Association of Metallothionein Expression and Lack of Apoptosis with Progression of Carcinogenesis in Barrett's Esophagus

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Barrett's esophagus is the transformation of normal esophageal squamous epithelium to specialized intestinal metaplasia (SIM). Among the Barrett's specialized cells, those that can develop protective mechanisms against apoptosis may have potential to become malignant. Studies have shown that overexpression of metallothionein (MT), low molecular protein that protects cells from apoptotic stimuli, appears to be associated with more advanced, highly malignant tumors. We thus investigated the relationship between MT expression and apoptosis in different stages of Barrett's carcinogenesis. Terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling and immunohistochemical dual-staining assay were performed in human biopsy samples of normal, SIM, dysplasia, and adenocarcinoma. Apoptotic index and MT expression were quantified by using an image system to analyze the converted digital data. A negative correlation between MT expression and apoptotic index was found. MT expression was significantly increased along with the histologic progression towards adenocarcinoma. This study thus suggests that MT may contribute to cytoprotection, thereby inhibiting apoptosis and leading to carcinogenesis of Barrett's esophageal cells. Exp Biol Med 228:286–292, 2003

Key words: Barrett's esophagus; apoptosis; metallothionein; carcinogenesis

Incidence of esophageal adenocarcinoma has increased dramatically over the past two decades at a rate exceeding that of any other cancers (1). Most of the esophageal adenocarcinoma are developed from Barrett's esophagus (BE), a premalignant disease characterized by replacement of the normal esophageal squamous epithelium with a specialized intestinal metaplasia (SIM; Ref. 2). Adenocarcinoma in BE dose not arise *de novo* but rather follows a multistep process in which the metaplastic epithelium sequentially develops low-grade dysplasia, high-grade dysplasia, and adenocarcinoma (3). However, the Barrett's epithelium is a heterogeneous metaplasia, and not all cases of BE follow the metaplasia–dysplasia–adenocarcinoma sequence (3, 4). Although only a subset of patients in BE develop cancer, the risk of esophageal adenocarcinoma has been estimated to be about 1 in 200 patient years, 30–125 times higher than the general population (2, 5). Numerous studies have assessed the prevalence of a variety of molecular changes in the evolution from BE to adenocarcinoma (6). However, the prognostic value of various molecular markers has not been determined.

It is widely accepted that proliferation, differentiation, and apoptosis are important events that all occur during the progression of Barrett's carcinogenesis (6). During the neoplastic transformation, cells that can evade apoptosis have a biologic advantage to follow the metaplasia–dysplasia–adenocarcinoma sequence. Apoptosis, a gene-directed cell death program, provides a mechanism of autodigestion for those surplus cells and cells that do not function properly. Defects in apoptotic pathways are believed to contribute to numerous disease processes including malignancy (7).

Suppression of apoptosis has been observed in BE (8–10). This anti-apoptotic capability is a potential mechanism for BE cells to survive and eventually develop adenocarcinoma. Interestingly, the regulation of apoptosis in BE is distinctly different from the apoptotic process in other tissues. For example, Bcl-2, an anti-apoptotic protein, is not over-expressed in the Barrett's carcinogenesis (8, 11). Fas

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protein, a stimulator of immunologic cell death, is enhanced rather than being suppressed (12). Hence, a unique mechanism exists to protect some BE cells from apoptosis.

Metallothionein (MT) is a thiol-rich protein and plays a major role in detoxification of toxic metals and in protection against oxidative damage (13). In the past decade, there is accumulating evidence that MT plays multiple functions in the initiation, progression, and metastasis of cancer (14). Many types of tumors have high concentrations of MT. MT overexpression seems to be associated with more advanced, highly malignant tumors in invasive ductal carcinoma of the breast (15), melanoma (16), and pancreatic carcinoma (17). Recent studies have shown that expression of MT positively correlates with tumor metastatic activity and proliferative potential in esophageal squamous-cell carcinoma (18). We postulate that MT also plays an important role in the carcinogenesis of BE.

In this study, we quantified apoptosis and expression of MT in different histologic stages of BE. Furthermore, we set forth to determine whether MT overexpression is associated with antiapoptosis and carcinogenesis of BE.

