Activity of Cathepsin B and D in Colorectal Cancer: Relationships with Tumour Budding

KATARZYNA GUZIŃSKA-USTYMOWICZ1, BOGDAN ZALEWSKI2, IRENA KASACKA3, ZDZISŁAW PIOTROWSKI2 and ELŻBIETA SKRZYDŁEWSKA4

Departments of 1General Pathomorphology, 2General Surgery, 3Histology and Embryology and 4Inorganic and Analytical Chemistry, Medical University of Białystok, Poland

Abstract. It has been reported that poorly-differentiated clusters of cancer cells at the invasive front, namely “tumour budding”, may reflect malignancy of colorectal cancer. The aim of the present study was to evaluate the activity of cathepsin D and B in tumours and in normal mucosa from pT3 and G2 colorectal cancers, and to analyse their association with tumour budding at the invasion front of colorectal cancer. Patients and Methods: A total of 40 patients classified as pT3, G2 underwent curative resection of colon cancer between 1997 and 2001. The fragments of tumours and normal colorectal tissue were obtained for biochemical examinations. We also categorized tumour budding (TB) at the front of invasion. Two groups were used for classification of the TB phenomenon: the first where no bud was observed- TB(-), and the second where at least one bud was found -TB(+) at the front of invasion in the examined slice. Results: The activity of cathepsins D and B was found to be statistically significantly higher both in the neoplastic tissue cytosol and homogenate, compared to the cytosol and homogenate of adjacent healthy tissue (p<0.05). There was, however, no significant difference between tumour budding and the activity of cathepsin D in tumour tissue, but we found a statistically significant difference between the activity of cathepsin B in the homogenate and cytosol of tumour tissue and budding-positive tumours (p=0.027, p=0.004, respectively). Conclusion: These results suggest that the activity of cathepsin D is not involved in tumour budding. In our opinion, much more attention should be paid to cathepsin B, as a potentially responsible factor in tumour progression, since it strongly increased with the presence of tumour budding.

Cancer cells are characterized by overexpression and secrete a large proportion of lysosomal proteases – cathepsins especially cathepsin B and D (1-3). Cathepsin D, as an endopeptidase, degrades many intracellular and endocytosed proteins as well as ECM (extracellular matrix) proteins and proteins of the basal epithelium. Cathepsin D can participate in limited proteolysis and it activates cysteine procathepsins B and L and also degrades and makes inactive their active forms (4). It has been shown that human cathepsin D stimulates tumour growth by acting – directly or indirectly – as a mitogenic factor on cancer cells independently of its catalytic activity (5). Cysteine cathepsins, mainly cathepsin B, may cooperate with cathepsin D in the proteolysis process. This cathepsin has a promotion role in carcinogenesis, which is connected with its proteolytic effects on basement membrane and interstitial stroma (6). In normal cells, these cathepsins are regulated at every level of their biosynthesis, including transcription, post-transcriptional processing, translation, post-translational processing and trafficking, thus maintaining their normal function in cell metabolism. In tumour cells, misregulation of this cathepsin at one or more of these levels results in increased mRNA and protein expression, increased activity and altered intracellular distribution (6). Increased expression, together with enhanced secretion and cell surface association of cathepsin B, was found in different types of tumour cells, especially in their more malignant variants (2,3,7). Recent studies have revealed that enhanced production and release of this cathepsin in tumour cells led to tumour cell growth, invasion and metastasis (8,9).

However, increased expression of cathepsin B in a premalignant lesion suggests that this enzyme may play a role in the transformation of the premalignant lesion to malignant tumours (10). Moreover, it has been found that cathepsin B expression is often increased specifically at the invasive edge of tumour cells (11), because in normal tissues granules containing cathepsin B are localized perinuclearly while during tumour progression they move to the inner basal surface of the plasma membrane (12). The redistribution of
cathepsin B to the basal membrane in cancer cells occurs coincidently with degradation of the underlying basement membrane. It is very important for degradation of the surrounding extracellular matrix in tumour progression, because tumour cell invasion involves local proteolysis.

