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Proenzyme therapy of sarcoma S-180 and melanoma B16-F10

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ABSTRACT

The aim of this study was to evaluate the effectiveness of individual (inactive) proenzymes and mixtures thereof in cancer treatment and to compare this treatment with more frequently used therapy based on active proteases. Experiments focused on explanation of possible mechanisms of proenzyme action against tumours are included.

Proenzyme therapy of sarcoma S-180 significantly reduced tumour growth and prolonged survival of mice. The effect of trypsinogen and chymotrypsinogen was synergistic. Proenzyme therapy of melanoma B16-F10 bearing mice reduced both tumour growth and prevalence of metastases. Active enzyme based therapy of melanoma B16-F10 was less effective. Severe combined immunodeficiency (SCID) mice bearing sarcoma S-180 did not respond to the proenzyme therapy, indicating that the effect of this therapy is dependent on fully developed acquired immunity. Measured decreased levels of TGF-β and an increased amount of alpha-2 macroglobulin in serum contributed to the elucidation of the cancer treatment mechanism.

Proenzyme therapy based on administration of a mixture of trypsinogen and chymotrypsinogen is effective in cancer treatment.

A R T I C L E  I N F O

Introduction

The beginnings of systemic pancreatic enzyme therapy of cancer date back to the early twentieth century. John Beard (1857–1924) noted a resemblance between embryonic/foetal trophoblastic cells and cancer cells and hypothesized that the growth of the former was eventually blocked by products of the foetal pancreas which becomes functional at about 56 days gestation. He further suggested that pancreatic enzymes were responsible for this 'anti-trophoblast' effect. Therefore, he proposed a new cancer therapy based on the administration of fresh pancreatic extracts. He called this treatment "trypsin therapy", assuming that the pancreatic protease trypsin was...
the major active ingredient when combined with pancreatic amylase as a supplemental component (Beard, 1911). This therapy had a wide range of successful followers (Cleaves, 1906; Golley, 1906; Campbell, 1907) but some scientists were not able to repeat the curative effect (Hald, 1907; Bainbridge, 1909) and trypsin therapy then fell out of use. We assume that Beard's early results could not be repeated by later investigators due to Beard's incorrect assumption that trypsin is the active ingredient. While Beard pointed out the need to use fresh pancreatic extracts (containing also mainly trypsinogen, chymotrypsinogen and amylase), the investigators using trypsin in isolation would not observe any effect. In later work, Wald and collaborators achieved a significant reduction in metastases using active proteases in mice intracutaneously inoculated with Lewis lung carcinoma (Wald et al., 1998) and B16 melanoma (Wald et al., 2001). In both cases, the enzyme mixture (trypsin, chymotrypsin and papain) was administered per rectum twice a day but the bioavailability of these proteases might be questioned. The use of proenzymes in cancer therapy was first described by Trnka et al. (1999) and Novak and Trnka (2005). In vitro experiments showed inhibitory effects on the migration and motility of cancer cells. In vivo experiments using mixtures of trypsinogen and amylase administered subcutaneously proved effective against methylcholanthrene-induced tumours and significantly reduced B16 melanoma metastases. The effects were achieved using trypsinogen concentrations ten or more times lower than that of the active enzymes used by Wald et al. (1998, 2001). This may reflect the selective conversion of proenzymes to the active form near the surface of tumour cells (Novak and Trnka, 2005).

In the present investigations, we chose a relatively slow growing transplantable sarcoma primarily because we intended to study the long term treatment of transplanted tumours. Sarcoma S-180, with low metastatic potential (Jones et al., 1939; Deodhar, 1971) is a good model for this purpose. As the second model, we used fast growing melanoma B16-F10. This model allowed us to evaluate the occurrence of metastases. We studied both the effect of proenzymes and that of a mixture of active proteases.

Materials and methods

Enzymes

Bovine trypsinogen, bovine alpha-chymotrypsinogen A, bovine trypsin, bovine alpha-chymotrypsin, and alpha-amylase from Bacillus sp. were obtained from Sigma.

Mice

SPF BALB/c and C57BL/6 mice were obtained from Charles River Laboratories (Sulzfeld, Germany). BALB/c and C57BL/6 mice were housed in plastic cages with wood-chip bedding situated in a specific-pathogen free room with a constant temperature of 22 °C and a relative humidity of 65%. SCID mice (C.B17/Icr-scid) of the BALB/c background were originally obtained from Charles River Laboratories (Sulzfeld, Germany) and were housed in plastic cages with sterilized wood-chip bedding situated in flexible film isolators (BEM Znojmo, Czech Republic) with high-efficiency particulate air filters. All mice were 18–20 g. Pellet diet and water were sterilized. All mice were housed in a 12/12-h photoperiod environment with free access to food and water.

