Elevations in Cathepsin B Protein Content and Enzyme Activity Occur Independently of Glycosylation during Colorectal Tumor Progression*

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Western blots, enzyme assays, protein glycosylation studies, and immunohistochemical staining were used to characterize cathepsin B expression at successive stages of colorectal tumor progression. In normal colon mucosa and premalignant adenomas, cathepsin B expression was predominantly due to mature two-chain protein detected on Western blots as the nonglycosylated 27-kDa form, with overexpression of this protein occurring in only 4 of 18 adenomas. Overexpression increased significantly in Dukes A and B carcinomas (26 of 37 cases), with cathepsin B protein generally detectable in carcinomas as a combination of both 27-kDa nonglycosylated and 28-kDa glycosylated mature two-chain forms. Glycosylated cathepsin B protein in carcinoma extracts was sensitive to PNGase F but resistant to Endo H, indicating a pattern consistent with complex rather than high mannose type glycosylation. When sorted by advancing tumor stage, peak expression of cathepsin B protein occurred in carcinomas involved in local invasion compared with adenomas or metastatic cancers. At all stages, cathepsin B activity correlated significantly with the levels of heavy chain mature cathepsin B protein ($r = 0.6682, p < 0.0001$) irrespective of glycosylation. Immunohistochemical staining of cathepsin B protein revealed fine diffuse cytoplasmic staining in both adenomas and carcinomas compared with coarse granular cytoplasmic staining (typical of lysosomes) seen in matched normal mucosa. Our results demonstrate several sequential, apparently independent changes in cathepsin B expression during colorectal tumor progression including early changes in subcellular localization, up-regulation of cathepsin B protein and activity in invasive cancers, and altered protein glycosylation detected in malignant tumors at all stages.

Among lysosomal cysteine proteinases, increased or altered cathepsin B expression has been particularly well documented in a variety of tumor cell types including colon, breast, pancreas, lung, and brain tumors (1–9). Cathepsin B has also been shown to be relocated to the plasma membrane (9) or secreted from tumor cells (10, 11), where it is believed to aid in degradation of components of the extracellular matrix and basement membrane (12). Furthermore, tumor cathepsin B has also been shown to retain greater proteolytic activity at or above neutral pH (1, 12) than the normal enzyme.

Previous studies of cathepsin B expression using a primary tumor model of colorectal cancer progression found cathepsin B enzyme activity to be frequently and significantly elevated in the early invasive stages of tumor growth (1, 2, 4). This finding was confirmed by Northern analyses of cathepsin B mRNA in colorectal tumors that also demonstrated greater increases in earlier than later stage carcinomas (3). Early elevation in cathepsin B expression, followed by an inverse correlation with cancer progression, suggested that cathepsin B activity might be particularly important in cancers associated with invasion of the colonic wall. These findings have been supported by the work of Leto et al. (13), who also observed significantly elevated cathepsin B enzyme activity in Dukes A stage colorectal carcinomas but not in Dukes D stage carcinomas. However, Campo et al. (14) made a different observation using immunohistochemical techniques, reporting a higher percentage of cells staining positively for cathepsin B protein in later compared with earlier stages of colorectal cancer. As the method of detecting cathepsin B expression by activity assay is quite different from protein expression detected by immunohistochemistry, it was possible that these different observations might have resulted from different assay techniques. For example, alterations in the expression of endogenous inhibitors of cathepsin B might affect enzyme activity detected in colorectal tumors such that a decrease in inhibitors would result in increased activity or vice versa. However, a change in inhibitor levels should not affect the amount of cathepsin B protein detected by immunohistochemical techniques. Decreases in plasma membrane-associated cysteine proteinase inhibitors have been detected in murine melanomas, resulting in increased effective activity of cathepsin B at the plasma membrane (15). However, no decrease in total endogenous cysteine proteinase inhibitor levels had been found in human colorectal carcinomas compared with normal mucosa, although the relative amounts of individual inhibitors have not been analyzed (1). Alternatively, increased expression of high molecular weight, inactive forms of cathepsin B protein in late stage tumors might explain an increase detected by immunohistochemistry, whereas an activity assay would not include enzymatically inactive forms.

Since previous studies of cathepsin B expression in colorectal tumors have not fully addressed the above questions, we have utilized Western blot analyses of 80 matched pairs of normal colorectal mucosa and adenoma (premalignant polyps) or carcinoma extracts to determine the relationship between expres-
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EXPERIMENTAL PROCEDURES

Materials—Z-Ala-Arg-Arg-MNA was purchased from Enzyme Systems Products (Dublin, CA); Fast Blue B, leupeptin, α-1 acid glycoprotein, and ovalbumin were from Sigma; PNGase F and Endo H from Genzyme (Cambridge, MA); E-64 from Calbiochem. Anti-human cathepsin B and D antibodies were purchased from BioGenex (San Ramon, CA) or Oncogene Science (Cambridge, MA).

Tissues—For these studies, 18 adenomas and 62 carcinomas together with matched normal mucosa were collected from 67 patients. All fresh tissue samples were collected from colectomy specimens at the Mallory Institute of Pathology or were obtained through the National Disease Institute of Pathology or were obtained through the National Disease Institute of Pathology. Tissue sections (5 μm) were fixed in 10% buffered formalin, embedded in paraffin, and processed onto 3-μm sections. Sections were deparaffinized, dewaxed, and placed on poly-L-lysine coated slides. After treatment with 70% ethanol for 2 min, sections were incubated at 37 °C for 1 h in 0.25 M sodium phosphate buffer, pH 8.6, containing 1.25% Triton X-100, 200 μg/ml Fast Blue B, and 250 milligrams of PNGase F. For removal of mannose oligosaccharides by Endo H (23), concentrated samples were boiled for 2 min with a 1.25% (w/v) excess of SDS and 10% 3-mercaptoethanol for 3 h at 37 °C in 50 mM sodium citrate buffer, pH 5.5, containing 2 μg/ml leupeptin and 10 milligrams of Endo H. All reactions were terminated by the addition of an equal volume of 2× sample buffer (2% SDS, 5% β-mercaptoethanol, 10% glycerol, 62.5 mM Tris, pH 6.8). Control samples were treated identically except for substitution of an equal volume of double distilled H2O in place of PNGase F or Endo H enzyme prior to incubation. Human α-1 acid glycoprotein and ovalbumin were used as positive controls for deglycosylation by PNGase F and Endo H, respectively (22, 23).

