Overexpression of *m*-Calpain in Human Colorectal Adenocarcinomas

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Abstract

Background: Calpains represent a well-conserved family of Ca^{2+}-dependent proteolytic enzymes. Recently, the importance of calpain in the metastatic process has received great attention. To investigate whether *m*-calpain contributes to the pathogenesis of colorectal cancer, we investigated the expression of *m*-calpain and its inhibitors, calpastatin and high-molecular-weight calmodulin-binding protein (HMWCaMBP), in human colorectal surgical specimens. Methods: Fifty cases of colon carcinoma were evaluated for this study. Of 50 cases evaluated, we presented in this report six cases for *m*-calpain, calpastatin and HMWCaMBP protein expression by Western blot analyses was done in both normal and invasive tumor components of human samples. In addition, immunohistochemistry analysis was also carried out in all patients. Results: The activity and protein expression of *m*-calpain was significantly higher in colorectal adenocarcinoma than in normal colonic mucosa. This finding was corroborated by immunohistochemical studies that showed strong cytoplasmic staining in the colon tumors with *m*-calpain antibody. The decreased expression of these calpain inhibitors (calpastatin and HMWCaMBP) paralleled increased activity and expression of calpain in colorectal adenocarcinoma and the well-documented involvement of this Ca^{2+}-dependent protease in colon tumor. Conclusion: Increased activity and moderate staining of *m*-calpain in polyps show the usage of this enzyme as a marker for the early detection of colorectal adenocarcinoma using immunologic approaches. These findings represent the first description of calpain overexpression in colorectal cancer. This has implications with regard to the design of chemotherapeutic drugs as well as in monitoring colorectal cancer in early stages of the metastatic process. (Cancer Epidemiol Biomarkers Prev 2004;13(10):1604–9)

Introduction

Calpains are Ca^{2+}-activated cysteine proteases, which are major mediators for Ca^{2+} signals in many biological systems (1, 2). There are two types of calpains, I or μ-calpain and II or *m*-calpain, which require micromolar and millimolar concentrations of Ca^{2+} for activation, respectively (1). Both calpains are heterodimers consisting of a common small subunit (28 kDa) with regulatory function and a distinct large catalytic subunit (80 kDa). Structural analysis has revealed that the large subunit of calpain is divided into four domains (I-IV). Domain II exhibits cysteine protease activity, domain III is involved in regulation of calpain activity by binding to phospholipids, and domain IV consists of a calmodulin-like structure containing four potential Ca^{2+} binding regions (1). Different mechanisms responsible for *m*-calpain regulation have been reported and an important role has been ascribed to the specific inhibitor calpastatin (3-5). In addition, a bovine cardiac high-molecular-weight calmodulin-binding protein (HMWCaMBP) was discovered in our laboratory (6). Based on sequence homology, amino acid analysis, antibody reactivity, and calpain inhibition, we showed that HMWCaMBP is homologous to calpastatin, an endogenous inhibitor of calpains (7).

Calpains result in the proteolysis of a broad spectrum of cellular proteins (2), including multiple signaling enzymes, protein kinase C, $pp60^{c\text{-}Src}$, and tyrosine phosphatase 1B (8-10). Most of the substrate proteins of calpains have been implicated in the pathogenesis of human tumors, suggesting an important regulatory role of calpains in malignant diseases. The role of calpains in carcinogenesis and tumor progression has yet to be explored. In human renal cell carcinomas, significantly higher levels of calpain I expression are found in tumors that have metastasized to peripheral lymph nodes compared with tumors that apparently had not metastasized (11). A recent report suggested that epigenetic activation of calpain II plays an important role in the invasion of human prostate cancer and can be targeted to reduce tumor progression (12). Gastric-specific calpain-9 is down-regulated in carcinomas from that tissue, although its relation to differentiation status or tumorigenesis is still unclear (13, 14). The activity and expression of μ-calpain was significantly higher in chronic lymphocytic leukemia cells than in nonmalignant cells, whereas the activity and expression of *m*-calpain and calpastatin are unchanged (15).

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In this study, we investigated the expression of *m*-calpain and its inhibitors, calpastatin and HMWCaMBP, in human colorectal cancer. Both Western blot and immunohistochemical analyses showed that increased expression of *m*-calpain in human colorectal adenocarcinoma paralleled decreased expression of calpain inhibitors, calpastatin and HMWCaMBP.

