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The immunohistochemical expression of metallothionein in inflammatory bowel disease. Correlation with HLA-DR antigen expression, lymphocyte subpopulations and proliferation-associated indices

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Summary. Metallothionein (MT) expression in intestinal resection specimens from 41 patients with ulcerative colitis (UC) and 10 patients with Crohn's disease (CD) was immunohistochemically studied by the avidin-biotin (ABC) method. In addition, the possible relationship of its expression with HLA-DR antigen expression, lymphocyte subpopulations and proliferation-associated indices was studied in order to elucidate the role of this molecule in inflammatory bowel disease (IBD). The MT immunoreactivity was recorded by staining and intensity-distribution scores. MT staining varied in and was mainly localized in the cytoplasm, although a combined nuclear/cytoplasmic reactive pattern was also seen in epithelial cells. MT expression was decreased in UC, and CD compared with normal mucosa. No difference in MT expression between UC and CD was noted. In UC, a gradually decreased expression from remission, to resolving and to active phase was observed. An inverse correlation of MT expression with HLA-DR antigen expression was detected (p=0.018) in the cases of UC. The data suggest that a low level of MT expression in inflammatory bowel disease and particularly in active phase of UC may indicate a decreased endogenous intestinal protection and it may be implicated in the pathogenesis of the disease.

Key words: Metallothionein, HLA-DR antigen, Lymphocytes subsets, Ki-67, PCNA, Inflammatory bowel disease, Immunohistochemistry

Introduction

The aetiology and pathogenesis of the ulcerative colitis (UC) and Crohn's disease (CD), which are the two main clinical forms of the inflammatory bowel disease (IBD), remain unknown. There is a general agreement that immune mechanisms are of importance. The pathogenesis of the immune and inflammatory responses that lead to tissue injury in IBD is unclear, although a number of potential mechanisms have been proposed (Shanahan and Targan 1992; Tsianos et al., 1994). It has been shown that oxygen- derived free radicals such as superoxide (O_2^-), hydrogen peroxidase (H_2O_2), and hydroxyl radicals ($OH^\cdot$) have a role in mediating intestinal damage in inflammatory bowel disease. The intestine is well endowed with enzymes capable of producing such free radicals (Parks 1989). Several studies suggest that peripheral blood monocytes and isolated intestinal macrophages from patients with inflammatory bowel disease produced increased amounts of free radicals (Mahida et al., 1989). Grisham and Granger (1988) hypothesised that in ulcerative colitis transient ischaemic episodes and subsequent reperfusion produce high levels of free radicals. Wakefield et al. (1989) presented evidence for multifocal infarctions in the intestine of patients with Crohn's disease, indicating that ischaemic episodes may also occur in this disease. It has also been shown that increased oxidative stress and decreased antioxidant defenses in mucosa of IBD may be important in the pathogenesis and/or perpetuation of the tissue injury of these lesions (Lih-Brody et al., 1996).

Metallothioneins (MTs) are small (M, 6000) stress-responsive, heavy metal-binding proteins that protect cells against metals (Hamer, 1986), genotoxic agents (Lazo and Pitt, 1995) and oxygen-and nitrogen-based reactive species (Tamai et al., 1993; Schwarz et al., 1994, 1995). There are two electrophoretically separable subgroups or isoforms of MT in mammals referred to as MT1 and MT2. These isoforms show small differences in their binding affinity for metal ions but appear to have similar biochemical properties (Masters et al., 1994). MTs appear to play a homeostatic role in the control of extracellular zinc and detoxification of copper and

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particularly cadmium (Masters et al,. 1994; Mullins and Fuentealba, 1998). MT synthesis is also induced by many endogenous factors such as glucocorticoids, growth factors, cytokines, interferon, interleukin 1 and Vitamin D (Hamer, 1986; Kagi, 1991). Other possible functions suggested for MT include participation in embryonic development, cellular differentiation, cell proliferation and growth and carcinogenesis (Hamer, 1986; Kagi, 1991). MTs are often associated with rapidly proliferating cells (Fuentealba and Mullins, 1999), such as fetal and neonatal liver (Fuller et al., 1990).