Materials and Methods

Study Subjects and Specimen Collection. Study subjects were recruited from the BE registry at the University of Louisville. Informed consent was obtained, and the study was conducted using endoscopic specimens from subjects with an established diagnosis of BE, the presence of SIM on histology. The length of the columnar-lined epithelium (CLE) was defined as the distance from the proximal margin of the gastric folds to the squamocolumnar junction. Short-segment BE was defined as CLE length $\leq$ 2 cm. Long-segment BE was defined as CLE length > 2 cm. The CLE was biopsied in a four-quadrant fashion every 2-cm interval, starting at the distal margin and proceeding proximally to the squamocolumnar junction. The jumbo biopsy forceps were used. Specimens were separated for each level and placed in bottles of 10% formalin solution.

A control group consisted of subjects without BE, defined by endoscopic findings of CLE but no pathologic evidence of SIM despite Alcian-blue staining on all of the four-quadrant biopsies. According to the American College of Gastroenterology practice guideline, subjects without SIM have essentially no neoplastic risk, and endoscopic surveillance is not recommended (19). This control group was selected to be compared with subjects exhibiting BE, a pre-neoplastic diagnosis. Subjects with erosive or ulcerative esophagitis were excluded from the study. Subjects were also excluded if there was a history of using bismuth compounds, radiotherapy, and chemotherapy, conditions that may induce MT expression and apoptosis. This study was approved by the Institutional Review Board for Human Study at the University of Louisville.

Pathology. The pathologic specimens were reviewed by two gastrointestinal pathologists independently. The pathologists were blinded to the subject's clinical history, the endoscopic findings, and the results of immunohistochemical dual-staining assay. Alcian-blue staining was used to identify any presence of SIM. Hematoxylin-eosin staining was used to identify any presence of dysplasia. Dysplasia was classified into negative, indefinite/low-grade dysplasia (I/LGD), high-grade dysplasia (HGD), and esophageal adenocarcinoma (EAC; Refs. 20, 21). Both pathologists had to concur on all specimens with HGD or EAC. A pathologic reading was determined for each biopsy slide. Each slide may contain several biopsy specimens, depending on the number of endoscopic biopsies taken at that particular level. An overall pathologic diagnosis was also determined for each subject.

Immunohistochemical Dual-Staining Assay for Apoptosis and MT Protein. Apoptosis and MT protein expression were quantified using an immunohistochemical dual-staining assay. The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP)-digoxigenin nick end labeling (TUNEL) method (ApopTag® *in situ* apoptosis detection Kit, Intergen Company, Purchase, NY) was used to detect the apoptotic cells, and MT primary antibody (ZYMED, San Francisco, CA) was used to detect MT protein expression.

Sections of 5 μm in thickness were cut from paraffin blocks. After deparaffinization and rehydration, endogenous peroxidase was blocked with H_2O_2 in methanol for 20 min. The sections then underwent proteinase K digestion for 15 min. DNA fragments were tailed using digoxigenin-dUTP along with anti-digoxigenin antibody conjugation with horseradish peroxidase along with substrate (DAB-H_2O_2) to develop a brown color. Normal serum blocking nonspecific antigen was reacted with specimen for 20 min and monoclonal Ab-MT was added and incubated overnight at 4°C.

The next day, biotinylated anti-mouse immunoglobulin Gb1 second antibody and AP-streptavidin were also incubated for 20 min, respectively. When MT is present, alkaline phosphatase substrate will develop a pink color. Counterstaining was performed with mythol green. Apoptosis, MT protein, and normal nuclei were identified by brown, pink, and green colors under light microscopy, respectively. Appropriate positive and negative controls were used. Immunohistochemical dual-staining was performed for each section of all biopsy specimens from each subject.

Computer Image Analysis. Quantification of apoptosis and MT expression was performed for each section using a computer image analysis. If a section contained dysplasia, the region with the highest grade of dysplasia or adenocarcinoma was selected for analysis. The imaging fields were chosen randomly from various section levels to ensure objectivity of sampling. Five to ten imaging fields were scanned for each specimen sample. All digital images were acquired with the microscope at 40× magnification using the Spot camera via the accompanying image analysis software (Diagnostic Instrument, Inc., Sterling Heights, MI) and stored as .JPG data files (the resolutions were fixed as 200 pixels/inch).

