Effect of vitamin E and human placenta cysteine peptidase inhibitor on expression of cathepsins B and L in implanted hepatoma Morris 5123 tumor model in Wistar rats

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INTRODUCTION

Lysosomal cysteine protease (CP) plays an important role in the intracellular protein turnover, proteolytic degradation of endocytoxed proteins, maturation cleavage of a number of precursor proteins[1]. Natural CP inhibitors (CPI) include members of the cystatin superfamily (stefins, i.e., family I cystatins, family II cystatins, and kininogens)[2]. Cystatins are distributed in different body fluids, suggesting a regulatory role against host or exogenous CP. In human blood plasma, CPI activities are represented by α2-macroglobulin, low-molecular mass kininogen and a small amount of cystatin C[3]. Cathepsin B (cat B) is released from lysosomes during tumor necrosis factor-alpha (TNF-α) cytotoxic signaling in hepatocytes and contributes to cell death. These data implicate a sphingosine-cat B interaction inducing cytoxic signaling in hepatocytes and contributes to cell death. These data implicate a sphingosine-cat B interaction inducing cytoxic signaling in hepatocytes and contributes to cell death. These data implicate a sphingosine-cat B interaction inducing cytoxic signaling in hepatocytes and contributes to cell death. These data implicate a sphingosine-cat B interaction inducing cytoxic signaling in hepatocytes and contributes to cell death. These data implicate a sphingosine-cat B interaction inducing cytoxic signaling in hepatocytes and contributes to cell death.

RESULTS: Cathepsins B and L activities were elevated by 16-fold in comparison with negative control tissues, and their endogenous inhibitor activity decreased by 1.2-fold before treatment. In several cases, tumors completely disappeared following vitamin E to rats with transplanted Morris-5123 hepatoma, on the expression of cathepsins B and L in tumor, liver, lung and blood sera after transplantation of Morris 5123 hepatoma.

CONCLUSION: The data indicate formation of metastasis significantly reduced in treated rats, which might provide a therapeutic basis for anti-cancer therapy.

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Key words: Morris-5123 hepatoma; Vitamin E; Human placenta cysteine peptidase inhibitor; Cathepsin B; Cathepsin L

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of liver cancer is therefore associated with induction of increased microsomal enzyme activity[11]. It was found that vitamin E could control the cellular formation of ras and myc oncogenes[12] and raise in vivo the level of autogenic cysteine peptidases inhibitors[13]. The information suggests the possibility of its application in adjuvant conventional oncological therapy. Vitamin E could also reduce a cytostatic level in patients after conventional chemotherapy[14]. The combined treatment of transplantable solid mammary carcinoma in Wistar rats using photodynamic therapy and proteinase inhibitor isolated from human placenta was done. The results indicated that in several cases tumors were completely disappeared following treatment HpD-PDT+CPI[15]. The aim of the present study was to investigate the new therapy in the effect of vitamin E and human placenta cysteine peptidase inhibitors on the expression of cathepsins B and L in implanted hepatoma Morris 5123 tumor model in Wistar rats.

MATERIALS AND METHODS

Animals

Male and female Wistar rats weighing 180-200 g (age: approximately 2 mo) were used. The rats were fed with normal diets during the experimental period according to Nirwana et al[16]. The food composed of (% w/w): crude protein 20.0, crude fibre 5.0, crude fat 2.5, moisture 13.0, ash 7.0, calcium 0.7-1.4, total phosphorus 0.6-1.2, and nitrogen-free extract 51.0.

Human placenta cysteine protease inhibitor (CPI)

The inhibitors were purified and identified using affinity chromatography on Sepharose 4B - papain, Sephadex G-75 chromatography column, and ion exchange chromatography on a DEAE-Sephacel column (Saleh et al[17] 2003). A highly purified preparation of cysteine protease inhibitors was obtained with a specific activity of 344.8 mEU/mg protein, and molecular weight of 67 kDa (by SDS-PAGE). The solution was concentrated on centricon (Amicon), lyophilized and stored at -20 °C until use. The pure and sterile inhibitors were dissolved in physiological saline and injected subcutaneously (sc.) at the doses of 200 μg and 400 μg per animal (in volume 0.1 mL).

Vitamin E

Vitamin E (400 mg/mL) solution was dissolved in corn oil and injected subcutaneously (sc.) at the doses of 10 and 20 mg/kg per animal (in volume 0.1 mL) obtained from Hasco, (Pharmaceutical Drugs Production, Wroclaw, Poland).

