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Decrease *in vivo* of cysteine endopeptidases in blood of patients with tumor of the larynx

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Since cysteine endopeptidase (cathepsins B and L) have been proposed to be implicated in tumor malignancy, we have attempted to decrease these *in vivo*. Large amounts of urine cysteine peptidase inhibitors (UCPI) are present in the urine of patients. Our results indicate protective effects of a UCPI preparation against human serum cysteine endopeptidases.

Key words: Cystatins, therapy, urine.

Introduction

The action of endopeptidases has been implicated in various aspects of cancer, including invasion of tissue,[1] formation of metastasis[2] and neoplastic transformation.[3] The action of specific endogenous cysteine peptidase inhibitors (CPI) is an ultimate control mechanism for tumor cysteine endopeptidases activity *in vivo*.[4] It has been proposed that endogenous cysteine peptidase inhibitors can limit the invasive and metastatic potential of human cancers.[5–8] Many laboratories have attempted to find non-toxic specific synthetic or microbial inhibitors to achieve inhibition *in vivo* of 'tumor' cysteine endopeptidase.[9] E-64, a specific inhibitor of cysteine peptidases, is effective in limiting the invasion and metastasis of cancer cells. Urinary cysteine peptidase inhibitors *in vivo* have been proposed to be implicated in inhibiting hydrolysis of laminin,[10] inhibiting tumor cathepsin B and L at the tumor–host border,[1] prevention of tumor cell induced platelet aggregation[2] and arrest of neoplastic transformation.[3]

The idea behind the present work was to use the cysteine peptidase inhibitors isolated from patient's urine by affinity chromatography for the inhibition *in vivo* of cysteine endopeptidases. Here we present data on the toxicity of urine cysteine peptidase inhibitors (UCPI) and on the possibility of reducing human serum cysteine endopeptidases *in vivo*. We also determined the optimal frequency of UCPI dose to regulate serum cysteine endopeptidases-like activity.

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Material and methods

Materials

Twenty-four hour urine of patients with laryngological cancer was condensed 20-fold by vacuum evaporation at 40°C, centrifuged at 3000 *g* for 20 min, dialyzed into water at 4°C and frozen at −20°C. UCPI were isolated by affinity chromatography on papain immobilized to Sepharose 4B from 1.0–2.0 l of condensed and dialyzed urine.[11] The obtained preparation was filtered on a Sartoruis Ministart filter NFL (0.2 μm) and then a microbiological test was performed. The sterile UCPI solution was frozen in 2.0 ml ampules at 4°C.

Patients

The UCPI injection was administered to six male or female larynx cancer patients (average age 40 years) who had received surgical intervention and chemotherapy and radiotherapy but showed progressive disease.

Treatment

Before the first injection, all patients were tested with a skin test for the UCPI preparation to exclude the possibility of any allergic reaction. The patients received a single muscle injection containing 30–40 units (defined below) of UCPI (one ampule) isolated from 48 h autologous urine (2.5–3.0 l). The patients received the treatment every day for a minimum of 2 weeks. During the UCPI therapy, the urine of the

patients was collected and UCPIs were isolated for the next cycle of therapy.

Monitoring of patients

Patients underwent a complete physical examination during which blood samples were drawn before the therapy and after each dose. The blood samples were drawn 10 times during the first 24 h after the first dose of UCPI. Alpha-macroglobulin was inactivated by incubation with 0.5 M methylamine at 37°C and pH 7.5 for 2 h.[12] Serum cysteine endopeptidase and papain-like activity were routinely assayed with *N*-α-benzoyl-DL-arginine-β-naphthylamide hydrochloride (BANA). The level of serum cysteine proteinase inhibitors was determined by measuring of inhibition of papain.[11,12,14] One unit of the enzyme activity corresponds to 1 μmol of substrate degraded per hour at 37°C and pH 7.5. An inhibitory unit corresponds to the inhibition of one unit of papain activity.

We presume the CPI^{37} value to be an active inhibitor of serum cysteine proteinases and applied the following procedure to determine CPI^{37}: 50 μl of serum were preincubated with 50 μl of water and 2.0 ml of 0.5 M methylamine in 0.01 M potassium phosphate, pH 7.5, for 2 h at 37°C, incubated with papain and BANA as a substrate. Inhibitory activity was determined when the inhibition of enzyme reached 20–80%.

We presume the CPI^{60} value to be the total activity of serum cysteine proteinase inhibitors. The procedure of assay of CPI^{60} was as follows: 50 μl of serum were preincubated with 50 μl of 0.03 M glycine/HCl buffer, pH 2.0, at 60°C for 20 min, then with 2.0 ml of 0.5 M methylamine, pH 7.5, at 37°C for 2 h. Samples were centrifuged, incubated with papain and then the activity of papain was determined with BANA as substrate and the activity of inhibitors was calculated.

ΔCPI, which represents the difference between CPI^{60} and CPI^{37}, was calculated and results are presented as complex forms of the inhibitors.[14]

Protein content was determined according to Bradford with bovine serum albumin as a standard.[15]

Results

Our investigation was an attempt to use a UCPI preparation for *in vivo* reduction of cysteine endopeptidases-like activity in the serum of the patients with larynx cancer with progressive disease. The patients received muscle injections (30–40 units of UCPI/day). During the first day after application of the first dose of UCPI two different periods were observed (Table 1 and Figure 1).