The procedures for the computer image analysis were as follows. The acquired color images from the immunohistochemical dual-staining assays were converted to binary shades of black-and-white, where the cell nuclei were represented as "black dots." Nonepithelial regions were demarcated on the computer image manually. The total number of epithelial cells, identified by the number of "black dots" on the image, was determined. The black-and-white images were then converted back to the original color image.

The apoptotic cells (brown) and area of MT expression (pink) were superimposed with a pseudo-red and pseudo-blue, respectively, according to the software specification. The computer program then quantified the number of pseudo-red and the area represented by pseudo-blue in the image. The apoptotic index for each specimen section was defined as the number of apoptotic cells (pseudo-red) divided by total number of epithelial cells (black-and-white image) multiplied by 100. MT protein expression was defined by the total number of pseudo-blue staining pixels with a defined imaging field (200 pixels/inch).

Statistic Analysis. Spearman rank correlation coefficient (R) was used to analyze the correlation between apoptosis and MT expression with the various histologic stages of BE. Multiple analysis of variance was used to determine the differences of apoptosis and MT expression, if any, between the different histologic stages of carcinogenesis. Differences between groups were regarded as statistically significant when P values were < 0.05.

Results

Sixty-six subjects (55M/11F) with BE were enrolled. The demographics of the study and control groups are presented in Table I. The study population consisted of 14 subjects with a diagnosis of SIM without dysplasia, 36 patients with I/LGD, 10 subjects with HGD, and 6 subjects with EAC. Within the control group consisted of 12 subjects with endoscopic BE without SIM (no pathologic evidence of SIM despite blue-blue staining), 33% had cardiac epithelium, and 67% had fundic epithelium on histology. Patients identified as having no Barrett's were mostly those with a short segment of CLE, whereas those identified with SIM, I/LDG, HDG, or EAC were mostly found to have a long segment of CLE.

MT Expression. MT protein was detectable in each histologic stage of BE and in the control subjects. The computer quantification of MT expression in control specimens was 3500 ± 258 pixels. Compared with the control specimens, MT expression in the SIM (3765 ± 297 pixels) was elevated; however, not to a significant level ($P > 0.05$). There were significant increases in MT expression in specimens with I/LGD (4398 ± 786 pixels), HGD (6547 ± 1026 pixels), and EAC (8441 ± 366 pixels) compared to control specimens ($P < 0.01$). Furthermore, MT expression appeared to increase with the histological severity of BE, and there was a significant positive correlation between MT expression and histopathologic changes from I/LGD to EAC ($R = 0.53$, $P < 0.001$; Fig. 1).

Apoptotic Index. Numerous apoptotic cells were seen in the Barrett's specimens, with or without dysplasia or EAC. However, few apoptotic cells can be seen in the control specimens. The apoptotic index was significantly greater in the specimens with BE than in the control subjects ($P < 0.01$). There is a trend that shows an inverse progression correlation between apoptotic index and histological severity in SIM, I/LGD, HGD, and ECA; however, the trend was not statistically significant. The correlation coefficient (R) between the histologic grade of BE and the apoptotic index was 0.2 ($P = 0.76$; Fig. 2).

Correlation Between Apoptosis and MT. Apoptotic cells and MT protein were determined in all sections with the dual-staining method. Representative sections stained by MT and TUNEL from SIM (A, B), I/LGD (C, D), HGD (E, F), and EAC (G, H) are shown in Figure 3. The results show that tissue with high expression of MT (left column) has low incidence of apoptosis, and vice versa (right column). A significant negative correlation between MT expression and apoptotic index was detected in the BE tissues ($R = -0.61$, $P < 0.001$; Fig. 4). It is important to note that squamous cell epithelium adjacent to HGD and ECA epithelium showed increased apoptosis with decreased MT expression.

