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Cathepsin B Expression in Colorectal Carcinomas Correlates with Tumor Progression and Shortened Patient Survival

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Cathepsin B is a lysosomal cysteine proteinase that has the ability to degrade several extracellular matrix components at both neutral and acidic pH and has been implicated in the progression of several human and rodent tumors. We have studied the expression of cathepsin B in human colorectal tissues using a monospecific polyclonal rabbit antibody raised against human liver cathepsin B. In immunoblots of normal and neoplastic colorectal tissues this antibody specifically recognized only cathepsin B. We studied 101 cases of formalin-fixed, paraffin-embedded tissue (15 normal mucosa, 17 adenomas, and 69 carcinomas). Epithelial cells of normal mucosa and adenomas were either negative or showed a weak granular reactivity located in the paranuclear and apical cytoplasm of superficial cells. Small clusters of histiocytes were also positive in the region of the superficial area of the lamina propria. In carcinomas, increased expression of cathepsin B correlated with advanced stage of the disease. Increased immunoreactivity of cathepsin B in malignant cells was associated with either a diffuse cytoplasmic staining or was polarized to the basal pole of the cells. This is in contrast to the punctate paranuclear staining pattern observed in normal colonic mucosal cells. In tumor stromal cells, increased expression of the enzyme correlated with neoplastic progression. Expression of high levels of cathepsin B in the tumor epithelial cells was associated with a significantly shorter survival of the patients. In conclusion, our results indicate that cathepsin B expression is up-regulated in human colorectal carcinomas compared with normal mucosa and adenomas and correlates with tumor progression. (Am J Pathol 1994, 145:301–309)

The ability of tumor cells to invade tissues and metastasize is thought to involve an increased expression of proteinases and/or a decrease in the levels of proteinase inhibitors.[1] Proteinases may facilitate metastasis in a number of different ways including detachment of individual cells from the primary tumor, invasion of surrounding tissues to allow contact with vascular channels, degradation of the basement membrane during both intravasation and extravasation, and invasion of tissues during formation of secondary tumor sites.

Several classes of proteinases have been implicated in this process including matrix metalloproteinases, cathepsins B and L (cysteine proteinases), cathepsin D (aspartic proteinase), and plasminogen activator.[2–7] The function of each of these enzymes in malignant human tumors is an area of active investigation. It is possible that human tumors may use combinations of these enzymes working synergistically to facilitate invasion, or, alternatively, one specific proteinase may play a dominant role in tissue invasion for a given cancer. Cathepsin B is a lysosomal cysteine proteinase that functions in the normal turnover of proteins in mammalian cells. The enzyme

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exhibits broad substrate specificity and has been shown to degrade several extracellular proteins at both acid and neutral pH.[8,9] Several human and rodent tumor cell lines exhibit quantitative and qualitative differences in the regulation of cathepsin B including increased synthesis, secretion of the proenzyme into the extracellular environment, and association of the active enzyme with the plasma membrane fraction of highly metastatic tumor cell lines.[4]

Colon carcinoma is the second leading cause of cancer deaths in the Western population. The majority of these deaths are attributable to either local tumor invasion or distant metastases.[10] Several proteinases have been implicated in these processes. Stetler-Stevenson et al[11] reported that the mRNA and protein levels of the 72-kd type IV collagenase increase with the advanced Dukes' stage of the tumors. Maciewicz et al[12] found that malignant colon tumor cells in culture secrete and process the precursor forms of both cathepsins B and L and that these purified enzymes were capable of degrading an isolated basement membrane matrix. Keppler et al[13] studied the immunohistochemical expression and localization of cathepsin B in normal human colon tissue and adenocarcinomas. They reported an increase in cathepsin B immunostaining in carcinomas in general, although they did not determine the levels of expression within the various tumor stages and the antibody used in their study also cross-reacted with cathepsins H and L.[13]

Defining the role of proteinases in the invasiveness of colon tumors is important with regard to understanding the biology of this process as well as in the search for potential prognostic markers and targets for therapeutic intervention. The objectives of this study were to examine the levels of cathepsin B in a series of colon carcinoma cases and in normal mucosa and adenomas. Using standard immunohistochemical techniques on formalin-fixed, paraffin-embedded tissues we determined that colon carcinoma shows an increased expression of cathepsin B, which further correlates with the stage of the disease. In contrast, both adenomas and normal colon mucosa showed only limited staining. Increased expression of the enzyme was also associated with a significantly shorter survival time.

