INHIBITION OF SELECTED ENZYMES WITH SPECIFIC INHIBITORS IN THE TISSUE HOMOGENATES FROM PATIENTS WITH COLORECTAL CANCER

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Large intestine malignancy is the second most common malignancy and second leading cause of cancer mortality in Poland. This is related to late detection of these lesions, e.g. due to lack of effective screening tests. Lesions found by a surgeon are clinically advanced, making the treatment often ineffective and sometimes even completely impossible. Discovery of a substance that would be able to stop key processes for the development of malignancy could change such situation. Activity of certain enzymes was found to increase in malignant cells and invasion of malignancy could be triggered by inadequate amount of endogenous inhibitors of these enzymes in the surrounding healthy tissues. Inhibitors identical with that produced in human cells were found in egg whites.

The aim of the study was to determine ability of cystatin isolated from egg whites to inhibit activity of cathepsin B and L.

Material and methods. Immunohistochemistry and histology of tissue specimen collected from malignant lesions resected from 60 patients diagnosed with large intestine adenocarcinoma, who underwent surgical treatment in 2nd Department of General and Oncological Surgery, Medical University of Wrocław between 2007 and 2009.

Results. Differences were fund between health tissues, margins and center of the malignant lesions with regard to amount and distribution of stained cathepsin B – cystatin complexes. The above mentioned inhibitors were able to inhibit 90% of primary activity of cathepsin B and L in malignant tissues.

Conclusions. Cystatins obtained from egg whites could be used as substances supporting anti-cancer therapy in the future.

Key words: malignancy, large intestine, cystatins, cathepsins, inhibitor therapy

Incidence of large intestine malignancy is growing and it is estimated that a lifetime risk of large intestine malignancy among the worldwide population is 6%; high percentage of them will die. Cancer of the large intestine (colorectal cancer) is the second most common malignancy and second leading cause of cancer mortality in Poland, both in women and men. Its incidence in our country is increasing at a rate of 2.5% per year. Colorectal cancer is diagnosed too late in our country. Currently no screening method that would result in early detection of colorectal cancer with high sensitivity and specificity, that would be cheap and accepted by patients, is available. Studies and analyses suggest that the most effective screening programs are based on colonoscopy.
The most common causes of gastrointestinal malignancies include: cigarette smoking, ingestion of spirits and detrimental dietary habits often related to high-fat diet. Adipose tissue is a site of accumulation of multiple toxic compounds that are insoluble in water, including aromatic compounds that initiate malignant transformation. Direct or indirect contact with chemical substances that can cause mutation of healthy cells, transforming them into malignant cells, is also significant. This phenomenon is most commonly observed in industrial regions where high emission of toxic substances to the atmosphere occurs. Results of previous epidemiological and statistical studies conducted in Poland confirm that approximately 60% malignancies are induced by carcinogenic substances related to environmental pollution. The principal cause of malignancies is overlapping of genetic factors and activity of carcinogenic substances. They contribute to activation of oncogenes that are the direct cause of development of malignancies (1, 2).

Probably, the first mutated gene that appears in gastrointestinal malignancies is a suppressor gene APC, encoding e.g. proteins responsible for cellular adhesion that regulate cellular interactions. Eventually these changes can result in development of a malignancy (3, 4, 5) (fig. 1).

Studies of selected proteolytic enzymes that have been performer for many years, suggest that they accompany key malignant processes. Results of these studies suggest that they constitute so-called cascade of malignant enzymatic changes — they undergo mutual autoactivation. They are found in a form of inactive precursors in a human body. They exhibit biological activity only after enzymatic activation. Cathepsin D is one of the first enzymes to initiate malignant changes. It appears in an active form following activation of its inactive precursor. Cathepsin D precursor appears in a cell as an effect of action of hormones, mainly estrogens, and undergoes activation as a result of action of enzymes from the group of serine proteases (6) (fig. 2).

An active cathepsin D catalyzes activation of cysteine peptidases (mainly cathepsins B and L). Appearance of these enzymes in their active forms triggers activation of multiple proteolytic enzymes, mainly metalloproteinases, plasminogen activator, elastases and many others. These enzymes along with cysteine peptidases cause autodegradation of own tissues as well as determine multiple processes that accompany malignant changes (7).

