Gut 1995; **37**: 690–695

Transglutaminases in Crohn's disease

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Abstract

Transglutaminases are a family of Ca-dependent enzymes involved in various biological events. Circulating transglutaminase (factor XIIIa) is decreased in blood of patients with inflammatory bowel diseases. There is evidence that factor XIIIa and tissue type transglutaminase, present in cell cytosol, bind to various proteins of the extracellular matrix. This study examined the value of serum transglutaminase assay in the treatment and follow up of Crohn's disease and then investigated the intestinal location of both forms of transglutaminases by immunohistochemistry in normal and abnormal tissues. Serum transglutaminase activity was assayed in 36 patients with active Crohn's disease (CDAI>150). Eighteen patients were studied prospectively from relapse into remission. A significant inverse correlation ($p<0.001$) was found between circulating transglutaminase and Crohn's disease activity index; a correlation was also found between serum transglutaminase and serum orosomucoid ($p<0.01$) and C reactive protein ($p<0.01$). Patients were prospectively studied until clinical remission showed improvement in both their CDAI score mean (SD) (230 (46) to 72 (34), $p<0.01$) and transglutaminase activity mean (SD) (0·61 (0·12) to 0·93 (0·13) mU/ml, $p<0.01$). The immunohistochemistry assessment showed a colocalisation of factor XIIIa and tissue transglutaminase to the extracellular matrix of damaged tissues. In conclusion, these data confirm the value of serum transglutaminase assay as marker of Crohn's disease activity, extend the utility of serum transglutaminase assay to follow up of the disease, and emphasised the role of different types of transglutaminases in extracellular matrix assembly in the damaged tissues.

(*Gut* 1995; **37**: 690–695)

Keywords: Crohn's disease, activity index, tissue transglutaminase, factor XIII, tissue repair.

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Accepted for publication 23 March 1995

Transglutaminases mediate covalent cross linking between proteins by forming amide bonds between the γ-carboxamide groups of peptide bond glutamine moieties and the ε-amino groups of specific peptide bond residues.[1] At least three genetically distinct forms of transglutaminases are known. Factor XIII (FXIII) is a plasma circulating haemostatic factor that represents an inactive form of transglutaminase.[2] Besides FXIII, a tissue transglutaminase that is present in cell cytosol of all tissues and organs,[3] and a membrane bound epidermal transglutaminase[4] present in keratinocytes have been described. As the final enzyme in the coagulation cascade, activated FXIII (FXIIIa) catalyses the intermolecular cross linking of fibrine chains to each other and to other haemostatic proteins.[5] The enzyme activity can be detected in Ca-activated plasma (FXIIIa)[6] and in serum after clot formation (serum transglutaminase).[7] FXIII circulates in blood as a heterotetrameric zymogen (a_2b_2) composed of two enzymatically active a-subunits and two b-subunits as carrier proteins.[8] Tissue transglutaminase participates in various processes such as programmed cell death,[9] wound healing,[10] and cell growth and differentiation.[11 12] Evidence is mounting that both tissue transglutaminase and FXIIIa bind to fibronectine and other extracellular proteins contributing to the wound healing process.[10 13–15] We found serum transglutaminase activity reduced in various intestinal disorders: coeliac disease with relation to the active and remission phases,[16] and widespread intestinal malignancies (for example, intestinal lymphoma and α-chain disease).[17] Circulating enzyme activities have also been found decreased during the acute phase of inflammatory bowel disease and they were strongly related to the activity indices.[6 7] Recently we showed in a rat model of chronic colitis that serum and tissue transglutaminase activities reflect the changed intestinal morphofunctional integrity suggesting that serum transglutaminase assay could be a simple marker of intestinal mucosal status in inflammatory bowel disease.[18] The clinical activity of Crohn's disease is based on clinical and laboratory evaluations representing the sum of scores from several variables (for example, CDAI and Harvey-Bradshaw score),[19 20] which do not always reflect the pathogenic processes taking place in the intestine. In a recent endoscopic study in Crohn's disease, Modigliani *et al* show that mucosal inflammation and ulceration may be present in patients with symptomatically quiescent disease.[21] In view of these findings, this study was performed with two main objectives. The first was to discover if serum transglutaminase assay could be useful in the treatment and follow up of Crohn's disease. The second objective was to investigate by immunohistochemistry the intestinal location of tissue transglutaminase and FXIIIa both in normal and abnormal mucosa in Crohn's disease.