Materials and Methods

Materials. Nitrocellulose membranes were purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). Polyclonal antibody specific for *m*-calpain was obtained from Chemicon International, Inc. (Temecula, CA). Anti-calpastatin was purchased from Sigma-Canada (Oakville, Ontario, Canada). The production and purification of the polyclonal antibody of HMWCaMBP has been described previously (16). All other reagents were of analytic grade and were purchased from either BDH (Toronto, Ontario, Canada) or Sigma-Canada.

Human Colorectal Specimens. The human colorectal cancer tissues were collected from the Royal University Hospital, University of Saskatchewan (Saskatoon, Saskatchewan, Canada). Surgical pathology specimens of 50 colorectal cancer patients who had undergone resection for colorectal adenocarcinoma were collected directly from the surgical operating room in a fresh state (before being immersed in tissue fixative). Following gross inspection, samples of tumors were dissected and immediately frozen at −80°C. Normal-appearing colonic mucosa at least 5 cm away from the cancer was similarly dissected and frozen. Most of the remaining tissue was fixed in neutral-buffered formaldehyde and processed for histologic and immunohistochemical evaluation.

Preparation of Tissue. All procedures were carried out at 4°C, unless otherwise stated. Tissues were homogenized in 100 mmol/L Tris-HCl (pH 7.4) containing 1 mmol/L EGTA, 1 mmol/L EDTA, 10 mmol/L 2-mercaptoethanol, 1 mmol/L benzamidine, 0.2 mmol/L phenylmethylsulfonyl fluoride, and 0.5 mg/mL leupeptin. The crude homogenate was centrifuged for 30 minutes at 10,000 *g* and the supernatant was filtered through glass wool.

Calpain Assay. The calpain assay was done according to the method of Yoshimura et al. (17) using Hammerstein-grade casein as a substrate. The assay mixture contained 20 mmol/L imidazole HCl buffer (pH 7.5), 5 mg casein, and 5 mmol/L Ca^{2+} in a total volume of 1 mL. The reaction mixture was incubated at 30°C for 30 minutes and the reaction was stopped by the addition of 1 mL of 5% trichloroacetic acid. After 10 minutes on ice, the samples were centrifuged at 1,000 *g* for 10 minutes and the supernatant containing trichloroacetic acid–soluble products was used for measurement by the dye binding method of Bradford (18). One unit of calpain activity is defined as the amount of enzyme that results in an increase of 1 absorbance unit at 595 nm after 30 minutes of incubation at 30°C.

Immunohistochemical Method. The study material consisted of 50 cases of human colorectal adenocarcinoma specimens. Two tissues were selected from each case: one from the tumor and the other from normal-appearing mucosa far removed from the cancer. Sections (~5 mm thick) were cut and subjected to the avidin-biotin complex method as described previously (19). Anti-*m*-calpain, anti-calpastatin, and anti-HMWCaMBP were used as the primary antibody at a dilution of 1:100, 1:2,500, and 1:1,000, respectively. The extent of staining was graded on a 5-point scale from 0 to 4 as described previously (20) and the intensity of staining was noted as mild, moderate, or marked.

SDS-PAGE and Western Blot Analysis. Proteins isolated from normal colon and cancer tissues were separated on 12.5% SDS-PAGE according to the procedure described by Laemmli (21). The protein expression of *m*-calpain, calpastatin, and HMWCaMBP in colon normal and cancerous tissues was determined by the immunoblotting method of Towbin et al. (22). The blot was incubated first with the anti-*m*-calpain and anti-HMWCaMBP polyclonal antibodies and anti-calpastatin monoclonal antibody at a dilution of 1:1,000, washed, and probed with either anti-rabbit or anti-mouse IgG horseradish peroxidase conjugate (Bio-Rad Laboratories) diluted 1:2,000. Membranes were then incubated in chemiluminescence reagent (NEN Life Science Products, Boston, MA) and exposed to Kodak X-OMAT Blue XB-1 film (Eastman Kodak, Rochester, NY) for detection.

Other Methods. Protein concentration was measured by the method of Bradford (18) using bovine serum albumin as a standard.

Statistical Analysis. All the data are reported as means ± SEM. The differences between the mean values were tested for statistical significance by the two-tailed Student's *t* test.

