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Expression of clusterin in Crohn's disease of the terminal ileum

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Summary. Crohn's disease (CD) is a chronic inflammatory intestinal disorder with disturbance and injury of the intestinal mucosal barrier, in which various proinflammatory molecules as well as molecules with antiinflammatory activity and cytoprotective function are found to be expressed. We investigated whether clusterin, a multifunctional cytoprotective protein, is upregulated in Crohn's disease, because augmented expression of clusterin is seen in many organs following various forms of tissue injury. Human actively and inactively inflamed ileal tissues from CD patients as well as normal intestinal specimens from control patients (normal ileum) were investigated by Western blot analysis, immunohistochemisty and *in situ* hybridization. As compared with controls, a strongly enhanced expression of clusterin was found in CD tissues, correlating with disease activity. Immunohistochemistry and *in situ* hybridization analysis revealed foci of crypts almost completely lined by clusterin expressing enterocytes in CD, a feature that was never seen in controls. Such crypts appeared especially within the morphologically intact mucosa apart from erosive or ulcerative lesions. Besides epithelia, clusterin was also expressed by inflammatory mononuclear cells. Enhanced expression of clusterin by crypt epithelia might reflect a cytoprotective function of the protein in order to prevent further injury of the intestinal mucosal barrier in CD.

Key words: Apolipoprotein J, Crohn's disease, Enterocytes, Ileum

Introduction

Crohn's disease (CD) is associated with increased permeability of the intestinal mucosal barrier (Olaison et al., 1990; Hollander, 1992; Peeters et al., 1994). Evidence is given that permeability changes in CD are related to disease activity (Pironi et al., 1990; Wyatt et al., 1993). In relatives of patients with CD, deranged intestinal permeability has been reported, pointing to the possibility of a hereditary defect in barrier function (Hollander et al., 1986; May et al., 1993; Hilsden et al., 1996; Söderholm et al., 1999a). In experimental animal models, bypassing (Yamada et al., 1993; Fries et al., 1999) or breaching (Hermiston and Gordon, 1995) the epithelial barrier can initiate disorders similar to CD. These data suggest that a defect in the intestinal barrier could be of etiological importance in CD (Söderholm et al., 1999b). On the other hand, it has been shown that molecules with cytoprotective function, such as activin, are upregulated at sites of inflammatory tissue damage (Hübner et al., 1999).

Clusterin - first described in 1983 as a major secretory glycoprotein produced by ram Sertoli cells (Blaschuk et al., 1983) - is physiologically expressed in a wide variety of tissues and shows a high degree of sequence conservation between species (for review see Rosenberg and Silkensen, 1995). Various functions for clusterin have been described, including induction of cellular aggregation (Silkensen et al., 1995), regulation of apoptosis (Buttyan et al., 1989), membrane remodeling (Fritz and Murphy, 1993), inhibition of complement function (Jenne and Tschopp, 1989), and modulation of fluid-tissue interfaces (Jordan-Starck et al., 1992). Moreover, augmented clusterin expression has been found in a variety of disease states and local overexpression of clusterin has been seen in tissue injuries (Silkensen et al., 1994), such as local acidosis, androgen ablation of the prostate gland, and diverse kidney diseases (summarized and cited in Rosenberg and Silkensen, 1995). Recently, it has been shown that clusterin has a cytoprotective function at sites of tissue damage through its genuine ability to prevent inappropriate precipitation of secreted or cell-surface proteins in a chaperone-like manner (Humphreys et al., 1999; Hochgrebe et al., 2000; Wilson and Easterbrook-

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Smith, 2000). Experimental data indicate that pH induced changes in the molecular structure of clusterin are responsible for its enhanced ability to bind ligands at mildly acidic pH (Hochgrebe et al., 2000; Wilson and Easterbrook-Smith, 2000). At sites of inflammation or tissue damage the local pH can drop to below 6, a phenomenon described as local acidosis (Hochgrebe et al., 2000; Wilson and Easterbrook-Smith, 2000). Such a state of local acidosis is likely to occur in inflamed gut in CD.

The expression of clusterin in the terminal ileum, a preferential site of CD in humans, has not been studied yet. The aim of the present work was to assess the expression of clusterin on the protein and on the mRNA level in ileal tissues of CD patients.