Polyacrylamide Gel Electrophoresis and Western Blotting—200 μg of matched normal and tumor extracts, as well as 20 μl of premixed Rainbow marker (Amersham Corp.) were prepared in 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 62.5 mM Tris, pH 6.8, and boiled at 100 °C for 2 min before electrophoresing on 10% polyacrylamide gels for 18 h at 70 V. Samples treated by PNGase F or Endo H were prepared in the same manner, followed by electrophoresing on 16% minipolyacrylamide gels 6 h at 80 V (24). After electrophoresis, the top portions of the separating gels containing proteins to be immunoblotted were transferred overnight (for large gels) or 12 h (for minigels) to nitrocellulose membranes (Schleicher and Schuell) in a Bio-Rad Tank Transblot unit at 200 μA per gel plus 15 μA per g of membrane at 250 V. Following transfer, the nitrocellulose membranes were blocked by incubation in a solution containing 5% nonfat dry milk, 0.1% SDS, and 0.1% Triton X-100 for 1 h at 37 °C, washed three times in PBS and primary antibody in the dilution suitable for the antibody (a 1:125 dilution of secondary antibody). For detection of human cathepsin B antibody, a 1:100 dilution of Oncogene Science monoclonal mouse anti-human cathepsin B antibody, a 1:1000 dilution of Oncogene Science polyclonal rabbit anti-human cathepsin D antibody). Blots were then washed 2 times in 5% milk TBST before incubation for 1 h with a horseradish peroxidase-conjugated secondary antibody (Sigma, Oncogene Science) diluted in 5% milk TBST. Blots were next washed overnight in 5% milk TBST followed by four 10-min washes in TBST without milk. Proteins were detected using an enhanced chemiluminescent system (NEB Life Science Products).

Denitroimetry—Scanning laser densitometry of autoradiographs was done using a Molecular Dynamics Personal Densitometer SI. To quantify the relative abundance of cathepsin B heavy chain protein in samples blotted, the 27- and 28-kDa protein bands as detected on autoradiographs were scanned by laser densitometry, and resulting adenoma/normal (A/N) or carcinoma/normal (C/N) ratios calculated for each of the 80 matched pairs were analyzed by Western blot. The calculated A/N or C/N ratio was then adjusted based on the Coomassie Blue-stained loading control for each matched pair to obtain a final adjusted A/N or C/N ratio.

Immunohistochemistry—Archival paraffin-embedded tissue sections of colorectal adenomas and carcinomas were obtained from the Mallory Institute of Pathology. Tissue sections (5 μm) were sliced using a microtome, mounted on polylysine-coated clean glass slides, dewaxed in xylene, and hydrated in graded concentrations of ethanol. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol, after

1 The abbreviations used are: Z-Ala-Arg-Arg-MNA, benzoyl-alanine-arginine-arginine-4-methoxy-2-naphthylamine; MES, morpholinoethanesulfonic acid; E-64, L-arginine-arginine-4-methoxy-2-naphthylamine; PNGase F, peptide-N*-N(α-acetyl-β-glucosaminyl)-asparagine amidase F; Endo H, Endoglycosidase H; PAGE, polyacrylamide gel electrophoresis; A/N, adenoma/normal; C/N, carcinoma/normal.
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RESULTS

Cathepsin B Protein Forms in Normal Colorectal Mucosa—The major form of cathepsin B detected in normal colon mucosa was the mature two-chain form, observed by Western blot in independent samples of normal colon mucosa from patients with 18 matched colorectal adenomas and/or 62 matched carcinomas and in five autopsy samples from patients without colorectal cancer. The 4-kDa light chain portion of mature two-chain cathepsin B was dissociated from the heavy chain prior to electrophoresis and migrated off the gel under the conditions required to visualize the higher molecular weight protein forms.

The heavy chain was detected as a doublet of nonglycosylated (27-kDa) and glycosylated (28-kDa) cathepsin B protein. The strongest cathepsin B protein band detected in normal colon mucosa was the 27-kDa nonglycosylated heavy chain of the two-chain form. However, small amounts of the 28-kDa glycosylated heavy chain were present in some cases (Fig. 1A). The uncleaved 31-kDa single chain form and the 47-kDa proform of cathepsin B protein were not detected in all cases, but when present they were usually seen in lesser amounts than the 27-kDa mature cathepsin B. The Oncogene Science and BioGenex antibodies were able to detect all protein forms of cathepsin B. However, the BioGenex antibody was found to be more sensitive at detecting the 47-kDa proform protein in colorectal extracts, while abilities to detect the 27/28-kDa and 31-kDa protein forms were approximately equal, as shown in Figs. 1A and 4.

To test for the possible autoactivation of the 47- or 31-kDa forms of cathepsin B to the 27-kDa/4-kDa two-chain form (25) during the tissue extraction process, a sample of normal colorectal mucosa was extracted in the presence of the cysteine proteinase inhibitor E-64, which abolished cathepsin B enzyme activity. Cathepsin B enzyme activity in the normal extraction was 42.40 pmol/mg/min; cathepsin B enzyme activity in the extraction in 10 μM E-64 was 0.17 nmol/mg/min. However, as seen in Fig. 1A (lanes 7 and 8), no increase was observed in the higher molecular weight forms of cathepsin B protein following extraction in the presence of E-64, indicating that the cathepsin B protein forms expressed in our tissue samples were not the result of autoactivation during the extraction process.