Methods

Patients

Thirty six patients with active Crohn's disease newly diagnosed at the gastrointestinal unit of

TABLE I *Clinical and laboratory summary of study group*

Crohn's patients		*Ileitis*	*Ileocolitis/colitis*
Patients (n)	36	15	21
Male	16	9	13
Female	20	6	8
Age (mean (SD))	40	40·7 (8·1)	38·3 (20·3)
CDAI	233 (49)	228 (37)	236 (57)
Serum transglutaminase mean (SD)	0·62 (0·11)	0·72 (0·14)	0·50 (0·08)
Packed cell volume mean (SD) (%)	37 (8)	38·8 (6·2)	37·5 (3·5)
White blood count mean (SD) (mm^3)	10·1 (3·2)	9·9 (2·1)	10·3 (4·2)
Albumin mean (SD) (g/d)	3·7 (0·50)	3·6 (0·40)	3·5 (0·7)
Orosomucoid mean (SD) (mg/dl)	134 (56)	147 (60)	103 (33)
Sedimentation rate mean (SD) (mm/h)	31 (19)	42 (25)	38 (28)
C reactive protein mean (SD) (mg/dl)	3·0 (3·9)	2·8 (3·5)	3·9 (4·1)

the University 'La Sapienza' of Rome were included in the study. The disease was confined to the distal ileum in 15 patients and to the colon with or without ileal involvement in 21 patients. Four patients with Crohn's disease of the ileum had bowel resection because of failure of medical treatment. The surgical procedure was ileocaecal resection with side to side ileocolonic anastomosis in all patients. In no patients did histological assessment show inflammation at the resection margins. The Crohn's disease group included 20 women and 16 men with a mean age of 40 years (range 19–72).

Disease activity was assessed by the Crohn's Disease Activity Index (CDAI),[19] supplemented by laboratory measurements. The CDAI score was evaluated in each patient on the day of blood collection for transglutaminase activity assay. Serum C reactive protein was measured by an electroimmunodiffusion technique,[22] orosomucoid by nephelometry[23]; the erythrocyte sedimentation rate was measured by the Westergren method.[24] Blood packed cell volume and white blood cell counts were performed by a Coulter counter and serum albumin concentrations by a colorimetric method.[25]

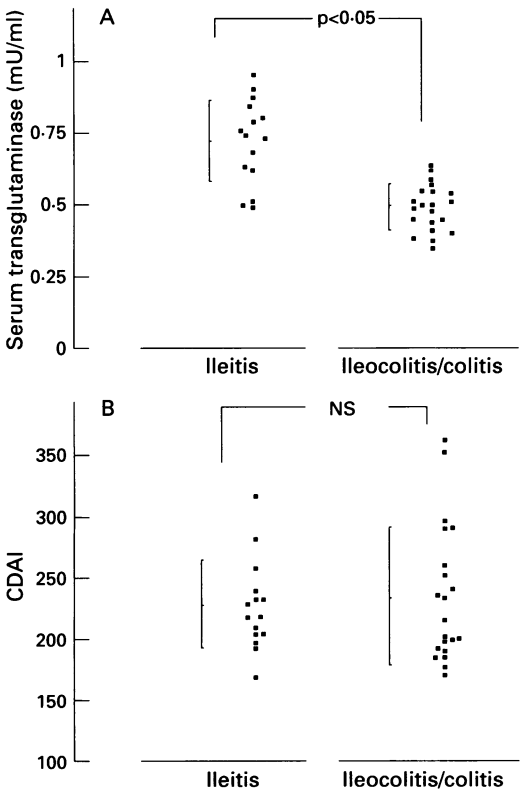

Figure 1: Serum transglutaminase (A) and CDAI score (B) in Crohn's disease patients with ileal or ileocolonic/colonic disease involvement.