Results

From the 50 patients analyzed, we present six cases in this report. To analyze the role of *m*-calpain in human

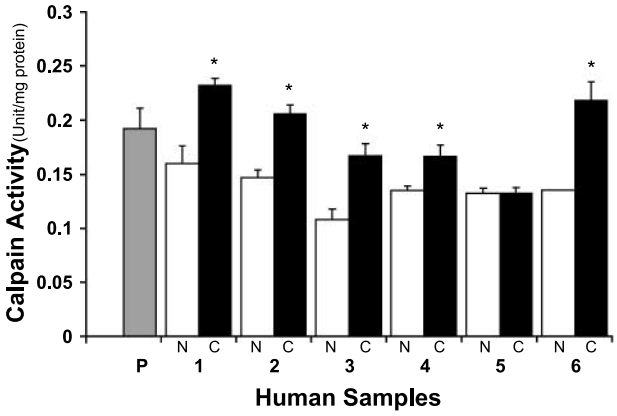

Figure 1. Calpain activity of human colorectal normal (□), polyps (□), and tumor (■) tissue. Human normal and colorectal tumor tissue extracts were analyzed for calpain activity as described under Materials and Methods. Data are representative of at least three separate experiments. *, $P < 0.05$, statistical significance determined using Student's *t* test.

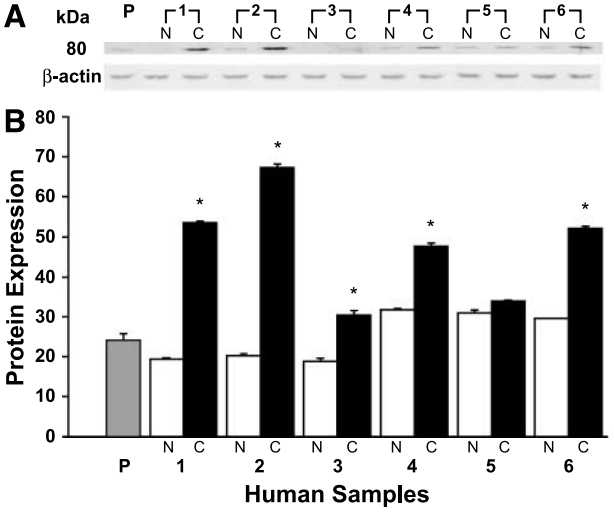

Figure 2. Expression of *m*-calpain in human colorectal adenocarcinoma. **A.** Western blot analysis of human colorectal normal (□), polyps (▫), and tumor (■) tissue extracts probed with anti-*m*-calpain. Equal amount (25 μg) of protein was loaded onto each lane of SDS-PAGE and immunoblotted with anti-*m*-calpain as described under Materials and Methods. **B.** Quantitation analysis of **A** was carried out using image software (NIH at http://rsb.info.nih.gov/nih-image/download.html). Data are representative of at least three separate experiments. *, $P < 0.05$, statistical significance determined using Student's *t* test.

colorectal adenocarcinoma, calpain activity and protein expression was carried out in tissue samples from human colorectal patients. Mucosa dissected far from cancerous tissue taken from respective patients was considered normal. In five of six patients, calpain activity was significantly higher in colorectal adenocarcinoma than in normal mucosa (83.3% of cases; $P < 0.05$; Fig. 1). Interestingly, *m*-calpain activity was higher in polyps than in normal tissues but less than in cancerous tissues (Fig. 1). Equal amounts of protein from human normal colorectal mucosal tissue and adenocarcinoma were subjected to immunoblotting and probed with polyclonal antibody to *m*-calpain. In colorectal tumors, this resulted in a prominent overexpressed immunoreactive band with an apparent molecular mass of 80 kDa (large subunit of calpain; Fig. 2A). However, the same 80 kDa was poorly expressed in samples from normal mucosa (Fig. 2A). Quantitative analysis of the 80-kDa band revealed 2- to 3-fold higher expression ($P < 0.05$) of *m*-calpain in colorectal tumors compared with the respective normal mucosa (Fig. 2B). However, no change in expression was observed for 28 kDa, small subunit of calpain (data not shown). In polyps, the expression of *m*-calpain was significantly higher than in normal tissues, whereas no significant change was observed in the remaining normal tissues (Fig. 2B). Furthermore, the immunohistochemical analysis showed strong staining for *m*-calpain in colorectal adenocarcinoma (Fig. 3C). In contrast, mild reactivity (<10% of protein expression) was observed in mucosal sections taken distant from the cancerous tissues (Fig. 3A). In polyps, a moderate staining was observed and the degree of immunoreactivity was less than in tumor tissue (Fig. 3B).