Discussion

The results obtained from this study demonstrate that there is an inverse correlation between MT expression and the extent of apoptosis in BE. Furthermore, MT expression increases with the progression of the histologic metaplasia–dysplasia–adenocarcinoma sequence. This is the first study to examine the relationship between MT expression and apoptosis in respect to metaplasia–dysplasia–adenocarcino-

Table I. Demographics of the 12 Control Subjects and 66 Study Subjects with BE

	ND	SIM	I/LDG	HDG	EAC
Subjects	12	14	36	10	6
Gender	10M/2F	11M/3F	30M/6F	8M/2F	6M/0F
Age (mean ± SD)	63 ± 8	59 ± 4	61 ± 2	62 ± 4	67 ± 4
Short-segment CLE	11	4	7	1	0
Long-segment CLE	1	9	29	9	6

Notes: ND = nondetectable; SIM = specialized intestinal metaplasia; I/LGD = indeterminate/low-grade dysplasia; HGD = high-grade dysplasia; EAC, esophageal adenocarcinoma; CLE = columnar-lined esophagus.

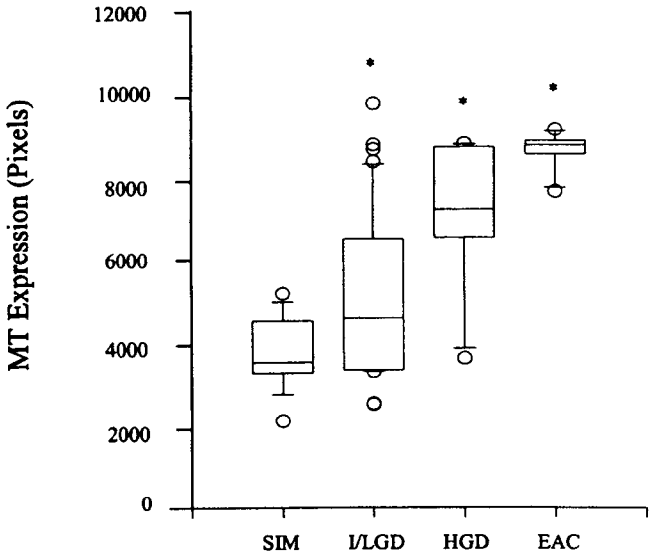

Figure 1. MT expression and the pathological severity of BE. MT expression (in pixels) is plotted against the four stages of BE. The line enclosed within the box represents the median value; the small circles indicate outlying data points. MT expression in specimens with I/LGD, HGD, and EAC was significantly increased in comparison with that in SIM (*$P < 0.01$). There is also a significant positive correlation between MT expression and histopathologic changes from I/LGD to EAC ($R = 0.53$, $P < 0.001$).

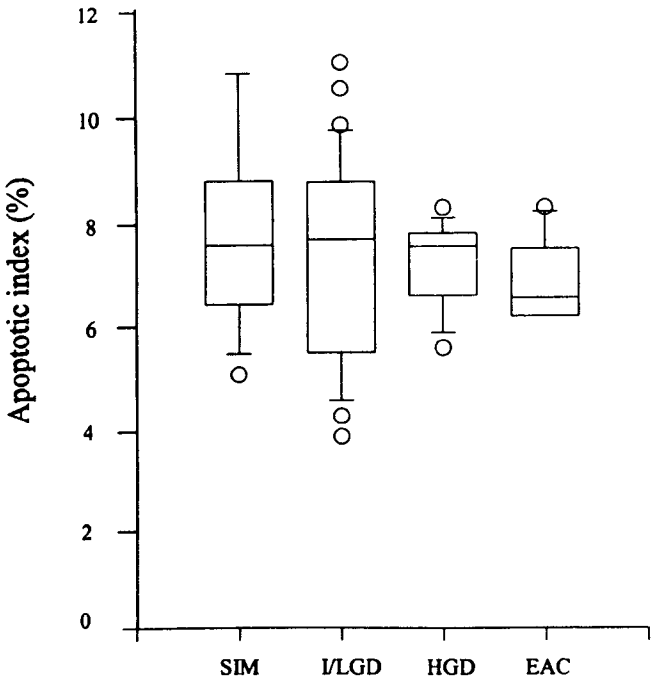

Figure 2. The apoptotic index along with different stages of BE. The line enclosed within the box represents the median value; the small circles indicate outlying data points. There is a trend that shows an inverse correlation between apoptotic index and the severity of BE pathology, however, it is not statistically significant ($R = 0.2$, $P = 0.76$)

ma sequence in BE. Several studies have demonstrated that MT is a potent antiapoptotic agent *in vivo* and it also functions in cell proliferation and differentiation (22). This study thus suggests that it is possible that overexpression of MT protects the BE cells from apoptotic stimuli and ensures these cells opportunity to proliferate and become adenocarcinoma.

