this is due to the fact that areas for measurements were randomly chosen, and that, therefore a considerable fraction of measurement points in fibrotic or cirrhotic tissues were occupied by fibrous tissue containing large amounts of interstitial collagens, but not type IV collagen, rather than by hepatic parenchyma containing sinusoids with Disse's spaces. There was, however, a trend for type IV collagen levels to increase with the amount of stored iron, which is of interest in the light of recent findings that iron can activate collagen genes in the liver of rodents (Gualdi et al., 1994). Increased type IV collagen levels were significantly correlated with higher grades of Cu/Zn-SOD immunoreactivity, whereas no relationship was found for the inducible Mn-SOD. Even though immunohistochemistry only reflects a distinct moment in the turnover of cellular enzymes, which may, in contrast to the situation of more stable structural proteins, not be representative for longer time periods, these findings suggest that Cu/Zn-SOD expression is generally high in situations where one key protein of the extracellular matrix is deposited, whereas Mn-SOD disclosed a weak reactivity throughout. Further studies will be necessary to test whether Mn-SOD is in fact downregulated in hepatic iron overload states, and whether this is associated with reduced antioxidant protection.

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