Although colonic enterocytes are negative for the expression of major histocompatibility complex antigen (HLA)-class II molecules, an increase of these antigen expressiones by colonic epithelial cells has been noted in both UC and CD (Selby et al., 1983; McDonald and Jewell 1987; Lih-Brody et al., 1996). It is likely that class II antigen expression on epithelial cells is induced by cytokines acting either as paracrine or autocrine factors on the lamina propria (Elson, 1988; Deem et al., 1991). In the present study the expression of MT on mucosal epithelial cells, in biopsy specimens obtained from 51 patients with IBD and its relationship to disease activity, the aberrant HLA-DR antigen expression, to the density of tissue-infiltrating lymphocytic subsets and the proliferation-associated indices were investigated. This study was performed using an immunohistochemical technique and various monoclonal antibodies, in order to elucidate the possible role of MT expression in the pathogenesis of these lesions.

Materials and methods

Forty-one patients (20 male and 21 female, mean age 48 years) with UC and 10 patients (6 male and 4 female) with CD were studied. The rarity of CD in our area was the reason for the relatively small number of CD patients investigated (Tsianos et al., 1994). Histologically normal tissue specimens from 5 patients who underwent surgery for colorectal cancer, were included as control (>10cm away from any histologically abnormal mucosa) according to the other studies (Mulder et al., 1991; Mckenzie et al., 1996).

Two-three tissue specimens from inflamed and noninflamed mucosa were obtained at colonoscopy from different anatomical sites of CD and UC (ileum, right or left colon, rectum) of intestine mucosa. All sections of specimens were processed by standard techniques in paraffin wax, after fixation in 10% formalin, stained with hematoxylin and eosin and were reviewed. The specimens with severe lesions were selected (usually rectal biopsies for UC and from the ileum for CD) for immunohistochemical evaluation. The diagnosis of IBD was based on clinical, radiological, endoscopic and histological evaluation (Binder et al., 1982). The activity, and severity of UC were defined according to Truelove's criteria (Truelove and Richards, 1965). Histologically, 18 cases of UC were in active phase, 11 in resolving and 12 in remission. 50% of the patients suffered from the disease <5 years, 26.3% 5 to 10 years, 21.1% 10 to 20 years and 2.6% >20 years. Seventeen patients were on 5-aminosalicylic acid (5-ASA) monotherapy (dose range 1.5-4.5 gr/day), 9 patients were on 5-ASA and corticosteroids P.O. (dose range 0.2-2 mg/Kg/day), 8 patients were on 5-ASA and azatheioprine, 11 patients on 5-ASA, azathioprine and corticosteroids and 4 patients on 5-ASA, corticosteroids and methotrexate (15mg/week).

For the immunohistochemical evaluation, additional 4 μm-thick sections were cut from paraffin-embedded tissue plassed on poly-L-lysine-coated glass slides and the avidin-biotin method as previously described (Hsu et al., 1981) was performed. A monoclonal antibody (E9) against a conserved epitope of I and II isoforms of MT was used on formalin-fixed, paraffin-embedded tissues. In brief, sections were deparaffinised in xylene and dehydrated. For the detection of Ki-67, slides were immersed in citrate buffer (0.1m, pH 0.6) in plastic Coplin jars and subjected to microwave irradiation twice for 15 min. The heat-mediated antigen-retrieval method was not used for MT, HLA-DR, PCNA, lymphocyte subsets and macrophage staining. Subsequently, all sections were treated for 30 min with 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity and then incubated with primary antibodies. The development of chromogen was performed with immersion of the slides in a diaminobenzidine-H_2O_2 substrate for 5 min. The slides were counterstained in Harris' haematoxylin, dehydrated and mounted. To assess the specificity of the reaction myoepithelial cells around background ducts and lobules, which stained strongly positive for MT from breast tissue, were used as positive control. Negative controls were included; sections subjected to the whole procedure expected for incubation without the primary antibody. The sources of primary antibodies and dilutions as well as the retrieval method used in this study are indicated in Table 1.