Implantation of tumor

Approximately 140 000 tumor cells of hepatoma Morris 5123 were implanted intramuscularly in the left limb of the Wistar rats. Two wk after implantation, the implanted tumor grew locally early in the course of diseases and eventually invaded the surrounding organs causing ascites and also metastasis in the lungs. Liver microangiography demonstrated that the tumor received blood supply mainly from the hepatic artery. The tumor was detected by histological examinations of these tissues using conventional hematoxylin-eosin (HE) staining.

Experimental design

The experiment was carried out on 100 male and female Wistar rats, which were divided into 10 groups: Group 1:10 rats, which were given normal diets without treatment during the experiment as control negative (healthy); group 2 :10 rats with hepatoma Morris 5123, which were given normal diets without treatment during the experiment as control positive (tumor); groups 3 and 4 : tumor rats were injected subcutaneously 10 and 20 mg/kg vitamin E in 0.1 mL corn oil during the experiment groups 5 and 6 : tumor rats were injected subcutaneously 200 and 400 μg human placenta purified cysteine protease inhibitor (CPI) in 0.1 mL physiological solution during the experiment groups 7 and 8 : tumor rats were injected subcutaneously 200 μg CPI plus 10 and 20 mg/kg vitamin E; groups 9 and 10 : tumor rats were injected subcutaneously 400 μg CPI plus 10 and 20 mg/kg vitamin E. All animals were injected for 10 d with human placenta purified cysteine protease inhibitors (CPI). From the eleventh day the rats were injected vitamin E for 30 d. After 8 wk, the experiment animals from all groups were narcotized, decapitated and the tumor, lungs, liver, and blood were taken and washed out of the blood with 0.9% NaCl and homogenized in electric Potter’s homogenizer according to the methods described by Malicka and Roth[18]. The activities of enzymes and histological examinations were performed.

Determination of cathepsin B and L activities

Cathepsin B activity was measured according to Barrett et al[19]. Fluorescence was measured in a luminescence spectrometer, Perkin Elmer LS 50 B at 370 nmol/L excitation and 440 nmol/L emission wavelengths using fluorescent substrate Z-Aeg-Arg-AMC for cathepsin B and Z-Phe-Arg-AMC for cathepsin L. Fluorescence readings of the sample assays were standardized with the reaction product 7-AMC (7-amino-4-methylcoumarin) [20]. One mEU of activity was defined as the quantity releasing 1 nmol/L of 7-AMC.

Inactivation of α-macroglobulin

α-macroglobulin (α-M) was inactivated by incubation with 0.5 mol/L methylamine at 37 °C and pH 7.5 for 2 h in samples and activity of CPI[3] was determined[21]. Fifty μL of sample was preincubated with 50 μL of water and 20 mL of 0.5 M methylamine in 0.1 mol/L potassium phosphate pH 7.5 for 2 h at 37 °C. Fifty μL of 0.1% papain was then added and the samples were incubated for 10 min at 37 °C. Then 50 μL of 0.66 mmol/L BANA solution was added and the free β-naphthylamine was measured. Inhibitory activity was determined[22]. CPI[3], the total activity of cysteine proteinase inhibitors was calculated. The procedure of assay was as follows. Fifty μL of serum was preincubated with 50 μL of 0.03 mol/L HCl at 80 °C for 20 min, then with 2.0 mL of 0.5 mol/L methylamine at 37 °C for 2 h. Samples were centrifuged and incubated with 0.01% papain solution, then the papain activity was determined and the activity of inhibitors was calculated. CPI: Difference between CPI[0] and CPI[3] was calculated, and the results were presented as complex (latent) forms of inhibitors CPI = CPI[0] - CPI[3]. The amount of vitamin E was determined by liquid chromatography, using a HPLC apparatus (“Philips”) and a Pye Unicam PU 4020 UV detector. The results were decoded with the use of Peak simple chromatography data system program[22].

Protein concentration

Total protein concentration was determined by the Bradford method[23] using bovine serum albumin as a standard.

Statistical analysis

The levels of variables in the cancer tissues and sera compared with the control were analyzed statistically. The results of cathepsin B and their inhibitor activity assays in the groups under study are given as median and mean±SD. To compare data of tumor and control tissues, Wallon’s rank test was used. P<0.05 was considered statistically significant. The calculation of survival probability was performed by the method developed by Kaplan and Meier. Checking the significance of a relationship between survival of rats and the levels of...
biochemical parameters was based on the Log-rank test. The discrimination levels were calculated by a computer program.