It was proved that colorectal cancer development, independent of changes in cathepsins distribution, is characterized by changes in histological features. At the invasive front of colorectal cancer, changes termed tumour budding were described. Morodomi et al. (13) defined tumour budding as bundles of five or more cancer cells occurring in a well-differentiated region (mainly the actively invasive area), which showed a tubular structure, and were classified as microtubular cancer nest. However, isolated cancer cells without a distinct structure were classified as undifferentiated cells. Since both of these appeared as if budding out from a large cancer gland, they called them budding. The buds appear to "drip" down from the main mass of more differentiated tumour (14). Some authors reported that the presence of tumour budding at the invasive front of the lesion is one of the most useful factors for identifying groups at high risk of haematogeneous or regional metastasis among patients with advanced colorectal cancer (15-21). Therefore, the aim of this paper was to check if the tumour budding is associated with the activity of the lysosomal proteases, cathepsin B and D.

**Patients and Methods**

The material for analysis consisted of 40 colorectal tumours obtained from patients surgically treated for primary colorectal cancer. The group of patients included 18 men and 22 women, aged 34-86.

Each neoplastic tumour was cut parallel to the longest axis to obtain at least one complete cross section 2 to 3 mm thick. It was then divided into small blocks, 1-1.5 cm in diameter, to obtain from each tumour 4-8 segments comprising the tumour and the adjacent macroscopically unchanged colon tissues.

The specimens were fixed in 10% buffered formaldehyde for up to 24 hours, and embedded in paraffin at 56°C. Sections, 5-μm thick, were cut and stained with hematoxylin-eosin. The microscopic picture was used to evaluate the histological type and differentiation, stage of clinical advancement (TNM classification) and presence of tumour budding.

**Method of determining budding (microtubular cancer clusters and undifferentiated cancer cells).** Budding was evaluated according to the criteria of Morodomi et al. (13). Two groups were used for classification of TB phenomenon. The first where no bud was observed – TB(−), and the second where at least one bud was found – TB(+), at the front of invasion in the examined slice. The front of invasion determined as a budding field was measured in a visual 500 μm × 2500 μm square at four locations in each slide. Figure 1 shows a representative photograph of tumour budding at the invasive margin (small nests of cancer cells with poorly-differentiated histology at the invasive margin of the tumour). All cases were at the same tumour stage pT3 and of histological grade G2.

---

**Table I. Activity of cathepsins D and B in normal colorectal mucosa and colorectal tumours.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal colorectal mucosa</th>
<th>Tumour tissue</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin D activity homogenate (nmol Tyr/ml)</td>
<td>160 ± 91.7</td>
<td>203.7 ± 104.3</td>
<td>0.0001*</td>
</tr>
<tr>
<td>Cathepsin D activity cytosol (nmol Tyr/ml)</td>
<td>129 ± 64.2</td>
<td>172.9 ± 96.7</td>
<td>0.0002*</td>
</tr>
<tr>
<td>Cathepsin B activity homogenate (nmol Tyr/ml)</td>
<td>69 ± 41.7</td>
<td>118 ± 58.9</td>
<td>0.0001*</td>
</tr>
<tr>
<td>Cathepsin B activity cytosol (nmol Tyr/ml)</td>
<td>63.1 ± 34</td>
<td>108.9 ± 42.1</td>
<td>0.0001*</td>
</tr>
</tbody>
</table>