Apoptosis is a critical event in autodigestion of surplus cells and in maintaining structural and functional integrity of multiple organ systems. In the highly proliferative BE cells, apoptosis would provide an important mechanism to prevent from carcinogenesis under a diversity of reflex stimuli. Only those cells that have developed anti-apoptotic mechanisms have the opportunity to follow the progress from LGD to HGD to adenocarcinoma. Therefore, many studies have focused on exploring mechanisms of anti-apoptosis in BE to develop diagnostic and preventive approaches to carcinogenesis of BE. One of the important mechanisms for cells to avoid apoptosis is the expression of Bcl-2 antiapoptotic protein (23). However, apoptotic cells often display expression of mutated tumor suppressor gene, p53 (24), and overproduction of death stimulator, Fas (25). Interestingly, many studies have ruled out the involvement of these mechanisms in the antiapoptotic process of the BE. Therefore, to explore possible alternative mechanisms, we examined the potential of MT in the present study.

The postulation for the involvement of MT in protection of BE cells from apoptosis is based on extensive studies of the relationship between MT expression and tumor development and progression in multiple organ systems. It has been shown that MT is overexpressed in various types of human neoplasms, such as breast, thyroid, testis, bladder, salivary gland, melanoma, pancreas, colon, cervical carcinomas, acute lymphoblastic leukemia, prostate, and liver cancer (14, 26). In studies of breast cancers (27), MT overexpression was positively associated with more malignant and higher grade tumors. Expression of MT in esophageal squamous carcinoma was also correlated with advanced malignancy (18).

It is important to understand the underlying mechanisms for MT involvement in carcinogenesis. We hypothesize the following possibilities: (i) MT protection of BE cells from reflex-induced apoptosis provides the opportunity for BE cells to undergo carcinogenesis, and (ii) MT functions as a zinc-donor in DNA replication and repair, thereby ensuring cell proliferation.

First, apoptosis involves a series of genetically preprogrammed events associated with endonucleolytic cleavage of DNA (28). It acts as an important cellular response to a variety of stimuli such as reflex stimulation. It plays a key role in the elimination of preneoplastic cells from the normal cell population. Failure to do so would favor tumor promotion (29). In BE, reflux esophagitis is believed to proceed formation of the metaplastic epithelium (6). A study (30) using chemiluminescence measurement has shown that oxidative stress, which is a critical stimulus for apoptosis, increases with the grade of esophagitis and was the highest in BE.