Materials and methods

Patients and tissues

Surgical resection of the ileocaecal region including the terminal ileum was performed in 10 patients with CD (mean age, 37 years; range, 22 to 59 years) and in 10 patients (mean age, 58 years; range, 39 to 69 years) with sporadic cancer of the ascending colon. The non affected terminal ileum of the 10 patients with sporadic cancer served as normal controls. In patients with CD, surgical resection was necessary due to stenosis of the intestinal segment and resistance to medical therapy (including corticosteroids in 6 patients; mesalazine in 5 patients). Diagnosis of CD was established by conventional clinical and histological criteria. All surgical specimens were opened longitudinally along the antimesenteric border. For subsequent analysis, samples of about 1 cm^2 in size were taken. One half of the unfixed material was used for molecular experiments (see below), the other half was postfixed in formaldehyde, subsequently paraffin embedded and used for morphological and immunohistological analysis.

Grading of inflammation

H&E-stained sections from paraffin-embedded tissues were used to define the inflammatory degree of CD tissues: the term 'inactive inflammation' (I) was used when the amount of lymphocytes and plasma cells in the stroma was increased (n=4) as compared with controls. 'Active inflammation' (A) designates intestinal CD tissues (n=6) where destructive lesions such as granulocytes, cryptitis, crypt abscesses, erosive defects and/or ulcers were additionally seen.

Preparation of riboprobes and in situ hybridization

A 1.36 kbp rat clusterin cDNA fragment subcloned in pBluescript SK- [a kind gift from R. Buttyan, (Buttyan et al., 1989)], was used to prepare digoxigenin-labeled sense and antisense riboprobes. The identity and the orientation of the insert was verified by sequencing. For generation of the antisense probe plasmid was linearized with Bam H1 and transcribed with T7 RNA polymerase; for generation of the sense probe, plasmid was linearized with Hind III and transcribed with T3 RNA polymerase. Because clusterin shows a high degree of conservation among species, a rat cDNA could be used for studies on human tissues. We have previously used the same cDNA to detect clusterin mRNA expression in rat polycystic kidneys (Obermüller et al., 1997). In vitro transcription was carried out using a commercially available kit (Roche Diagnostics, Mannheim Germany) according to the manufacturer's suggested protocol. To improve the penetration of the probes during the in situ hybridization experiments, the transcripts were shortened to a calculated average length of 250 bases by alkaline hydrolysis.

In situ hybridization was carried out on tissues of three randomly-selected CD-patients (n=3) and controls (n=3), as described previously, with slight modifications (Obermüller et al., 1998). In brief, deparaffinized sections were postfixed in 4% paraformaldehyde/PBS for 10 min, rinsed three times in PBS and then treated with proteinase K (8 μg/ml in PBS) for 30 min at 37 °C. Thereafter sections were washed briefly in PBS and fixed again for 1 minute in 4% paraformaldehyde/PBS and washed. To reduce background, slides were acetylated for 15 minutes in 0.1M triethanolamine, pH 8.0, containing 0.25% acetic anhydride, added immediately before starting this step. After rinsing in PBS slides were dehydrated in 70, 80, and 95% ethanol and air-dried.

Prehybridization and hybridization as well as the immunological detection of the hybridized riboprobes were performed as described previously (Obermüller et al., 1998). Hybridization was carried out overnight at a temperature of 46 °C; the hydrolysed clusterin probe was used at a concentration of 2 to 4 ng per μl hybridization mixture. Control experiments included incubations with sense probes on alternate sections. In addition, other sections were hybridzed without antisense probe or by omitting the anti-digoxigenin antibody. All controls yielded completely negative results.

Antibodies and immunohistochemistry

For immunohistological staining experiments (IH) and immunoblotting analyses (IB) the following primary and secondary antibodies, diluted as indicated in brackets, were used: goat polyclonal anti-clusterin antibody (IH, 4 μg/ml; IB, 0.4 μg/ml); mouse anti-ß-actin antibody (IB, 0.2 μg/ml); and horse-radish-peroxidase (HRP) conjugated affinity-purified anti-goat as well as anti-mouse antibodies (IB, 1:5000); all antibodies were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, USA. The biotin-coupled sheep anti-goat antibody (IH, 1:200) was purchased from Dianova, Hamburg, Germany. For ABC immunostaining, an ABC detection kit with DAB as the chromogen (DAKO, Hamburg, Germany) was used in

accordance to the manufacturer's suggested protocols. Finally, immunostained sections were counterstained with hematoxylin and mounted. Immunohistochemistry was carried out on tissues of all CD-patients (n=10) and controls (n=10).