Fig. 1. Characterization of anti-human cathepsin B antibodies. A, cathepsin B protein in soluble protein extracts (200 μg of protein/lane) was subjected to SDS-PAGE analysis (16%). Proteins were blotted onto nitrocellulose sheets and detected with either the BioGenex sheep polyclonal anti-human cathepsin B antibody (lanes 1, 3, and 4) or the Oncogene Science mouse monoclonal anti-human cathepsin B antibody (lanes 2 and 5–8). Autoradiographs show cathepsin B protein bands detected in extracts of colorectal tissues obtained either from autopsy (lanes 1 and 2) or surgery (lanes 3–8). In lanes 1 and 2, the arrows indicate the major 27-kDa nonglycosylated heavy chain protein form (bottom arrow) as well as small amounts of the 28-kDa glycosylated form (top arrow) present in the same extract of normal colon mucosa detected on the same blot probed sequentially by each antibody. Predominant expression of the 27-kDa form in normal mucosa together with an increase in the amount of the 28-kDa form in cancer was also detected similarly by each antibody used to probe independent blots of the same matched extract pair of normal mucosa and carcinoma run on two different gels. Lanes 3 and 4, normal and cancer samples on blot 1, probed with the BioGenex antibody; lanes 5 and 6, normal and cancer samples on blot 2, probed with the Oncogene Science antibody. Lanes 7 and 8, cathepsin B protein forms detected with the Oncogene Science antibody after extraction of soluble proteins from colorectal tissue in the absence (lane 7) or presence (lane 8) of 10 μM E-64. B and C, Western blots of four types of purified human liver cathepsins (100 μg/lane) including cathepsin B (lane 1), cathepsin D (lane 2), cathepsin H (lane 3), and cathepsin L (lane 4) were probed with the Oncogene Science (C) and BioGenex (D) anti-cathepsin B antibodies to confirm antibody specificity.

To test the specificity of the BioGenex and Oncogene Science antibodies in detecting cathepsin B, a Western blot was done using these cathepsin B antibodies against four lysosomal cathepsins purified from normal human liver. (Fig. 1, B and C). Both antibodies detected only cathepsin B protein and not cathepsin D, H, or L.

Cathepsin B Protein Overexpression in Colorectal Carcinomas—Cathepsin B protein overexpression (C/N ≥ 1.4) was
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Quantitative and qualitative changes of cathepsin B protein forms in adenomas and carcinomas compared with matched normal mucosa. Matched pairs of normal colon mucosa (NCT), adenoma (Ad), and carcinoma (CA) tissue extracts were subjected to SDS-PAGE (16%), and proteins were transferred to nitrocellulose sheets and probed with the Oncogene Science cathepsin B antibody. Mature protein expression in matched sets of tissue extracts was detected on autoradiographs and scanned by laser densitometry to calculate adenoma/normal or cancer/normal ratios. A, increased cathepsin B protein was detected in both adenomas 92 AdS (intermediate stage adenoma) and AdL (late stage adenoma) compared with matched normal mucosa, but only the 27-kDa cathepsin B heavy chain protein was expressed in either normal mucosa or the two adenomas (AdS and AdL). B, detection of cathepsin B protein in a late stage adenoma and carcinoma from case 7 (Dukes stage A) compared with matched normal mucosa demonstrates only the 27-kDa cathepsin B heavy chain protein in normal mucosa and adenoma, but both the 28-kDa (top arrow) and 27-kDa (bottom arrow) heavy chain proteins were present in the cancer. Most of the CN increase in cathepsin B in the cancer was due to the appearance of the 28-kDa band. C, the amount of cathepsin B heavy chain protein detected in carcinoma 29 (Dukes stage B) was less than that detected in the matched normal mucosa, but cathepsin B heavy chain protein still demonstrated a shift from predominant expression of the 27-kDa heavy chain form in normal mucosa to more equal amounts of 27- and 28-kDa heavy chain cathepsin B forms in the corresponding cancer.

Detected in 38 of 62 (61%) carcinomas analyzed on Western blots. Of these 38 cases with overexpression, 32 (84%) showed a major increase in expression of the glycosylated 28-kDa form (Fig. 2B, case 7, and Fig. 4, cases 64, 31-1, 104, 65, and 110), while the remaining six carcinomas (16%) predominantly overexpressed the 27-kDa nonglycosylated heavy chain form (Fig. 4, cases 55 and 31-2).

In 24 of a total of 62 carcinomas analyzed that did not contain a quantitative increase in the total amount of cathepsin B mature protein, a change was often observed in the ratio of cathepsin B protein forms. For 17 of these 24 carcinomas in which total mature protein levels were the same or decreased compared with normal, a shift in the ratio of 27-kDa to 28-kDa cathepsin B heavy chain forms had occurred due to increased amounts of glycosylated 28-kDa heavy chain cathepsin B in the cancer samples (Fig. 2C, case 29, and Fig. 4, case 46). Thus, 49 of 62 colorectal carcinomas (79%) demonstrated increased expression of the glycosylated 28-kDa heavy chain mature two-chain cathepsin B protein in tumor samples compared with matched normal mucosa from the same patient. In one case, (Fig. 4, case 26), increased expression of the glycosylated 28-kDa heavy chain was not detected in a Dukes D primary carcinoma compared with matched normal mucosa, but increased expression with glycosylated form was seen in the corresponding metastatic lesion from this patient. A shift to greater expression of the glycosylated 28-kDa form of cathepsin B in cancers compared with normal was detected with the same approximate frequency at all stages of colorectal carcinoma (64% Dukes A cancers, 81% Dukes B cancers, 82% Dukes C cancers, and 63% of Dukes D cancers).