RESULTS

The best results were obtained after combination of 20 mg of vitamin E and a higher dose of CPI were used, i.e., 400 µg, in control groups. In these cases the animals survived for longer than 8 wk. In numerous cases the tumors were completely disappeared following CPI and vitamin E application. Table 1 shows that the number of complete tumor responses was higher when 400 µg of CPI was used plus 20 mg of vitamin E, i.e., 7/10 rats, and survived more than two mo. Whereas application of 10 mg of vitamin E and a lower dose of CPI (200 µg) resulted in only 4/10 total tumor responses, and the rats survived for about 26 d. The difference between these two groups was significant ($P\leq0.0001$). No complete tumor response was achieved in all the other experimental groups. The vitamin E after the last 10 mg of vitamin E and a lower dose of CPI (200 µg) resulted in only 4/10 total tumor responses, and the rats survived for about 26 d. The difference between these two groups was significant ($P\leq0.0001$). No complete tumor response was achieved in all the other experimental groups. The vitamin E after the last treatment with human placental CPI was injected subcutaneously at the doses of 10 and 20 mg per animal for one month. Cathepsin B and L activity was measured in all animals with tumor homogenates and in untreated animal tissues (control). The mean or median activity of cat B and L was much higher in tumor homogenates in comparison with that in the negative control homogenates and the differences were highly significant ($P\leq0.0001$) before treatment. While no significance was observed in the activity of cathepsins B and L in tumor tissues after the rats were given-high doses of inhibitors plus vitamin E ($P\leq0.0005$).

The total cat B and L activity was 88.5 mEU/mg proteins in tumor tissue homogenates compared with 5.8 mEU/mg protein in negative control tissue ($P\leq0.0005$). Cathepsins B and L activities were elevated 16-fold in comparison with negative control tissue. Table 1 shows that the inhibitory activity of CPI was significantly decreased from 10.5 mEU/mg protein in control tissue to 8.5 mEU/mg protein in tumor tissue ($P\leq0.0005$), thus it decreased 1.2-fold in tumor tissues. The complex form of CPI was also decreased from 14.2 mEU/mg protein in control tissue to 9.0 mEU/mg protein in tumor tissue ($P\leq0.0005$), with a decrease of 1.6-fold in tumor tissues.

Cathepsin B and L activity decreased in all vitamin E groups by 1.0-1.3 fold, while in the rats obtained only CPI, the cathepsin B and L activity decreased by 1.2-1.8 folds. By the results from Table 1 also showed that the activities of cathepsins B and L decreased by 5.8 and 11.8 fold after the rats received 20 mg vitamin E plus 200 and 400 µg CPI in tumor tissues. While the