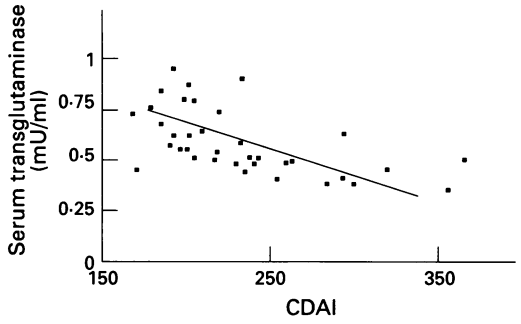

Figure 2: Correlation between serum transglutaminase and CDAI score in active Crohn's disease patients (r=−0·62, n=36, p<0·001).

Serum transglutaminase and haematological parameters were also evaluated in 18 patients studied prospectively during the active phase (CDAI>150) and subsequent inactive phase (CDAI<150) of the disease.

In the four patients undergoing surgery, serum transglutaminase concentrations and haematological parameters were evaluated before and 15 and 60 days after bowel resection.

The control group included 21 patients with disease other than inflammatory bowel disease but also leading to debility (liver cirrhosis, cardiovascular disorders, non-intestinal malignancies). Serum transglutaminase from 38 healthy subjects was also assayed.

Transglutaminase activity

Venous blood samples were collected after an overnight fast and left at room temperature for two hours to avoid the influence of coagulation. Serum samples were stored at −20°C until the assay. Transglutaminase activity on serum was assayed using a modified method of Lorand *et al*[26 27]: 30 µl of the sample were added to 45 µl of reaction mixture containing a final concentration of 0·25 mM of ^{14}C-putrescine (Amersham, UK), 50 mM of dithiothreitol, 10 mM of $CaCl_2$, and 4% (w/v) dimethylcasein in TRIS-HCl buffer (50 mM) pH 9·0 with 0·1% Triton X100 and incubated in a shaking bath at 37°C for 20 minutes. Twenty µl were spotted onto 3MM Whatman round paper filters (2 cm) and immediately plunged into 10% ice cold trichloroacetic acid for 15 minutes. Two consecutive 15 minutes washings were performed in 5% ice cold trichloroacetic acid followed by a brief washing in ethanol-acetone (50% v/v) and then in acetone. The dried paper filters were counted in 6 ml of Aquasure scintillant (Dupont-NEN). A similar procedure was adopted for blanks, standards, and controls. Transglutaminase units were expressed as 1 mU=1 nmol of putrescine into acceptor protein at 37°C, pH 9.

Western blotting

Tissue transglutaminase from guinea pig liver (Sigma, St Louis, MO) and purified FXIIIa (gift from Behring, Marburg, Germany) were run on SDS/polyacrylamide gels according to

the method of Laemmli,[28] and than transferred to nitrocellulose with Biorad transblot apparatus. The nitrocellulose was blocked by incubation with 3% bovine serum albumine in TTBS (50 mM TRIS, pH 7·9, 150 mM NaCl and 0·05% TWEEN 20). Primary antibodies, anti-tissue transglutaminase (generously given by Dr Vittorio Gentile 2nd University of Naples) or anti-FXIIIa (Behring) were added and incubation continued overnight at room temperature. After the nitrocellulose had been washed three times in TTBS, the appropriate avidin conjugated secondary antibody was added in TTBS for 60 minutes. After washing, immunoreactive proteins were detected by development with the ABC Vectastain kit, according to the manufacturer's directions.