Of the 62 cases analyzed, only 12 carcinomas (19%) contained increased expression of the 31-kDa single chain form, and each of these 12 cases also showed increased expression of the 27/28-kDa heavy chains of the mature two-chain protein (see Fig. 4). In these 12 cases, the relative increase in the 31-kDa single chain form was generally much less than the increase in the 27/28-kDa heavy chain forms. In addition, 9 of these 12 cases were among those with the largest increases in mature two-chain protein expression and were Dukes stage A and B cases (n = 3 and n = 6, respectively). Thus, increases in the single chain form of cathepsin B were primarily detected in tumors with very high total cathepsin B protein levels.

With respect to the 47-kDa proform, 10 of 62 cases (16%) contained increased expression of the proform in the carcinoma. Of these 10 cases, 9 were cases that also contained increased expression of the mature 27/28-kDa forms, and 2 of these 9 cases also contained increased expression of the 31-kDa single chain form of cathepsin B. These cases with increased expression of the proform in cancer were distributed among Dukes stages A, B, and C (n = 2, n = 4, and n = 4, respectively).

Cathepsin B Protein Expression in Colorectal Adenomas—Expression of cathepsin B protein in 18 colorectal adenomas was also determined by Western blot. Unlike carcinomas, cathepsin B protein in colorectal adenoma extracts was detected primarily as the 27-kDa nonglycosylated heavy chain of the mature two-chain form, similar to that found in normal mucosa. Overexpression of the mature heavy chain forms of cathepsin B protein was detected in 4 of 18 (22%) adenomas analyzed on Western blots. Of these four adenomas, one contained increased expression of the glycosylated 28-kDa heavy chain protein, and the other three overexpressed only the nonglycosylated 27-kDa heavy chain protein (Fig. 2A, cases 92 AdS and 92 AdL; Fig. 2B, case 7). In 17 of 18 adenomas analyzed (94%) the 27-kDa nonglycosylated heavy chain form was the major form of cathepsin B protein detected (e.g. Fig. 4, cases 100 Ad and 10 Ad). Thus, 1 of 18 adenomas (6%) analyzed on Western blots demonstrated increased expression of the 28-kDa glycosylated heavy chain form, compared to 49 of 62 (79%) of colorectal carcinomas analyzed, which expressed increased amounts of the 28-kDa glycosylated heavy chain form (p = 0.0001, Fisher’s exact test).

In a small number of adenoma cases, the 31-kDa and/or the 47-kDa proform of cathepsin B were also detected, but they were detected in small amounts and did not account for a significant amount of cathepsin B protein expression in adenomas.

Glycosylation of Cathepsin B in Colorectal Tumors—Previous reports (26–28) have shown that the cathepsin B heavy chain protein doublet represents glycosylated and nonglycosylated heavy chain forms of cathepsin B. To test the nature of the increased 28-kDa form detected in colorectal carcinomas, one case of normal colorectal mucosa and four carcinomas were selected (two Dukes A and two Dukes C) for incubation with PNGase F, which cleaves asparagine-linked glycans of both the high mannose and complex types, or Endo H, which hydrolyzes high mannose oligosaccharides preferentially (29). As seen in Fig. 3A, incubation of normal mucosa and carcinoma extracts in the presence of PNGase F resulted in increased mobility of cathepsin B protein detected as a shift from the 28- to the 27-kDa form of cathepsin B, confirming that the mature 28-kDa cathepsin B protein form represents an asparagine-linked glycosylated protein chain (22). However, incubation of these identical tissue extracts with Endo H, shown in Fig. 3B, did not result in a shift of the 28-kDa form to the 27-kDa form in either normal mucosa or carcinomas of any stage, suggesting that the asparagine-linked oligosaccharide of glycosylated mature two-chain cathepsin B in these cases is a complex type oligosaccharide and not a high mannose oligosaccharide (Fig. 3B).

The 32-kDa heavy chain form of cathepsin D has been shown...
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FIG. 3. Characterization of the glycosylation of cathepsin B heavy chain protein in normal colorectal mucosa and colorectal carcinomas. Purified control proteins were compared with normal colorectal mucosa and four colorectal cancer extracts (two Dukes A and two Dukes C carcinomas) for deglycosylation by PNGase F and Endo H. Treated and untreated samples were analyzed by 16% SDS-PAGE, and results were detected either by Coomassie Blue protein staining (A and B, lanes 3–12) or by transferring proteins to nitrocellulose membranes and probing with the Oncogene Science anti-cathepsin B antibody (A and B, lanes 3–12) or with the Oncogene Science anti-cathepsin D antibody (C, lanes 3–12). A, human α1 acid glycoprotein (lanes 1 and 2), normal colorectal mucosa (lanes 3 and 4), Dukes A colorectal carcinoma extracts (lanes 5–8), and Dukes C colorectal carcinoma extracts (lanes 9–12) were incubated in the absence (−) and presence (+) of PNGase F enzyme prior to SDS-PAGE. Increased mobility of the 27/28-kDa glycosylated heavy chain doublet was seen in treated (+) extracts (lanes 4, 6, 8, 10, and 12) compared with untreated (−) extracts (lanes 3, 5, 7, 9, and 11). B, human transferrin (lanes 1 and 2) and the same normal mucosa and carcinoma extracts shown in panel A (lanes 3–12) were incubated in the absence (−) and presence (+) of Endo H enzyme prior to SDS-PAGE. On membranes probed with anti-human cathepsin B antibody, the 27/28-kDa glycosylated heavy chain doublet for cathepsin B was seen in both untreated (−) extracts (lanes 3, 5, 7, 9, 11) and treated (+) extracts (lanes 4, 6, 8, 10, and 12), unlike human transferrin (lanes 1 and 2), which demonstrated increased mobility after treatment (+) with Endo H. C, human transferrin (lanes 1 and 2) was incubated in the absence (−) and presence (+) of Endo H, while the identical nitrocellulose membranes containing samples shown in panel B were reblotted with anti-human cathepsin D antibody. In both normal mucosa (lanes 3 and 4) and colorectal carcinomas (lanes 5–12), the 32-kDa glycosylated heavy chain form of cathepsin D seen in untreated samples (−) demonstrated increased mobility to a 29–30-kDa form after deglycosylation by Endo H (+), as did the 46-kDa proform of cathepsin D in these carcinomas (lanes 6 and 10).