Preparation of tissues, SDS-PAGE, and Western blot analysis: Specimens of the terminal ileum (about 300 mg) of five randomly-selected CD-patients (n=5) and controls (n=5) were homogenized in 10 mM Tris-HCl, pH 7.4 containing 10% glycerol using the Ultra Turrax equipment (IKA Labortechnik, Staufen, Germany) in an ice bath with a tissue to buffer ratio of 1:3. The homogenates were centrifuged at x42,000g at 4 °C for one hour. The supernatants in reducing Laemmli buffer were subsequently used in Western blot analysis. This procedure was adapted from the method of Grima et al. (1990). Protein measurements were performed using the BioRad assay reagent (BioRad, München, Germany). Samples were stored at -20 °C until use.

One-dimensional SDS-PAGE (10%) was performed according to the method of Laemmli (Laemmli, 1970). Molecular weight markers were purchased from Amersham (Amersham International plc, Little Chalfont, UK). 15 μg of total protein were loaded for each sample, separated under reducing and denaturing conditions and transferred to a PVDF Immobilon-P membrane (Millipore Corporation, Bedford, MA, USA) using a semi-dry blot equipment. The ECL substrate (Amersham International plc, Little Chalfont, UK) was applied for subsequent detection. For antibody stripping, blots were immersed in methanol, washed in water and incubated in stripping buffer consisting of 2% SDS, 100 mM ß-mercaptoethanol, and 62.5 mM Tris-HCl (pH7.5) for 30 min at 50 °C. Subsequently, blots were processed as described above in order to evaluate ß-actin as loading control. Negative controls included blotting experiments in which the primary antibody was omitted.

Results

The expression of immunoreactive clusterin was increased in tissue homogenates of the terminal ileum of CD patients when compared with the normal controls (non affected terminal ileum of 5 randomly selected patients with sporadic cancer). Two bands specific for clusterin were detected at a molecular mass of about 40 kDa, which is in agreement with the reported molecular mass of the alpha and beta chains of human clusterin (Murphy et al., 1988). In CD, expression of clusterin was strongly increased in actively inflamed tissues of the terminal ileum as compared with inactively inflamed ileal specimens (moderate increase) or in control ileal tissues (Fig. 1). Thus, the amount of immunoreactive clusterin in ileal samples paralleled the severity of the inflammatory bowel disease.

Using immunohistochemistry in CD and control tissues (non affected terminal ileum of the 10 patients with sporadic cancer), expression of clusterin was found in individual enterocytes of the apical villus as well as in intestinal lymphatic follicles (Figs. 2, 3A). Interestingly, in CD clusterin was additionally expressed by enterocytes lining crypts, a phenomenon clearly different from that seen in normal controls (Fig. 3). Exclusively in CD, foci of crypts almost completely lined by clusterin expressing enterocytes were found, as seen in the overview in figure 3A. Although in many cases clusterin protein expressing enterocytes intermingled with unreactive epithelial cells, occasionally, various crypts were almost completely lined with immunostained epithelial cells (Fig. 3B). Notably, histomorphological signs of apoptosis were not encountered in these cells. At sites of erosive defects or ulcers, the epithelium of very few crypts occasionally showed immunostaining for clusterin, but the overwhelming number of crypts was devoid of specific detection signals. Also in the surface epithelium bordering such mucosal defects, immunostained epithelial cells were regularly lacking (Fig. 3C). In CD, clusterin was also strongly expressed by inflammatory cells within the lamina propria (e.g., granulocytes, lymphocytes) and by lymphocytes of intestinal lymphatic follicles (Fig. 3A). Inflammatory cells at sites of erosive defects and ulcers were regularly positive for clusterin (Fig. 3C). However, immunoreactivity of clusterin expressed in leucocytes showed interindividual differences. In the majority of CD tissues (n=7), expression of clusterin in leukocytes was strong. In the other CD tissues (n=3), the