Immunohistochemical evaluation: The evaluation of MT immunostaining (nuclear and cytoplasmic) was assessed as the total percentage of positive cells. Then the percentages of weakly, moderate, and strongly staining cells were assessed, as the sum of these categories equaled the overall percentage of positivity. A staining score was then calculated as previously described (Douglas-Jones et al., 1997). HLA-DR antigen

Table 1. Antibodies used.

ANTIBODIES	SUPPLIER	DILUTION	INCUBATION TIME
Metallothionein, E9	Dako	1: 50	One hour
HLA-DR	Dako	1: 50	One hour
CD4	Dako	1: 25	One hour
CD8	Dako	1: 25	One hour
CD68#	Dako	1: 50	One hour
PC-10	Dako	1: 50	One hour
MIB1 (Ki-67)*	Dako	1: 50	Overnight

*: With microwave oven antigen retrieval. #: Incubation with pronase.

expression (cytoplasmic) was calculated as the percentage of positive epithelial cells in relation to the total number in representative fields in superficial epithelium and the epithelium of the crypts. Nuclear staining for Ki-67 and PCNA was estimated as the percentage of positive cells to the total number of superficial epithelium and the epithelium of the crypts. The distribution (intraepithelial and extraepithelial-stromal cells) and the relative number of B cells, T helper/inducer (T4, CD4), T cytotoxic/suppressor cells, (T8, CD8) and macrophages (CD68) were calculated. Lymphocyte subpopulations were in aggregates or diffusely in the lamina propria and were separately evaluated. The frequency of these cells was expressed as means, estimation for each sample section by counting the positive cells in at least 5 high-power representative fields (HPF x40 obsective). All slides were reviewed and scored in a blind test by two pathologists (EI, MM) Differences in interpretation were reconciled by rereview of slides separately or jointly at a double-headed microscope.

Statistical analysis

All data were entered into a microcomputer and statistical analysis was performed using SPSS statistical package. The association of continuous variables was confirmed using Kendall Tau correlation coefficients and for two independent samples with Mann-Whitney u-test. P-Values less than 0.05 were considered as statistically significant.

Results

Epithelial metallothionein expression

Normal mucosa showed intense MT expression in the nucleus or cytoplasm of epithelial cells present at the luminal surface or crypts (Fig. 1). MT expression was detected in the majority of the cases examined showing both nuclear and cytoplasmic immunoreactivity (Figs. 2, 3). This expression was usually located at the crypts. The specimens with severe lesions showed lower MT expression compared with “normal” or non-inflamed mucosa and these specimens were evaluated. Decreased MT expression in UC and CD compared to the control group (Fig. 4) was found (p=0.004 and p=0.04 respectively). MT expression was correlated with disease activity in CD. In particular, the cases of active phase of UC showed lower MT expression (Fig. 5) than the cases of resolving and remission phase (p=0.0014). Epithelial MT expression was inversely correlated with aberrant epithelial HLA-DR antigen expression in the cases of UC (p=0.018). No correlation of MT expression with proliferation-associated indices or lymphocyte subpopulations was found in either UC or CD. The intestinal epithelial MT expression in the specimens of the patients with IBD did not correlate with the