FIG. 4. Cathepsin B protein expression in colorectal adenomas and carcinomas representing the stages of colorectal tumor progression. Protein patterns in 12 sets of matched normal (NCT), adenoma (Ad), and primary carcinoma (CA) tissue extracts plus one metastatic lesion (TM) are shown, as detected by autoradiography of Western blots. Soluble protein extracts (200 μg/slot) were subjected to SDS-PAGE (16%), transferred to nitrocellulose sheets, and probed with anti-human cathepsin B antibody. Overexpression of cathepsin B protein was seen to occur in adenomas and all stages of colorectal carcinoma. However, the greatest amounts of overexpression occurred more frequently in earlier stage carcinomas, compared with adenomas and later stage carcinomas. To determine A/N and C/N ratios for cathepsin B mature heavy chain protein in matched sample pairs, both samples were always run on the same gel and compared on the same autoradiograph scanned by laser densitometry in the region of the 27- and 28-kDa bands. Intensity of protein bands on different autoradiographs does not reflect absolute amounts of protein present in different samples, since optimal autoradiograph development for detection of the protein bands varied with the range of cathepsin B protein present in a given matched pair. Cases 100, 48, and 26 were blotted with BioGenex antibody, while the remaining samples where blotted with Oncogene Science antibody. *, this patient had two primary tumors at the time of surgery, one Dukes A and one Dukes B stage tumor.
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Correlation of Cathepsin B Mature Protein Expression with Enzyme Activity—Cathepsin B enzyme-specific activity levels were determined, and adenoma/normal or cancer/normal ratios for cathepsin B activity were calculated for the same 80 matched pairs of colorectal tissue used to analyze cathepsin B protein levels on Western blots. Related enzyme activity data have been described in greater detail in other publications.

Although the mean A/N ratio for cathepsin B protein content increased with adenoma size, the percentage of adenomas expressing significantly increased cathepsin B protein was approximately the same for all stages. Thus, 1 of 4 early adenomas (25%), 2 of 8 intermediate adenomas (25%), and 1 of 6 late adenomas (17%) contained an A/N ratio > 1.4. However, at the transition from adenoma to early stage carcinoma, both the elevation in cathepsin B protein levels (see Fig. 5) and the percentage of cases expressing elevated protein increased markedly and remained high in Dukes A and B cancers, with a subsequent decrease in cathepsin B protein in later stage carcinomas. Significant cathepsin B mature protein overexpression (C/N ratio > 1.4) was found in 7 of 11 Dukes A carcinomas (64%), 19 of 26 Dukes B carcinomas (73%), 9 of 17 Dukes C carcinomas (52%), and in 0 of 8 Dukes D carcinomas (0%).

Correlation of Cathepsin B Mature Protein Expression with Enzyme Activity—Cathepsin B enzyme-specific activity levels were determined, and adenoma/normal or cancer/normal ratios for cathepsin B activity were calculated for the same 80 matched pairs of colorectal tissue used to analyze cathepsin B protein levels on Western blots. Related enzyme activity data have been described in greater detail in other publications (1–4). The cancer/normal ratios calculated for all 62 cases of carcinoma analyzed in this report demonstrated a tumor-specific pattern of cathepsin B enzyme activity similar to that described in our previous studies (1–4). Cathepsin B enzyme activity was highest in Dukes A and B carcinomas and decreased with stage such that Dukes D carcinomas demonstrated significantly less enzyme activity than earlier stage tumors. The percentage of carcinoma cases demonstrating increased enzyme activity was also observed to be inversely correlated with Dukes stage. As graphed in Fig. 5 and discussed above, patterns reported here for cathepsin B mature protein content are very similar to the patterns previously reported for cathepsin B enzyme activity, showing progressive changes with different colorectal cancer stages.

Quantitation of changes in cathepsin B enzyme activity in 18 colorectal adenomas compared with matched normal mucosa also revealed a pattern similar to that reported in prior enzyme activity studies. We had previously shown that cathepsin B enzyme activity was elevated in 10% of adenomas compared with approximately 60% of Dukes A colorectal carcinomas (2). In this current study, increased cathepsin B enzyme activity was detected slightly more often in colorectal adenomas, possibly reflecting an increase in the number of larger adenomas sampled in this study. When sorted by stage of adenoma, increased cathepsin B enzyme activity was found to occur more frequently in adenomas >1 cm in size (33%) compared with adenomas <1 cm in size (0%), similar to the pattern found for mature protein expression. However, no statistically significant correlation was found between the exact size of an adenoma and its cathepsin B activity A/N ratio (p = 0.15, Spearman rank correlation coefficient).

In Fig. 6, the correlation between cathepsin B heavy chain protein expression and cathepsin B enzyme activity in 80 individual colorectal tumors is shown by using the scatter plot method to compare the cathepsin B enzyme activity A/N or C/N ratios (y axis) with the cathepsin B mature protein A/N or C/N ratios (x axis). These results show that the quantitation of changes in cathepsin B mature protein correlates significantly with changes in cathepsin B enzyme activity in those same cases (p = 0.0001, Spearman rank correlation coefficient).

