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Involvement of calpain in colorectal adenocarcinomas (Review)

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Abstract. Calpains represent a well-conserved family of Ca^{2+}-dependent proteolytic enzymes. Recently, the importance of calpain in the metastatic process has received a great deal of attention. Various reports have suggested that *m*-calpain contributes to the pathogenesis of various cancers, including colorectal cancer. The activity and protein expression of *m*-calpain was significantly higher in colorectal adenocarcinoma than in normal colonic mucosa as revealed by Western blotting and immunohistochemical analysis. In addition, the decreased expression of calpain inhibitors (calpastatin and high molecular weight calmodulin-binding protein) was correlated with the increased activity and expression of calpain in colorectal adenocarcinoma. This has implications with regard to the design of chemotherapeutic drugs as well as for the monitoring of colorectal cancer in the early stages of the metastatic process. In this review, we summarize some of the recent findings from our laboratory regarding calpain and its inhibitors in human colon cancer.

Contents

1. Introduction

Calpain is an intracellular Ca^{2+}-dependent cysteine protease that is ubiquitously distributed throughout the body. Calpain plays a significant role in various cellular functions such as signal transductions and cell morphogenesis (1,2). Various reports have suggested that humans have 15 genes that encode a calpain-like protease domain. These generate diverse types of calpain homologues with combinations of several functional domains, such as Ca^{2+}-binding and the Zn-finger domains (1,2). The purpose of this review is to summarize the cross-talk between *m*-calpain and its inhibitors, calpastatin and high molecular weight calmodulin-binding protein (HMWCaMBP), in human colorectal cancer.

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2. The calpain family

Calpains are Ca^{2+}-activated cysteine proteases that act as major mediators for Ca^{2+} signals in many biological systems (1,2). There are two types of calpains, I or μ- and II or *m*-calpain, which require a micromolar and millimolar concentration of Ca^{2+} for activation, respectively (1,2). Both calpains are heterodimers consisting of a common small subunit (28 kDa) with a regulatory function and a distinct large catalytic subunit (80 kDa). 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, an endogenous calpain inhibitor, HMWCaMBP, was identified and characterized in our laboratory (6). Based on sequence homology, amino acid analysis, antibody reactivity and calpain inhibition, we demonstrated that HMWCaMBP is homologous to calpastatin, an endogenous inhibitor of calpains (7).

Calpain has catalytic and regulatory subunits that can be divided into 4 and 2 domains, respectively (1-5). The N-terminus of domain I of the large subunit is autolyzed upon activation by Ca^{2+} in order to have a lower Ca^{2+} requirement, and the autolysis results in the dissociation of the subunits. Therefore, autolysis is involved in the regulation of calpain activity and specificity (1). Three-dimensional structural studies revealed that the protease domain in the absence of Ca^{2+} is divided into two sub-domains, domains IIa and IIb, which are folded into one domain upon Ca^{2+} binding (1-4). This domain is most conserved among calpain family members, suggesting it has indispensable functions. The protease domain of μ- and *m*-calpains without other domains showed Ca^{2+}-dependent protease activity. This is supported by 3-D-structural studies of the protease domain in the presence of Ca^{2+}, which showed Ca^{2+} binding to domains IIa and IIb. Thus, the Ca^{2+}-dependency of calpains is controlled as a whole molecule, since all domains (IIa, IIb, III, IV and VI) bind at least one Ca^{2+} with different affinities (1-4).

3. Calpain in pathophysiology

Recently, the importance of calpain in the metastatic process has received a great deal of attention. Calpain may be involved in cell adhesion, spreading, migration, myoblast fusion, cell cycle control and mitosis (8-10). 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). Calpains cause limited proteolysis of substrates, resulting in the alteration of substrate activity. PEST sequences are believed to be the intramolecular signals for rapid proteolytic degradation by *m*-calpain. Lakshmikuttyamma *et al* (11,12) reported that the increase in calcineurin (CaN) activity and strong immunostaining in ischemic/perfused rat hearts may be due to the *m*-calpain-mediated proteolysis of CaN. In addition, the interaction of CaN with *m*- and μ-calpains was strong in epileptic chickens compared to normal birds (13,14). It has also been reported that N-myristoyltransferase (NMT) interacts with *m*-calpain in epileptic chickens (14,15). Among two forms of NMTs (NMT1 and NMT2), a higher interaction of *m*-calpain with NMT2 was observed (14,15).

