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Ethanol-induced alterations of matrix network in the duodenal mucosa of chronic alcohol abusers

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Abstract Excessive consumption of alcoholic beverages may be associated with gastrointestinal symptoms, including dyspepsia and diarrhoea. It is not clear whether or not chronic alcohol ingestion damages the mucosa of the small intestine. We investigated the effect of chronic alcohol abuse on the duodenal mucosa, and particularly on its extracellular matrix (ECM) network. Duoenal biopsy specimens were obtained during upper gastrointestinal endoscopy from 50 chronic alcoholics without cirrhosis and 10 healthy subjects. Morphological studies were performed by routine histology, immunohistochemistry and electron microscopy. Morphometry of duodenal tissues was performed with a computerized image analyser. No significant duodenal epithelial changes were found in alcoholics, despite an evident reduction in the enterocyte turnover. Myofibroblast-like cells were significantly increased in the villus stroma of alcoholics in comparison to controls. These cells stained positively for desmin, α-smooth muscle actin and for several ECM components. In alcohol abusers the thickness of the mucosal basement membrane was greater and the staining for collagen I and III was enhanced both in the basement membrane and in the villus stroma. A higher expression of tenascin was also seen at the base of villi of alcoholics. Chronic alcohol abuse may induce fibrosis of duodenal villi which is associated with a transformation of villus juxta-parenchymal cells into active subepithelial myofibroblast-like cells able to produce different ECM components.

Key words · Ethanol · Intestine · Duodenum · Collagen · Matrix

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Introduction

Excessive consumption of alcoholic beverages may be associated with gastrointestinal symptoms including dyspepsia and diarrhoea. Malnutrition sometimes occurs in alcohol abusers, but varies with the severity and duration of ethanol abuse. Malnutrition may be due to poor dietary intake (typical of advanced alcoholics), but also to altered digestion and absorption of nutrients [25].

Prolonged ingestion of high doses of alcohol induces significant alterations in the small intestine by affecting mucosal enzyme activity [4], permeability [3], and bacterial growth [6]. Whether these alterations result from the direct toxic effect of ethanol or are secondary to malnutrition is not yet clear [30, 49]. In particular, it remains controversial whether or not chronic alcohol ingestion damages the mucosa of the small intestine. Some studies have led to reports of normal histology revealed by light microscopy [35, 36, 50], whereas others have shown significant histological alterations [25, 51, 52]. Duodenal and jejunal biopsies, taken after a period of heavy drinking, have usually shown normal histology on light microscopy [3, 19, 35, 38]. Conversely, ultrastructural changes of the small intestine mucosa, such as mitochondrial abnormalities, dilatation of endoplasmic reticulum and alteration in the surface of enterocytes, have been reported after chronic alcohol abuse [34, 38]. In studies based on morphometric methods a reduced villus height in the jejunal mucosa has been reported in alcoholics [5, 43]. A constant finding in recent studies is reduced enterocyte turnover by mitotic inhibition [24, 51, 52].

None of these studies has investigated possible effects of ethanol on the extracellular matrix (ECM) network of the jejunal mucosa. It is well established that ethanol and

its metabolities can affect the metabolism of collagens and other components of the ECM significantly in other organs, such as the liver [7–12, 31, 32] and the pancreas [44, 45].

The aim of this study was to investigate whether chronic alcohol abuse induces alterations of the duodenal mucosa, and particularly of its ECM network.

Materials and methods

Fifty chronic alcohol abusers (39 men, 11 women; age 47.2±12.4 years, mean±SD, range 27–76 years; 186.3±61.7 g absolute ethanol/day, range 80–300 g, for over 10 years) and 10 healthy controls (5 males, 5 females; age 37.8±18.4 years, range 26–57 years; less than 30 g ethanol/day) were studied. All subjects underwent upper gastrointestinal (GI) endoscopy. Informed consent was obtained from all patients prior to the endoscopy. The present study was approved by the local ethical committee. Patients with a history of renal disease, chronic liver disease, diabetes or previous GI surgery were excluded. Routine haematology, liver and kidney chemistry, and fasting blood sugar were carried out in all patients at the time of the study. The D-xylose absorption test (5-h urine test) was performed in all subjects for evaluation of malabsorption [33].

Six to eight biopsy specimens were obtained during upper gastrointestinal endoscopy from the distal duodenum. Three were fixed in 10% buffered formalin and embedded in paraffin for conventional histological examination. Deparaffinized sections were stained by haematoxylin and eosin (H-E), Masson trichrome technique, and alcian blue pH 2.5/PAS. For immunohistochemistry, additional samples were immediately snap-frozen and stored in liquid nitrogen until sectioning. For electron microscopic (EM) studies, the remaining samples were fixed with 0.8% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/l cacodylate buffer (pH 7.6) for 1 h at room temperature. After a brief rinse in cacodylate buffer, samples were left overnight at 4°C in cacodylate buffer containing 2.5% sucrose. Post-fixation was then performed with 1% osmium tetroxide in 0.1 mol/l cacodylate buffer for 1 h. Samples were dehydrated in ascending series of ethanol and embedded in Epon/Araldite. Sections 1 µm thick were examined under the light microscope for selection of well-oriented samples. Ultrathin (60 nm) sections were taken on copper grids and examined under the Philips 201 EM after staining with lead citrate.