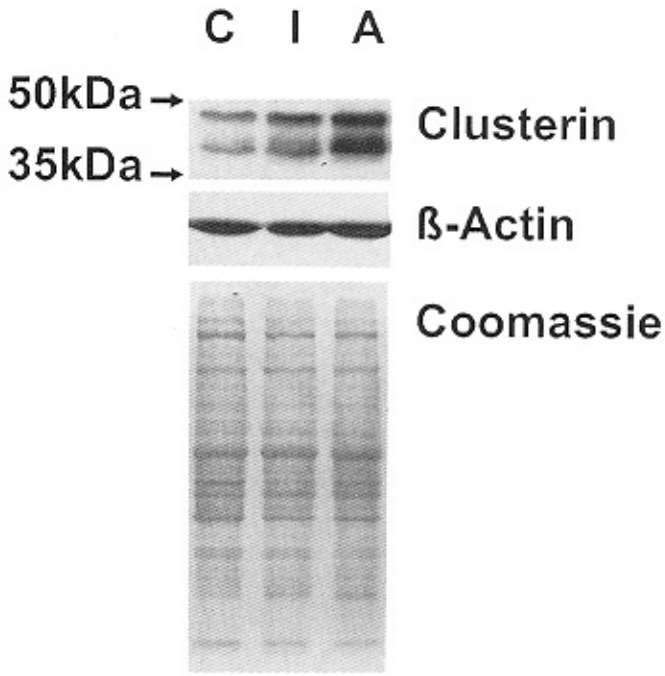

Fig. 1. Western blot analysis of clusterin protein expression in actively **(A)** and inactively **(I)** inflamed ileal tissues of Crohn's disease and in unaffected ileal tissues of controls **(C)**. Clusterin expression is increased in Crohn's disease (A and I) in comparison to normal controls. ß-actin labeling and a Coomassie-stained PVDF membrane are shown as loading controls.

lymphocytes exhibited only a moderate staining, whereas no expression was seen in granulocytes.

The findings obtained by immunohistochemistry were corroborated by the results of the *in situ* hybridization experiments performed on CD tissues. The overview in figure 4A shows a strong expression of clusterin mRNA by epithelial cells, preferrably located in crypts, but also in some cells of the apical part of the villi. In addition, clusterin mRNA was found to be expressed by inflammatory cells present in the villous stroma and in cells of lymphatic follicles (Figs. 4A,B). The detailed analysis in figure 4C shows that especially epithelial cells lining crypts exhibited a strong expression of clusterin mRNA.

Discussion

CD is a chronic inflammatory bowel disease histologically characterized by segmental and transmural inflammation. The intestinal segment most commonly involved in CD is the terminal ileum, but all sites of the gastrointestinal tract may be affected discontinuously (Desreumaux et al., 1999). One interesting hypothesis assumes a primary defect in the intestinal barrier (Söderholm et al., 1999b), because increased intestinal permeability has been found in CD patients (Olaison et al., 1990; Hollander, 1992; Peeters et al., 1994) and in their relatives (Hollander et al., 1986; Hilsden et al., 1996; Söderholm et al., 1999a).

Clusterin is a multifunctional protein showing a wide physiological tissue distribution as well as a marked induction in a variety of states of tissue injury and remodeling (for review see Rosenberg and Silkensen, 1995). Ample evidence suggests that clusterin exerts cytoprotective functions, although the expression of this protein under different pathophysiological conditions still remains puzzling.

Our data reveal that the expression of clusterin is upregulated in the small intestine of CD patients in comparison to ileal control tissues. The data obtained by Western blot analysis show that the amount of the clusterin protein expressed correlated with disease