Cells

Murine cell lines sarcoma S-180 and melanoma B16-F10 (both donated by Prof. Řihová, Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague) were used. The cells were cultivated in the same conditions – RPMI 1640 with 20 mM HEPES and l-glutamine (Sigma) supplemented with 10% foetal calf serum (FCS) ≤10 EU/ml endotoxin (Sigma), and Antibiotic/Antimycotic Solution (PAA). The cells were maintained at 37 °C in humidified air with 5% carbon dioxide.

Tumour transplantation

4 × 10^5 S-180 or B16-F10 cells in 0.1 ml RPMI without serum per mouse were inoculated subcutaneously (s.c.) in a shaved area on the right flank. We used BALB/c and SCID mice for transplantation of S-180 and C57BL/6 for B16-F10 cells.

Treatment

Therapeutic preparations (proenzymes and enzymes dissolved in saline), sterilized by filtration and stored frozen were injected daily i.m. in the semitendinosus muscle of the left hind leg (0.1 ml per mouse). Doses correspond to the original treatment (Beard, 1911) reevaluated by Novak and Trnka (2005).

Evaluation of treatment

Tumours were measured twice a week using callipers. Volume was calculated according to the method of Inaba et al. (1986) using the formula V = π/6 AB^2 (A denotes the largest dimension of tumour mass and B stands for the smallest dimension).

Photographic documentation of tumour development and of side effects was also performed. All dead mice were dissected.

Lung metastases

In melanoma B16-F10 experiments, all lungs were examined with the aid of a dissecting microscope. The presence of metastases (black points) was evaluated.

Histology

Tumours, liver, kidney, spleen and lungs were fixed by a 4% neutral solution of formaldehyde. Paraffin blocks were prepared. Sections were stained by haematoxylin/eosin.

Determination of total TGF-β in serum

We used ELISA READY-SET-GO! Human/Mouse TGF-β 1 kit (eBIO SCI ENCE).
Determination of trypsin activity

The method developed by Erlanger et al. (1961) was used. This method uses N-alpha-benzoyl-DL-Arg-p-nitroanilid (BAPNA, Sigma) as a substrate.

Proenzyme therapy of sarcoma S-180 bearing mice

40 female BALB/c mice were injected with sarcoma S-180 cells. The mice were randomly divided into 4 groups:

- Group TG + CHG + A (9 mice): 7500 BAEE units of trypsinogen (after activation) + 31 BTEE units of alpha-chymotrypsinogen A(after activation) + 820 maltose units of alpha-amylase per ml in saline.
- Group TG + A (9 mice): 15,000 BAEE units of trypsinogen (after activation) + 820 maltose units of alpha-amylase per ml in saline.
- Group CHG + A (10 mice): 62 BTEE units of alpha-chymotrypsinogen A(after activation) + 820 maltose units of alpha-amylase per ml in saline.
- Group Control (10 mice): saline, control group.

The 2 remaining mice died during or shortly after tumour injection and were not considered in the analysis.

All solutions (0.1 ml per mouse) were injected daily from the fourth day after tumour cell transplantation. The mice were observed daily for 100 days. Tumour volumes were measured twice a week.

Proenzyme and enzyme therapy of melanoma B16-F10 bearing mice

55 female C57BL/6 mice were injected with melanoma B16-F10 cells. All mice were monitored to document tumour onset. On day eleven after transplantation the mice were randomly divided into 4 groups. The therapy was started on the same day. Mice received daily doses of 0.1 ml of a solution composed as follows:

- Group P “proenzyme therapy” (14 mice): 7500 BAEE units of trypsinogen (after activation) + 31 BTEE units of alpha-chymotrypsinogen A(after activation) + 820 maltose units of alpha-amylase per ml in saline.
- Group E = P “enzyme therapy” (14 mice): 7500 BAEE units of trypsin + 31 BTEE units of alpha-chymotrypsinogen A + 820 maltose units of alpha-amylase per ml in saline.
- Group E = 1/10P “enzyme therapy, low concentrations” (14 mice): 750 BAEE units of trypsin + 3.1 BTEE units of alpha-chymotrypsinogen A + 820 maltose units of alpha-amylase per ml in saline.
- Group Control “control group” (13 mice): saline.