For immunohistochemistry, thin sections (5 µm) were collected onto clean slides and dried overnight at room temperature. Sections were fixed with acetone and chloroform (30 min each) and then blocked with phosphate-buffered saline (PBS) containing 10% serum appropriate for the secondary antibody. After subsequent washing in PBS (pH 7.3) for 10 min, the sections were incubated with primary antibodies diluted in PBS/bovine serum albumin (BSA, 1%, w/v) for 30–60 min.

A variety of monoclonal and polyclonal antibodies were used. All were titered for optimal reactivity, and minimal incubations periods had been determined in preceding experiments. Monoclonal antibodies directed against human desmin (D33) and α-smooth muscle actin (αSMA; IA4) were purchased from Dakopatts (Copenhagen, Denmark). Affinity-purified rabbit antibodies specific for monkey type I procollagen (pIp), human type III procollagen (pIIIp), and both human laminin (P1 fragment) and monoclonal antibodies directed against human NC1 domain of collagen type IV, fibronectin (FN) and undulin (Un; collagen XIV) were kindly provided by Dr. Detlef Schuppan. Their characterization as monospecific by a sensitive radioimmuno-inhibition assay and Western blotting has been described elsewhere [2, 41]. Anti-tenascin (Tn) antibody was a gift from Dr. Ruth Chiquet-Ehrismann and was raised in rabbits using Tn purified from conditioned medium of primary chick embryo fibroblast cultures [13].

The slides were rinsed again for 10 min in PBS/BSA and then incubated sequentially with affinity-purified mouse anti-rabbit or rabbit anti-mouse immunoglobulin G (Dakopatts) as linking antibodies (30 min each) and monoclonal peroxidase anti-peroxidase (PAP) immune complex (Dakopatts) diluted 1:50 in PBS. Endogenous peroxidase was blocked with 3% hydrogen peroxide in absolute methanol, and 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, Mo.) was used as chromogen. Finally, the sections were weakly counterstained with Mayer's haemalum and coverslipped using Kayser's gelatin. Negative controls included replacement of the primary antibodies by either PBS or nonimmune mouse (or rabbit) serum.

Immunohistochemistry for MIB-1, a marker of cell replication [23] was performed on formalin-fixed, paraffin-embedded sections. Sections 5 µm thick were cut and mounted on poly-L-lysine (Sigma)-coated slides. They were then dewaxed, and endogenous peroxidase was blocked with 3% hydrogen peroxide in distilled water. After microwave treatment (5 min ×2 in citrate buffer, pH 6.0, in a household microwave oven, 750 W), immunohistochemical staining was carried out according to a standard labelled streptavidin–biotin peroxidase method by using a mouse anti-human Ki-67 monoclonal antibody (clone MIB-1, Immunotech, Marseille Cedex, France). Negative controls were performed by substituting the primary antibody with nonimmune mouse serum.

The evaluation of enterocyte turnover was carried out by counting the MIB-1-positive cells in the crypts. Measurements were done with a micrometre scale inserted in the ocular of the microscope. Five different fields, each containing at least five crypts, were evaluated for each subject studied. Values were averaged and expressed as mean±SD of positive cells per patient.

Quantitative analysis was performed with a Leitz microscope equipped with a computerized image analyser (Videoplan, Kontron Electronics, Munich, Germany). The thickness of the duodenal mucosal basement membrane was measured on Masson trichrome-stained biopsies by evaluating five different villi and five crypts per patient. Ten different measurements were performed in each crypt. Villi were divided into two equal parts (top and bottom), and 10 measurements were performed in each part. Villus height was evaluated by measuring the distance (mm) between the tip and the base (defined as the idealized line passing through the upper part of the crypts beside the villus) of each of 10 well-oriented villi per subject. The parenchymal extension of desmin- and αSMA-positive cells was evaluated by measuring the area of positive cells with the image analyser and expressing the results as percentages of the total area of the villus stroma.

Statistical evaluation of the data was performed by analysis of variance.

Table 1 Nutritional parameters and daily ethanol intake of the 50 chronic alcohol abusers studied (*BMI* body mass index)

	Mean	SD	Range	Reference values
Consumption (g/day)	186.34	±61.76	80–300	
Albumin (g/dl)	4.28	±0.36	3.5–5	3.6–4.9
Prealbumin (mg/dl)	27.45	±5.76	18–37	18–37
β-Carotene (µg/dl)	87.22	±40.27	50–225	50–250
BMI				
M	23.26	±2.28	19.32–29.74	20–24.9
F	20.37	±1.65	18.49–23.43	18.6–23.9
Folate (ng/ml)	6.10	±3.59	2.60–10.50	3.0–17
Vitamin B_{12} (pg/ml)	465.27	±34.51	290–710	200–950