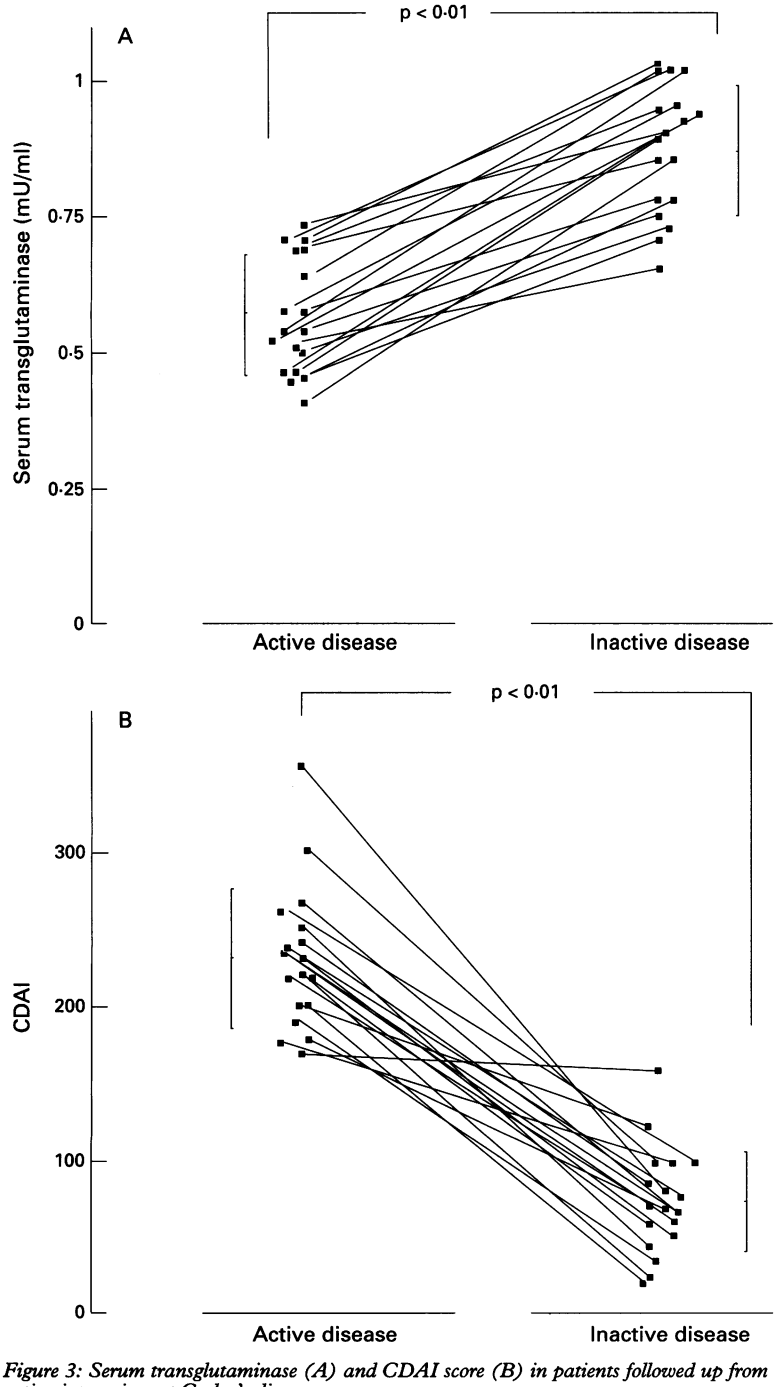

Figure 3: Serum transglutaminase (A) and CDAI score (B) in patients followed up from active into quiescent Crohn's disease.

Immunohistochemistry

Routinely processed, formalin fixed, and paraffin wax embedded specimens were taken from the ileum of four Crohn's disease patients and from the colon of four Crohn's disease patients who were operated on during 1993 and were drawn from the files of the Institute of Pathologic Anatomy at the School of Medicine, Naples. Uninvolved bowel of patients undergoing surgery for carcinoma or large polyps were used as a 'normal' control tissue. Immunohistochemical examination was performed on normal and abnormal ileum and colon using either anti-tissue transglutaminase or anti-FXIIIa antibodies. The ematoxilin-eosine stained specimens were immunoprobed with the antibodies and visualised using a peroxidase anti-peroxidase system,[29] according to the manufacturer's directions.

Statistics

Student's *t* test and linear regression were used to perform statistical evaluations. Results, expressed as mean (SD), were considered statistically significant when $p<0·05$.