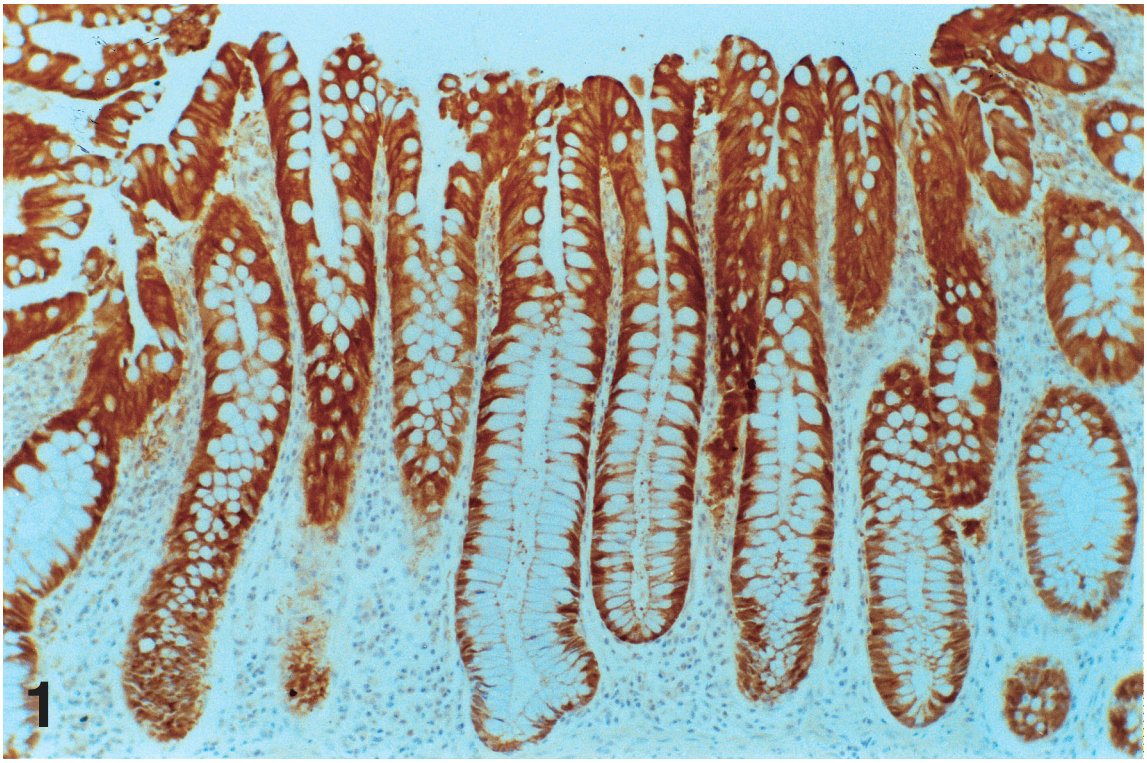

Fig. 1. Strong MT expression in the normal colonic epithelial cells present at the luminal and crypts. ABC, x100

