

## The activities and subcellular localization of cathepsin B and cysteine proteinase inhibitors in human ovarian carcinoma

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**Ovarian carcinomas exhibited a higher cathepsin B activity than did normal ovaries. The plasma membrane fractions of ovarian cancers were enriched in the activities of both cathepsin and cysteine proteinase inhibitors. The ratio of cathepsin to the inhibitors in these fractions was higher at stage IV than at stage III of the disease, classified according to the Federation of International Gynecology and Obstetrics.**

Human ovarian cancer is characterized by its tendency to extend over the peritoneal surface of the abdominal cavity, resulting in a wide-spread disease. At its terminal stages, multiple metastatic foci appear in distant organs, possibly with the involvement of proteolytic enzymes.

Proteinases could facilitate metastasis in different ways, including detachment of individual cells from the primary tumor, their invasion to surrounding tissues to allow contact with blood vessels, degradation of the basement membrane during both intravasation and extravasation, and invasion of tissues by formation of secondary tumor sites [1]. Several classes of proteinases, including cysteine proteinases, have been implicated in this process.

Cathepsin B (CB) (EC 3.4.22.1), one of the most active cysteine proteinases, was reported to have an important physiological

role in tumor invasion and metastasis. This enzyme, which is expressed at higher levels in invasive tumors than in normal or benign tissues, can degrade three of the major components of the basement membrane, i.e. laminin, fibronectin and type IV collagen [2]. Membrane-associated cathepsin B may also substitute plasmin in the activation of soluble or tumor-cell receptor-bound prourokinase-type plasminogen activator [3].

Ovarian cancer cell lines express detectable and reproducible levels of surface urokinase-type plasminogen activator and cathepsin B [4]. In ovarian cancer, primary tumors differ from metastases in their content of urokinase-type plasminogen activator, its receptor, and the inhibitor types 1 and 2 [5]. A latent, high molecular mass form of cathepsin B, presumably pro-cathepsin B, has been shown to accumulate in malignant ascitic fluids, among others from patients

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**Abbreviations:** CB, cathepsin B; CPIs, cysteine proteinase inhibitors; RSA, relative specific activity.

with ovarian cancer [6]. This form can be activated by pepsin, cathepsin D or neutrophil elastase [6, 7]. The activities of cysteine proteinases are controlled by endogenous inhibitors (CPIs). Alterations in the balance between endogenous cysteine proteinase inhibitors and cysteine proteinases have been postulated to contribute to malignant progression [8]. Cysteine proteinase inhibitors of three families, i.e., stefins, cystatins and kininogens were found in ascites fluid from patients with ovarian carcinoma [9].

To our knowledge, cysteine proteinase and CPIs activities so far have not been measured in ovarian cancer. The purpose of the present study was to characterize the enzyme-inhibitor relationship in ovarian carcinoma and normal ovary. The activities of cathepsin B and cysteine proteinase inhibitors as well as their subcellular distribution were examined.

## MATERIALS AND METHODS

**Materials.** Tissue samples were obtained from patients undergoing surgery at the Clinic of Gynaecology, Wrocław University of Medicine. Samples of 7 primary ovarian carcinomas from patients at stage III ( $n = 4$ ), and IV ( $n = 3$ ) were studied. The age of the patients ranged from 14 to 61 years. The stage of the ovarian cancer was classified according to the Federation of International Gynecology and Obstetrics (FIGO). The control group consisted of 6 normal human ovary tissues. Samples were taken from parts of surgical specimens that were normal and showed no significant abnormality on histological examination. The age of the donors ranged from 30 to 55 years. The samples were obtained immediately after surgical removal and stored at  $-20^{\circ}\text{C}$  until use. A few sections from each tumor were examined.