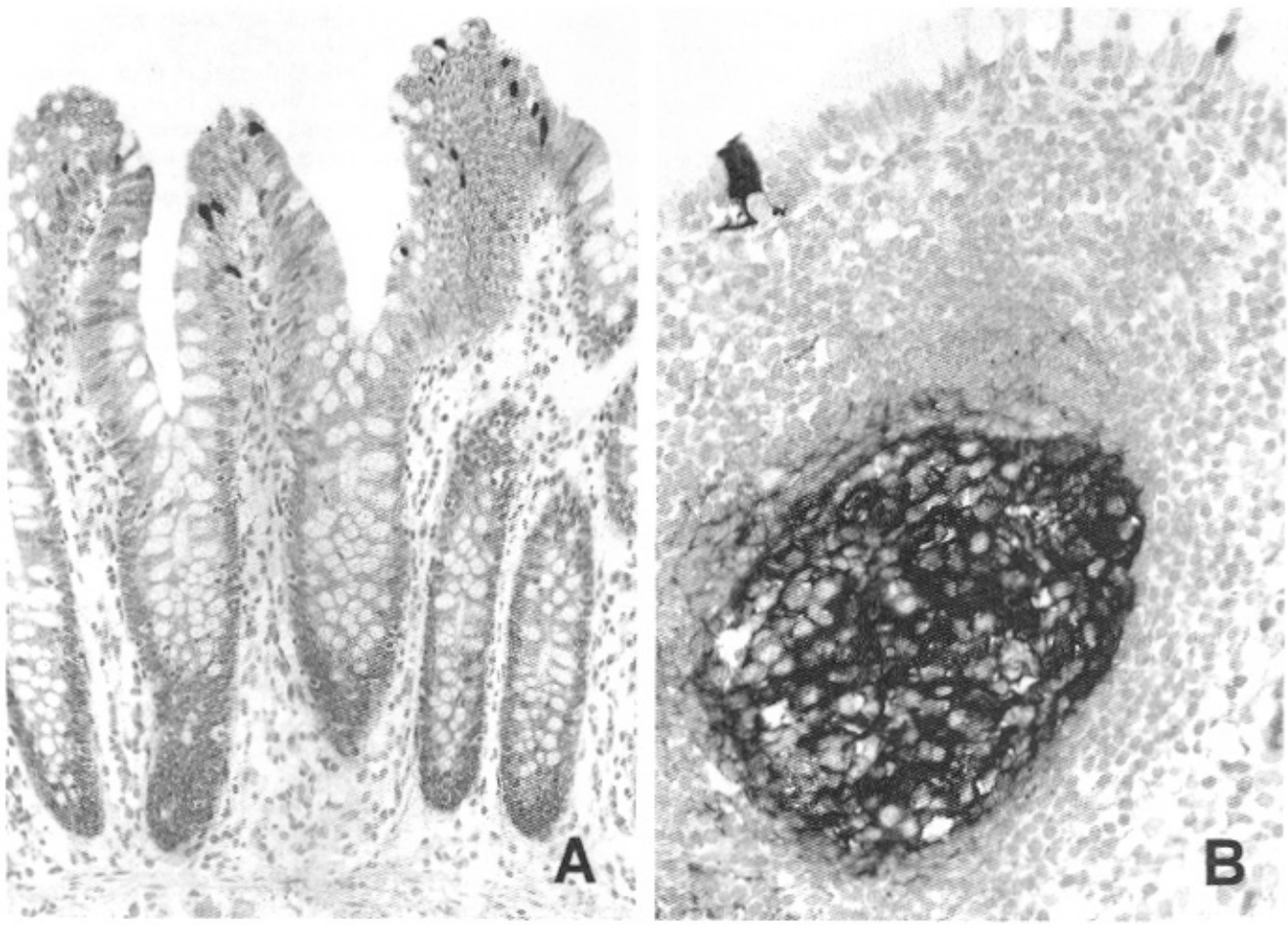

Fig. 2. Immunohistochemical detection of clusterin using the ABC technique in unaffected control tissue of the terminal ileum. **A.** Ileal mucosa with slender and short villi lined by columnar epithelial cells and goblet cells. At the apical tip few enterocytes are expressing clusterin. **B.** High magnification of the surface epithelium in the vicinity of a lymphoid follicle. Expression of clusterin is found within the follicle and in single epithelial cells of the surface epithelium. A, x 80; B, x 250

activity. The fact that clusterin is also detectable in tissue homogenates of control samples demonstrates that different cells in the normal intestine also produce clusterin at probably low levels. Previous reports demonstrated expression of clusterin in the small intestine of rat and mouse (Agarwal et al., 1996; Arai et al., 1996) pointing to a role in tissue differentiation and remodelling (Ahuja et al., 1994). Likewise, in the human terminal ileum clusterin might be secreted during the normal differentiation of e.g. enterocytes. It must also be considered that a variable small number of mononuclear cells present in the intestine as well as clusterin-positive cells from lymphatic follicles may contribute to the overall clusterin expression seen in controls of the western blot analysis.