4. Calpain in colon cancer

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 (16-18). 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 μ-calpain expression were found in tumors that had metastasized to peripheral lymph nodes compared to tumors that apparently had not metastasized (19). It has been reported that the epigenetic activation of calpain II plays an important role in the invasion of human prostate cancer and can be targeted to reduce tumor progression (20). Gastric-specific calpain-9 is down-regulated in carcinomas, and its relation to differentiation status or tumorigenesis remains unclear (21,22). The activity and expression of μ-calpain were significantly increased in chronic lymphocytic leukemia cells compared to non-malignant cells, whereas the activity and expression of *m*-calpain and calpastatin were unchanged (23).

The 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 (FAK; a non-receptor kinase) are substrates for calpain (24-26). Calpain-mediated cleavage of FAK and focal adhesion disassembly accompany v-Src-induced morphological transformation (26). v-Src-induced oncogenic transformation is characterized by alterations in cell morphology, adhesion, motility, survival and proliferation (27,28). In response to v-Src activation, Carragher *et al* (29) demonstrated an increase in the total protein levels of calpain II and decreased levels of calpastatin in chicken embryo fibroblasts. Furthermore, the data suggested a feed-back loop mechanism of calpain activation initiated in response to the activation of the v-Src oncogene (29). Activation of Src, which has intrinsic tyrosine kinase activity, has been demonstrated in human solid tumors such as colorectal and breast carcinomas (30,31). We observed that the increased activity and expression of *m*-calpain corresponded to a decrease in the expression of calpastatin and HMWCaMBP in adenocarcinoma (32). Selvakumar *et al* (33) reported that the protein-protein interaction of NMTs revealed that *m*-calpain interacts with NMT1, while caspase-3 interacts with NMT2 in human colorectal adenocarcinomas. Previously, our laboratory reported that *m*-calpain proteolyses NMT1 and abolishes the enzyme activity (34).

Cell death by apoptosis is a fundamental process controlling the normal development and homeostasis of multicellular organisms. Decreased apoptotic susceptibility contributes to the pathogenesis of several diseases including cancer (1-5). 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. A central regulator of apoptotic susceptibility is the tumor suppressor protein p53, whose level is regulated by several stress conditions, cell adhesion and the expression of several oncogenes (35,36). It has also been reported that p53 is proteolytically cleaved *in vitro* by calpains (25,37). The elevated expression of *m*-calpain in colorectal cancer may act on p53, and is followed by a decrease in the event of apoptosis. Likewise, the calpain-mediated cleavage of Bax promotes the pro-apoptotic effect of Bax (38), and the calpain cleavage of pro-caspase-7 and pro-caspase-3 leads to the activation of these proteases (39,40). Chen *et al* (41) reported a reduction in the protein levels of caspase-3, -7 and -9 in human colon cancer specimens. The apoptosis promoting caspase system is activated after calpain inhibition with calpain inhibitor II in neoplastic lymphoid cells (42). Cross-talk between calpain and caspases appears to be important for the regulation of apoptosis in colon tumors.

Various reports, including those from our laboratory, have suggested that *m*-calpain is involved in the progression of metastasis (26,32). In order to analyze the role of *m*-calpain in human colorectal adenocarcinoma, calpain activity and protein expression was examined in human tissue samples. In most of the cases, the calpain activity was significantly higher in human colorectal adenocarcinoma than in normal mucosa (83% of cases, $P<0.05$) (Fig. 1). Notably, *m*-calpain activity was higher in polyps than normal tissues, though not as high as in cancerous tissues (Fig. 1A). Furthermore, a higher expression of *m*-calpain was found in cancerous tissues, whereas it was poorly expressed in normal mucosa as determined by Western blot analysis (Fig. 1B, panel I). Quantitative analysis of the 80-kDa band revealed a 2- to 3-fold higher expression ($P<0.05$) of *m*-calpain in colorectal tumors compared to their respective normal mucosa (Fig. 1B, panel II). However, no change in expression was observed at the 28 kDa small subunit of *m*-calpain (unpublished data). In polyps, the expression of *m*-calpain was higher than in normal tissues, while no significant change was observed in the remaining normal tissues (Fig. 1B, panel II).