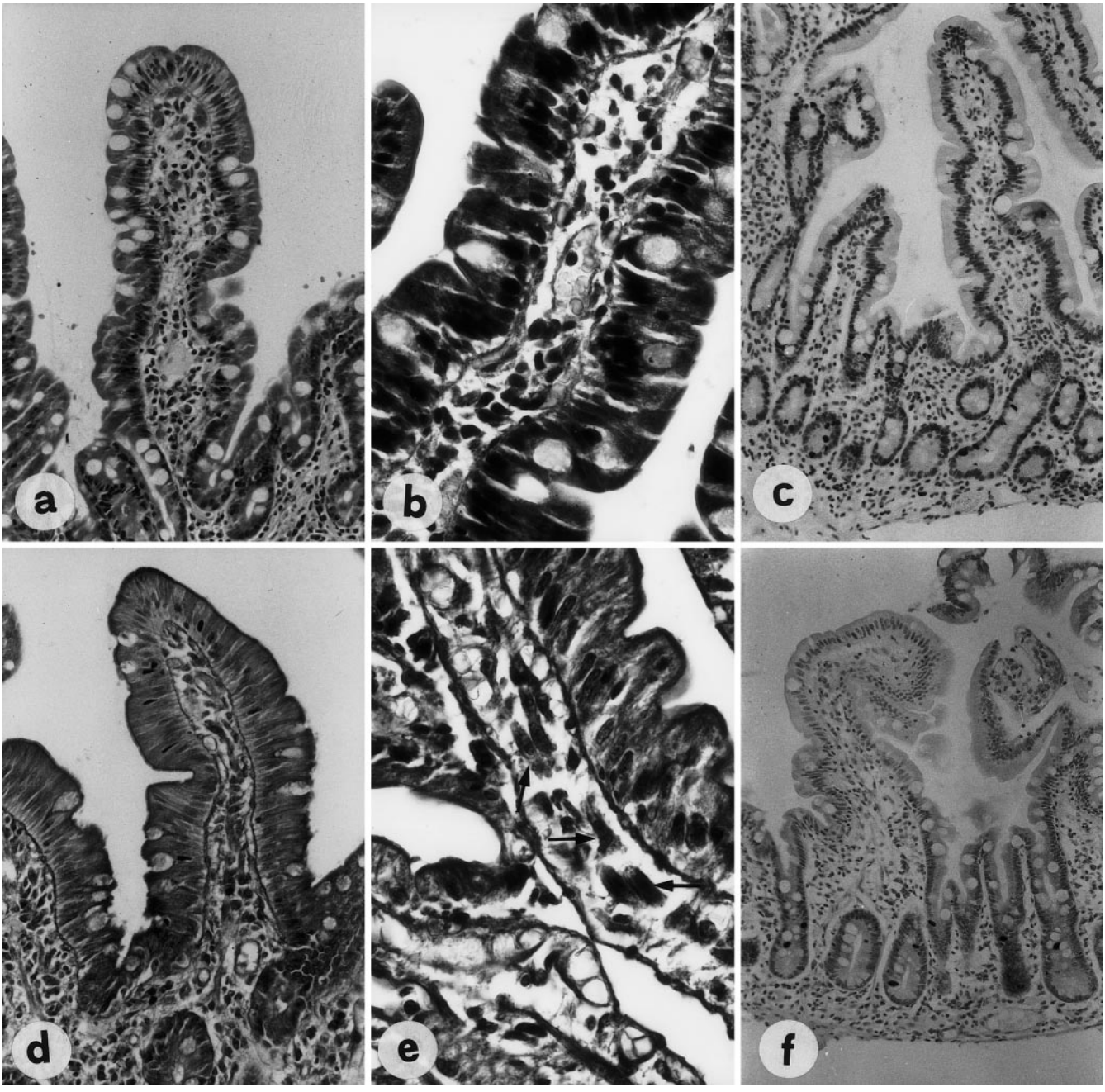


Fig. 1 Representative Masson trichrome light micrographs and immunostaining for MIB-1 of distal duodenal mucosa from **a–c** control subjects and **d–f** chronic alcohol abusers. A significant increase in the thickness of both epithelial and capillary basal membrane is evident in the villus of alcohol abusers **d** compared with normal gut **a**. Several myofibroblast-like cells are present in the villus lamina propria of alcoholic patients (**e** *arrows*), where they are more numerous and larger than in control subjects **b**. The MIB-1-positive cells in the crypts of duodenal glands are significantly reduced in alcohol abusers **f** compared with controls **c**. Original magnification **a, c, d, f** ×85, **b, e** ×260

Results

None of the 50 alcohol abusers had the clinical picture of severe liver disease. Abdominal ultrasonography demonstrated either a normal or a "bright"/fatty liver. Twenty-one patients whose ultrasonography and serum findings suggested possible chronic liver disease underwent needle liver biopsy that showed only fatty changes (n=16) or fatty liver with mild fibrosis (n=5). All the alcohol abusers showed normal nutritional variables. Five patients had borderline low levels of folate (2.6, 2.8, 2.9, 3.0, 3.0 ng/ml) (Table 1).

Light microscopic examination of the duodenal mucosa showed no significant epithelial changes in alcoholics compared with controls (Fig. 1a, d). In addition, the pattern of alcian/PAS staining of globet cells in patients overlapped that of controls (not shown). Evident dilatation of capillaries, particularly at the villus tip, was seen in alcohol abusers.