Apoptosis in BE is thus most likely caused by reflux-

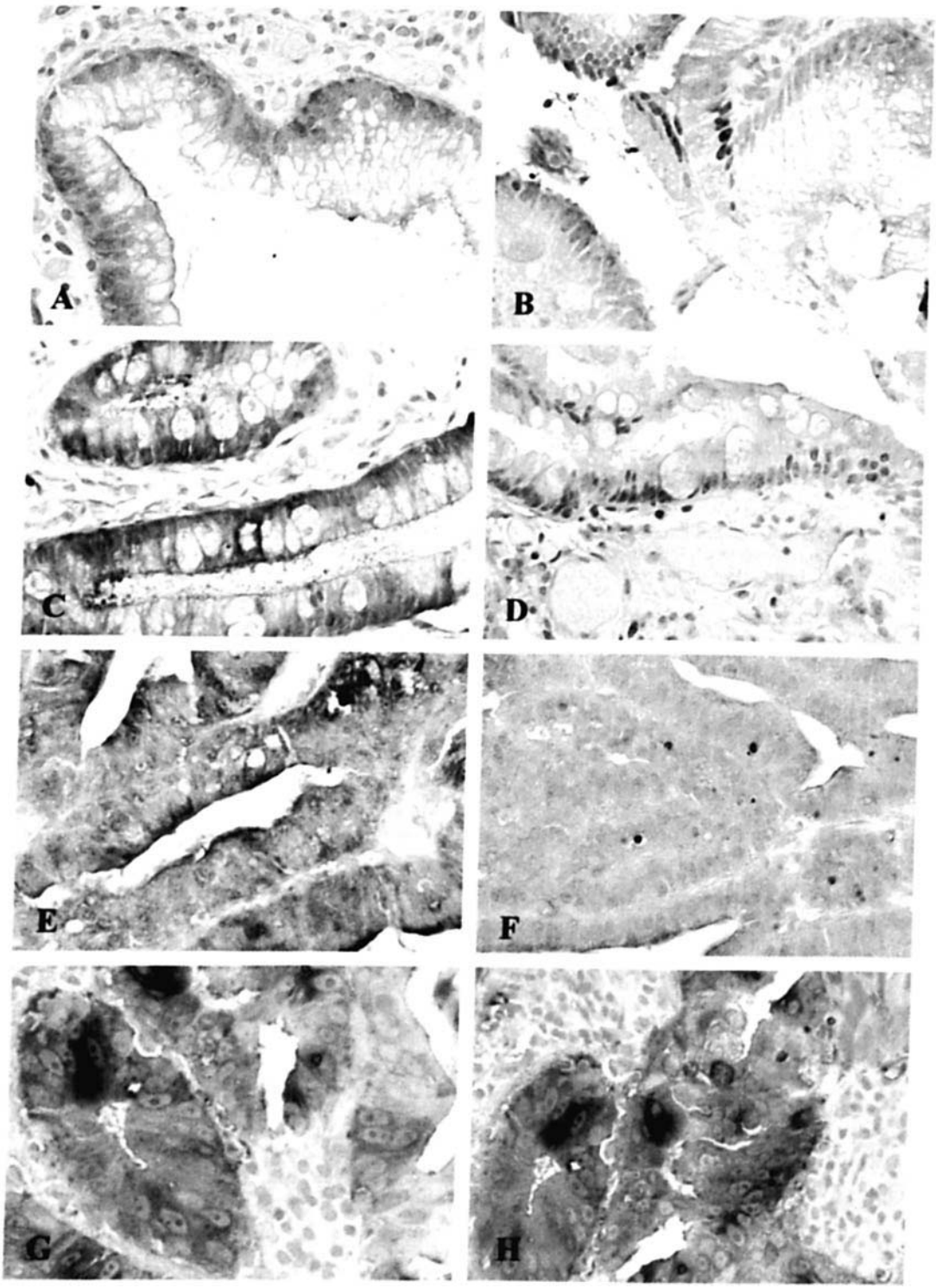

Figure 3. Representative MT and TUNEL dual staining in the sections from different stages of BE. In the same tissue obtained from SIM, TUNEL-positive cells were barely detected in the strong MT staining (A) and TUNEL-positive cells were dominant in the area with weak MT staining (B). The same pattern of staining was observed in the tissue obtained from I/LGD (C, D), HGD (E, F), and EAC (G, H). The MT staining was found in both cytoplasm and nuclei.

induced free radicals. MT has been shown to function as an antioxidant to protect cells from apoptosis (13). Furthermore, downregulation of MT expression can increase apoptotic activity in breast cancer cells (31). Importantly, suppression of oxidative stress-induced apoptosis may favor the initiation of carcinogenesis (29). Hence, MT protection from reflux-induced apoptosis is likely a mechanism for the initiation of Barrett's cells to develop the metaplasia-dysplasia-adenocarcinoma sequence.