Results

Patients

Table I shows the clinical and haematological characteristics of Crohn's disease patients. As shown, the mean CDAI score in the total group of patients was 233 (range 169–366), while the mean (SD) serum transglutaminase activity was 0·62 (0·11). When patients were grouped according to localisation of the disease, serum transglutaminase concentrations were found to be significantly higher in patients with ileitis (transglutaminase=0·72 (0·14) mU/ml) compared with patients with ileocolitis/colitis (transglutaminase=0·50 (0·08); $p<0·05$). No differences were found, however, between CDAI scores from patients with ileitis versus ileocolitis/colitis (228 (37) *v* 236 (57) respectively) (Fig 1).

When the whole group of patients was considered, a strong correlation ($r=-0·60$; $p<0·001$) was found between serum transglutaminase and CDAI scores (Fig 2). A significant correlation was also found between circulating transglutaminase and serum orosomucoid ($r=-0·55$; $p<0·01$) and C reactive protein ($r=-0·52$; $p<0·01$).

Eighteen patients were followed up until remission, which mainly occurred after 4·1 months (range 1–9). Their CDAI scores improved from 230 (46) to 72 (34) during the active and inactive phases respectively ($p<0·01$). Furthermore, the decreased circulating transglutaminase values seen during the active phase returned toward normal values during the subsequent inactive phase of the disease (0·61 (0·12) *v* 0·93 (0·13), $p<0·01$) (Fig 3). In the four patients who had intestinal resection, the CDAI significantly decreased 15 days after surgery, while increased serum transglutaminase was seen only at 60 days (Table II). As expected, the mean serum

TABLE II *CDAI and serum transglutaminase (mU/ml) in patients who had a resection*

Resection	*Before*	*15 Days after*	*60 Days after*
CDAI	265 (57)†	64 (23)*	85 (31)*
Serum transglutaminase	0·64 (0·08)§	0·74 (0·10)	1·05 (0·09)‡

*p<0·01 *v* †; ‡p<0·01 *v* §.

enzyme values in the control and healthy volunteer groups fell to within the normal range (1·80 (0·57) mU/ml).[7]

Western blotting

Antiserum raised against FXIIIa recognised purified FXIIIa and cross reacted with tissue transglutaminase on western blots as shown in Fig 4 (lane 1 and 2). Anti-tissue transglutaminase antiserum showed a specific immunoreactivity for tissue transglutaminase (lane 4), while did not recognise FXIIIa (lane 3).

Immunohistochemical studies

Tissue transglutaminase antibody – the staining pattern showed that the enzyme is present in the basal region of the crypts in normal ileum (Fig 5 (A)) while strongly positive areas involving the whole crypt and to a lesser extent the extracellular matrix appeared in ileal Crohn's disease (Fig 5 (B)). In normal colon the positivity to tissue transglutaminase antibody was found along the crypt surface (Fig 5 (C)). In colonic Crohn's disease the staining pattern showed that the enzyme is mainly localised to the extracellular matrix but also within the crypts (Fig 5 (D)).

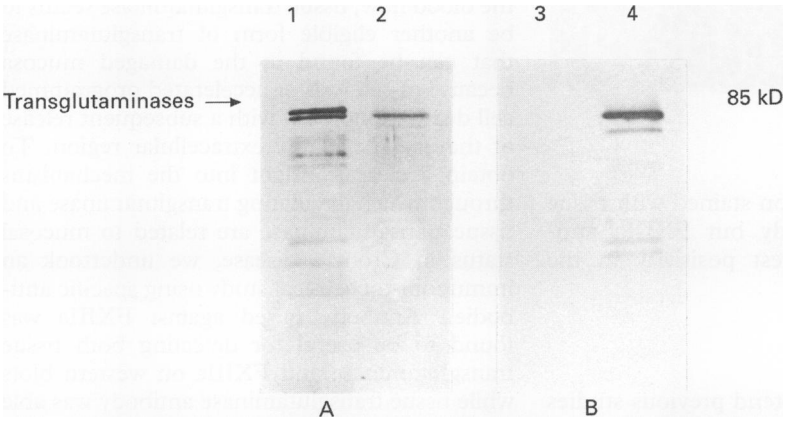

Figure 4: Western blots of purified factor XIIIa (lane 1 and 3) and tissue transglutaminase (lane 2 and 4) after denaturing SDS-gel electrophoresis. (A) Blot immunoprobed with factor XIIIa antibody; (B) blot immunoprobed with tissue transglutaminase antibody.