Our results are in good agreement with other recent experimental data showing that clusterin is expressed at sites of active inflammation and tissue damage (Hochgrebe et al., 2000; Wilson and Easterbrook-Smith, 2000). In the diseased ileal tissues investigated by immuno-histochemistry, expression of clusterin was seen in epithelial cells on the one hand and inflammatory cells on the other hand. The high number of inflammatory cells expressing clusterin mRNA may largely account for high amounts of clusterin seen in the Western blot analysis. However, the particular expression of clusterin by enterocytes of crypts was a unique and prominent feature detected exclusively within CD tissues. Of note, when estimating the amount of clusterin mRNA expression by the time-dependent appearance of the hybridization signal during colour development, is that the strongest signals were obtained

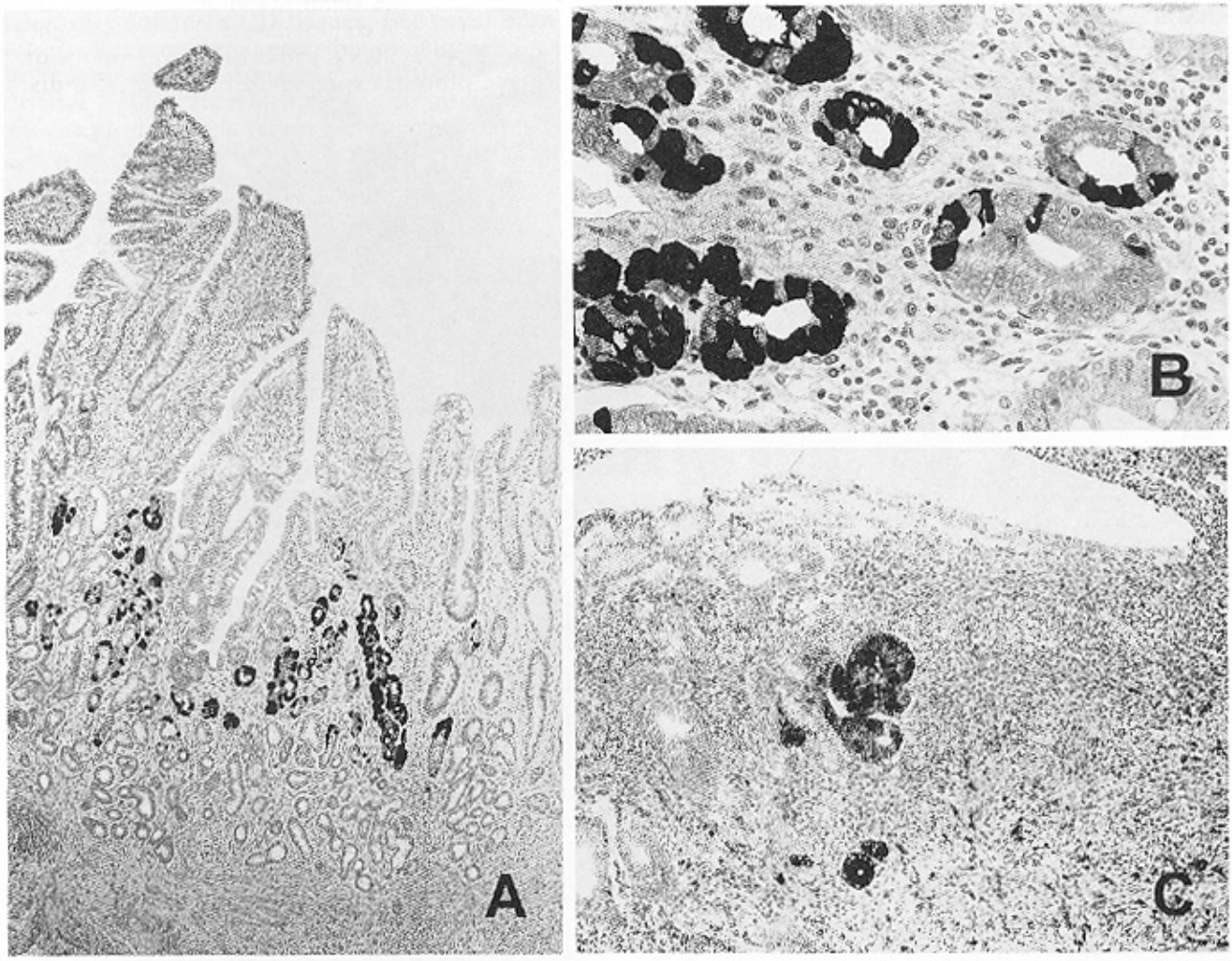

Fig. 3. Immunohistochemical detection of clusterin using the ABC technique in actively **(A, C)** and inactively **(B)** inflamed ileal tissues with Crohn's disease. **A.** In the overview, expression of immunoreactive clusterin can be seen in epithelial as well as in inflammatory cells (actively inflamed tissue). Strong immunoreactivity for clusterin is apparent in strings of epithelial cells located in crypts. **B.** Clusterin protein expressing enterocytes intermingled with unreactive epithelial cells are also existent in inactively inflamed Crohn's disease. **C.** Actively inflamed ileal tissue with a large ulcerative lesion (right upper corner). Only few crypts adjacent to the ulcerative injury are immunostained. Note that the surface epithelium is without any staining. Several inflammatory cells in the stroma are immunostained for clusterin. A, x 30; B, x 240; C, x 150