**Subcellular fractionation.** The procedure was performed according to Rozhin *et al.* [10]. The tissue (5 g) was minced in ice-cold homogenization buffer (250 mM sucrose, 25 mM Hepes, 1 mM EDTA, pH 7.3). All further manipulations were carried out at  $4^{\circ}\text{C}$ . The minced tissue was homogenized by

two 5-s bursts of a Unipan 309 homogenizer at maximum speed (1200 r.p.m.), separated by a cooling period in an ice slurry. The homogenate was filtered through four layers of cheesecloth, its volume adjusted to be 10% (w/v) with the homogenization buffer, and then it was centrifuged at  $500 \times g$  for 11 min. The obtained nuclear pellet was washed in 20 ml of the buffer and recentrifuged. The two post-nuclear supernatants were pooled and centrifuged at  $7000 \times g$  for 7 min to yield the mitochondrial pellet; this pellet was washed and recentrifuged. The pooled supernatants were centrifuged at  $15\,000 \times g$  for 19 min (ultracentrifuge UP 65) to yield a post-mitochondrial pellet, which was washed and recentrifuged.

The final post-mitochondrial pellet was further subfractionated by density gradient centrifugation on 30% isoosmotic Percoll prepared in the homogenization buffer. The self-forming gradient was generated by centrifugation for 16 min at  $60\,000 \times g$ . Density marker beads were centrifuged in parallel gradients. Two visible bands collected by aspiration were separated from the Percoll medium after being diluted in the homogenization buffer, by recentrifugation at  $100\,000 \times g$  for 55 min. The upper band was enriched in plasma membrane marker ( $\text{Na}^+, \text{K}^+$ -ATPase), and the lower one in lysosomal marker ( $\beta$ -glucuronidase). Fractions were resuspended in, or diluted with, the homogenization buffer, frozen rapidly, and stored for enzyme assays.

In all fractions activities of cysteine proteinase, CPIs and marker enzymes were determined. Triton X-100 (0.1%, v/v, final concentration) was added to all aliquots except those used in the assay of  $\text{Na}^+, \text{K}^+$ -ATPase. Samples were stored at  $-20^{\circ}\text{C}$  until the assays.

**Enzymes and inhibitor assays.** Cathepsin B activity was measured according to Barrett *et al.* [11] using the fluorogenic synthetic substrate (0.02 mM) CBZ-Arg-Arg-NMec (Sigma, U.S.A.). Fluorescence of the released free 7-amino-4-methylcoumarin was determined with a spectrofluorimeter (Perkin Elmer LS-3B) at 370 nm excitation and 460 nm emission wavelengths.

Activity of ouabain-sensitive ( $\text{Na}^+/\text{K}^+$ -activated) ATPase (EC 3.6.1.3) was determined according to Jorgensen [12].

$\beta$ -Glucuronidase (EC 3.2.1.31) activity was measured using *p*-nitrophenyl- $\beta$ -D-glucuronide as a substrate according to Fishman [13].

One unit of the enzyme activity was defined as 1 nmol of the reaction product released per minute.

To determine the activity of endogenous cysteine proteinase inhibitors, we dissociated inhibitor-cysteine proteinase complexes by heating at 100°C for 5 min. After centrifugation, the heat stable inhibitors were assayed against papain (Sigma, U.S.A.) the plant cysteine proteinase using *N*- $\alpha$ -benzoylarginine- $\beta$ -naphthylamide as substrate according to Barrett & Kirschke [14].

One unit of cysteine proteinase inhibitors activity was defined as the amount required to inhibit 1  $\mu\text{g}$  papain.

**Protein assay.** Protein concentrations were determined by the Bradford procedure [15], using bovine serum albumin as a standard.

## RESULTS

### Subcellular fractions

The subcellular distribution of CB, CPIs, and marker enzymes ( $\beta$ -glucuronidase, and

$\text{Na}^+,\text{K}^+$ -ATPase) was analyzed initially after separation of homogenates by differential centrifugation into four fractions: nuclear, mitochondrial, post-mitochondrial, and supernatant [11]. The recovery of protein ranged from 59–79% and the recovery of the enzyme activities from 55–106%.