Materials and Methods

Tissues

One hundred and one cases representing normal colon tissue, adenomas, and stages I through IV colon carcinomas[14] were obtained from the files of the Hospital Clinico Provincial de Barcelona. The cases were randomly selected from a series of patients operated on between 1986 and 1989 with fully documented clinical history, staging investigations, and follow-up. Slides and paraffin blocks were obtained in all cases. The cases included normal mucosa (n = 15), adenomas (n = 17), stage I (n = 8), stage II (n = 20), stage III (n = 30), and stage IV (n = 11). The carcinomas included well differentiated (n = 5), moderately differentiated (n = 47), poorly differentiated (n = 11), and mucinous tumors (n = 6).[15] Adenomas were obtained from both surgically removed specimens and fiberoptic biopsies. Normal mucosa samples were selected from normal areas of surgical specimens. All tissues were routinely fixed in 10% buffered formalin and embedded in paraffin.

Preparation of Monospecific Anticathepsin B IgG

Antisera were raised in rabbits (New Zealand white male) against the native double chain form of human liver cathepsin B, as we have described.[16] An IgG fraction was purified by our described procedures[16] and stored at −20 C. The specificity of the IgG for cathepsin B has been confirmed by slot blotting using purified cathepsin B and cathepsin L and by immunoblotting using purified cathepsin B and extracts (acetone fractions) of normal human liver and human sarcoma.[16] The monospecific anticathepsin B IgG recognizes single chain and double chain forms of the mature enzyme[16] and immunoprecipitates mature and pro forms of the enzyme.[17]

Immunoblotting

Four matched pairs of normal and cancerous human colon tissues were processed as for partial purification of protein (ie, acid precipitation and acetone fractionation steps) using our published protocols.[16] The partial purification of protein is necessary for subsequent electrophoresis and does not appreciably increase the percentage of cathepsin B protein in the sample.[16] Acetone fractions were dialyzed and concentrated in Centricon microconcentrators (Amicon, Danvers, MA). For each pair, all of the concentrated sample was electrophoresed in 12% polyacrylamide gel slabs and transferred to nitrocellulose membranes. Human liver cathepsin B, purified according to our published protocol,[16] also was electrophoresed in 12% polyacrylamide gel slabs and transferred to nitrocellulose membranes. Membranes were developed with an enhanced chemiluminescence

Western blot detection system using dry milk (10%) and Tween as blocking agents.

Immunohistochemistry

Immunohistochemical staining was performed using the avidin-biotin-peroxidase complex technique (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). The slides were dewaxed in xylene and then rehydrated. The endogenous peroxidase was blocked with 3% H_2O_2 for 8 minutes. The sections were next incubated with normal goat serum for 20 minutes, followed by an overnight incubation with the primary antibody at 4 C. The biotinylated goat anti-rabbit IgG and the avidin-biotin-peroxidase complex were diluted in the same buffer and incubated for 30 and 45 minutes, respectively, at room temperature. Slides were washed three times with Tris-buffered saline after each incubation. Peroxidase activity was developed by a solution of 5 mg of 3–3′ diaminobenzidine tetrahydrochloride (DAB; Sigma Chemicals St. Louis, MO) dissolved in 10 ml of 0.05 M Tris buffer, pH 7.6, and 0.03% of H_2O_2. The DAB solution was filtered and the sections were incubated under microscopic control. Harris' hematoxylin was used to counterstain the slides. Control slides were produced by replacing the primary antibody with the IgG fraction of normal rabbit serum.