Subcellular Localization of Cathepsin B Protein in Colorectal Adenomas and Carcinomas—Since altered glycosylation of cathepsin B has been suggested to alter its subcellular localization (31), seven colorectal adenomas and seven early stage (Dukes’ A or B) colorectal carcinomas embedded in paraffin were immunohistochemically stained for cathepsin B protein using...
epithelial cells (e.g. diffuse and/or coarse, granular) were not related to the intensity of cathepsin B staining in adenomas and carcinomas. Thus, at the microscopic level, cathepsin B protein was observed to have a more general cytoplasmic distribution within epithelial cells of all positively staining adenomas and carcinomas compared with normal mucosa, but differences in subcellular localization between adenomas and carcinomas were not detected.

**DISCUSSION**

This study of cathepsin B expression patterns in a large set of primary human colorectal tumors provides evidence for a temporal sequence of events that occurs in the regulation of cathepsin B expression in colorectal tumor progression. Characterization of a large set of adenomas and carcinomas, each with patient-matched normal colon mucosa, demonstrated that tumor-specific increases in cathepsin B activity and mature two-chain protein forms occurred frequently in the early invasive stages of colorectal carcinoma. Increases in cathepsin B protein or activity also occurred, although infrequently, in adenomas. Furthermore, we provide novel data showing that cathepsin B protein was modified at the post-translational level in carcinomas, but not in adenomas, as detected by increased glycosylation of the mature two-chain forms. This shift at the transition from adenoma to carcinoma to increased expression of glycosylated mature cathepsin B was then maintained at all stages of colon cancer, independent of increases or decreases measured in cathepsin B activity or protein expression. Finally, our data indicate that alterations in cathepsin B subcellular localization occur similarly in both adenomas and carcinomas compared with normal colon mucosa, suggesting possible changes in vesicle trafficking early in colorectal tumor progression.

The protein forms of cathepsin B that we have observed in normal colorectal mucosa are in agreement with those described by Mach et al. (27) in human Hep G2 cells and by Hanewinkel et al. (28) in human fibroblasts. In each of these pulse-chase studies, cathepsin B protein was detected as a proform that was processed into a mature single chain plus a two-chain form or completely into the two-chain form. The relative amounts of each protein form differed in Hep G2 cells compared with fibroblasts, possibly due to tissue-specific processing (27). In our studies of human colorectal tissue extracts, each of these reported forms of cathepsin B protein was detected, although the primary protein form present was mature, fully processed two-chain cathepsin B. Keppler et al. (32) also observed only mature, fully processed forms of cathepsin B in cultured cell homogenates of colon carcinoma and hypothesized that the homogenization of whole tissue samples during the extraction procedure might cause autoactivation of latent forms of the enzyme. Yet this did not appear to explain our observations, since extractions in the presence of E-64, resulting in inhibition of cathepsin B activity, did not result in detection of increased amounts of either procathepsin B or single chain mature cathepsin B.

Several detailed studies of cathepsin B enzyme-specific activity levels, mRNA content, and immunohistochemical staining by various research groups analyzing colorectal carcinomas have generated both similar and conflicting information on whether this proteolytic enzyme is predominantly up-regulated in the early or late stages of colorectal tumor progression. In our current expanded study of colorectal adenoma and carcinoma extracts, we have found that both the amount and frequency of elevated cathepsin B expression changed sequentially from small adenomas (<1 cm) to larger adenomas (>1 cm) and from larger adenomas to Dukes A stage carcinomas. The up-regulation of cathepsin B expression was gradual dur-
ing premalignant progression of colorectal tumors followed by a major surge in expression at the transition to invasive cancer. Our current observation that cathepsin B protein was elevated in 22% of adenomas supports our previous enzyme activity studies that showed infrequent elevation of cathepsin B activity levels in adenomas (2). However, in contrast to adenomas, cathepsin B protein expression was strikingly high in early stage cancers and then dropped off gradually in tumors with lymph node metastases (Dukes C cases) and fell very dramatically in late stage primary cancers having distant metastases (Dukes D cases). These results support our prior conclusions that elevated cathepsin B-specific activity and mRNA content in colorectal tumors occurs early in the development of colorectal cancer, with high expression levels associated with invasion of the colon wall (Dukes stages A and B) rather than metastatic spread (Dukes stages C and D) (1–4). Leto et al. (13) have also reported significantly higher levels of cathepsin B enzyme activity in Dukes stage A colorectal tumors compared with Dukes stage D tumors as well as in cancers <5 cm in diameter. Ferinati et al. (33) recently observed that increased levels of cathepsin B and L protein content as measured by enzyme-linked immunosorbent assays were also an early change in gastric cancers. These authors suggested that these cathepsins may play a role not only in the process of cancer invasion but also in the progression of precancerous changes into cancer. Furthermore, using tissue microdissection to determine enzymatic activity in different areas of colorectal tumors, Emmert-Buck et al. (34) demonstrated increased cathepsin B enzyme activity in invasive areas of tumors compared with matched normal epithelial cells from the same patient. In a related study, Leto et al. (35) found that continuous 24-h administration to mice of the cysteine proteinase inhibitor E-64 inhibited spontaneous metastasis formation (in which tumor cells must first invade tissues surrounding the primary tumor to reach blood vessels and spread to distant sites) but did not inhibit experimental metastasis formation (in which tumor cells are directly introduced into the blood stream), supporting a role for cathepsin B in the early steps of the metastatic process associated with invasion of tissues and less with spread to distant sites. However, these data remain at odds with those of Campo et al. (14), who, using immunohistochemical techniques, did not typically observe a high percentage of cells positive for cathepsin B staining in adenomas or early stage carcinomas but saw frequent positive staining of both epithelial and stromal cells in later stage tumors. Although we have not done a large scale immunohistochemical study for this paper, we have analyzed seven adenomas and seven early stage carcinomas by immunohistochemical techniques to assess changes in cathepsin B subcellular localization at the transition from adenoma to carcinoma. In the process, we observed intense and widespread cathepsin B immunohistochemical expression primarily localized to tumor epithelium in both adenomas and early stage colorectal carcinomas. We have also previously reported minimal cathepsin B immunohistochemical staining in late stage cancers in which we had also measured low cathepsin B activity levels (36). One possible explanation of the Campo et al. (14) data may be that the cathepsin B antibody used for that study recognized a new or altered epitope present in cathepsin B protein in late stage cancers but not in adenomas or early stage colorectal tumors and thus resulted in a different observed pattern of expression of cathepsin B.