The studies confirmed that malignant cells produce increased amounts of cathepsin B and L as compared to normal cells, both in vivo and in vitro. These enzymes produced by malignant cells are much more stable than their analogues produced by normal cells. Activity of proteolytic enzymes associated with malignant cells is regulated by their autogenous, specific inhibitors that are found in the body. Probably, both initiation and development of malignant processes depend on overexpression of proteolytic enzymes in view of too low expression of their autogenous inhibitors. Correlation between active metalloproteinases, elastases and plasminogen activators versus their autogenous inhibitors has been understood in detail. Inhibitors of cysteine peptidases were also studied. Inhibitors of cysteine peptidases, by inhibiting cathepsin B and L activity, can inhibit formation of active forms of collagenases, elastases, collagen activator and metalloproteinases, i.e. key proteolytic enzymes involved in degradation of normal body tissues by malignant cells (6, 8, 9).

Increased expression of active cysteine peptidases in malignant cells makes autogenous inhibitors unable to inhibit their excess. There have been suggestions that this process could be controlled by using artificially isolated inhibitors. Recently it has been found out and presented in publications that cysteine peptidase inhibitors isolated from urine, placenta

![Fig. 1. Multistage process of malignant changes in gastrointestinal malignancy (normal cell → polyp → cancer) involving changes in genetic base of normal cells](image-url)
and amniotic fluid of patients are non-toxic for living organisms and inhibit activity of these peptidases both in vivo and in vitro. Inhibitors isolated from the egg whites have been shown to exhibit similar properties (10, 11).

The aim of this study was to assess a change of cathepsin B and L activity following the use of their specific inhibitors.

**MATERIAL AND METHODS**

Determinations were done in malignant tissues obtained from resected malignant lesions from 60 patients diagnosed with colorectal adenocarcinoma. Presence of malignancy was confirmed with histopathological examination of the resected tissue. The results were compared to 20 specimen (control group) obtained from healthy colorectal mucosa collected from the same patients. 60 tissue specimens in which adenocarcinoma cells were found, were collected from patients who underwent surgical treatment in 2\textsuperscript{nd} Department of General and Oncological Surgery, Regional Specialist Hospital in Legnica between 2007 and 2009. Age of the patients ranged from 35 to 73 years. The tissues were collected from 32 (53\%) men and 28 (47\%) women, including 40 patients with large intestinal cancer (67\%) and 20 with rectal cancer (33\%).

Immediately after the surgical procedure, the collected tissues were selected, frozen and underwent histopathological examination. Then the specimens were divided into two parts: one was embedded in paraffin for immunohistochemistry, and the other was subjected to homogenization according to a method reported by Malicka-Blaszkiewicz and Roth (12). Then the homogenates were centrifuged and cytosolic fraction was frozen. Activity of cysteine peptidases and degree of its inhibition, using cystatins obtained from egg whites, was measured with a spectrofluorimetric method. One inhibitor unit was defined as an amount of an inhibitor that completely inhibited 1 unit of activity of cathepsin B and L.

Three specimen from a single patient were always selected, collected from: center of the tumor, border of the invasion (malignant infiltrate) and healthy tissues. Immunohistochem-

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Fig. 2. Hypothetical mechanism of a cascade of enzymatic changes in malignant processes (6)
ical staining was performed in these specimens, to determine site of occurrence of complexes cathepsin B – autogenous cystatin by using mouse anti-cathepsin B – cystatin from egg whites. It was possible due to similarity of structure of cystatin C and cystatin from egg whites. Rabbit anti-mouse antibodies, labeled with peroxidase, were used in another stage of the assay. Finally, active peroxidase was visualized in the tissues, indirectly indicating sites of the presence of complexes cathepsin B – autogenous cystatin, specific for malignant processes.

Calculations were done using Statgraf software. Distribution of obtained results was assessed with Kolmogorow – Smirnow test. All tests provided results exhibiting normal distribution and therefore parametric tests were used in subsequent statistical analyses. To compare two studies parameters, t-Student test, nonparametric Wilxocon test or Kruskal-Wallis test was used. Correlation coefficients were calculated using t-Student test for correlation coefficients. All hypotheses were tested at a significance level of $p = 0.05$.