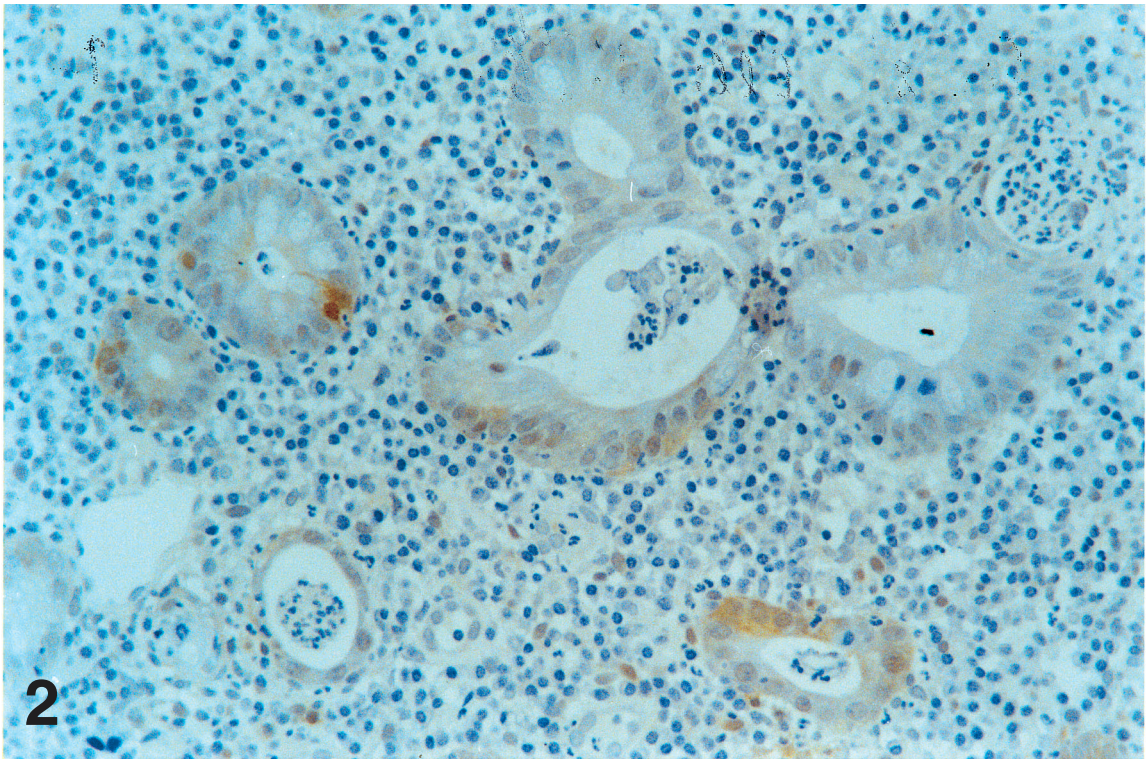

Fig. 2. Weakly cytoplasmic and nuclear MT immunostaining in intestinal epithelial cells in a case of UC in active phase. ABC, x 200

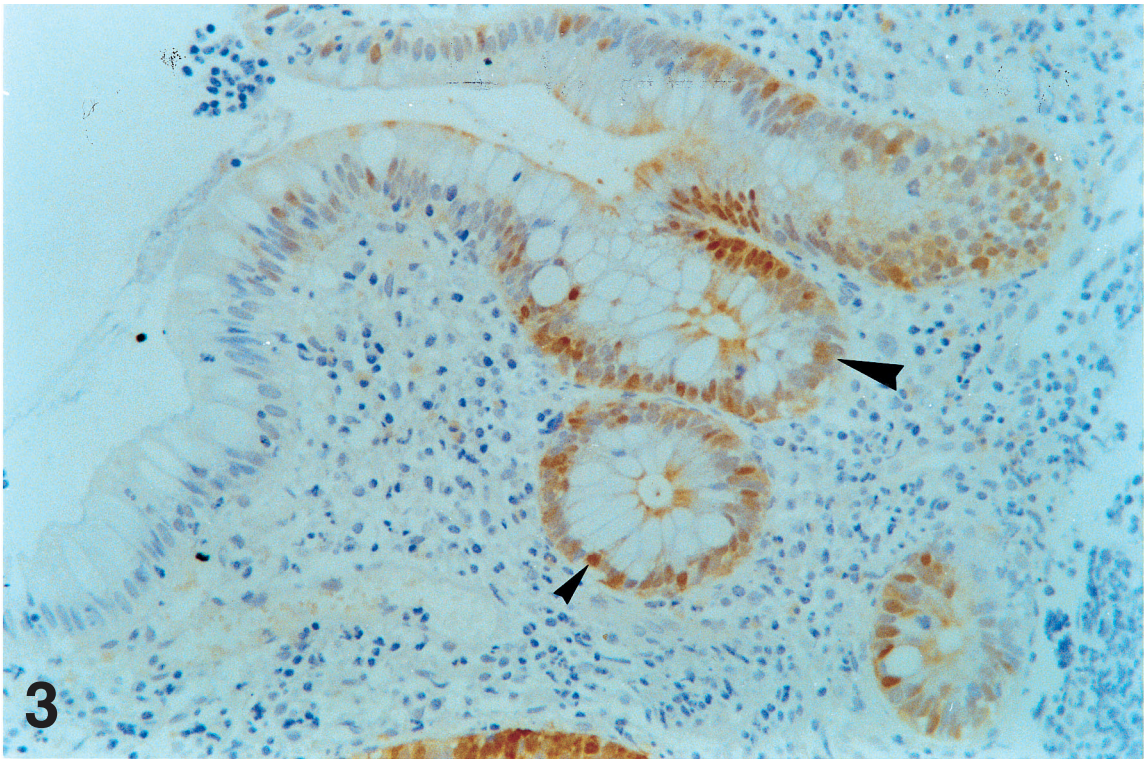

Fig. 3. Moderate MT immunostaining in the nucleus (small arrow) and the cytoplasm (big arrow) of epithelial cells usually present at the crypts in a case of CD. ABC, x 200

corticosteroid dependence or long-standing disease.