Tables 1 and 2 illustrate the distribution of CB and heat stable cysteine proteinase inhibitors in normal human ovary (control tissue), and in ovarian carcinoma. The degree of enrichment in each fraction is represented by the ratio of the specific activity in a given fraction to that in the homogenate (RSA), and corresponds to changes in the activity of measured parameter in that fraction. The mitochondrial fraction (RSA = 1.7) of the control tissue showed higher CB activity than the post-mitochondrial fraction (RSA = 1.0). In contrast, in the ovarian tumors (stages III and IV) CB activity was found primarily in the post-mitochondrial fractions. This shift was not related to the stage of the disease (RSA = 1.6) (Table 1).

Most of CPIs were found in the post-mitochondrial and supernatant fractions of all the examined tissues (normal and cancerous). This activity in the post-mitochondrial fraction of ovarian tumors was lower than in normal ovaries. In the supernatants, activity of the inhibitors was lower at stage IV (RSA = 1.1) than in normal ovary (RSA = 1.7) (Table 2).

**Table 1. Distribution of cathepsin B activity in subcellular fractions of normal ovaries and ovarian cancer**

Fraction	Ovaries		
	Normal (n = 6)	Stage III (n = 4)	Stage IV (n = 3)
Homogenate	507 $\pm$ 20 <sup>a</sup> 1.0 <sup>b</sup>	1072 $\pm$ 291 1.0	1300 $\pm$ 212 1.0
Nuclear	554 $\pm$ 138 1.1	607 $\pm$ 138 0.6	1287 $\pm$ 212 1.0
Mitochondrial	854 $\pm$ 219 1.7	1326 $\pm$ 813 1.2	1351 $\pm$ 210 1.0
Post-mitochondrial	510 $\pm$ 138 1.0	1697 $\pm$ 225 1.6	2052 $\pm$ 211 1.6
Supernatant	340 $\pm$ 99 0.7	645 $\pm$ 85 0.6	892 $\pm$ 151 0.7

<sup>a</sup>The activity is expressed in units per mg of protein. <sup>b</sup>The relative specific activity in each fraction was referred to the specific activity of the homogenate. The ratio above 1 indicates the enrichment of the fraction in CB. The results are mean values  $\pm$ S.D. from 3 or 4 determinations.

**Table 2. Distribution of cysteine proteinase inhibitors in subcellular fractions of normal ovaries and ovarian cancer**

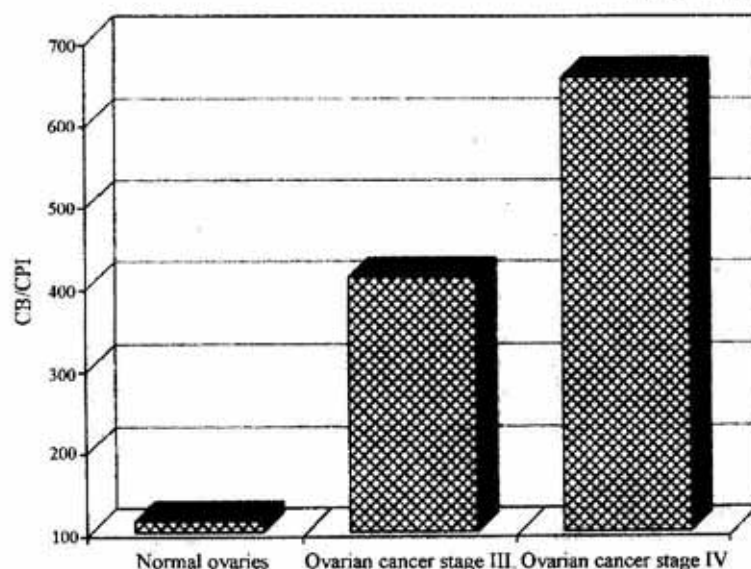
Fraction	Ovaries*		
	Normal (n = 6)	Stage III (n = 4)	Stage IV (n = 3)
Homogenate	2.1 ± 1.5 <sup>a</sup> 1.0 <sup>b</sup>	1.8 ± 0.3 1.0	2.7 ± 0.9 1.0
Nuclear	1.8 ± 1.0 0.9	1.9 ± 0.4 1.0	1.7 ± 0.7 0.6
Mitochondrial	1.5 ± 1.2 0.7	1.6 ± 0.4 0.9	1.3 ± 0.9 0.5
Post-mitochondrial	4.4 ± 2.0 2.1	2.6 ± 0.9 1.4	3.3 ± 1.0 1.2
Supernatant	3.6 ± 1.8 1.7	3.1 ± 1.0 1.7	3.0 ± 1.3 1.1