| Cathepsins B, L | Inhibitor (CPI) | Complex form | Survival time (d) | Cure (yes/No) | Value ($P$) |
|---------------|---------------|--------------|-----------------|--------------|-------------|
| (mEU/mg)      | (mEU/mg)      | (mEU/mg)     |                |              |             |
| Control negative | 5.8           | 24.7         | 10.5           | 14.2         | 0.0005      |
| (healthy)     | 6.5±1.7       | 25.4±8.9     | 11.2±2.8       | 14.2±6.1     | 60.0        |
| (Tumor)       | 88.5          | 17.5         | 8.5            | 9.0          | 09-12       |
| (Tumor+ 10 mg | 85.4±12.6     | 18.8±8.6     | 9.3±1.2        | 9.5±7.4      | (10.5)      |
| Vit E         | 81.8±20.6     | 29.5±12.0    | 17.0±5.5       | 12.5±6.5     | (19.0)      |
| (11.0-99.8)   | (6.7-35.7)    | (3.9-20.3)   | (2.8-15.4)     | (2.6-10.4)   |
| Tumor+20 mg   | 78.7          | 35.6         | 20.3           | 15.3         | 15-30       |
| Vit E         | 79.4±20.4     | 36.6±15.0    | 21.5±4.6       | 15.1±10.4    | (22.5)      |
| (10.7-97.4)   | (7.3-40.1)    | (4.2-22.0)   | (3.1-18.1)     | (2.8-12.8)   |
| Tumor+ 200 µg | 56.5          | 46.3         | 35.8           | 10.5         | 16-30       |
| CPI           | 57.4±15.1     | 47.7±20.0    | 37.0±17.6      | 10.7±2.4     | (23.0)      |
| (22.1-80.6)   | (12.3-52.9)   | (9.0-40.0)   | (3.3-12.9)     | (3.3-25.0)   |
| Tumor+ 400 µg | 47.9          | 67.8         | 46.8           | 21.0         | 18-35       |
| CPI           | 48.7±18.6     | 69.0±25.2    | 47.3±16.0      | 21.7±9.2     | (21.5)      |
| (23.2-68.9)   | (20.6-88.5)   | (14.4-60.1)  | (6.2-28.4)     | (6.2-43.5)   |
| Tumor+ 10 mg Vit E | 41.8      | 71.8         | 52.3           | 18.5         | 27-38       |
| +Tumor+ 200 µg CPI | 43.0±10.6 | 73.0±25.4    | 53.6±22.1      | 19.4±3.3     | (32.5)      |
| (12.0-46.7)   | (22.7-82.8)   | (20.4-63.3)  | (2.3-11.5)     | (2.3-25.3)   |
| Tumor+ 10 mg Vit E | 30.2      | 82.2         | 61.0           | 21.2         | 30-40       |
| +Tumor+ 400 µg CPI | 31.6±12.0 | 83.5±29.8    | 62.4±25.5      | 21.1±4.3     | (35.0)      |
| (9.8-38.7)    | (26.9-93.7)   | (24.6-70.2)  | (2.3-23.5)     | (2.3-25.3)   |
| Tumor+ 20 mg Vit E | 15.4      | 94.4         | 74.2           | 10.2         | 38-60       |
| +Tumor+ 200 µg CPI | 16.5±2.8   | 95.8±35.4    | 75.6±33.1      | 10.2±2.3     | (49.0)      |
| (6.4-20.8)    | (37.8-112.7)  | (32.3-94.5)  | (5.5-18.2)     | (5.5-18.2)   |
| Tumor+ 20 mg Vit E | 7.5       | 120.8        | 105.5          | 15.3         | 45-60       |
| +Tumor+ 400 µg CPI | 8.4±1.8    | 122.0±43.6   | 107.0±39.2     | 15.0±4.4     | (52.5)      |
| (2.6-10.2)    | (45.8-135.5)  | (35.6-118.2) | (10.2-17.3)    | (10.2-17.3)  |

Table 1: Cathepsins B, L and their inhibitor activities, survival time and the total cure responses of hepatoma morris-5123 in Wistar rats before and after treatment in comparison with negative control (median, mean±SD, range)

The significance of the differences in Median values of tumor and control tissues was calculated by Wilcoxon matched pair’s signed-rank test.
endogenous inhibitor activity was increased 7.4 and 10.5 folds respectively in the same groups, and the complex form ΔCPI activity obtained in the same range to negative control group.

The results in Table 2 showed that the mean±SD of cat B and L activities in the tissue homogenates were decreased from 68.9±23.6 mL/mg protein and 28.8±10.4 mL/mg protein before treatment to 8.4±2.5 mL/mg protein and 3.8±1.6 mL/mg protein, respectively, after the rats received 20 mg vitamin E plus 400 μg CPI. Highly significant differences were observed between the activities of cat B and L in tissue homogenates before and after treatment with 20 μg vitamin E plus 400 μg CPI (P≤0.0001), and this was similar to control tissues. The activities of cat B and L were decreased by 8.2 and 7.6 fold, respectively. The endogenous inhibitors were increased from 9.0±23.6 mL/mg protein before treatment to 79.4±22.5 mL/mg protein after the rats were administered 20 mg vitamin E plus 400 μg CPI. This increased by about 9.0-fold. The activities of cat B and L were also decreased significantly in liver and lung tissue homogenates and in sera after treatment (P≤0.0001) in comparison with negative control group. While the endogenous inhibitors also increased significantly in comparison with negative control group (P≤0.0001). The activities of cat B and L were decreased 6.2 fold and 7.0 fold in liver tissues, and 9.3 fold and 6.0 fold in lung tissues, while 8.2-fold and 7.6-fold in sera, respectively after treatment. The endogenous inhibitors were increased 11.6, 10.2-folds, and 72.0-fold in liver, lung tissues and in sera after treatment, respectively. No significance was observed in cat B and L activities in tissue homogenates and in blood sera after the rats obtained high doses of CPI plus vitamin E in comparison to negative control group.