The immunostained sections were evaluated independently by two observers. A case was considered negative when no immunoreactivity was seen or it was detected in less than 1% of the cells, low cathepsin B expression when less than 25% of the tumor cells were positive, and high cathepsin B expression when immunoreactivity was found in more than 25% of the tumor cells. Epithelial and stromal cells were evaluated separately. Interpretation was reproducible because the two observers only disagreed in the evaluation of the epithelium in five (6%) cases and in the stroma in 8 (9%) cases. Discrepant results were reviewed and an agreement was reached. For statistical analysis, negative cases were included in the low cathepsin B expression group and compared with high cathepsin B expression cases.

Statistical Analysis

Contingency tables and the χ^2 test were used for comparison between frequencies. The Kaplan-Meier method was used to calculate the probability of survival as a function of time.[18] The Mantel-Haenszel procedure was used to evaluate the significance of the difference between a pair of Kaplan-Meier curves.[19]

Results

Antibody Specificity

Our results indicate that the antibody against cathepsin B specifically recognizes only cathepsin B in samples of human liver and colon carcinomas. Immunohistochemical staining with normal human liver sections revealed punctated cytoplasmic staining in hepatocytes consistent with the localization of a lysosomal enzyme and intense staining of Kupffer cells (data not shown). Immunoblotting against samples from different stages of purification of cathepsin B from normal human liver revealed cross-reactivity with mature cathepsin B (single chain and double chain forms) (Figure 1).

Immunoblotting against four matched sets of extracts from normal colon mucosa and carcinoma from the same patient revealed similar specificity of the antibody (Figure 2). Three of the four tumor samples showed an increase of cathepsin B protein levels compared with the normal mucosa (Figure 2, samples 1 to 3). The increased staining of tumor samples 1 to 3 on the immunoblot correlated with the positivity of the tissue section immunostaining for each case, whereas the tumor from sample 4 showed little increase in immunostaining compared with the normal mucosa. The higher molecular weight bands represent the single chain form of cathepsin B. The lower

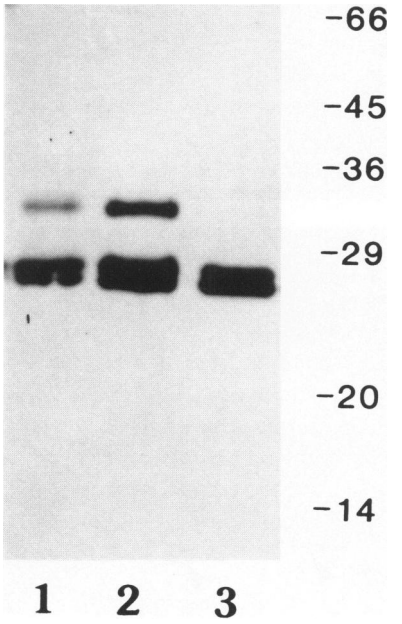

Figure 1. *Immunoblot of normal human liver. Samples (<1 μg/lane) from human liver were electrophoresed in 12% polyacrylamide gels and electrophoretically transferred to a nitrocellulose membrane. The membrane was developed using an antibody to human liver cathepsin B. Lanes represent: 1, acetone fraction; 2, pooled affinity fraction; 3, mono-S fraction. Molecular masses (M) are indicated at the right.*

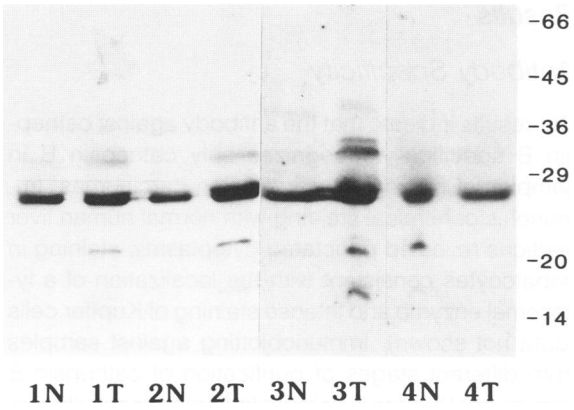

Figure 2. *Immunoblot of four matched sets of colon tumor and normal colon mucosa. Four matched pairs of normal and cancerous human colon tissue were processed as for partial purification of cathepsin B. For each pair, all of the concentrated sample was electrophoresed in 12% polyacrylamide gel slabs and transferred to nitrocellulose membranes. The membrane was developed using an antibody to human liver cathepsin B. Lanes 1 to 4 represent the four patient samples of normal colon tissue (N) and colon tumor (T), respectively. Molecular masses (M) are indicated to the right.*

molecular weight bands exhibiting cross-reactivity with the antibody are most likely proteolytic fragments of cathepsin B because the enzyme has been shown to undergo autolysis while working at or near neutral pH.[9]