Our characterization of identical tumor extracts for cathepsin B protein content and enzyme activity has also made it possible to test whether activity levels reflect protein levels in a particular adenoma or carcinoma. We found a statistically significant correlation between A/N and C/N ratios for mature protein expression and A/N and C/N ratios for enzyme activity in the same cases (p = 0.0001). Thus, increased enzyme activity measured in colorectal adenomas and carcinomas was primarily due to the overexpression of mature protein compared with normal colon mucosa. Only a small number of adenoma and carcinoma cases did not show a correlation of enzyme activity with mature protein expression. In these outlier cases, other mechanisms may dictate the final amount of cathepsin B enzyme activity expressed. In studies of murine melanomas, increased enzyme activity appeared to be explained by decreased expression of endogenous cysteine proteinase inhibitors (9, 15). However, Sheahan et al. (1) observed no significant difference in the levels of endogenous cysteine proteinase inhibitors measured in normal colorectal mucosa versus carcinoma. Thus, the mechanisms that generate cathepsin B overexpression may differ in different types of cancer or in particular cases of the same cancer type.

Although a much greater number of colorectal carcinomas than adenomas demonstrated increased cathepsin B expression, an even more significant difference between adenomas and carcinomas was observed in the amount of glycosylated cathepsin B heavy chain protein form detected. In adenomas, any increased cathepsin B protein levels were typically detected as overexpression of the nonglycosylated 27-kDa chain of the mature two-chain form, also detected as the major form in normal colon mucosa. However, in carcinomas, regardless of increased or decreased C/N ratios, cathepsin B protein was typically detected as a combination of both the glycosylated 28-kDa and nonglycosylated 27-kDa heavy chains of two-chain cathepsin B. This shift in cancers to proportionately greater amounts of glycosylated cathepsin B protein was highly significant (p = 0.0001) and raised the possibility that the glycosylated form of cathepsin B in malignant tissues may differ with respect to stability and enzymatic activity (27), cellular localization (31), affinity for endogenous inhibitors (37, 38), or different substrate specificities (37, 39). However, in addressing the question of stability and enzymatic activity against a synthetic substrate, Mach et al. (27) found that neither were significantly affected by the carbohydrate moiety of the cathepsin B enzyme. This is consistent with our findings that increased cathepsin B activity in carcinomas and adenomas measured in matched tissue extracts correlated with the combined amount of mature heavy chain protein (27 kDa plus 28 kDa), irrespective of the ratio of glycosylated to nonglycosylated forms. Hence, high cathepsin B protein and activity levels could be observed in adenomas or some carcinomas that did not demonstrate much glycosylated cathepsin B (e.g. see Fig. 4, case 55). Low cathepsin B protein and activity levels could also be observed in carcinomas that nonetheless demonstrated a significant shift to the glycosylated cathepsin B protein band (e.g. Fig. 2C, case 29 and Fig. 4, case 46). Furthermore, the percentage of cases with increased glycosylation was approximately the same in all Dukes stages, while the total amount of mature protein and activity were greatest in earlier Dukes stages. This change in expression to more glycosylated forms of cathepsin B occurred slightly later in tumor development than the increase in protein and activity levels but represented a more permanent alteration in cathepsin B expression. Thus, it is possible that this form of glycosylated cathepsin B is advantageous to the malignant phenotype in the colon at all cancer stages, while high levels of both glycosylated and nonglycosylated mature cathepsin B may optimize the process of local invasion through the bowel wall.

In addition to this novel finding of increased amounts of glycosylated cathepsin B in colorectal carcinomas, we have also...
observed that cathepsin B is glycosylated with a complex oligosaccharide as indicated by the resistance of cathepsin B to deglycosylation by Endo H but not PNGase F. Lysosomal enzymes are typically glycosylated with mannose-6-phosphate residues to facilitate their sorting in the Golgi complex to lysosomal compartments, although cathepsin B may contain very little high mannose oligosaccharide compared with related lysosomal enzymes and may not even demonstrate much total glycosylation in some tissues (40, 41). These reports are supported by our detection of predominantly 27-kDa nonglycosylated cathepsin B in normal mucosa. Once an enzyme is in the lysosome or late endosome, mannose residues are trimmed off by endogenous endoglycosidasases or as a consequence of the decreased pH (42). However, glycosylation patterns of proteins destined for the plasma membrane or for secretion tend to be complex type oligosaccharides (43). Our studies have shown not only that expression of the 28-kDa glycosylated form of cathepsin B is increased in colorectal carcinomas but also that it is a complex type of oligosaccharide. We did find one case of normal mucosa that was unique in that it expressed amounts of the 28-kDa glycosylated cathepsin B protein form similar to that seen only in carcinomas. The 28-kDa form in this case of normal mucosa was also resistant to deglycosylation by Endo H (case 121 in Figs. 3 and 4) and suggests that this case of histologically normal colonic mucosa may contain alterations similar to those in carcinomas, which are detectable as overexpression of cathepsin B 28-kDa heavy chain protein. Pagano et al. (31) also found that the procathespin B purified from ovarian adenocarcinoma ascites fluid contained a more complex type of oligosaccharide typical of secreted proteins rather than the simple mannose-containing oligosaccharide found on lysosomal proteins. Alterations in glycosylation of cathepsin B in cancers may represent increased amounts of cathepsin B protein containing novel complex carbohydrate structures that have been reported as minor forms of glycosylated cathepsin B in some normal tissues (40, 41). Similarly, Fernandes et al. (44), in a study of general glycosylation patterns (not cathepsin B specifically), demonstrated increased branching and complexity of asparagine-linked oligosaccharide structures in human colorectal tumors.