For details see the legend to Table 1.

### Submitochondrial fractions

The post-mitochondrial fractions which showed the greatest differences between the normal and tumor tissues, were further fractionated by density gradient centrifugation on a self-generated gradient of iso-osmotic 30% Percoll. Two visible bands were aspirated and assayed. The upper band was designated as the plasma membrane fraction and the lower one as the lysosomal fraction.

membrane and the lysosomal fractions. However, the relative specific activity of CB in the lysosomal fraction was about twice that in the plasma membrane fraction (stage III 4.2 *vs* 2.6, stage IV 5.7 *vs* 3.0, respectively). The lysosomal fraction of normal ovary was enriched in the inhibitor activity more than was the plasma membrane fraction as referred to the homogenate (RSA = 2.6). However, in ovarian carcinoma the plasma membrane fraction was found to be more enriched in the



**Figure 1. The ratio of cathepsin B to cysteine proteinase inhibitors activity in the plasma membrane fractions of normal ovary and ovarian cancer.**

The results are expressed as the ratio of specific activities.

Table 3 illustrate the distribution of CB, CPIs and marker enzymes in those subfractions. Only the lysosomal fraction of normal human ovary was enriched (RSA = 1.4) in CB activity. However, in ovarian cancers, the increase in CB activity involved both plasma

inhibitor activity than the lysosomal fraction. The relative specific inhibitory activity amounted to 3.7 at stage III, and 2.1 at stage IV of the disease. The marker enzymes Na<sup>+</sup>,K<sup>+</sup>-ATPase and β-glucuronidase were also determined (Table 3).

Since cysteine proteinases bind to cystatins at a 1:1 molar ratio, we can arbitrarily estimate the potential "working capacity" of cathepsin B by determining the ratio of the activity of cathepsin B to that of the heat stable cysteine proteinase inhibitors in the plasma membrane fraction. This ratio increased in the following order (Fig. 1): normal ovary (113), stage III (411), and stage IV (655).

made with animal amelanotic melanoma B16, and Lewis lung carcinoma tissues [10, 28]. The authors postulated that the change in localization of cathepsin B was due either to the movement of lysosomal vesicles to the cell periphery or altered trafficking of CB with a subsequent association of the enzyme to the plasma membrane, or both. Our results also suggested association of CB with the plasma

**Table 3. Relative specific activities of cathepsin B, cysteine proteinase inhibitors and marker enzymes in the plasma membrane and lysosomal fractions of normal ovaries and ovarian cancer**

Fraction	Enzymes and CPIs distribution	Ovaries		
		Normal (n = 6)	Stage III (n = 4)	Stage IV (n = 3)
Plasma membranes	CB	0.7	2.6	3.0
	CPIs	1.5	3.7	2.1
	$\beta$ -Glucuronidase	1.3	1.5	1.7
	$\text{Na}^+, \text{K}^+$ -ATPase	3.6	4.3	7.5
Lysosomal	CB	1.5	4.2	5.7
	CPIs	2.6	2.1	1.8
	$\beta$ -Glucuronidase	2.7	2.3	2.7
	$\text{Na}^+, \text{K}^+$ -ATPase	1.3	1.2	2.7