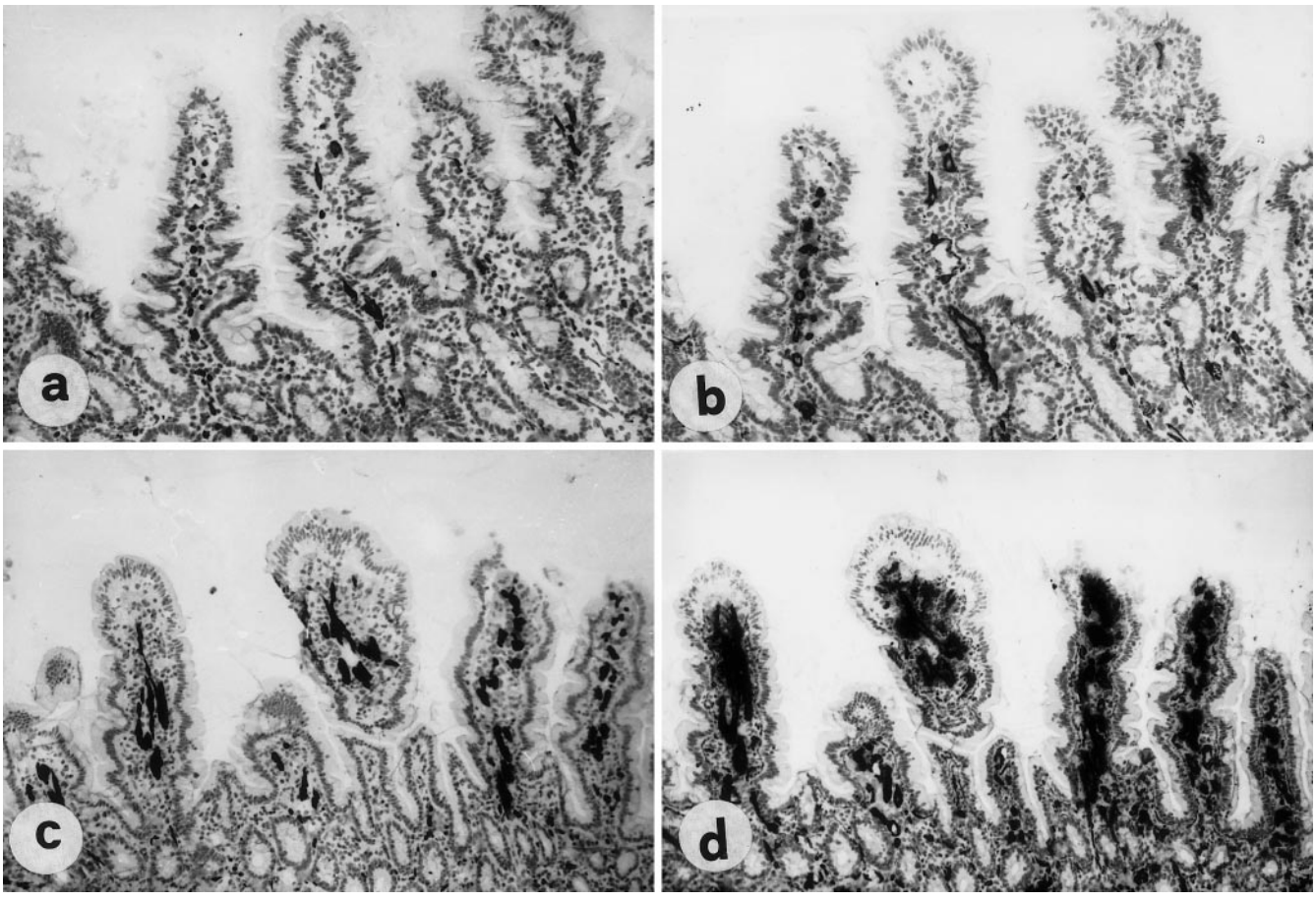


Fig. 2 Immunostaining for **a, c** desmin and **b, d** α-SMA (PAP method) on adjacent sections of distal duodenal mucosa from a control subject and an alcoholic patient. **a–d** A marked increase in number and size of subepithelial and pericryptal myofibroblast-like cells is evident at low magnification in the intestinal mucosa of alcohol abusers **c, d** compared with normal gut **a, b**. Original magnification **a, b** ×85, **c, d** ×65

There were no evident differences in the duodenal villus height between alcohol abusers (1.36±0.29 mm) and control subjects (1.38±0.22 mm; mean±SD). Measurement of the MIB-1-positive cells in the crypts showed that the enterocyte turnover was significantly lower in patients than in controls [5.1±3.2 vs 33.0±6.1 cells, respectively (mean±SD; $P<0.001$); Fig. 1c, f].

The thickness of the basement membrane of duodenal villi, evaluated on Masson trichrome-stained biopsies, was significantly higher in alcohol abusers than in controls (Fig. 1a, b, d, e); this phenomenon was particularly evident at the villus bottom and also extended to the crypts (Table 2).

Several nonparenchymal cells with abundant eosinophilic cytoplasm, exhibiting a myofibroblast-like elongated shape, were present in the villus stroma and underlay the crypts and surface epithelium; these cells were more numerous and larger in alcoholics than in control subjects (Figs. 1b, e, 2).

The subepithelial and pericryptal cells of villus stroma expressed both desmin and αSMA with the typical microfilamentous pattern, suggesting a myofibroblastic phenotype (Fig. 2). The parenchymal extension of αSMA-positive cells was much higher in alcohol abusers than in controls [54.6±3.5% vs 9.6±1.1%, respectively (percentage of total area of the villus stroma, mean±SD, $P<0.001$); (Fig. 2b, d)]. A similar difference, although less evident, occurred in the desmin-positive cells (27.5±1.5% vs 10.9±1.3% for alcoholics vs controls, respectively, $P<0.001$; Fig. 2a, c).