*P<0.05
Table II. Activity of cathepsins D and B in budding-positive and budding-negative colorectal tumours.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tumour budding</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Budding-positive</td>
<td>Budding-negative</td>
</tr>
<tr>
<td>Cathepsin D activity (nmolTyr/ml)</td>
<td>204.1±110.4</td>
<td>201.7±66.8</td>
</tr>
<tr>
<td>Homogenate</td>
<td>201.7±66.8</td>
<td>204.1±110.4</td>
</tr>
<tr>
<td>Cytosol</td>
<td>172.6±102.9</td>
<td>174.9±55.7</td>
</tr>
<tr>
<td>Cathepsin B activity (nmolTyr/mL)</td>
<td>167.1±69.3</td>
<td>110±53.6</td>
</tr>
<tr>
<td>Homogenate</td>
<td>152.7±35.7</td>
<td>101.1±38.6</td>
</tr>
<tr>
<td>Cytosol</td>
<td>172.6±102.9</td>
<td>174.9±55.7</td>
</tr>
</tbody>
</table>

*p<0.05

Biochemical procedure. The fragments of tumours and normal colorectal tissue, obtained for biochemical examinations, were perfused with 0.15 mol/L NaCl and homogenized (1:9 w/v) in a glass homogenizer in 0.25 mol/L sucrose with and without 0.2% Triton X-100. The homogenates were centrifuged at 18,000 x g for 20 min to settle the lysosomes and membranes. The supernatant received from homogenates prepared in sucrose was named cytosol, while the supernatant received from homogenates prepared in sucrose with Triton X-100 was named full homogenate. The activity of cathepsin D was measured with Bz-DL-arginine-pNA as a substrate at pH 6.0 (22), while the cathepsin D activity was determined with urea-denatured hemoglobin (pH 4.0) (23). The protein concentration was determined by the Lowry et al. (24) method.

Statistical analysis. The data obtained in this study are expressed as mean±SD. The data were analysed by use of standard statistical analyses, one way ANOVA with Scheffe’s F-test for multiple comparisons to determine significance between different groups. The values for p<0.05 were considered significant.

Results

Activity of cathepsins D and B in normal colorectal mucosa and colorectal tumours (Table I). We revealed a significantly higher activity of cathepsins D and B both in cytosol and in homogenate in tumour tissue, compared to unchanged colorectal mucosa (p<0.05).

Activity of cathepsins D and B in budding-positive and budding-negative colorectal tumours (Table II). The budding-positive tumours were observed in 34/40 (85%) cases, whereas budding-negative tumours were found in 6/40 (15%) cases. We found a statistically significant difference between the budding-positive tumours and the activity of cathepsin B in cytosol (167.1±69.3) and homogenate (152.7±35.7) in tumour tissue (p=0.027, p=0.004, respectively). However, the activities of cathepsin D in homogenate in budding-positive tumours (204.1±110.4) and in budding-negative tumours (201.7±66.8) were not statistically significant. Also, the activities of cathepsin D in cytosol associated in budding-positive tumours (172.6±102.9) and in budding-negative tumours (174.9±55.7) were similar and no statistically significant difference was observed.

Discussion

Studies have revealed that enhanced production and release of cathepsin B in tumour cells led to tumour cell growth, invasion and metastasis (8,9,25-29). Results obtained in this paper confirmed the above data by showing the significant increase in cathepsins D and B activities in the cytosol, as well as in full homogenate of colorectal carcinoma cells. However, the most important is finding the connection of the above increase in activity of cathepsin with the probability of metastases formation. The correlation between the increase in the activity of cathepsin B and the specific histological feature – budding in tumour cells – may link cathepsin activity with metastases. The presence of isolated single cells or small cell clusters (up to five), that are scattered in the stroma at the invasive margin of the tumour, referred to as tumour budding, appears to have a prognostic value in colorectal cancer (16). In an earlier study, it was demonstrated that tumour budding was strongly correlated with lymph node metastases (30). Hase et al. (16) examined 663 patients with colorectal cancer for tumour budding. They suggested that tumour budding is an important predictor in patients with colorectal cancer. Morodomi et al. (13) reported that tumour budding represents the neoplastic cells which are directly involved in host tissue invasion. According to these authors, if the differentiation degree of colorectal adenocarcinoma is moderate (G2), lymph node involvement is highly probable. However, even in these histologically moderately-differentiated tumours, with undetected poorly-differentiated clusters of cancer cells, lymphatic involvement is probable but relatively less likely. In well-differentiated adenocarcinomas (G1), tumour budding and lymphatic invasion are usually not observed. If tumour budding occurs in such cases, then lymph node metastases are very likely. Gabbert (31), in his in vivo observations in the rat with 1,2-dimethylhydrazine-induced colonic cancer, noted that the first and essential step in tumour invasion is tumour dedifferentiation and dissociation at the invasive front. In his opinion, this phenomenon in experimental colon cancer corresponds well to the budding of human colorectal cancer. Ueno et al. (17) suggests that tumour budding would be a good index to estimate the aggressiveness of rectal cancer.