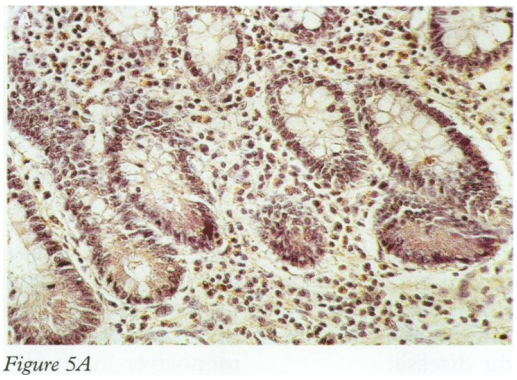

Figure 5A

FXIIIa antibody – the positivity to FXIIIa antibody found in normal ileum (Fig 6 (A)) and colon Fig 6 (C)) was similar to that obtained with tissue transglutaminase antibody. Immunostaining in ileal (Fig 6 (B)) and colonic (Fig 6 (D)) Crohn's disease showed

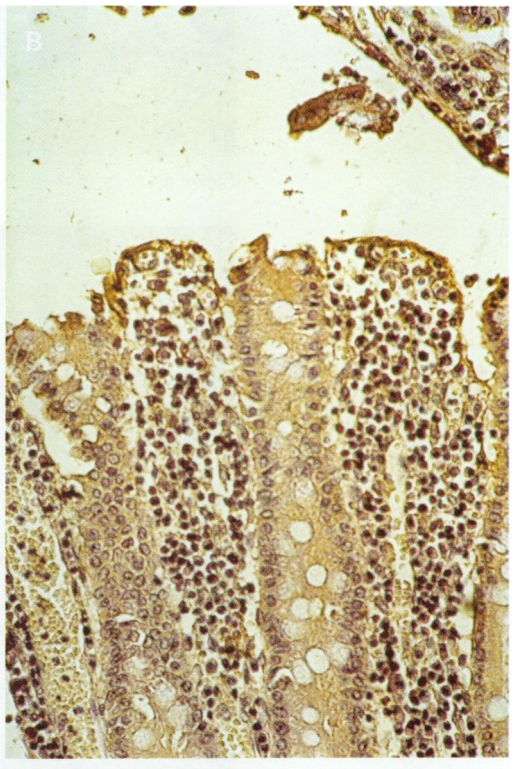

Figure 5B

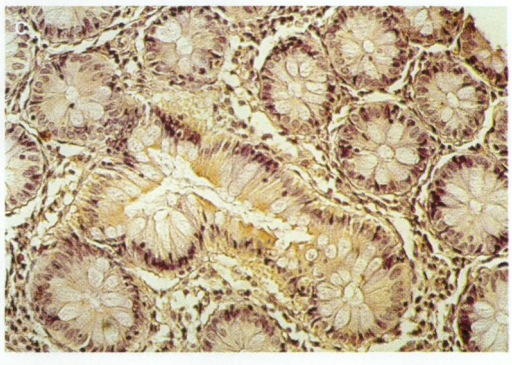

Figure 5C

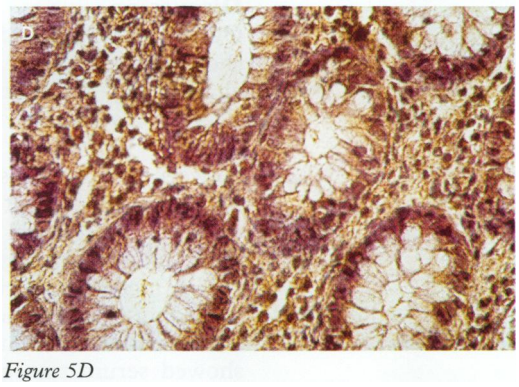

Figure 5D

Figure 5: Immunohistochemistry of specimens from normal controls and Crohn's disease: tissue transglutaminase staining using tissue transglutaminase antibody and peroxidase-antiperoxidase system; (A) normal ileum; (B) Crohn's of the ileum; (C) normal colon; (D) Crohn's of the colon.

