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Comparison of Cathepsin B Activity and the Ratio of Cathepsin B to Cysteine Peptidase Inhibitor in Human Colorectal Cancer Tissue

Porównanie aktywności katepsyny B i indeksu katepsyna B/inhibitory peptydaz cysteinowych w ludzkiej tkance nowotworów okrężniczo-odbytniczych

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Abstract

Background. Cathepsin B (CB), which is inhibited by cysteine peptidase inhibitors (CPIs), plays an important role in the process of cancer invasion and metastasis.
Objectives. The aim of this study was to compare CB and low-molecular-weight CPI activities in the extracts of human colorectal carcinoma tissues from the center of the tumor and the tumor-free area 2 cm and 5 cm from the tumor border in order to examine their significance in this type of cancer.
Material and Methods. CB activity was measured using a flurogenic substrate and CPI using a colorimetric substrate as anipapain activity. The CB:CPI ratio was also calculated.
Results. The activities were significantly higher in the tumor tissue extracts than in the controls ($p \leq 0.0001$). CB was elevated 26-fold and CPI 2-fold. Only cathepsin B activity decreased significantly with distance from the tumor border ($p \leq 0.0001$). CB and CPI activities as well as the CB:CPI ratio increased with the stage of tumor tissue differentiation grade.
Conclusion. The results provided convincing evidence that the distribution of CB and CPI may be important for colorectal cancer invasion. CB activity of cancerous tissue extracts can be an additional marker of tissue differentiation grade. For patients with marked elevation of CB activity in the cancer tissue, relatively extensive resection may be necessary (**Adv Clin Exp Med 2009, 18, 1, 41–45**).

Key words: colorectal cancer, cathepsin B activity, cysteine peptidase inhibitors, cystatins.

Streszczenie

Wprowadzenie. Katepsyna B (CB), hamowana przez inhibitory peptydaz cysteinowych (CPI), odgrywa istotną rolę zarówno w powstawaniu, jak i inwazji procesu nowotwowego.
Cel pracy. Porównanie i poznanie istotności ekspresji CB oraz CPI w ekstraktach ludzkich nowotworów okrężniczo-odbytniczych z tkanek pobranych ze środka guza (0) oraz w odległości 2 i 5 cm.
Materiał i metody. Aktywność CB oznaczano metodą fluorometryczną, a aktywność CPI metodą kolorymetryczną jako aktywność antypapainową. Obliczano również indeks CB/CPI.
Wyniki. Oznaczane aktywności były istotnie wyższe w ekstraktach tkanek nowotworowych w stosunku do grupy kontrolnej ($p < 0{,}001$). CB rosła 26-krotnie, a CPI 2-krotnie. Jedynie aktywność CB malała istotnie statystycznie ($p < 0.001$) wraz z odległością od środka guza. Wszystkie oznaczane wskaźniki rosły wraz ze stopniem zróżnicowania guza, ale różnice nie miały charakteru istotnie statystycznego.
Wnioski. Rezultaty pracy wskazują, że dystrybucja ekspresji CB i CPI jest istotna dla inwazji nowotworów okrężniczo-odbytniczych. Oznaczanie aktywności CB może być pomocne w określeniu stopnia zróżnicowania tkanek guza. U chorych ze znacznym wzrostem aktywności CB w ekstraktach tkankowych byłaby wskazana jak największa resekcja guza (**Adv Clin Exp Med 2009, 18, 1, 41–45**).

Słowa kluczowe: rak okrężniczo-odbytniczy, aktywność katepsyny B, inhibitory peptydaz cysteinowych, cystatyny.

Colorectal cancers (CRC) are a significant cause of morbidity and mortality [1]. Numerous investigations have shown that proteolytic enzymes, such as lysosomal cysteine peptidases (e.g. cathepsins B and L), are involved in the growth, invasion, metastasis, and angiogenesis of cancer [2–4]. Of the cysteine cathepsins, the most investigated is cathepsin B (CB). Increased CB expression, measured as enzyme activity or antigen concentration (ELISA) as well as by proteomic screens, has been observed in colorectal neoplastic tissues compared with normal mucosa [4–6]. The expression and activity of CB and cathepsin L (CL) antigen is associated with early CRC progression. Survival of patients with potentially curable disease was inversely related to both CB and CL activities in tumor tissue [7]. High activities of these enzymes were found particularly in cells at the invasion front, namely in the "tumor budding" region, which is generally characterized by a high expression of proteases [8], which suggested a more aggressive tumor phenotype [9]. It has been also shown that overexpression of CB in cancer cells of colon adenocarcinoma correlates with the intensity of angiogenesis [10].