As for the role of glycosylation affecting the cellular localization of cathepsin B (31), we did not observe any notable difference by immunohistochemistry between the subcellular localization of cathepsin B in adenomas, which typically did not contain glycosylated cathepsin B, and carcinomas, which typically did contain glycosylated cathepsin B. Increased glycosylation of cathepsin B in carcinomas may thus reflect the presence of an enzyme with differing endogenous substrate activity or susceptibility to inhibition compared with adenomas (37–38). Evidence for this scenario has been suggested by Speiss et al. (37) using an in situ enzymatic activity assay to characterize cathepsin B activity and subcellular localization in two lung carcinoma cell lines of differing metastatic abilities. In both lung carcinoma cell types, cathepsin B enzyme activity was found in the lysosome, at the plasma membrane, as well as at the nuclear membrane and endoplasmic reticulum, but cathepsin B of the more metastatic cell line exhibited significantly different substrate cleavage rates and rates of inhibition by different inhibitors. In a related study by Krepela et al. (38), multiple forms of cathepsin B protein were identified by isoelectric focusing in human lung carcinomas that were not present in normal lung tissue, with these tumor-specific forms containing a more acidic pH than found in normal lung tissue. These acidic protein forms expressed in the lung carcinomas were more resistant to inactivation by the cysteine proteinase inhibitor E-64 than the cathepsin B forms from normal lung. The type of cathepsin B protein expressed in the lung carcinoma cells (glycosylated or nonglycosylated) was not investigated in either of these studies. In breast carcinomas, cathepsin D was also found to have a more acidic isoelectric point compared with normal breast tissue, due to modifications of the oligosaccharides expressed on the cathepsin D protein (45). Thus, our data may suggest a similar occurrence to that observed by others in that both nonglycosylated cathepsin B protein in adenomas and glycosylated cathepsin B protein in carcinomas have similar subcellular localizations, but in carcinomas the altered processing of cathepsin B protein, detected as increased amounts of glycosylation, may act to modify the endogenous rate of inhibition at a particular intracellular site or the ability to cleave subcellular or extracellular substrates.

Although we have observed no difference in the subcellular localization of cathepsin B between adenomas and carcinomas, we did observe the relocation of cathepsin B from a punctate distribution in normal mucosa to a more diffuse distribution in adenomas and carcinomas. Since this observation was not correlated with increased glycosylation of the cathepsin B enzyme, other factors that occur in both adenomas and carcinomas may be responsible for these trafficking alterations, such as those that are suggested to occur in the presence of ras mutations. Ras mutations have been shown to occur in up to 50% of carcinomas and in colorectal adenomas >1 cm in size (16, 46). Furthermore, Ras mutations have been shown to cross-talk with the Rac and Rho families of Ras-related proteins, which are involved in regulation of the actin cytoskeleton (47). In human breast epithelial cells, increased membrane association of cathepsin B has been shown to be induced by the c-Ha-ras mutant oncogene, although mRNA for cathepsin B was unchanged (48). Studies have also shown that the intracellular distribution of cathepsin B is dependent on an intact microtubular network and could be altered by an acidic pericellular pH (49). This shift in cathepsin B to the plasma membrane has been suggested to place the enzyme in the vicinity of the extracellular matrix, which can result in focal dissolution of extracellular matrix proteins and enable the tumor cell to invade and metastasize (9). However, we have observed cathepsin B located diffusely throughout the cytoplasm and along the basement membrane of adenomas, which are not invasive tumors, suggesting that shifting of cathepsin B to the plasma membrane alone is not sufficient to facilitate invasion. Qualitative changes in the cathepsin B protein expressed, including increased glycosylation of a complex type as we have shown here, may permit carcinoma cathepsin B to degrade the extracellular matrix at an increased rate compared with adenoma cathepsin B. Alternatively, an increase in glycosylated cathepsin B in cancers may alter the association of secreted cathepsin B with extracellular matrix components with subsequent, as yet unexplored, effects on cell motility.

In summary, our data provide strong evidence of a temporal sequence of events that occurs during the regulation of cathepsin B enzyme in colorectal tumor formation and progression. An early event in this sequence is the relocalization of cathepsin B enzyme at the subcellular level from a coarse, granular pattern seen in normal mucosa to the qualitatively fine and diffuse granular distribution throughout the cytoplasm seen in all adenomas and carcinomas irrespective of the intensity of cathepsin B staining. This subcellular relocalization may indicate changes in vesicle trafficking that have been shown to occur early in colorectal tumor progression (16, 46, 47).

A second early event appears to be a gradual up-regulation of cathepsin B protein and activity in adenomas that is related to adenoma size (approximately 1.3-fold in adenomas >1 cm) and dysplasia. At the transition to invasive cancer, however, a
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further increase in cathepsin B protein and activity levels occurs (approximately 2–3-fold over normal mucosa). At this point, there also occurs an increase in the amount of heavy chain cathepsin B protein glycosylated with complex type oligosaccharides, a posttranslational modification that may enhance the ability of cathepsin B to associate with and/or cleave extracellular matrix components and thus facilitate the invasion of colorectal tissues. This increase in glycosylation was detected in the majority of carcinomas at all stages, suggesting a selective pressure in malignancies to maintain the glycosylation of cathepsin B even as total protein and activity levels fall in late stage primary cancers. Thus, glycosylated cathepsin B may be particularly important in maintaining the malignant phenotype, whether invasive or metastatic. Further studies are warranted to elucidate the exact role(s) of glycosylated cathepsin B in malignant tumor progression.

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