Immunohistochemical analysis showed strong staining for *m*-calpain in colorectal adenocarcinoma (Fig. 2A-c) by the avidin-biotin complex method graded as described previously (43,44). Mild reactivity (<10% of protein expression) was observed in mucosal sections taken distant from the cancerous

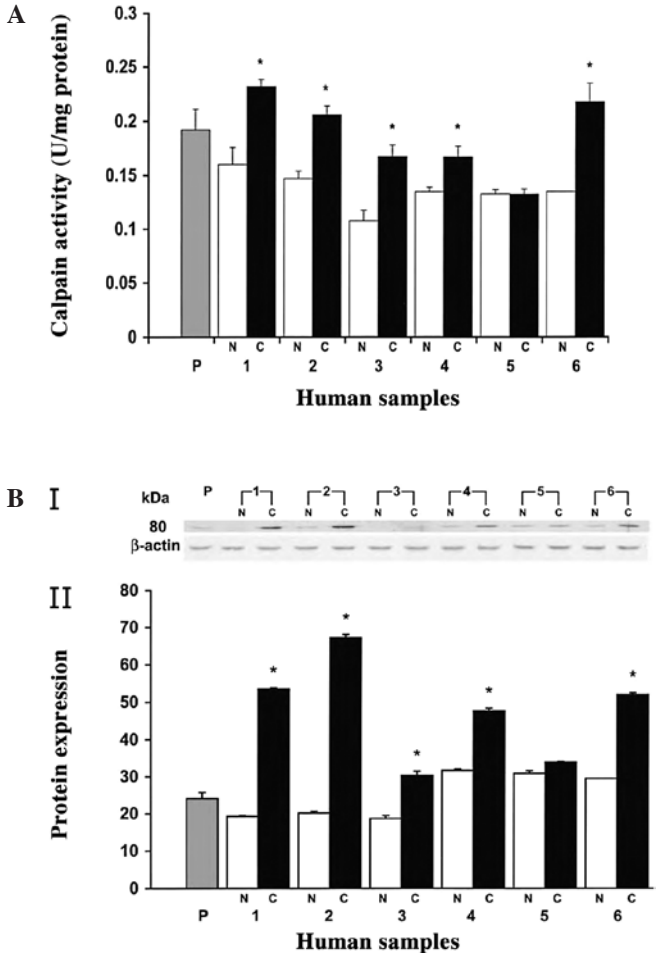

Figure 1. (A) Calpain activity of human colorectal normal (□), polyp (▨) and tumor (■) tissue. (B) Expression of *m*-calpain in human colorectal adenocarcinoma. Panel I, Western blot analysis of *m*-calpain in human colorectal normal (□), polyp (▨) and tumor (■) tissue extracts; panel II, quantitative analysis of Fig. 1B, panel I (32).