Immunostaining was performed by using monospecific antibodies to collagen type I, III, IV, laminin, fibro-

Table 2 Morphometric evaluation of the thickness of the jejunal villus basement membrane in alcohol abusers and control subjects. Thickness of the jejunal mucosa basement membrane was measured on Masson thrichrome stained biopsies by evaluating five different villi and five crypts per patient. Ten different measurements were performed in each crypt. Villi were divided in two equal parts (top and bottom), and 10 measurements were performed in each part (*n* number of measurements, *P* statistical significance according to analysis of variance)

	Entire villus (μm)	Villus top (μm)	Villus botton (μm)	Crypt (μm)
Controls	1.6±0.2 *n*=1000	1.5±0.2 *n*=500	1.6±0.2 *n*=500	1.6±0.2 *n*=500
Chronic Alcohol Abusers	2.7±0.6 *n*=5000	2.4±0.5 *n*=2500	3.0±0.6 *n*=2500	2.6±0.4 *n*=2500
	P<0.001	*P*<0.001	*P*<0.001	*P*<0.001

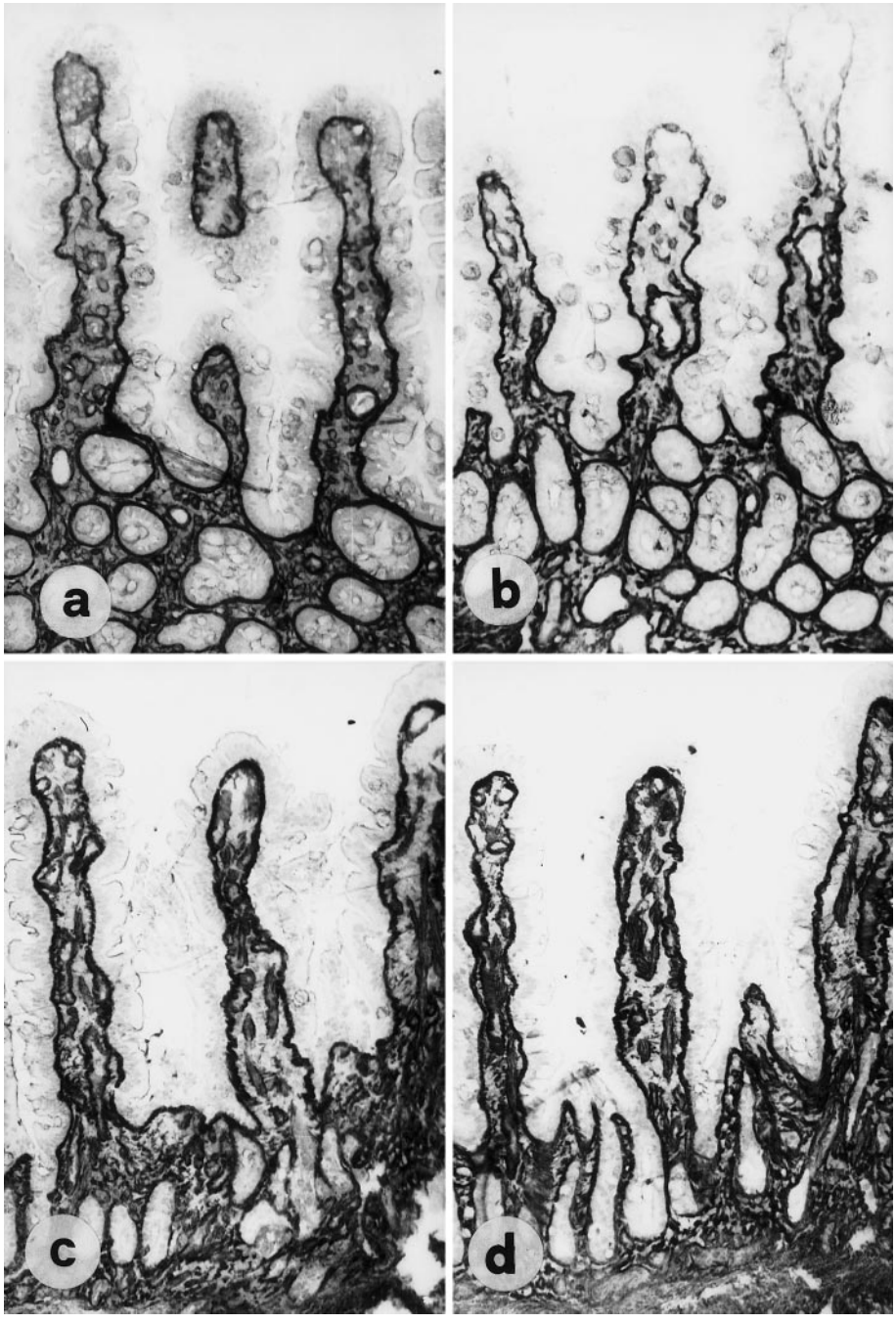


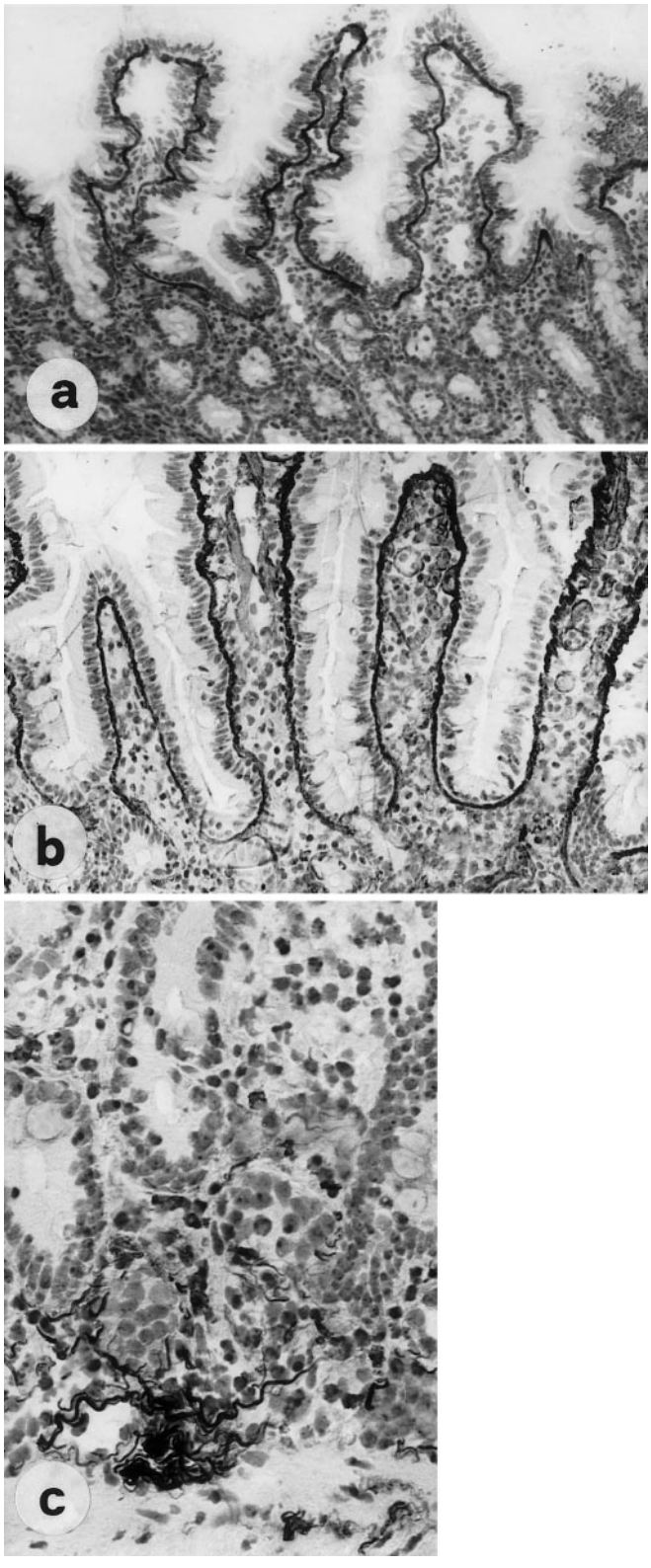
Fig. 3a–d Immunostaining for collagen type I and III. In normal intestine, collagen types I **a** and III **b** form a continuous band underneath the epithelial basal membrane; in addition, a sparse interstitial meshwork of small fibres is observed in the lamina propria of the villi and around the crypts. The staining for collagen type I is moderately enhanced in the intestinal mucosa of alcohol abusers **c**, whereas the amount of collagen type III **d** is markedly greater than in normal gut, particularly in the stroma at the base of the villi. Original magnification ×85

nectin, undulin and tenascin (pIp, pIIIp, NC1, LamP1, FN, Un and Tn) with the PAP method. In normal intestine pIp and pIIIp were expressed as a continuous band underneath the epithelial basal membrane; in addition, a sparse interstitial meshwork of small fibres was observed in the villus lamina propria and around the crypts (Fig. 3a, b). In alcohol abusers the staining for