Epithelial HLA-DR antigen expression

Aberrant HLA-DR antigen expression in UC and CD compared to control group (p=0.03 and p=0.03 respectively) was observed. No statistically significant difference in HLA-DR antigen expression between UC and CD was found. Increased HLA-DR antigen expression in active and resolving phase compared to remission phase (p=0.0001 and p=0.004 respectively) was observed in the cases of UC. A positive correlation of epithelial HLA-DR antigen expression and $CD4^+$ subpopulation (p=0.05) and CD4 aggregates (p=0.004) was found in the cases of UC. The HLA-DR antigen expression in the epithelium of the crypts was positively correlated with the proliferating-associated index MIB1 at some places in the cases of UC (p=0.006) and a trend of correlation in the cases of CD (p=0.07). The correlations of MT and HLA-DR antigen expression with various parameters studied in the intestinal biopsies of IBD patients are shown in Table 2.

Table 2. Correlation of the immunohistochemical expression of MT and HLA-DR antigen in UC, CD and control between them and PCNA, MIB1, CD4. CD8 and CD68.

Compared with	MT p value	HLA-DR p value
HLA-DR UC*	p=0.018	
HLA-DR CD*	NS	
HLA-DR control		
PCNA UC*	NS	NS
PCNA CD *	NS	NS
PCNA control	p<0.0001	
MIB1 UC *	NS	p=0.006
MIB1 CD *	NS	p=0.07
MIB1 control	p<0.0001	
CD4 UC #	NS	p=0.05
CD4 UC agr• #	NS	p=0.004
CD4 CD #	NS	
CD4 control	NS	
CD8 UC #	NS	NS
CD8 CD #	NS	NS
CD8 control	NS	
CD68 UC #	NS	NS
CD68 CD #	NS	NS
CD68 control	NS	

*: Percentage of positive epithelial cells. #: Mean per high power field positive cells, at the lamina propria. • agr: Aggregates of CD4 positive cells. HLA-DR antigen did not express in control specimens

Proliferating-associated indices

No statistically significant difference of MIB1 and PCNA score between UC and CD was found. The values of MIB1 were higher in active phase compared to resolving (p=0.02) and remission phase (p=0.01) in the cases of UC.

Lymphocyte subpopulations and CD68

The mean value of $CD4^+$ subpopulation was higher in the cases of CD compared to the cases of UC (p=0.003). No statistically significant difference of the subpopulations CD8 and B cells as well as CD68 in these two lesions was found. The mean values of CD4 subpopulation and CD4 aggregates were higher in active phase compared to the remission phase of UC (p=0.06 and p=0.006).

Discussion

In this study we have detected decreased epithelial

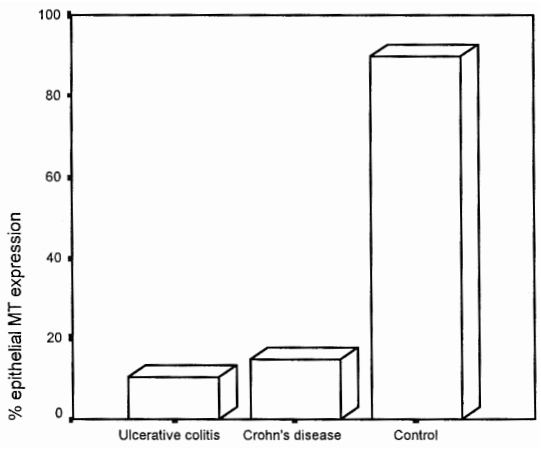

Fig. 4. MT expression in UC, CD and control (Mean values).