## DISCUSSION

We have found that CB activity is higher in ovarian cancer tissues than in normal ovaries, with a tumor/normal ratio of 2.0–2.5. The increased expression of proteolytic systems, including CB, is one of the characteristics of transformed and malignant cells and has been observed in such human cancer tissues as: breast [16], cervix uteri [17], colorectal [18], lung [19], gastric [20], pancreas [21], prostate [22], gliomas [23], and melanoma [24] tumors. Elevated CB activity in ovarian lesions has not been reported, although cysteine proteinase inhibitors have been isolated from ascites fluid and ovarian cancer tissue [9, 25]. It has been suggested that, like cathepsin D for breast cancer, cytosol CB activity may appear as a prognostic factor for some other cancer types [26].

Although normal and tumor cathepsins B are similar in their enzymatic properties and amino-acid composition [27], they differ in their intracellular distribution. In normal cells CB is localized in lysosomes, whereas in tumor cells it was also detected in plasma membranes. Such observations have been

membranes of human ovarian tumors which was not significantly correlated with the stage of the disease. Unfortunately, the small number of examined tissues as well as the biochemical techniques and cell fractionation procedures used in our study did not allow to draw definite conclusions. Therefore more specific methods will be used in further experiments.

Kobayashi *et al.* [3] who studied the ovarian cell line, HOC-I, have shown using the ELISA technique that CB becomes membrane associated. These authors demonstrated that the membrane-associated CB was able to activate the receptor-bound prourokinase and suggested that this proteolytic cascade was responsible for the ability of ovarian tumor cells to invade through reconstituted basement membrane *in vitro*. Recent evidence on this redistribution in human tumors paralleling malignant progression and increasing invasiveness [22, 23], is suggestive of a yet undefined role for cathepsin B in tumor cell invasion. Like in animal tumors [10] in the plasma membrane fractions of ovarian tumors the activity of the nonproteolytic hydrolase ( $\beta$ -glucuronidase) was not higher than in normal tissue.

The literature on cysteine proteinase inhibitors from human tumors is not extensive, particularly with regard to normal and tumor tissues or cells. Nevertheless, the balance between cysteine proteinases and their inhibitors seems to be associated with the increased metastatic potential in some experimental tumors [28, 29].

In our experiments the ovarian cancer homogenates or supernatants showed inhibitors activity similar to that in normal ovarian tissue. Such results have been also obtained for colorectal cancer tissue [18]. In human breast specimens, 2/3 of the breast carcinomas exhibited low inhibitors activity as compared to normal breast tissue [8].

However, we have observed an increased content of CPIs in the plasma membrane fractions of ovarian cancer tissue. Such a phenomenon was observed earlier in murine amelanotic melanomas (B16a) [29] and murine hepatomas [30]. According to Moin *et al.* [30] the cysteine proteinase inhibitor associated with Hepa CL9 membranes would be a modified form of stefin A. The ratio of cathepsin B activity to that of the heat stable cysteine proteinase inhibitors in the plasma membrane fractions of ovarian cancer was 6 times as high at stage IV and 4 times as high at stage III as in normal ovary (Fig. 1).

Nevertheless, it should be stressed, that we assessed the inhibitor activity in tissue homogenates toward the plant cysteine proteinase, papain, which as a model enzyme expresses inhibition of mammalian cysteine proteinases. Although this method does not discriminate between various inhibitors and insufficiently describes the actual situation *in vivo*, still it is the most accessible method for such determinations.

According to Kastelic *et al.* [25] ovarian cancer tissue contains kininogens and stefins. Tumor stefin B isolated from ovarian cancers does not differ from normal stefin B. The very low amount of stefin A in ovarian cancer, a tissue of epithelial origin, may be related to the malignancy.

Testing of inhibitory activities of stefins versus CB activity should provide better insight into the impaired regulation of CB activity in ovarian cancers.

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