pIp and pIIIp was enhanced in the basement membrane and in the stroma of duodenal villi compared with that in control subjects (Fig. 3c, d). This difference was particularly evident for pIIIp, whose deposition was markedly increased in alcoholics both at the base of villi and in the mucosal basement membrane. Conversely, no significant differences were evident in the extracellular deposition of NC1, LamP1 and FN between patients and controls (not shown). Un (collagen XIV) expression in the mucosa was low both in healthy and in alcoholic subjects; its extracellular deposition was sometimes enhanced in areas where some inflammatory cells were present, (Fig. 4c). In the distal duodenal mucosa of normal subjects Tn content was limited to a continuous band underneath the villus epithelium, which started at the base and progressively broadened toward the villus tip; Tn expression was seldom expressed around the crypts (Fig. 4a). Tn was distributed differently in alcohol abusers; in fact, its expression was higher at the mucosal surface and an

uninterrupted positive band was frequently seen at the base of villi, associated with a scattered distribution in the crypts (Fig. 4b).

The myofibroblast-like cells of the villus stroma described above showed positive staining for all the ECM components tested except Un (collagen XIV), whose staining was constantly negative (not shown).

In control tissues prepared for EM, numerous tapering cells were observed in the submucosa, underlying and closely apposed to the basal aspect of the epithelium. These cells were variable in shape and size, but exhibited common features including elongated cell bodies with attenuated cytoplasmic processes, elongated nuclei and prominent endoplasmic reticulum (Fig. 5a). In alcohol abusers, these cells appeared less tapering and exhibited a thicker cell body with a large, indented nucleus; bundles of thin filaments and subplasmalemmal densities were prominent (Fig. 5b).