Cathepsin B Expression in Normal Mucosa and Adenomas

Epithelial cells of normal mucosa were either negative or revealed a minimal granular staining located in the paranuclear and apical area of the cytoplasm of superficial columnar cells (Figure 3, A). Small clusters of immunoreactive macrophages were also seen in the upper area of the lamina propria in all the cases of normal mucosa. Similarly, adenomas were negative or showed a weak staining with the same paranuclear pattern observed in normal mucosa (data not shown).

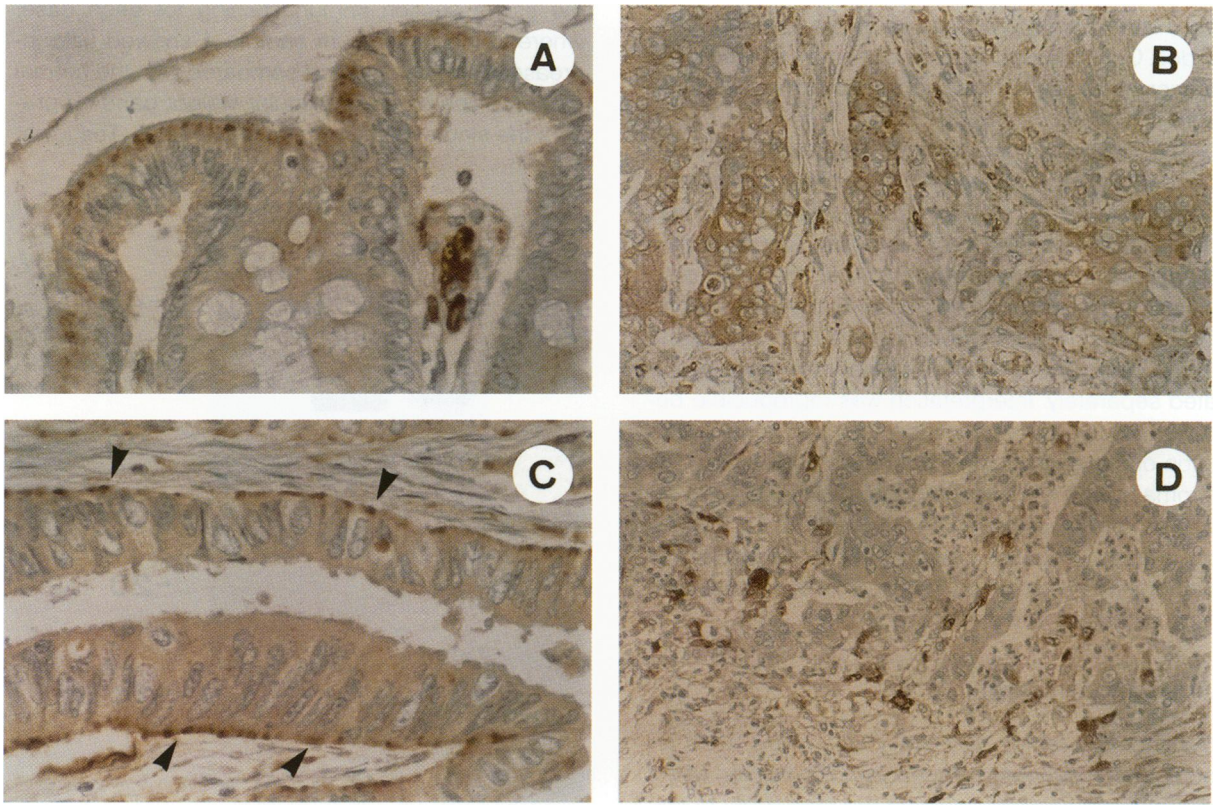

Figure 3. *Cathepsin B immunostaining in normal colon mucosa* (A) *and colorectal carcinomas* (B-D). A: *The normal mucosa shows granular staining in paranuclear and apical areas of the cytoplasm in the superficial epithelial cells. Clusters of macrophages in the upper area of the lamina propria are also positive (immunoperoxidase-hematoxyline, ×400).* B: *Cathepsin B positivity in infiltrating epithelial cells shows a diffuse cytoplasmic pattern (immunoperoxidase-hematoxyline, ×200).* C: *Basal polarization of cathepsin B immunostaining in malignant epithelial cells (arrows) (immunoperoxidases-hematoxyline, ×400).* D: *Cathepsin B immunoreactivity in peritumoral stromal cells. Adjacent tumor epithelial cells are negative (immunoperoxidase-hematoxyline, ×200).*

Cathepsin B Expression in Colorectal Carcinomas

When compared with normal mucosa and adenomas, cathepsin B expression in carcinomas showed an increase that correlated with the stage but not the histological classification ($P = 0.9$) (Tables 1 and 2). In well differentiated areas the pattern of staining was similar to normal cells with a granular paranuclear distribution. However, in the majority of the positively staining tumors, neoplastic cells showed a granular staining that was either diffuse throughout the cytoplasm (Figure 3, B) or polarized to the basal pole of the cells (Figure 3, C). Epithelial cathepsin B immunostaining correlated with the progression of the tumors because none of the 8 stage I carcinomas, 25% (5 of 20) of stage II carcinomas, 44% (13 of 30) of stage III carcinomas, and 72% (8 of 11) of stage IV carcinomas showed a high cathepsin B immunoreactivity ($P = 0.001$) (Table 1). No correlation was found between cathepsin B immunostaining and tumor size or location.

Immunoreactivity was observed in stromal fibroblasts and macrophages in almost all of the cases. These cells were seen throughout the tumors but were often associated with nests of malignant cells at the infiltrating edge of the tumor (Figure 3, D). The frequency of positive staining stromal cells varied between the cases. A correlation was observed with the stage of the carcinomas ($P < 0.05$) but not with the histological classification of the tumors (Tables 1 and 2). Cathepsin B expression in stromal cells also correlated with the expression of the enzyme in the tumor epithelial cells ($P = 0.045$) (Table 3).