in epithelial cells of crypts before the appearance of the signal in other cell types. This raises some interesting questions about the role of clusterin especially in those locations. We have previously shown in a rat model of polycystic kidney disease, that clusterin mRNA is strongly upregulated in distinct renal epithelial cells, also including cystic epithelia. This prompted us to speculate that clusterin serves to maintain cell integrity, therefore being protective rather than being a mediator of cell injury (Obermüller et al., 1997). Thus, it is possible that clusterin might promote cell-cell interactions which are perturbated in the setting of CD. Whether this is in response to primary or secondary events in CD must remain speculative.

Interestingly, increased clusterin expression was not an evident feature of cells in the vicinity of erosive and ulcerative lesions. It is possible that clusterin is induced much more earlier in enterocytes, that is, before ulcerative lesions have been formed. Clusterin expression in crypts may therefore indicate an early type of epithelial injury. Notably, CD is associated with an increased intestinal permeability (Hollander, 1992), indicating disturbance of the intestinal barrier including defects of the epithelial cellular layer. Therefore upregulated clusterin may function to protect cell membranes as has been recently suggested (Rosenberg and Silkensen, 1995).

Finally, it cannot be excluded that e.g. a persistent disturbance in differentiation of enterocytes leads to increased clusterin production, since it is well known that this protein is induced spatially and temporarly during organogenesis and differentiation of cells (French et al., 1993). Moreover, a previous study demonstrated upregulated expression of clusterin mRNA in the small