Table 1. Level of cysteine peptidase inhibitors and cysteine endopeptidase-like activity in the serum of patients with larynx cancer—effect of treatment with UCPI

Time (h) after UCPI injection	Cysteine peptidase inhibitors (U/mg of protein)			Cysteine endopeptidase (U/mg of protein)
	CPI^{37}	CPI^{60}	ΔCPI	
0.0	58.5	84.5	26.3	0.897
1.0	60.5	84.5	24.0	1.025
2.0	70.2	81.9	11.7	1.181
3.0	65.7	78.0	12.3	1.120
4.0	53.3	72.8	19.5	1.110
5.0	48.1	75.4	27.3	0.986
6.0	36.4	78.0	41.6	0.726
12.0	33.8	81.9	48.1	0.592
18.0	18.2	70.2	52.0	0.512
24.0	41.6	67.6	26.4	0.854

(i) Between the second and fourth hour an increase of serum cysteine endopeptidases and some decrease of cysteine peptidase inhibitors in complex forms was observed; all patients suffered mild chills.

(ii) Between the fourth and 24th hour a decrease of cysteine endopeptidases-like activity and an increase of cysteine peptidase inhibitors-like complex forms was observed. During this period and after the next doses of UCPI preparation applied in the treatment cycle the feeling of the patients was very good.

The adverse reaction between the second and fourth hour was observed only after the first dose of the UCPI preparation in the 10 day cycle of this experimental treatment.

Discussion

There is support for the notion that cysteine peptidase inhibitors might be useful as potential anticancer agents.[14,16] The difference between the level of the tumor cathepsin B activity and its inhibitors could be important in the progression or regression of tumor diseases.[5–8] Cysteine endopeptidase inhibitors are natural peptides produced by an organism, mainly to inhibit cysteine

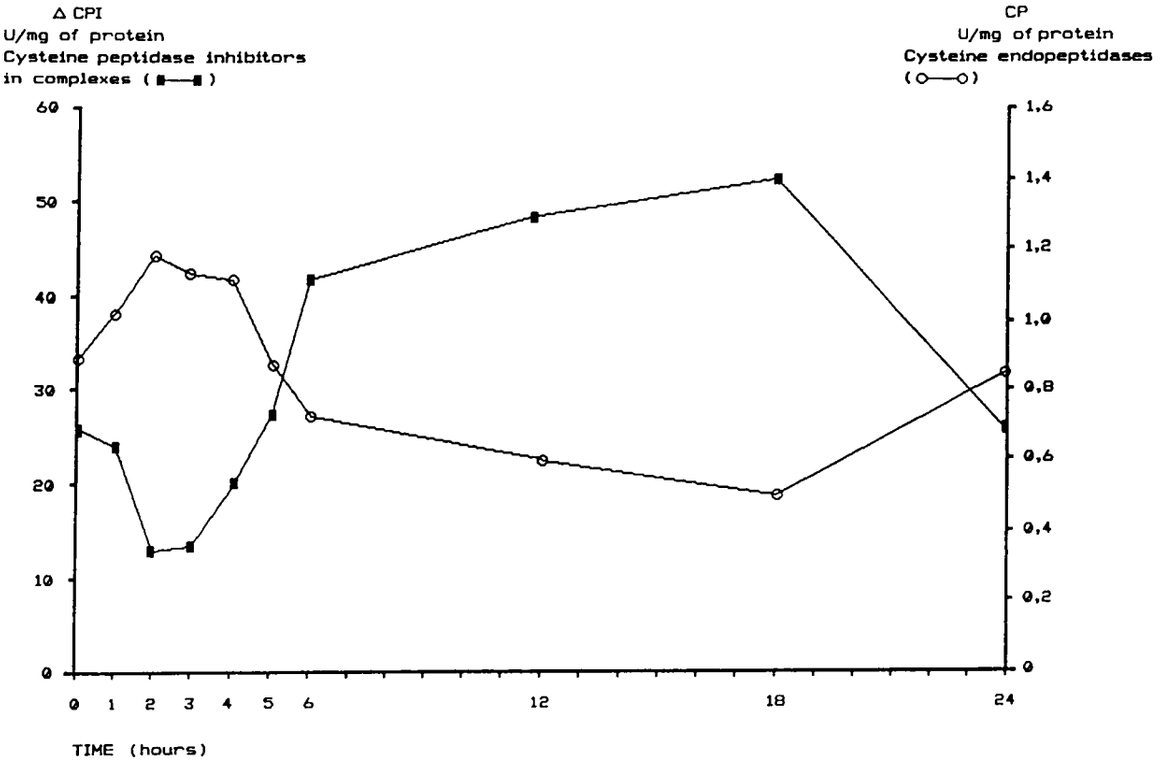

Figure 1. Level of cysteine endopeptidases and cysteine peptidase inhibitors as complex in the serum of patients with larynx cancer 24 h after injection of UCPI.

endopeptidases. The role of these inhibitors is probably to protect normal proteins and tissues against enzymes released under abnormal conditions.[4] In the urine of patients with neoplastic diseases the level of cysteine peptidase inhibitors is increased as compared with healthy controls.[16]

UCPI are naturally occurring peptides secreted from tissues to blood which accumulate in urine.[17] In our experiments we isolated cysteine peptidase inhibitors from the urine of patients with malignant tumors and used them in the same patients as a potential antitumor agent.

The patient's own urine (autologous urine) is a convenient and inexpensive source of cysteine peptidase inhibitors (of low toxicity and not antigenic) with which to inhibit cysteine endopeptidases *in vivo*.[17] Our results suggest that there is some protective effect of the UCPI preparation against serum cysteine peptidases *in vivo*, probably via specific inhibitors complexed with the serum 'tumor peptidases'. UCPI preparations as a potential antitumor agents are currently administered to six patients diagnosed with larynx carcinoma (stage III–IV). A report of this study will be published elsewhere.

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