Cathepsin B Expression and Survival

High cathepsin B expression in tumor epithelial cells was associated with a significantly shorter overall survival of the patients ($P < 0.001$) (Figure 4). Although a high stromal cell staining also showed a trend toward a shorter survival, the difference did not reach statistical significance ($P < 0.1$). Stratification of the cases into stages I/II (nonmetastatic tumors) and III/IV (metastatic tumors) for low *versus* high cathepsin B expression resulted in the survival curves shown in Figure 5, A and C. In stage I/II lesions cathepsin B expression did not correlate with survival. However, in stage III/IV lesions survival was significantly shorter in cases with high cathepsin B expression ($P < 0.02$). Similarly, in the group of patients in stage II/III with an intermediate risk of recurrence, the high expression of cathepsin B was associated with a shorter survival ($P < 0.05$) (Figure 5, B). The correlation between cathepsin B expression and patient survival was also independently evaluated within the stage II, III, and IV cases. In all the groups the patients with high cathepsin B expression had a shortened survival time when compared with the low cathepsin B expression cases. However, these differences did not reach statistical significance due in part to the small number of patients within each subset.

Discussion

The cellular regulation of proteinases in tumor invasion and metastasis appears to be complex. Ultimately, an invasive tumor requires proteinases that are active and properly exposed to the extracellular environment. Invading tumors maintain control over the activity of these enzymes at multiple levels including synthesis, secretion, extracellular activation/localization, and inactivation by endogenous inhibitors. It seems reasonable to assume that although tumor invasion is facilitated by up-regulation of proteinases, the tumor must retain tight control over the activity of these enzymes to avoid the deleterious effects of unrestricted proteolysis. Once secreted into the extracellular environment, the enzymes may be activated/associated with either tumor cells or stromal cells. There is an increasing body of evidence that the invasive front of an advancing tumor represents a coordinated effort among multiple cell types to produce and regulate these enzymes.[20–24]

Cathepsin B is secreted in its proenzyme form by tumors and tumor cell lines in culture.[12,25–27] This is a similar phenomenon to that observed with cathepsin L, a related lysosomal cysteine proteinase.