The activities of cysteine peptidases are controlled within cells by the cytosolic inhibitors stefin A and B [11]. In colon cancers expressing CB, the surrounding tissue also demonstrated increased amounts of cystatin C, a secreted inhibitor [12]. Alteration of the balance between cysteine peptidases and their endogenous inhibitors has been postulated to contribute to malignant progression and may participate in the modulation of the invasive phenotype of tumors [4, 11, 13]. Recombinant cystatin C, characterized by high inhibitory activity against CB, effectively and dose-dependently retards the growth and invasiveness of human colon carcinoma [14].

Based on the hypothesis that an imbalance between cysteine peptidases and their inhibitors leads to cancer invasion, the current study investigated the activity of CB and the antipapain activity of cysteine peptidase inhibitors (CPIs) and calculated CB:CPI ratio in extracts of human colorectal carcinoma tissues obtained at different distances from the tumor border. Antipapain activity reflects the expression of all low-molecular-weight inhibitors (cystatin C and stefins A and B).

Material and Methods

Thirty-eight tissue samples of colorectal carcinomas and of 30 normal mucosa were obtained from patients undergoing surgery at the Silesian Center of Oncology, Wrocław, Poland. The patients were informed of the aim of this investigation and gave their permission to participate in the study. The ages of the patients ranged from 38 to 68 years. These tissue samples were classified according to the routine histological hematoxylin and eosin (H&E) staining as colorectal adenocarcinoma and carcinoma (Table 1). Tumor-node-metastasis (TNM) staging was done in accordance with the American Joint Committee on Cancer

Table 1. Cathepsin B activity and the CB:CPI ratio in normal and colorectal tissue extracts with cancer of different histology type and cell differentiation stage shown as mean ± *SD*

Tabela 1. Aktywność katepsyny B i indeks CB:CPI w tkance prawidłowej i w ekstraktach nowotworów okrężniczo-odbytniczych różnego typu histologicznego i stopnia różnicowania komórek – średnia ± *SD*

Histology type Tissue differentiation grade (No of patients) (Typ histologiczny (Stopień zróżnicowania tkanki) (Liczba pacjentów)	Cathepsin B – U/mg of protein (Katepsyna B – U/mg białka)	CPI – U/mg of protein (CPI – U/mg białka)	CB:CPI Ratio (Wskaźnik CB:CPI)
Normal tissue (30)	6.6 ± 5.8	0.2 ± 0.1	33
Carcinomas G_1 (5) G_2 (10) G_3 (3)	 22.8 ± 8.7 103.2 ± 95.6 286.9 ± 158.4	 0.3 ± 0.1 0.6 ± 0.3 0.9 ± 0.4	 76 172 319
Adenocarcinoma G_2 (7)	457.5 ± 275.2	0.5 ± 0.2	915
Invasive adenocarcinoma G_3 (13)	621.4 ± 345.7	0.8 ± 0.3	777

All values for the cancer tissues were significantly higher ($p < 0.001$) than for the normal tissue. In both histopathological types the differences in CB and CPI between subgroups were not statistically significant.

Wszystkie wartości dla tkanki raka były istotne statystycznie ($p < 0{,}001$) większe niż dla tkanki prawidłowej. W obu typach histopatologicznych różnice między CB i CPI nie były istotne statystycznie.

(AJCC). Five carcinoma tissues (13%) were well-differentiated (grade G1), ten (26%) moderately differentiated (grade G2), and three (8%) poorly differentiated (grade G3). Seven tubular adenomas with high-grade dysplasia/carcinoma in situ (18%) of grade G2 and thirteen invasive adenocarcinomas (34%) of grade G3 were also collected. Moreover control mucosa samples taken from normal tissue located at least 10 cm from the tumor site were analyzed.