RESULTS

Microscopic examination was used to demonstrate location and pattern of distribution of cathepsin B – autogenous cystatin complexes. Antibody conjugated peroxidase was demonstrated to occur in a healthy tissue in a disseminated pattern, in the whole tissue, i.e. was probably not bound to any specific site in the tissue, indicating lack of cathepsin B – autogenous cystatin complexes. Significant increase of number of complexes was found in tissues collected from the border of invasion and from the center of the tumor; even contours of malignant invasion could be seen. Below representative images demonstrating the biggest differences, are provided: healthy tissue (fig. 3), border of invasion (fig. 4), malignant tumor (fig. 5).

In another series of tests of specimens collected from malignant tissues, cathepsin activity was inhibited with a specific inhibitor of cysteine peptidases, isolated from egg whites according to patent owned by Medical Univer-
sity of Wroclaw. This inhibitor inhibited activity of cathepsin B and L by as much as 90% of their initial activity.

**DISCUSSION**

We examined activity of cathepsin B and L in both malignant tissues and healthy tissues isolated from patients with colorectal cancer and if these enzymes could be inhibited with cysteine peptidases isolated from egg whites. Similar examinations related to control of changes of activity of these enzymes in gastric cancer have already been performed in Medical University of Wroclaw. These enzymes have been purposefully selected for this study since information obtained from the literature indicates that they play a key role in basic processes of initiation and development of malignancies, such as: malignant transformation, invasion, metastases and angiogenesis. These enzymes were found to play a significant role in processes that accompany apoptosis while abnormalities or apoptosis determine high stability of malignant cells and their resistance to current anti-cancer drugs (6, 13-17).

For many years there was a search for inhibitors that would be non-toxic for the body while concurrently would be able to inhibit enzymatic processes that accompany malignant processes. Recently these inhibitors have been treated as proteins that could be a primary component of new generation of drugs in so called “inhibitor therapy”. Structure of inhibitors obtained from egg whites resembles that of autogenous proteins found in tissues and other human body fluids. This suggests low risk of immune reactions and their toxicity.

Egg inhibitor was reported several years ago by Davis as a potential factor controlling malignant changes. He emphasized the principal problem that must be solved by pharmaceutical industry with regard to these inhibitors, was very high cost of their isolation which prevented their widespread use (18).

A team of researchers from Medical University of Wroclaw prepared a new method of isolation of these inhibitors that could be used on a commercial scale which hopefully would eliminate the problem mentioned by Craig Davis (19). Presumably this inhibitor, formulated as a drug, could be given both orally in enteric capsules, as enemas or through appropriately modified colonoscope that could be used to inject drugs into the tumor, thus limiting its growth and ability to form metastases. These methods could be a supplement to conventional therapies, including surgical treatment and chemotherapy or radiotherapy. The most probable method of administration of cystatins isolated from egg whites may be per rectum, using a colonoscope or as a component of targeted drugs to inhibit in vivo enzymes responsible for malignant processes.

Results of this study support these suggesting by confirming that activity of cathepsin B and L can be inhibited. Further studies can demonstrate that inhibition of activity of these enzymes with their specific and non-toxic inhibitors could result in controlling of processes accompanying malignancies.

These presumptions are based on results of studies conducted in Medical University of Wroclaw that demonstrated that cysteine peptidases could be inhibited by inhibitors obtained from various sources. Specific inhibitors of cysteine peptidases were also tested in animal models implanted with human breast or liver tumor. In such situations, these inhibitors were a significant supplement of photodynamic therapy as well as vitamin E therapy (20-23).

**CONCLUSIONS**

1. Activity of studied enzymes (cathepsin B and L) is significantly higher in tissues collected from the center of colorectal cancer versus in healthy tissue.
2. Activity of cathepsin B and L can be inhibited with inhibitors of cysteine peptidases obtained from egg whites by as much as 90% of their initial activity.
3. Immunohistopathological staining can detect cathepsin B – autogenous cystatin complexes. There is a significant difference with regard to microscopic image between healthy tissues and tissues collected from border of malignant invasion and the center of the tumor.
4. Immunohistochemistry confirms that cystatins isolated from egg whites could be used interchangeably with autogenous inhibitors in the human body, including probably the most common cystatin C.
5. Cystatins isolated from egg whites could be used to inhibit one of the most pathogenic
proteolytic enzymes that initiate pathogenic processes, including malignant processes.

6. It could be possible to use cystatins from egg whites as components of new generation of anticancer drugs.

REFERENCES


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