In addition, we monitored the expression of calpain inhibitor, calpastatin, to determine whether these endogenous inhibitors regulate the calpain activity in colorectal adenocarcinoma. Western blot analysis of equal amounts of protein with monoclonal antibody to calpastatin revealed strong expression of a immunoreactive band with an apparent molecular weight of 140 kDa in normal mucosa, whereas weak expression was observed in colorectal tumors (Fig. 4A). Quantitative analysis of calpastatin revealed a ~2-fold increased expression in normal tissues compared with cancerous tissues (Fig. 4B). The weak expression of calpastatin in colon tumors was further confirmed by immunohistochemical analysis (Fig. 5B). Moderate to strong staining was observed for normal mucosa, whereas a weak cytoplasmic positivity was observed for calpastatin in the invasive carcinoma with decreased intensity in the invasive component (Fig. 5A).

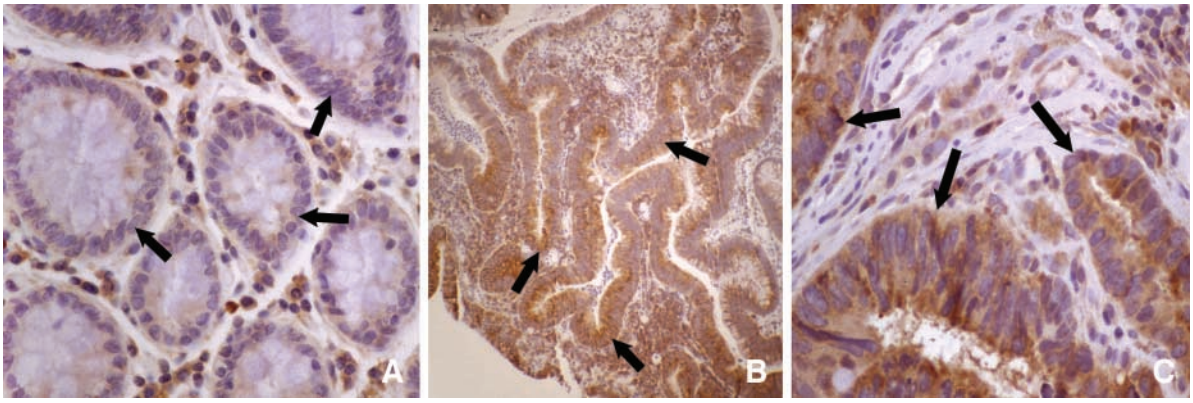

Figure 3. Immunohistochemical staining for *m*-calpain using anti-*m*-calpain. **A.** Section from normal mucosa far removed from tumor showing a mild degree of focal staining with anti-*m*-calpain (*arrows*; immunoperoxidase; original magnification, ×120). **B.** Section from polyps showing a moderate degree of anti-*m*-calpain reactivity (immunoperoxidase; original magnification, ×120). **C.** Section from colorectal adenocarcinoma showing a marked degree of anti-*m*-calpain reactivity in most of the tumor cells (immunoperoxidase; original magnification, ×120).

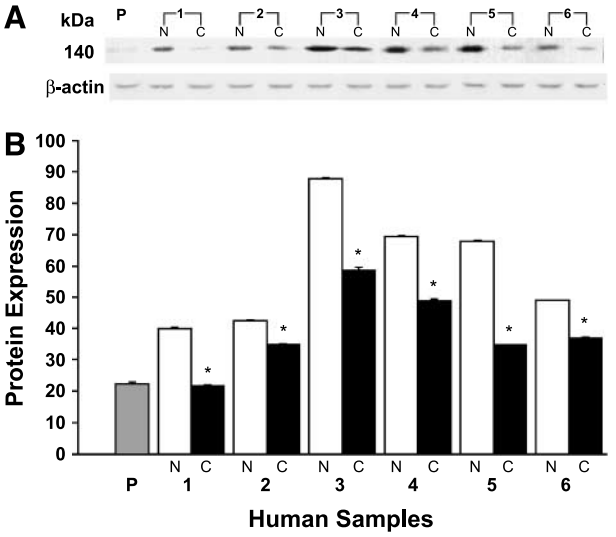

Figure 4. Expression of calpastatin in human colorectal adenocarcinoma. **A.** Western blot analysis of human colorectal normal (□), polyps (▣), and tumor (■) tissue extracts probed with anti-calpastatin. Equal amount (25 μg) of protein was loaded onto each lane of SDS-PAGE and immunoblotted with anti-calpastatin as described under Materials and Methods. **B.** Quantitation analysis of **A** was carried out using image software (NIH at http://rsb.info.nih.gov/nih-image/download.html). Data are representative of at least three separate experiments. *, $P < 0.05$, statistical significance determined using Student's t test.