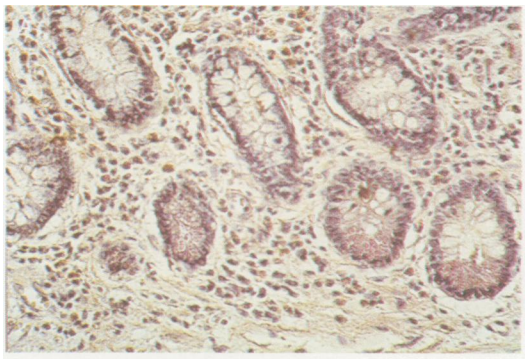

Figure 6A

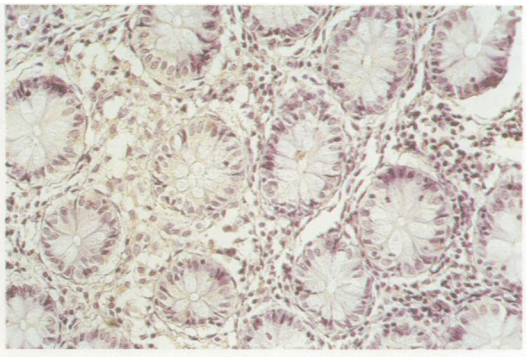

Figure 6C

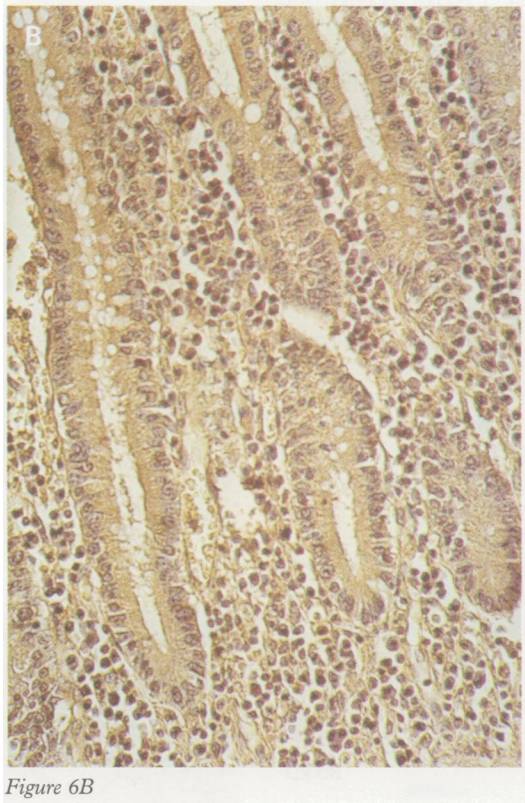

Figure 6B

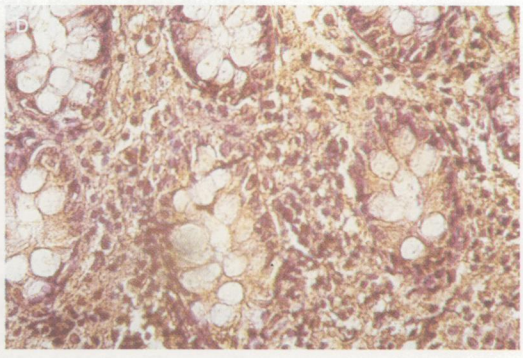

Figure 6D

Figure 6: Immunohistochemistry of specimens from normal controls and Crohn's disease: FXIIIa staining using FXIIIa antibody and peroxidase-antiperoxidase system; (A) normal ileum; (B) Crohn's of the ileum; (C) normal colon; (D) Crohn's of the colon.

the same pattern of section stained with tissue transglutaminase antibody but FXIIIa antibody produce the highest positivity in the extracellular matrix.