**DISCUSSION**

Cancer cells are characterized with high motility, loss of function which makes them unite with neighbouring cells, invasiveness, and lack of sensibility to contact inhibition and general change of cell shape. Adhesion and locomotion are the main capabilities of cells in tumor growth and metastasis. Proteases are attractive targets for drug development for therapeutic application. The use of novel inhibitors in clinical practice is dependent not just on their specificity and effectiveness, but mainly on the knowledge of their precise role in mechanisms of the proteolysis system in the development of malignant disease[29]. The combination of vitamin E with other cancer chemopreventive agents appears to be a reasonable procedure[28]. Association of the balance between various proteases and their inhibitors with cancer diseases has been widely studied to understand the molecular mechanisms of development and metastasis of tumors. It is an often presented result in literature that cysteine peptidase activity in various malignant tumor tissues and body fluids of cancer patients was altered compared to physiological levels[27]. It has been widely noted that vitamin E showed numerous beneficial effects through and beyond its antioxidative properties. Consequently, vitamin E is expected to prevent degenerative diseases[26]. In the present study, we investigated the effectiveness of vitamin E in combination with human placental cysteine proteinase inhibitors (CPI). The results showed that the number of complete tumor responses was higher when the rats obtained 400 μg of CPI plus 20 mg of vitamin E, i.e., 7/10 rats. Whereas application of 10 mg vitamin E and a lower dose of CPI (200 μg) resulted in only 4/10 total tumor responses. The present study could be explained in such a way that CPI damaged the cancer cells. The number of total (complete) tumor responses was distinctly higher after application of 400 μg CPI than 200 μg CPI. This indicated that in the same conditions of vitamin E, the efficacy of treatment of CPI was dose-dependent. Saleh et al[35] indicated that in several cases tumors completely disappeared following the treatment with mammary carcinomas using HpD-PDT+CPI. Another independent research direction was the therapeutic indications of vitamin E in carcinogenesis processes[29].

The data obtained in this work involving hepatoma Morris-5123 carcinoma in model Wistar rats were compared with those in non-cancerous tissues as a negative control before and after treatment. High activity of cathepsins B and L with a noteworthy simultaneous decrease of the activity of endogenous cysteine protease inhibitors was observed before treatment in tumor.
liver, lung tissues and in sera in comparison with negative control tissue homogenates. Cathepsin B and L activities were elevated 16-fold as against negative control tissues before treatment. The endogenous inhibitor activity also decreased 1.2 fold, and the complex form ΔCPI decreased 1.6-fold in tumor tissues. They provided further evidence for the alteration of the protease/inhibitor balance that occurred during the process of tumor cell invasion. Since the level of proteinases and inhibitors in extracellular fluids may reflect not only their local expression in tumors but also the systemic response to the disease, the mechanisms of the alteration of protease/inhibitor balance are not solved. The highly significant difference was observed between the levels of cathepsins and/or their precursor in malignant tumor tissue before treatment. Tumor tissue activity of cat B and L might be related to the severity of cancer disease and there are some prognostic aspects of such studies since it previously showed that patients with higher content or increased proteolysis activities of cat B and its precursor in tissue homogenates of breast, ovarian and gastric cancer had significantly higher risk of recurrence or death than the cases with low content of the enzyme. After the rats were injected with 20 mg vitamin E plus 400 µg CPI, the cat B and L were expressed significantly in tumor, liver, lung tissues and in sera in parallel to the increasing of the endogenous inhibitor activity (P ≤ 0.0001). The rats survived for a longer period and they returned to normal life without any stress. Nyandieka et al. suggested that vitamins could inhibit liver cancer by inducing hepatic microsomal enzymes that metabolise aflatoxins to non-carcinogenic products.

Welss et al. demonstrated that hurpin was an intracellular, differentially spliced member of the serpin superfamily that has been linked to differentiation and apoptosis of human keratinocytes. It can be transiently down regulated by UV light and overexpressed in psoriatic skin lesions. It is a potent and selective inhibitor of the archetypal lysosomal cysteine proteinase cathepsin L.

Sakamoto et al. suggested that vitamin E administration could stimulate macrophages tosynthesize interleukin 1 and then, acted on fibroblasts and lymphocytes, thus raising the interleukin 6 levels. As a final effect it could increase T-kininogen then, acted on fibroblasts and lymphocytes, thus raising the proteinase cathepsin L.

and overexpressed in psoriatic skin lesions. It is a potent and selective inhibitor of the archetypal lysosomal cysteine proteinase cathepsin L.

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