To determine whether HMWCaMBP was involved in the colorectal adenocarcinomas, the expression of HMWCaMBP was analyzed in the present study by Western blot and immunohistochemical analyses. Similar to calpastatin, immunoblot analysis showed a weakly expressed immunoreactive band with an apparent molecular mass of 140 kDa in colon tumors. Significantly higher staining with anti-HMWCaMBP was observed in normal mucosa (data not shown). Furthermore, immunohistochemical studies revealed mild reactivity of HMWCaMBP in colorectal adenocarcinoma (Fig. 6B). However, the mucosal sections taken distant from the tumor showed strong staining (Fig. 6A).

Discussion

The major finding in this article is an increased expression of *m*-calpain in human colorectal adenocarcinomas. Calpain activity is tightly regulated by its ubiquitously expressed endogenous inhibitor calpastatin (3-5). The protein expression of *m*-calpain was 2- to 3-fold greater in colonic tumor. However, the enzyme activity of *m*-calpain increased only ~1.5-fold in colonic tumor. This slight difference in activity and protein expression may be due to the presence of endogenous inhibitors, calpastatin and HMWCaMBP, in the crude tissue extract. Marked increase in the staining of *m*-calpain in immunohistochemical studies indicated that elevated levels of calpain in colon cancer are due to increased production of the enzyme rather than an alteration in the conformation of the enzyme.

Calpain-mediated proteolysis represents a major pathway of post-translational modification that influences various aspects of cell physiology, including apoptosis, cell migration, and cell proliferation (1). Recently, the importance of calpain in the metastatic process has received great attention. Calpain may be involved in cell adhesion, spreading, migration, myoblast fusion, cell cycle control, and mitosis (23-25). Proto-oncogenes c-*fos* and c-*jun*, several cytoskeletal proteins, the tumor suppressor protein p53, and signaling molecules protein kinase C and focal adhesion kinase (a nonreceptor kinase) are substrates for calpain (26-28). Calpain-mediated cleavage of focal adhesion kinase and focal adhesion disassembly accompany v-Src-induced morphologic transformation (28). v-Src-induced oncogenic transformation is characterized by alterations in cell morphology, adhesion, motility, survival, and proliferation (29, 30). In response to v-Src activation, Carragher et al. (31) noted an increase in the total protein levels of calpain II and decreased levels of calpastatin in chicken

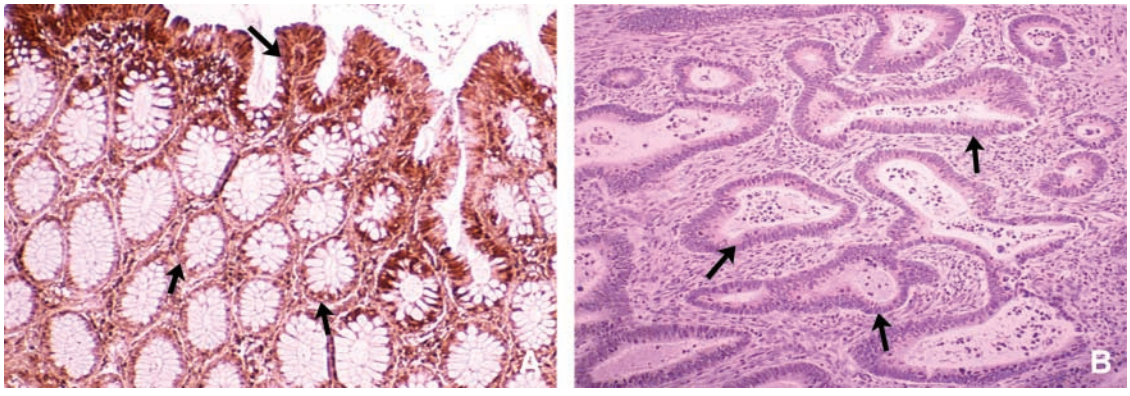

Figure 5. Immunohistochemical staining for calpastatin using anti-calpastatin. **A.** Section from normal mucosa far removed from tumor showing a marked degree of focal staining with anti-calpastatin (*arrows*; immunoperoxidase; original magnification, ×120). **B.** Section from colorectal adenocarcinoma showing a mild degree of anti-calpastatin reactivity in most of the tumor cells (immunoperoxidase; original magnification, ×120).