Discussion

Chronic alcohol abuse may alter the matrix network of the duodenal mucosa in the sense of inducing a mucosal fibrosis, a term that indicates an excessive deposition of ECM components within a tissue. The pIp and pIIIp deposition was enhanced both in the basement membrane and in the stroma of duodenal villi in alcoholic patients compared with that in control subjects. This fibrosis was particularly evident in the stroma at the base of villi, where several crypts appeared reduced in number and strangled by excessive collagen deposition (see Fig. 3c, d). Interestingly, type III collagen was the most abundant in the "fibrotic" duodenal villi; these morphological features are reminiscent of one of the early stages of the alcoholic liver fibrosis, where an increased accumulation of type III collagen in the perivenular and perisinusoidal areas has been described [20, 40]. The increased deposition of collagen type I and, particularly, type III may explain the basement membrane thickening of duodenal villi shown in alcoholics by morphometry. Among the ECM components studied, chronic alcohol abuse also induced a different distribution of Tn, whose deposition was increased at the mucosal surface and, even more, at the base of villi. Tn, a hexameric glycoprotein ("hexabrachion") with a molecular mass of 1,000 kDa whose structure contains several fibronectin type III and EGF-like repeats [13], may be a modulator of such cell functions as cell-matrix adhesion and migration, through a direct interaction with cellular receptors [14, 15]. Nu-

Fig. 4 Immunostaining for **a, b** tenascin and **c** undulin (collagen XIV). **a** In the distal duodenal lamina propria of normal subjects tenascin content is limited to a continuous band underneath the villus epithelium, which starts at the base and becomes progressively broader towards the tip of the villus; some scattered immunostaining is also observed in a few crypts. **b** Tenascin distribution is quite different from normal in the lamina propria of alcohol abusers: tenascin content is high at the mucosal surface, while an uninterrupted positive band is frequently seen at the base of the villi and in some crypts. **c** A small focal deposit of undulin (collagen XIV), with the typical staining pattern consisting of undulating uniform fibres with parallel alignment is shown in the mucosa of an alcoholic patient at the site of a mild inflammatory infiltrate. Original magnification **a** ×85, **b** ×130, **c** ×260