Table 1. *Correlation Between Cathepsin B Expression in Tumor Epithelial and Stromal Cells and Stage in 69 Colorectal Carcinomas*

	Epithelial Cells			Stromal Cells		
	(−)	Low	High	(−)	Low	High
Stage I ($n = 8$)	5 (62%)	3 (38%)		2 (25%)	5 (63%)	1 (12%)
Stage II ($n = 20$)	1 (5%)	14 (70%)	5 (25%)		11 (55%)	9 (45%)
Stage III ($n = 30$)	3 (10%)	14 (46%)	13 (44%)		9 (30%)	21 (70%)
Stage IV ($n = 11$)		3 (28%)	8 (72%)		6 (55%)	5 (45%)
Total ($n = 69$)	9 (13%)	34 (49%)	26 (38%)	2 (3%)	31 (45%)	36 (52%)

Table 2. *Correlation Between Cathepsin B Expression in Tumor Epithelial and Stromal Cells and the Histological Type of 86 Colorectal Tumors*

	Epithelial Cells			Stromal Cells		
	(–)	Low	High	(–)	Low	High
Adenomas	14 (82%)	3 (18%)		6 (35%)	9 (53%)	2 (12%)
Carcinomas						
Well differentiated (n = 5)		4 (80%)	1 (20%)		3 (60%)	2 (40%)
Moderately differentiated (n = 47)	7 (15%)	22 (47%)	18 (38%)	2 (4%)	21 (45%)	24 (51%)
Poorly differentiated (n = 11)	1 (9%)	5 (46%)	5 (45%)		4 (36%)	7 (64%)
Mucinous (n = 6)	1 (17%)	3 (50%)	2 (33%)		3 (50%)	3 (50%)
Total (n = 86)	23 (27%)	37 (43%)	26 (30%)	8 (9%)	40 (47%)	38 (44%)

Table 3. *Correlation Between Cathepsin B Expression in Tumor Epithelial and Stromal Cells*

	Epithelial Cells	
Stromal Cells	Low Cathepsin B Expression	High Cathepsin B Expression
Low cathepsin B expression	25	8
High cathepsin B expression	18	18
Total	43	26

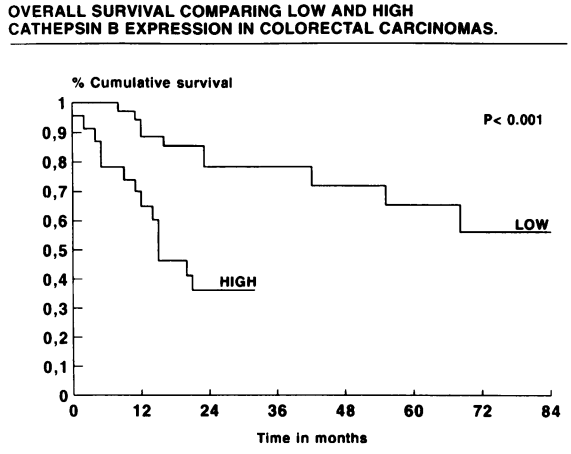

Figure 4. *Overall survival of patients with colorectal carcinomas comparing low* versus *high cathepsin B expression. Tumors with no cathepsin B expression (negative cases) are included in the low expression group.*

Gottesman et al[28] observed that transformed NIH-3T3 cell lines secrete large amounts of procathepsin L into the medium. The secretion of both procathepsin B and procathepsin L is in marked contrast to the normal processing of these enzymes by nontransformed cells, where the proenzymes are targeted to lysosomes and processed intracellularly.[29] Currently, the mechanism of extracellular activation of both procathepsin B and procathepsin L and potential substrates that may be involved in tumor invasion and/or progression remain speculative.

In addition to secretion of the proform of cathepsin B, several tumors show a shift in the distribution of the active enzyme from a lysosomal to a plasma membrane location.[30,31] The exact pathophysiological significance of this process is not clear. The simplest explanation is that the change in subcellular location may facilitate exposure of the active enzyme to extracellular substrates.