Immediately after surgery a pathologist selected the tissues and washed them of blood with 0.9% NaCl. For CB determinations the frozen tissues (0.5 g) were homogenized with 2.5 ml of buffer (20 mM Tris-3 mM EDTA-1 mM dithiothreitol, pH 7.6) at 4°C. The homogenates were centrifuged for 6 min at 13,000 × *g* at 4°C. To determine the antipapain activity of the low-molecular-weight cysteine proteinase inhibitors (CPIs), tissue homogenates were prepared according to Lenney et al. [15]. Dissociation of the cysteine peptidase inhibitor complexes was done by heating the homogenates at 100°C for 5 min and subsequent centrifugation. Appropriate extracts (supernatants) were used for determining CB and antipapain activity.

CB activity was measured according to Barrett et al. [16] using the fluorogenic synthetic substrate benzyloxycarbonyl-arginyl-arginyl-AMC (Z-Arg-Arg-AMC). Fluorescence of the released 7-amino-methylcoumarin (7-AMC) was measured by a spectrofluorimeter (Perkin-Elmer LS-3B) at 370 nm excitation and 440 nm emission wavelengths. One unit of enzyme activity was defined as 1 nM of 7-AMC released per minute. Antipapain activity of heat-stable low-molecular-weight inhibitors was assayed using N-α-benzylarginine-β-naphthylamide as the substrate according to Barrett [17]. One unit of CPI activity was defined as the amount required to inhibit 1 unit of papain activity, which was previously determined by active site titration with E-64 [18]. The protein concentration was determined by the Bradford method using bovine serum albumin as the standard [19].

The biochemical analysis results are presented as the mean values ± *SD*. Analyses of the differences between the results obtained in tumor and control tissue homogenates were performed with the Wilcoxon signed rank test; a level of probability lower than 0.05 was taken as significant.

Results

Figure 1 presents the median values of CB and CPI activity and the CB:CPI ratios in the extracts of malignant and nonmalignant colorectal tissues. All the parameters were significantly higher in the

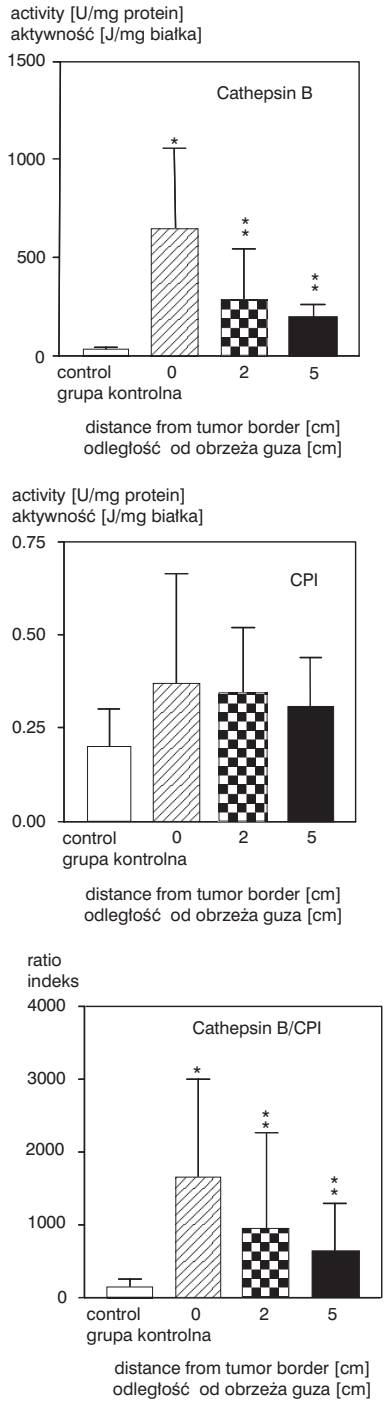

Fig. 1. Cathepsin B and antipapain activity of CPI and the CB:CPI activity ratio in colorectal cancer tissue extracts. The significance of the differences (*p*) in median values of tumor and control tissue were calculated by Wilcoxon matched pair signed-rank test.
* $p < 0.001$ compared with control tissue
** $p < 0.001$ compared with 2 or 5 cm from the tumor border