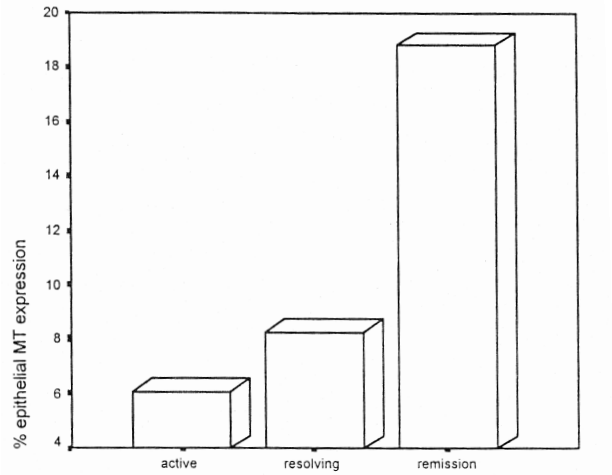

Fig. 5. MT expression in histological phases of UC (Mean values).

MT expression in IBD compared to the normal colonic mucosa. In previous studies it has been shown that MT concentration (measured using radioimmunoassay in the homogenate tissue) was reduced in IBD and in UC (Mulder et al., 1991; Sturniolo et al., 1998) and these results were in accordance to our findings (using immunohistochemical method). Recently, it has been shown that an imbalance in the formation of reactive oxygen and decreased level of zinc and cooper were found in IBD and was considered to be implicated in the pathogenesis and/or perpetuation of the tissue injury in these lesions (Lih-Brody et al., 1996). Another study provides direct evidence of in vivo oxidant injury in colon epithelial crypt cells from inflamed mucosa of IBD patients. It has been suggested that oxidation and inhibition of essential protein function by inflammatory cells is a potential mechanism of tissue injury that may contribute to the pathogenesis of the disease and supports the exploration of compounds with antioxidant activity as new therapies for IBD (McKenzie et al., 1996). In a recent study, MT overexpression which was found in the fibroblasts of IBD indicated a protective role, while MT overexpression in intestinal epithelial cells correlated with the grade of inflammation (Bruwer et al., 2001), supporting the hypothesis that MT may act as an oxygen free radical scavenger to provide cytoprotection in inflamed mucosa. In our study, we found lower MT expression in the active phase of UC, in which there are more inflammatory cells, particularly granulocytes that can produce a large amount of free oxygen radicals, in comparison to the resolving and remission phase. The dense inflammatory cells which characterized the mucosal lesions in IBD secrete many inflammatory mediators including cytokines (IL-1, IL-6, IL-8, IL-10) (Fiocci, 1992; Pullman et al., 1992; Kappeler and Mueller, 2000). On the other hand, it has been shown that MT synthesis may also be induced by endogenous factors such as cytokines (Hamer, 1986; Kagi, 1991). A possible explanation of these contradiction hypotheses is that in the active phase initially a decrease of epithelial MT content may occur in response to this injury accompanied with the presence of MT in fibroblasts. In addition, MT epithelial synthesis may be induced from the chronic inflammatory cells through the pathway of cytokines.