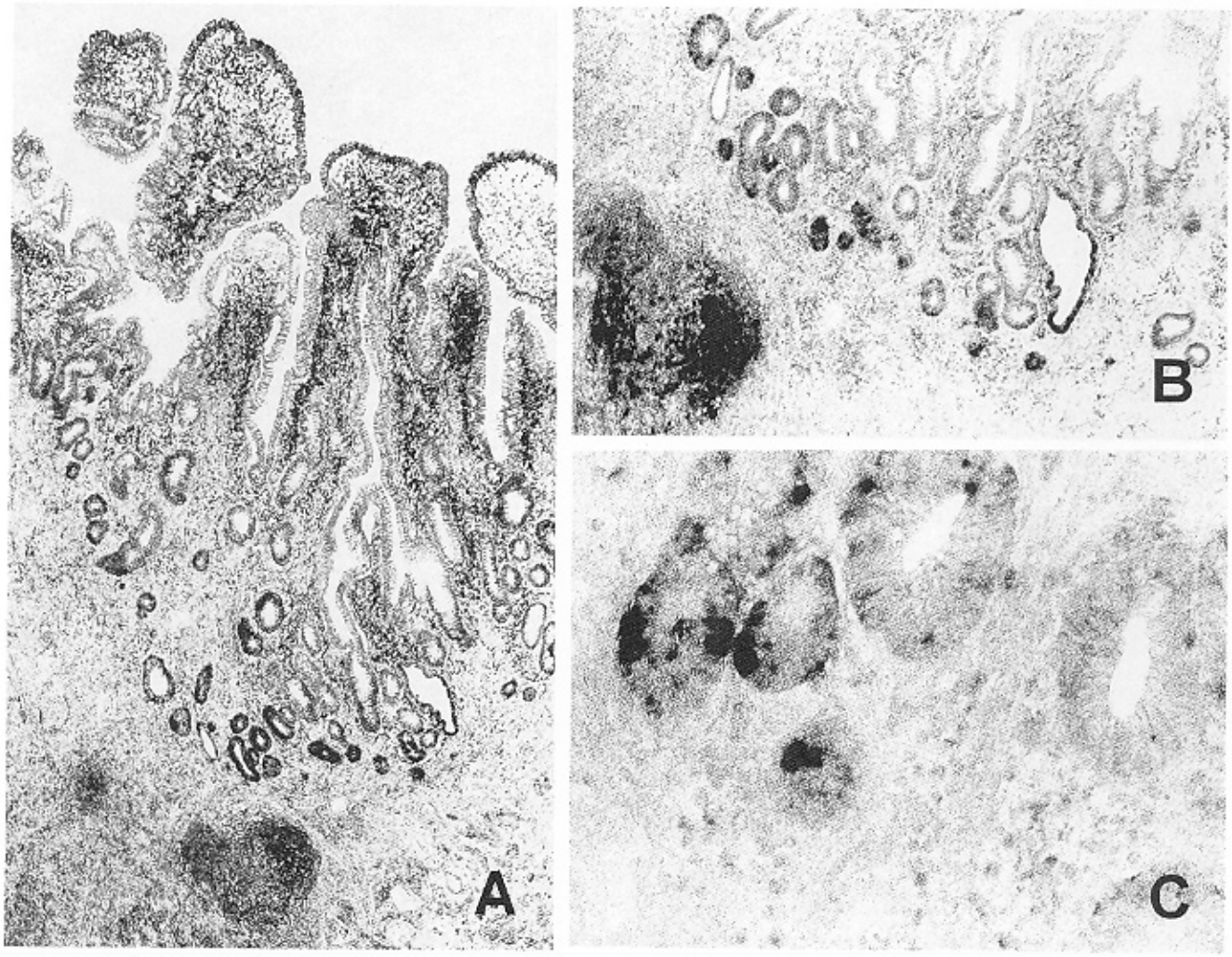

Fig. 4. In situ hybridization for clusterin mRNA using digoxigenin-labeled riboprobes in actively **(A, B)** and inactively **(C)** inflamed ileal tissues with Crohn's disease. **A.** In the overview, clusterin mRNA expression is seen in crypt epithelium and in inflammatory stromal cells. **B and C.** Detailed views of clusterin mRNA expression in crypts; (B) is a higher magnification of (A); (C) shows the expression pattern in an inactively inflamed specimen. Note the clusterin mRNA expressing lymphatic follicle in the left lower corner of (B). A, x 30; B, x 50; C, x 240

and the large intestine of the rat after irradiation (Arai et al., 1996). That investigation showed a temporal link between apoptosis and clusterin expression in enterocytes. In our study, however, we did not find histomorphological characteristics of apoptosis in clusterin-positive epithelial cells, which were located at the basis of mucosal crypts, whereas apoptotic enterocytes are typically seen at the most apical parts of the mucosal villi, as demonstrated recently (Arai et al., 1996). Future studies will have to address whether the sites of clusterin expression in CD are associated with abnormalities of epithelial differentiation.

In summary, we found that the expression of clusterin in CD of the terminal ileum is upregulated as compared with normal controls. The protein was basically expressed by enterocytes and inflammatory mononuclear cells. Foci of crypts almost completely lined by clusterin expressing enterocytes were exclusively found in CD tissues, but never seen in controls. Such crypts appeared especially within the morphologically intact mucosa apart from erosive or ulcerative lesions. Increased expression of clusterin by crypt enterocytes might reflect a cytoprotective function of the protein in order to prevent further disturbances of the intestinal mucosal barrier in CD.

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