Second, MT may function as a zinc-donor for DNA

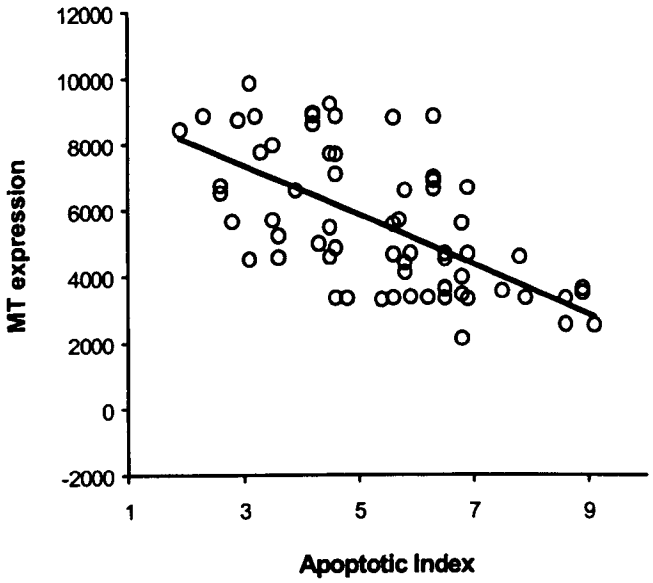

Figure 4. Relationship between MT expression and apoptotic index in 66 BE patients. A significant negative correlation between MT expression and apoptotic index was found ($R = -0.38$, $P < 0.001$).

replication and repair in BE. Zinc is required for the rapid proliferation of tumor cells (26). Increased MT levels, particularly in the nuclei, were observed in breast cancers (26), and in regenerated liver cells after partial hepatectomy (32). This is caused by the requirement for zinc in DNA synthesis during cell proliferation. In contrast, downregulation of MT genes by antisense oligodeoxynucleotide inhibits the growth of tumor cells (31) and delays the hepatic regeneration in rats after partial hepatectomy (32). Recent *in vitro* studies using two myoblast cell lines (L6 and H9C2) have demonstrated that MT and zinc were localized mainly in the cytosol in myoblasts but were translocated into the nuclei of proliferating myotubes. After the myotubes have fully developed and cell proliferation has ceased, MT and zinc contents were decreased and were localized in cytosol (33). These results strongly suggest that MT may be required for the delivery and donation of zinc to certain metalloenzymes for DNA synthesis and cell growth. Therefore, MT overexpression during rapid tumor growth may be a phenomenon in response to mitogenic signals (34).

However, it is also possible that tumor proliferation and invasion may stimulate MT synthesis in BE. MT can be induced by a variety of stresses including metals and non-metal factors (22). MT is also considered as a stress protein because almost all factors that can induce heat-shock protein can also induce MT (35). For example, surgical stress can induce a significant increase in MT in multiple organs (36). Chronic diseases, such as diabetes, can induce MT synthesis in multiple organs (37). Cytokines, such as interleukin-6 and tumor necrosis factor, are able to induce MT synthesis (38). Pretreatment with dexamethasone, a strong inhibitor of inflammation, can prevent MT synthesis induced by inflammatory agents (39). Therefore, it is possible that rapid growth and invasion of cancer cells in BE would stimulate local inflammation to release cytokines such as tumor necrosis factor, which in turn would induce MT synthesis.

Regardless of whether MT overexpression is involved in carcinogenesis in BE or whether BE cell carcinoma leads to MT production, it is worthy to note that MT overexpression in the BE may potentially serve as a marker for diagnosis of carcinoma of BE. This will be explored in our future studies.

In summary, we studied the relationship between expression of MT and apoptosis in the progression from metaplasia to dysplasia to adenocarcinoma in subjects with BE. We found that Barrett's cells with low apoptotic activity had high levels of MT expression. This suggests that MT may contribute to cytoprotection, thereby inhibiting apoptosis and increasing the likelihood of Barrett's cells to undergo abnormal cell cycling or gene expression. Therefore, MT may be involved in the initiation of Barrett's carcinogenesis. We also found that MT expression is enhanced along with the histological progression of BE toward adenocarcinoma. We hypothesize that enhanced expression of MT may either act as zinc donor in favor of tumor proliferation or be induced by the rapid growth of the tumor. Further studies are needed to correlate MT expression with clinical outcome. Animal models are required to determine the exact mechanism of MT action in the metaplasia-dysplasia-adenocarcinoma sequence of BE.

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