The results described in this paper show an increase in the expression of cathepsin B in the progression of colorectal carcinomas. The increased staining was observed in both the tumor cells and the associated stromal cells, although the increased tumor cell staining was more closely correlated to the stage of the disease. The increased staining of stromal cells is consistent with tumors using other cell types besides themselves to produce and/or process proteinases during tumor invasion, similar to the findings of Dano et al[21] with urokinase-type plasminogen activator. The correlation observed in this series between cathepsin B expression in epithelial and stromal cells may suggest a cooperation between these two types of cells in the progression of the tumor.

We also observed a qualitative difference in the staining between the normal colonic mucosa and the highly invasive tumors. Normal mucosal cells showed characteristic punctate paranuclear staining, consistent with a lysosomal distribution of cathepsin B. In contrast, the invasive tumors showed either a diffuse cytoplasmic pattern or localization of the enzyme in the basal pole of the cells adjacent to the stroma. One can speculate that this staining pattern represents either plasma membrane-associated enzyme or enzyme associated with small vesicles in the cell periphery; however, resolution of this question depends on ultrastructural immunolocalization studies.

Patients with colorectal tumors expressing high levels of cathepsin B had a significantly shortened survival time. This relationship was limited to those patients with metastatic lesions (stage III and IV) (Figure 5, B) and patients with an intermediate risk of recurrence (stage II/III). In the group of advanced tumors (stage III/IV), after 2 years of follow-up, 25% of the patients with high expression of cathepsin B were