Ryc. 1. Aktywność katepsyny B i antypapainowej CPI i wskaźnik CB:CPI w ekstraktach nowotworów okrężniczo-odbytniczych. Istotność różnic (p) mediany wartości dotyczących guza i grupy kontrolnej obliczono za pomocą testu Wilcoxona.
* $p < 0{,}001$ w porównaniu z grupą kontrolną
** $p < 0{,}001$ w porównaniu z tkanką pobraną w odległości 2 lub 5 cm od obrzeża guza

tumor tissues than in the control tissues ($p \leq 0.0001$). CB was elevated 26-fold, CPI 2-fold, and CB:CPI 10-fold. Both CB activity and the CB:CPI ratio were significantly lower at distances of 2 and 5 cm from the tumor border ($p \leq 0.0001$). The differences in antipapain activity were not statistically significant.

The analysis of the results with respect to histology and stage of cell differentiation are shown in Table 1. It is evident that CB and CPI activity increase with the stage of disease. In carcinomas, CB activity rose from 22.8 ± 8.7 in G1 to 103.2 ± 95.6 in G2 to 286.9 ± 158.4 U/mg of protein in G3. In adenocarcinomas, CB activity was even greater than in carcinomas, with 457.5 ± 275 in G2 and 621.4 ± 345.7 U/mg of protein in G3. The antipapain activity of the cystatins in carcinomas was 0.3 ± 0.1 in G1, 0.6 ± 0.3 in G2, and 0.9 ± 0.4 U/mg of protein in G3. The differences between the groups were not statistically significant. The differences between the inhibitory activity obtained for G2 adnocarcinomas and invasive G3 adenocarcinoma were not statistically significant, in contrast to the CB:CPI ratio ($p \leq 0.0001$).

Discussion

Cysteine peptidases (e.g. CB) of various tumor cells, including colorectal carcinoma, were shown to be involved in cancer progression and metastasis [2–5]. It is now evident that, depending on their location, they can be involved in two apparently opposing mechanisms: tumor invasion and apoptotic regression. Enzymes that are secreted or associated with the plasma membrane participate in tumor invasion through proteolytic cascade activation, ECM degradation, and inactivation of cell-adhesion proteins. In addition, intracellular proteolysis contributes to progression by degradation of endocytosed collagen in lysosomes. On the other hand, cytosol-translocated cysteine cathepsins trigger apoptosis via BH3-interacting death domain cleavage followed by cytochrom c release from mitochondria. This cascade activated downstream executioner caspases, resulting in tumor cell death [20].

In the present study, CB was elevated (26-fold) in colorectal carcinoma tissue extracts compared with normal mucosa. CB activity increased with the tissue differentiation grade, from G_1 to G_3, and was higher in adenomas than in carcinomas. Such results are in accordance with other observations [2, 5, 21]. The present study also searched for CB activity in extracts of tissue taken 2 and 5 cm from the tumor center and observed decreased activity with distance, but the values were still significantly higher than in the control tissues ($p \leq 0.0001$). Such results might provide some suggestion in designating the extent of a CRC operation.

In the present study, CPI antipapain activity was also found to be increased compared with the control tissue extracts ($p \leq 0.0005$), but the differences in the distance from the tumor border and between histology or tissue differentiation grade were not statistically significant. The observed changes indicate a shift in the CB-CPT balance, which may enhance carcinogenesis. Others found altered mRNA expression of cystatin C in 55% of examined colon cancers [22]. Interestingly, they also established cystatin C as a novel TGF-β receptor antagonist.

The present authors' previous study demonstrated immunochistochemically, using anti-chicken cystatin antibodies, higher expression of cystatin C in CRC tissue than in controls. The inhibitor was found in the cytoplasm and on the cell surface in CBC tissue [23]. Altered extracellular levels of stefin A and B and cystatin C correlated significantly with a high risk of adverse outcome in cancer patients [24]. Determination of cystatin C-cathepsin B complex concentrations in serum has been suggested as a prognostic indicator for cancers, for example CRC [25].

The present results provide convincing evidence that an imbalance of CB and its tissue inhibitors may contribute to CRC invasion. Determining CB activity in tissue extracts seems to be more suitable than CPI antipapain activity in the monitoring of CRC progression and could be an additional marker of tissue differentiation grade in this cancer.

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