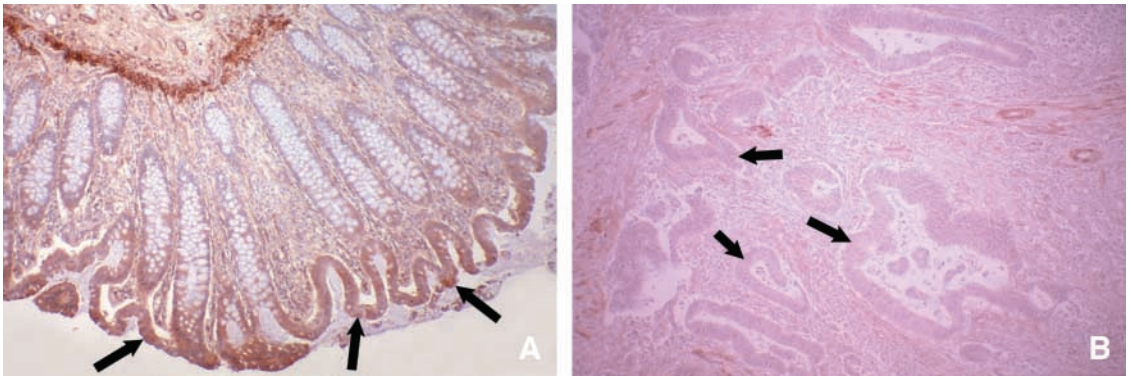

Figure 6. Immunohistochemical staining for HMWCaMBP using anti-HMWCaMBP. **A.** Section from normal mucosa far removed from tumor showing a marked degree of focal staining with anti-HMWCaMBP (*arrows*; immunoperoxidase; original magnification, ×120). **B.** Section from colorectal adenocarcinoma showing a mild degree of HMWCaMBP antibody reactivity in most of the tumor cells (immunoperoxidase; original magnification, ×120).

embryo fibroblasts. Furthermore, data suggested a feedback loop mechanism of calpain activation that is initiated in response to activation of oncogene v-Src (31). Activation of Src, which has an intrinsic tyrosine kinase activity, has been shown in human solid tumors such as colorectal and breast carcinomas (32, 33). In our present study, the increased activity and expression of *m*-calpain paralleled decreased expression of calpastatin and HMWCaMBP in adenocarcinoma.

Cell death by apoptosis is a fundamental process controlling normal development and homeostasis of multicellular organisms. Decreased apoptotic susceptibility contributes to the pathogenesis of several diseases including cancer. Calpains are involved in controlling the level and duration of transduction signals leading to either proliferation or apoptosis in multiple cell systems (1, 3). However, their role in the development and course of apoptosis is controversial. The role of calpains in the development of colon cancer has not been postulated until today. A central regulator of apoptotic susceptibility is the tumor suppressor protein p53, the level of which is regulated by several stress conditions, cell adhesion, and expression of several oncogenes (34, 35). It has also been reported that p53 is proteolytically cleaved *in vitro* by calpains (27, 36). The elevated expression of *m*-calpain in colorectal cancer may act on p53 and followed by a decrease in the event of apoptosis. Likewise, calpain-mediated cleavage of Bax promotes the proapoptotic effect of Bax (37) and calpain cleavage of pro-caspase-7 and pro-caspase-3 leads to activation of these proteases (38, 39). Chen et al. (40) reported a reduction in protein levels of caspase-3, caspase-7, and caspase-9 in human colon cancer specimens. The apoptosis-promoting caspase system is activated after calpain inhibition with calpain inhibitor II in neoplastic lymphoid cells (41). Cross-talk between calpain and caspases seems to be important for the regulation of apoptosis in colon tumors.

Our data, added to the known regulatory functions of calpains, suggest that increased *m*-calpain expression may directly contribute to the development of cell progression in colorectal adenocarcinoma. The determination of the mechanisms causing the increase in calpain expression and its action on cell signaling will yield data critical for addressing many unanswered questions about cell proliferation in colon tumors. Increased activity and moderate staining of *m*-calpain in polyps show the usage of this enzyme as a marker for the early detection of colorectal adenocarcinoma using immunologic approaches. The overexpression of calpastatin or HMWCaMBP, which are specific for inhibiting the calpains, may be used as a specific molecular target for the treatment of colon cancer.

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