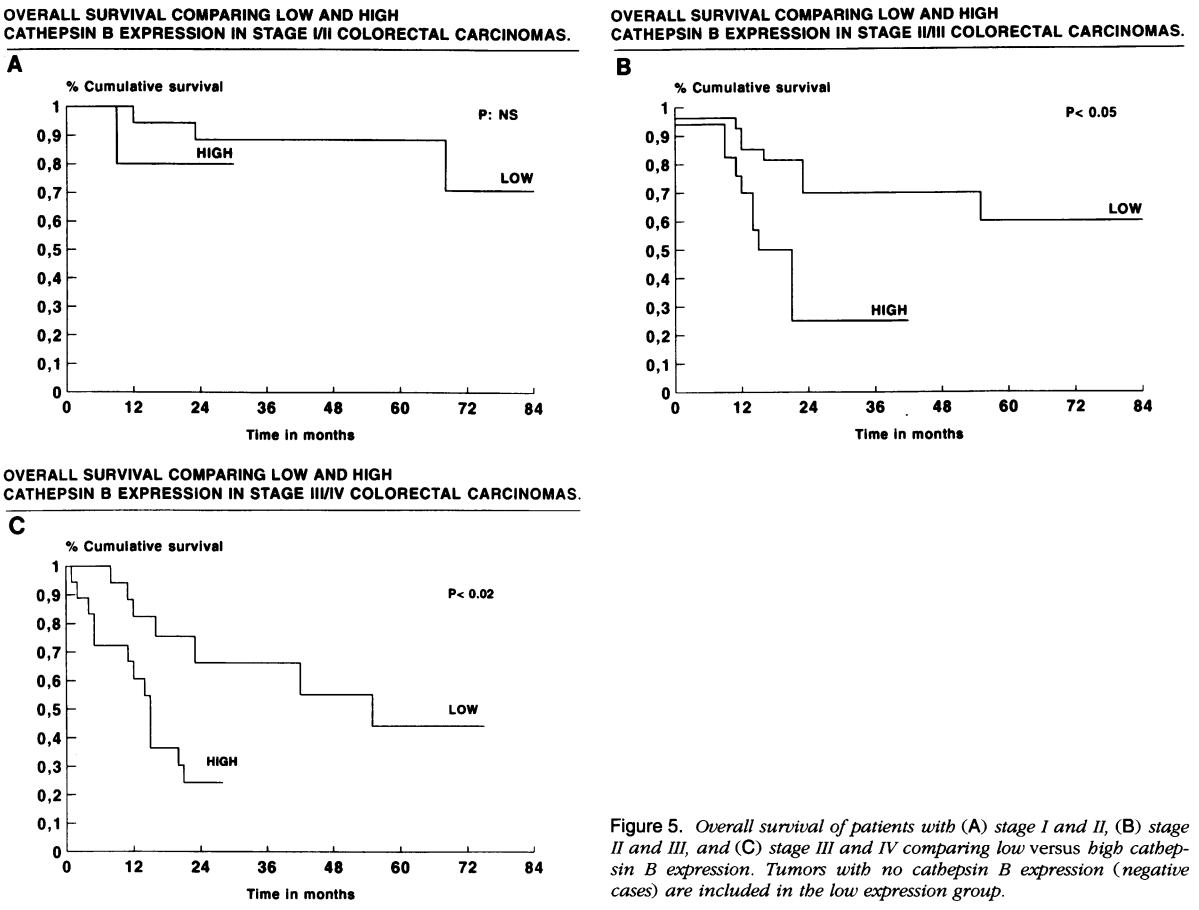

Figure 5. *Overall survival of patients with* (A) *stage I and II,* (B) *stage II and III, and* (C) *stage III and IV comparing low* versus *high cathepsin B expression. Tumors with no cathepsin B expression (negative cases) are included in the low expression group.*

alive compared with 65% of those with low expression of the enzyme. We did not see a difference in survival curves for the patients with nonmetastatic lesions (stage I and II) (Figure 5, A). At 3 years of follow-up 75% or more of the patients with nonmetastatic lesions from both the low and high expressors of cathepsin B were alive. Surgical resection is generally a curative procedure in these patients, thus it is not possible to assess whether the tumors with high cathepsin B expression represent more aggressive lesions. Based on these findings we cannot suggest that cathepsin B is an independent prognostic marker in colorectal tumors, however, we can conclude that increased cathepsin B expression correlates with aggressive tumor behavior and tumor progression.

Similar to our findings with increased cathepsin B expression, anomalies in the deposition of laminin and type IV collagen are associated with the progression of colorectal carcinomas[32–34] and with shortened patient survival in metastatic tumors (stage III/IV) but not in nonmetastatic lesions (stage I/II).[32,33] The mechanism(s) leading to basement membrane anomalies in tumors is not known; however, it may include either a decrease in the synthesis or secretion of basement membrane components by tumor cells, or, alternatively, an increase in degradation of basement membrane components by specific proteinases elicited by tumor and/or stromal cells. Our findings suggest that cathepsin B in colorectal carcinomas could play a role in degrading extracellular matrix proteins, including basement membrane laminin[9] and contribute to the progression of these tumors.

The results of this study appear to disagree with the findings of Murnane et al[35–37] who have reported decreased levels of cathepsin B activity and mRNA in Dukes' stage C and D tumors compared with stage A and B lesions. The reasons for this discrepancy are not clear. Our study used immunohistochemical staining of fixed tissues to examine the total cathepsin B protein in these tumors, including proenzyme, mature enzyme, and mature enzyme complexed with inhibitor. Thus, the level of protein detectable by immunostaining in these sections may not necessarily correlate with the level of active enzyme. The decrease in mRNA observed by Murnane et al[35] in these tumors compared with the increased immunostaining that we

observed may indicate a difference in the translational control and/or posttranslation processing of the enzyme once it has been synthesized in these more advanced lesions.

There are many features of cathepsin B regulation and localization within tumors that remain to be determined. The proper activation and placement of an enzyme within a tumor is critical to its role in tumor invasion. Studies of cathepsin B activity with homogenized tissue, as conducted by Murnane et al,[35,36] allow for determination of overall levels of enzyme activity within a tumor but may not reflect the specific events occurring in the region of actual tumor invasion. Studies on fixed tissue sections, such as the one we report here, provide information regarding the levels of cathepsin B protein in the different regions and cell types within a tumor but do not provide information regarding the particular form of the enzyme, its relationship to endogenous inhibitors, or its subcellular location. Future studies to address these concerns are needed to fully understand the role of cathepsin B in the biology of human colorectal tumors.

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