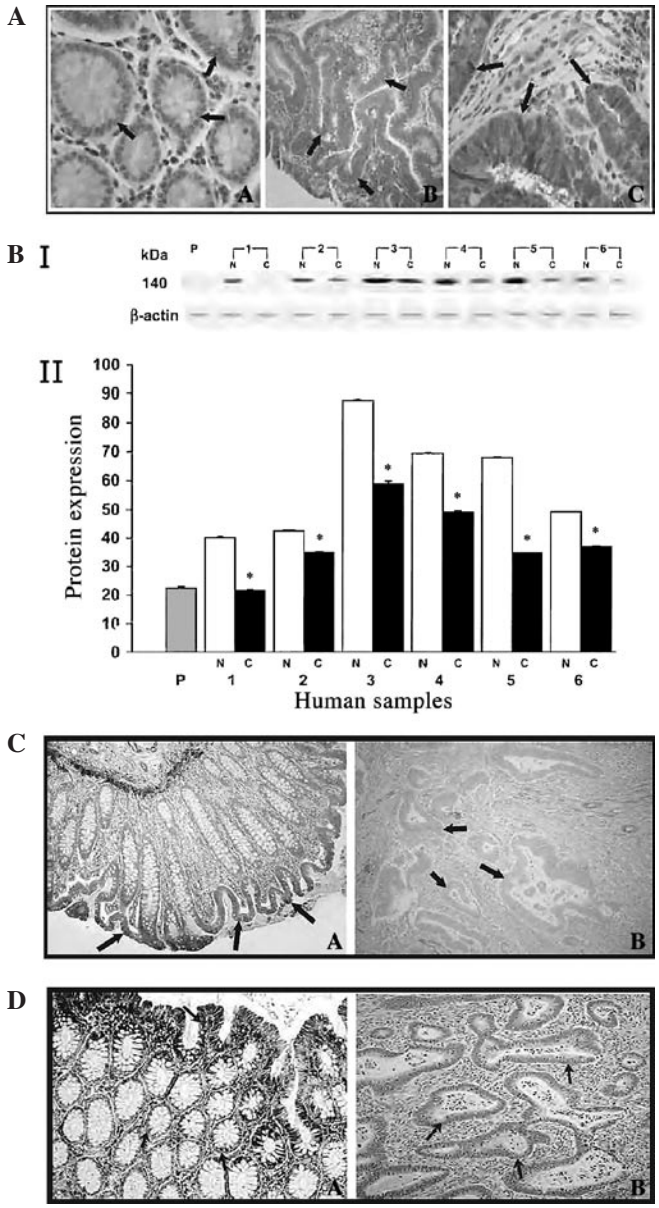

Figure 2. (A) Immunohistochemical analysis of *m*-calpain. Section from (a) normal mucosa, (b) polyps and (c) colorectal adenocarcinoma. (B) Expression of calpastatin in human colorectal adenocarcinoma. Panel I, Western blot analysis of calpastatin in human colorectal normal (□), polyp (▨) and tumor (■) tissue extracts; panel II, quantitative analysis of Fig. 2A(a). (C) Immunohistochemical staining for calpastatin using anti-calpastatin. Sections from (a) normal mucosa and (b) colorectal adenocarcinoma. (D) Immunohistochemical staining for HMWCaMBP using anti-HMWCaMBP. Sections from (a) normal mucosa and (b) colorectal adenocarcinoma (arrows, immunoperoxidase; original magnification, x120).

tissues (Fig. 2A-a). In polyps, moderate staining was observed, and the degree of immunoreactivity was less than in tumor tissue (Fig. 2A-b). From these studies and various reports, it is clear that *m*-calpain is a major player during the process of various diseases, including cancer, cardiovascular and neurological diseases.

5. Biological inhibitors of calpain

Calpain activity is tightly regulated by its ubiquitously expressed endogenous inhibitor, calpastatin (3-5). We also monitored the expression of the calpain inhibitor, calpastatin, to determine whether these endogenous inhibitors regulate calpain activity in colorectal adenocarcinoma (32). Western blot analysis of calpastatin revealed strong expression in normal mucosa, whereas weak expression was observed in colorectal tumors (Fig. 2B, panel I). Quantitative analysis of calpastatin revealed an approximately 2-fold increased expression in normal tissues compared to cancerous tissues (Fig. 2B, panel II). The weak expression of calpastatin in colon tumors was further confirmed by immunohistochemical analysis (Fig. 2C-b). Moderate-to-strong staining was observed for normal mucosa, while a weak cytoplasmic positivity was observed for calpastatin in invasive carcinoma, with decreased intensity in the invasive component (Fig. 2C-a).

We also investigated the role of another calpain endogenous inhibitor, HMWCaMBP, which was identified and characterized at our laboratory in human colorectal adenocarcinomas (32). Similar to calpastatin, Western blot analysis was carried out using anti-HMWCaMBP and 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 (unpublished data). Furthermore, immunohistochemical studies

revealed mild reactivity of HMWCaMBP in colorectal adenocarcinoma (Fig. 2D-b). However, the mucosal sections taken distant from the tumor showed strong staining (Fig. 2D-a). We observed that the increased activity and expression of *m*-calpain was correlated with the decreased expression of calpastatin and HMWCaMBP in human colorectal adenocarcinoma.

6. Conclusion

Apart from the known regulatory functions of calpains, various reports, including ours, 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 may yield data critical for addressing many unanswered questions about cell proliferation in colon tumors. Increased activity and moderate staining of *m*-calpain in polyps demonstrate the usage of this enzyme as a marker for the early detection of colorectal adenocarcinoma using immunological approaches. The overexpression of calpastatin or HMWCaMBP, which are specific for calpain inhibition, may be used as a specific molecular target for the treatment of colon cancer.

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