Although the relationship between budding and lymph node metastases have been observed (13,17-20,30), the relationship of budding with the expression of oncoproteins, oncoproteins and expression of proteolytic enzymes, have still not been investigated. Only one study indicates a correlation between tumour budding in colorectal cancer and immunohistochemically-detected expression of (matrix
metalloproteinase) MMP-7 and MMP-2 (21,32). It was observed that tumour budding at the invasive margin and matrilysin expression were more useful in identifying a high-risk group for adverse outcome in patients with early invasive colorectal cancer. Adenis et al. (33) presented an association between high expression of cathepsin B and progression of colorectal cancers. Also a strong correlation between the immunohistochemical expression of cathepsin D and the grade of differentiation was reported by Saku et al. (34). In our previous study, we found no such correlation (35). Similar results were obtained by Adenis et al. (33), who reported a lack of difference between high activity of cathepsin D in the neoplastic tissue and the stage of tumour advancement (according to Astler-Coller), tumour differentiation grade (G1-3), lymph node involvement, DNA ploidy, tumour site or sex of patients. Considering literature data and the present results, the cathepsin B activity may indicate the existence of metastases.

Overexpression of cathepsin B mRNA has been reported in several human tumours including brain, colon, prostate and thyroid (9,11,36,37). Increased expression of cathepsin B in a premalignant lesion suggests that this enzyme may play a role in the transformation of the premalignant lesion to malignant tumours (10). Furthermore cathepsin B may actively participate in the development of metastases. Cathepsin B can directly affect the extracellular connective matrix causing its proteolytic degradation or indirectly via activation or amplification of other ECM-degrading proteases. This cathepsin may act at the contact regions of tumour cells and basement membrane or interstitial stroma. These places are often acidified by tumour cells, which is conducive to the activation of secreted precursors to active forms which degrade the protein components of basement membranes and interstitial connective matrix including laminin, fibronectin, elastin and various type of collagen (37-39). In such a way, cathepsin B assists tumour cells in their detachment from ECMs and metastasis.

Moreover, it has been found that cathepsin B expression is often increased specifically at the invasive edge of tumour cells (11). In normal epithelial cells, the lysosomes containing mature cathepsin B are perinuclear (40). However, immunohistochemical studies of human carcinomas reveal a different pattern of cathepsin B localization. In the 69 colorectal carcinomas analyzed, immunostaining for cathepsin B showed that a redistribution of cathepsin B to the cell periphery parallels the malignancy of the tumours (41). In normal colonic epithelium, cathepsin B staining is vesicular and distributed throughout the apical region of these polarized cells. At some point during progression to late adenoma and early carcinoma, cathepsin B moves to the inner basal surface of the plasma membrane immediately adjacent to the underlying basement membrane. These data also revealed that changes in localization of cathepsin B seem to precede the increase in cathepsin B protein, thus indicating that alterations in cathepsin B trafficking are independent of increased expression.

The results of this study suggest that the activity of cathepsin D is not involved in tumour budding. In our opinion, much more attention should be paid to cathepsin B, as a potentially responsible factor in tumour progression, since it strongly increased with the presence of tumour budding.

According to our results, it seems that immunohistochemical detection of cathepsins B in tumour budding is suitable for determination of whether the phenomena of tumour budding formation takes place.

References


Received April 15, 2004
Accepted July 15, 2004