Discussion

Our data confirm and extend previous studies showing the presence of a correlation between circulating transglutaminase values and Crohn's disease activity as assessed by CDAI score.[7 30] A strong correlation was found between serum transglutaminase values and inflammatory mediators as serum orosomucoid and C reactive protein. When patients were followed up, serum transglutaminase values increased during the quiescent phase according to the improved CDAI. Furthermore, patients with ileocolitis/colitis showed serum transglutaminase values lower than the subgroup with ileitis, suggesting that this parameter could be helpful not only in the assessment of the severity of inflammation but also in providing some information about the extension/localisation of the disease.

The decreased serum transglutaminase activity led us to look for a possible enzyme requirement in mucosal recovery. Besides FXIII, which can be delivered to the damaged tissue by the blood flow, tissue transglutaminase seems to be another eligible form of transglutaminase that can be found in the damaged mucosa because of cell lysis or accelerated programmed cell death (apoptosis) with a subsequent release of the enzyme in the extracellular region. To obtain a clearer insight into the mechanisms through which circulating transglutaminase and tissue transglutaminase are related to mucosal status in Crohn's disease, we undertook an immunohistochemical study using specific antibodies. Antibody raised against FXIIIa was found to be useful for detecting both tissue transglutaminase and FXIIIa on western blots while tissue transglutaminase antibody was able to recognise only the protein derived from tissue. These immunoreactivities allowed us to investigate the intestinal pattern of distribution of tissue transglutaminase and FXIIIa both in normal and abnormal mucosa. The same immunostaining pattern was seen in normal tissues using either FXIIIa or tissue transglutaminase antibodies. In Crohn's disease the two antibodies showed a similar staining pattern in the crypts but when FXIIIa antibody was used the highest positivity was detected in extracellular matrix. These findings, for the first time, show the presence of both types of transglutaminases in extracellular matrix of damaged tissues. Tissue transglutaminase activity has been seen in wound healing process in the cell monolayer in vitro[10] and in mucosal repair in

the intestine,[31] using proteins of the extracellular matrix such as fibronectin and collagen, which are suitable substrates for the enzyme.[15 32 33] On the other hand, it has been suggested that FXIIIa and plasma fibronectin may localise to the extracellular matrix contributing to the healing process.[31] In 1989 Allan *et al*[34] showed that plasma fibronectin concentrations were low in patients with extensive or severe Crohn's disease while increased tissue deposition has been shown in experimental animal models in a variety of circumstances.[35 36] It is probable that plasma transglutaminase (FXIII) in its active form FXIIIa is locally utilised for tissue repair with a subsequent reduced circulating concentration. Recent studies[6] also showed a significant decrease of FXIIIa in ulcerative colitis suggesting that the enzymatically active fraction plays a part in haemostatic events during the active phase of the disease. We previously showed that transglutaminase activity significantly decreases in serum being closely related to the severity of inflammation in experimental colitis in rats.[18] In the same model, FXIII intravenous treatment, improved the induced colitis.[31] These findings show that circulating transglutaminase values are related to the presence of intestinal inflammatory lesions. Even if serum transglutaminase cannot be considered a specific test for the diagnosis of inflammatory bowel disease, our data suggest that serum transglutaminase could represent a very easy test for monitoring the intestinal inflammatory process in Crohn's disease. Patients undergoing bowel resection, showed an improved CDAI 15 days after surgery while serum transglutaminase increased toward control values by 60 days. It is possible that the delayed normalisation of serum transglutaminase when compared with CDAI scores could be related to a slow reconstitution of the normal transglutaminase circulating pool or to the local need of the enzyme for the mucosal morphofunctional recovery after intestinal resection, or both.[37]

In conclusion our data further confirm the usefulness of serum transglutaminase assay as a marker of Crohn's disease activity, extend the utility of serum transglutaminase assay to the follow up of the disease, and underline the role of different types of transglutaminases in the extracellular matrix assembly in the damaged tissue.

The authors thank Dr H Metzner (Behring) for generously providing purified factor XIII and Dr V Gentile (2nd University of Naples) for kindly supplying the tissue transglutaminase antibody.

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