Aberrant HLA-DR antigen expression was detected in both UC and CD according to the previous studies (Selby et al., 1983; McDonald and Jewell 1987; Fais et al., 1987). The significance and function of HLA-DR molecules expressed on epithelial cells of the intestine are unknown. It has been claimed that the significant function of HLA-DR antigens is to mediate the communication among immunocompetent cells acting as antigen presenting cells to T cells. The induction of activated helper T lymphocytes requires the presentation of specific antigens by HLA-DR-positive cells such as macrophages, B lymphocytes and certain T lymphocytes (Ko et al., 1979; Fais et al., 1987; Mayer and Schlien, 1987; Deem et al., 1991). It is proposed that aberrant epithelial HLA-DR positive cells possibly interfere with the presentation of preexisting autoantigens to the immunocompetent cells and thus an autoimmune disorder begins. In our study we found a positive relationship of HLA-DR antigen expression with the CD4 subpopulation in UC. Thus, the aberrant expression of HLA-DR antigen by the intestinal epithelial cells might function as antigen-presenting cells to $CD4^+$ T lymphocytes according to the findings of other investigators (Selby et al., 1981; Fais et al., 1987; Hershberg et al., 1997). These observations supported the suggestion that mucosal $CD4^+$ T cells are implicated in the pathogenesis of IBD (Powrie 1995). In vitro experiments revealed that γ–interferon as well as other stimuli can induce aberrant HLA-DR antigen expression (Bottazo et al., 1983; Todd et al., 1985; Hoang et al., 1992). So, the inappropriate colonic epithelial HLA-DR antigen expression can be the result of the activated helper $CD4^+$ T cells. Recently, it has been suggested that intestinal epithelial cells use two distinct pathways for HLA class II antigen processing with differential immunomodulatory properties in the presence of the proinflammatory cytokin γ-interferon or in the absence of mucosal inflammation with a conventional antigen-presenting cell (Hershberg et al., 1997).

The interesting finding of this study is the inverse correlation of epithelial HLA-DR antigen with the MT expression. An additional possible role of the aberrant HLA-DR production by the intestinal epithelial cells is the perpetuation of the mucosal inflammation, which in the active phase is necessary for the secretion of cytokines or other growth factors, in order to increase MT synthesis, being decreased in this phase of the disease. Thus, it is possible that the aberrant epithelial HLA-DR antigen expression may protect the mucosa, interfering in the immunopathological mechanism which takes place, mediating in communication among immunocompetent cells. On the other hand, it has been shown that MT have been implicated in the transient cellular response to tissue injury such as inflammation or irradiation, possibly through attenuation of the potentially damaging effects of oxygen-derived free radicals (Chubatsu and Meneghini, 1993; Fu et al., 1997). This is also supported by the increase of MT and decrease of HLA-DR antigen expression during the process of disease. So, these two events seem to be transient and may play a protective role in the process of IBD.

Although the number of CD patients in this study was relatively small, reflecting perhaps the reported low incidence of CD in our area (Tsianos et al., 1994) we found no significant difference in MT or HLA-DR antigen expression in either UC or CD. Nonetheless the inverse correlation of MT with HLA-DR antigen expression as well as the positive correlation of epithelial HLA-DR antigen expression with $CD4^+$ T subpopulation were observed only in the cases of UC. In addition, $CD4^+$ T cells were more in UC than CD. These findings may reflect different endogenous pathogenetic

mechanisms of these two diseases. In a previous study, it has been suggested that the contribution of the HLA class II genes to the disease susceptibility, is quite different for the two disorders, on genetic backgrounds (Toyoda, 1993).

It has been shown that MTs are often associated with rapidly proliferating cells (Fuller et al., 1990; Fuentealba and Mullins, 1999). On the other hand it has been found that increased cell proliferation characterizes both UC and CD (Franklin et al., 1985; Niffsinger et al., 1998). In the current study a strong relationship of MT expression with the two proliferating-associated indices (MIB1 and PCNA) in control specimens was found, while in either UC or CD did not find such a correlation. So, the MT synthesis seems not to be correlated with the cell proliferation, but possibly with the immune activation and inflammation in IBD.

Non-steroidal anti-inflammatory drugs as well as corticosteroid (Summer et al., 1989; Lee and Archer 1998) are known to induce MT-synthesis. However, we found no relationship of MT expression and the received therapy. Experimental studies showed that different mechanisms of MT-regulation implicate within various organs in response to corticoids (Hadalgo et al., 1988). So, the MT-regulation seems to be more complex and other factors possibly genetics may implicate in this process.

In conclusion our data of decreased intestinal epithelial MT expression and aberrant increased HLA-DR antigen expression during the process of IBD may indicate defective and protective endogenous mechanisms, respectively, in response to the local injury of the tissue. In addition, the differences in the interrelationship of HLA-DR antigen expression with MT expression or the $CD4^+$ lymphocyte subpopulation in UC and CD may reflect different pathogenetic mechanisms of these two disorders.

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