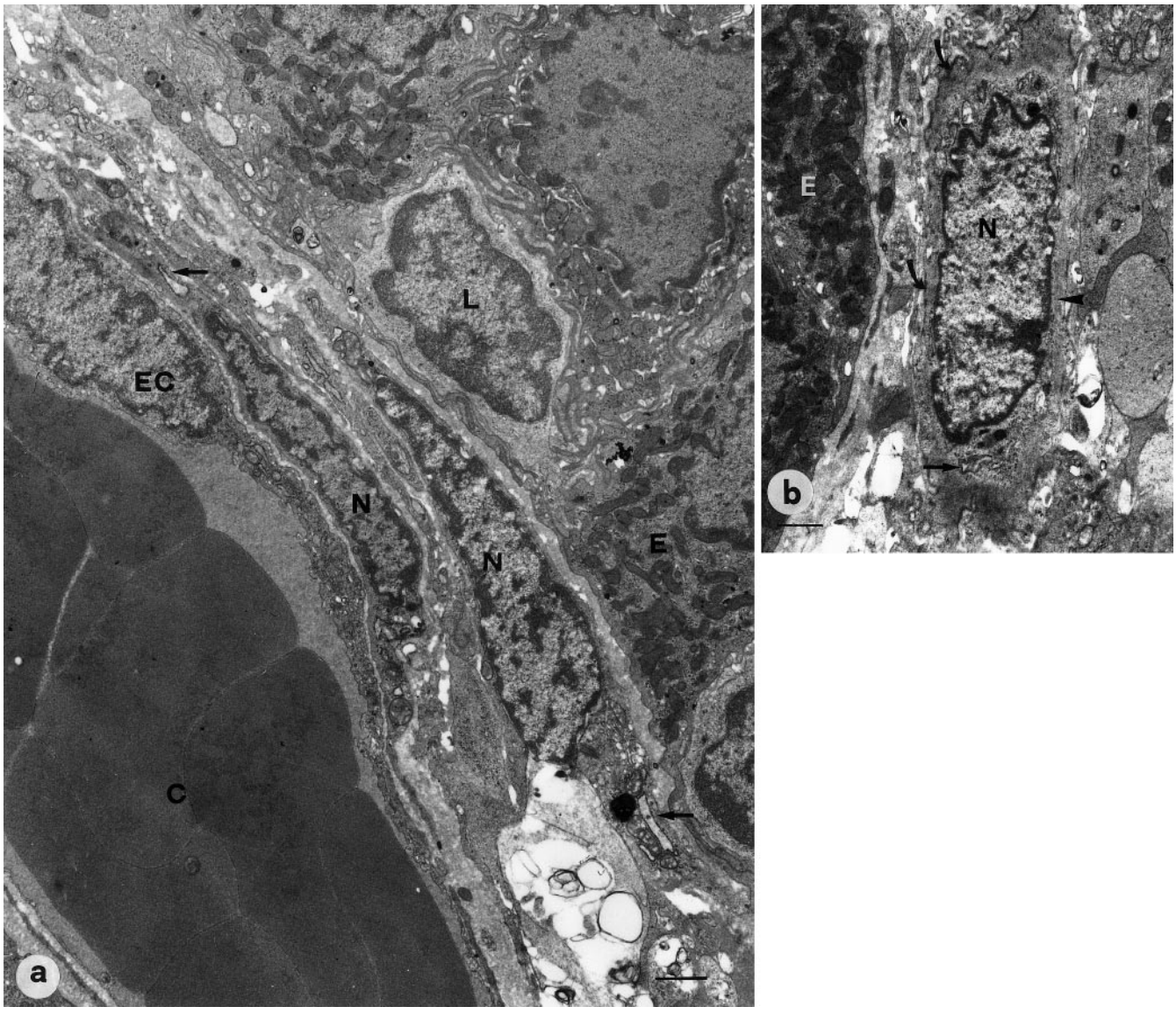


Fig. 5a The electron micrograph shows a general view of the relationship between the intestinal epithelium (*E*) and the adjacent subepithelial components in a control subject. A large capillary (*C*) filled with red blood cells is seen in the lower left corner, lined with thin cytoplasmic extensions of an endothelial cells (*Ec*). The area between the capillary and the epithelium is occupied mainly by two elongated fibroblast-like cells, running parallel to the basal aspect of the epithelium. They exhibit large, elongated nuclei (*N*), abundant rough endoplasmic reticulum (*arrows*) and cytoplasmic extensions running deep in the lamina propria. An intraepithelial lymphocyte (*L*) is present. **b** Representative electron micrograph of a myofibroblast-like juxta-parenchymal cell closely apposed to the duodenal epithelium (*E*) of a chronic alcohol abuser. The juxta-parenchymal cell exhibits a large indented nucleus (*N*); the cytoplasm contains numerous cisternae of rough endoplasmic reticulum (*arrows*) and bundles of microfilaments (*arrowheads*). Especially prominent are the numerous thickenings of the cytoplasmic membrane, which are suggestive of hemidesmosome-like structures (*curved arrows*). *Bar* 1 μm

merous nonparenchymal cells staining positively for both α-SMA and desmin were present in the stroma of villi in tight junction with the surface epithelium. These cells were five times as frequent in alcoholics as in control subjects and extended their cytoplasmic processes subepithelially. The microfilamentous pattern of α-SMA/desmin staining and the ultrastructural characteristics of these cells are typical of myofibroblasts [39]. Moreover, these nonparenchymal cells also showed positive staining for all the ECM components studied except undulin (collagen XIV) whose expression is almost absent in the mucosa. Considering the close apposition of some of these cells to the epithelium, we have assumed that they are responsible for the increased ECM deposition in both the basement membrane and the stroma of duodenal villi. Features of these cells are similar to those reported in other mammals, although various names have been used for them, including pericryptal fibroblastic sheath [22], subepithelial reticulohistiocytic complex [18] and subepithelial fibroblast sheath [29]. Recent data

suggest that the fibroblast-like cells of the intestinal mucosa are capable of a contractile function [21] and that they are myofibroblast-like cells: they are present in a number of tissues, located between parenchymal and stromal cells, and have been collectively designated juxta-parenchymal cells [47]. Interestingly, these stromal cells were not only less numerous in control subjects but also exhibited more fibroblastic features, similar to the juxta-parenchymal cells with contractile function described in the normal duodenal mucosa of other species [21, 47]. Thus, chronic alcohol abuse is also associated with a transformation of these cells into a more active synthetic phenotype, as happens in the liver, where alcohol induces the transformation of juxta-parenchymal stellate cells (Ito cells) into activated myofibroblasts [28]. In patients affected by chronic alcohol abuse the enterocyte turnover of the distal duodenum was significantly lower than in control subjects, as indicated by a smaller number of MIB-1-positive cells in the intestinal crypts of alcoholics. Despite this phenomenon the villus height and the enterocyte column size were unaffected. A similar effect has been described previously in rats chronically fed ethanol [24, 51, 52**].** It is known that enterocytes differentiate functionally during transit, becoming mature cells only in the upper portion of the villus [27]. It is possible that chronic alcohol abuse is associated with an increased residence time on the crypt–villus axis, resulting in a greater proportion of mature cells on the villus surface.

Previous reports have described an inhibitory effect of ethanol on liver regeneration [46, 48]. In particular, ethanol is able to impair both protein synthesis at the level of RNA transcription [36] and DNA synthesis [17]. More recently, ethanol has been shown to inhibit parenchymal cell regeneration through different mechanisms [1, 16]. The formation of acetaldehyde adducts with tubulin, previously shown in different organs [37], may interfere with the induction of enterocyte mitosis. Local production of acetaldehyde in the duodenal mucosa is suggested by the presence of both alcohol dehydrogenase and cytochrome P450 in enterocytes [25, 42]. Thus, the presence of acetaldehyde in the villus microenvironment might also be responsible for the alteration of the mucosal ECM [7–12, 31, 32].

Alcohol-related nutritional disturbances do not play any significant part in modification of the mucosal matrix network. In fact, none of the 50 chronic alcohol abusers considered in our study had either clinical evidence of malnutrition or severe liver disease. This is in agreement with previous reports (for review see [26]) that indicated the presence of alcohol-induced malnutrition associated with the occurrence of either severe liver disease or important alcohol-related disturbances of dietary intake. The intestinal fibrosis observed in our alcoholics may be mild compared with that found in many cases of chronic liver disease. However, it is noteworthy that none of the 50 alcoholics had clinical evidence of any chronic liver disease. Thus, the present study evaluated the effect of chronic alcohol abuse on the intestinal mucosa without possible bias from the presence of significant liver disease. The present data, although obtained with qualitative and semiquantitative methodologies, suggest that chronic alcohol abuse may induce fibrosis of the intestinal mucosa. Such fibrosis is associated with a transformation of villus juxta-parenchymal cells into active subepithelial myofibroblast-like cells able to produce different ECM components.

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