Tumour volumes were measured twice a week.

Proenzyme therapy of tumour bearing immunodeficient mice

We studied proenzyme therapy of female SCID mice bearing sarcoma S-180. We used proenzyme therapy based on mixture of trypsinogen (7500 BAEE units after activation/ml saline), alpha-chymotrypsinogen A (31 BTEE units after activation/ml saline) and alpha-amylase (820 maltose units/ml saline); details are given in section “Results”.

Proenzyme therapy of melanoma B16-F10 bearing C57BL/6 mice treated by proenzyme therapy

The experimental design was the same as the above mentioned proenzyme therapy of melanoma. Details are given in the chapter “Results”. Levels of alpha-2 macroglobulin, contrapsin and alpha-1 antitrypsin in pooled serum samples were determined using mass spectrometry (MS). Samples were diluted 500 times using ammonium bicarbonate buffer (c = 100 mmol/l, pH = 8.0, Sigma). We used enolase from yeast Saccharomyces cerevisiae (Sigma) as an internal standard. Diluted samples were digested by trypsin (c = 100 ng/ml, Sigma) for 12 h at 37 °C and analyzed using Q-ToF Premier mass spectrometer (Waters Corporation, ESI-Q-ToF mass spectrometer coupled with a Nano Acquity Liquid Chromatography device). The chromatographic column was filled by hydrophobic compound (Bridged Ethyl Hydrid) with an eighteen-carbon chain. The chromatography analysis took 90 minutes. During the first 60 minutes we increased the ratio of acetonitril to MilliQ Water (Millipore) from 3% to 60%. The flow was 400 nanoliters per minute.

We used the MS² Identity method to obtain raw data, which were later processed by the PLGS 2.3 software (Waters). We used species-specific protein database containing mouse (Mus musculus) entries from UniProt. The Expression Analysis function of the PLGS 2.3 software was used for quantitative analysis. Absolute quantitative values of all analytes were obtained on the basis of juxtaposition with a concentration of murine albumin. All measurements were triplicated.

All the experimental in vivo procedures were carried out in accordance with rules which are valid in the EU and USA.

Statistics

Statistical analysis was performed using the two-tailed Student’s t-test and the software STATISTICA VII, Survival Analysis (StatSoft, Inc., Tulsa, OK 74104, USA) at the significance level α = 0.05.

Results

Proenzyme therapy of sarcoma S-180 bearing mice

Roughly 30% of the mice in each group failed to develop tumours, which corresponds with data published by others (Jones et al., 1939). Hence, the final number of mice in groups TG + CHG + A, TG + A, CHG + A and Control was 6, 7, 7 and 7, respectively. As shown in Fig. 1, the administration of combined trypsinogen, chymotrypsinogen and amylase significantly reduced tumour growth. Tumour volumes in this group averaged 40.3% of those of the control group over a period of 14–61 days (mean of all measurements). The difference between group TG + CHG + A and Control increased over the course of the experiments and was significant at the
significance level $\alpha = 0.05$ on days 49 and 61. Individual proenzymes showed substantially less effect than their mixture.

The effect of trypsinogen and chymotrypsinogen was synergistic. In the case of one mouse which survived for 115 days (group TG + CHG + A), tumour volume decreased from 4000 mm$^3$ on day 89 to 224 mm$^3$ on day 115 indicating cytoreduction consequent to proenzyme therapy.

![Fig. 1 - Growth of sarcoma S-180 tumours in mice treated with trypsinogen (TG), chymotrypsinogen (CHG), amylase (A) and combinations thereof. Mice were inoculated with $4 \times 10^5$ S-180 cells s.c., the treatment started on day 4 after tumour cell transplantation. Mice were injected daily (0.1 ml i.m.) with following solutions. Group TG + CHG + A (9 mice): 7500 BAEE units of trypsinogen (after activation) + 31 BTEE units of alpha-chymotrypsinogen A (after activation) + 820 maltose units of alpha-amylase per ml in saline. Group TG + A (9): 15000 BAEE units of trypsinogen (after activation) + 820 maltose units of alpha-amylase per ml in saline. Group CHG + A (10): 62 BTEE units of alpha-chymotrypsinogen A (after activation) + 820 maltose units of alpha-amylase per ml in saline. Group Control (10): saline. * Statistically significant as compared with control.](image)

In all three groups injected with proenzymes, extensive surface lesions and tumour necrosis were observed. The surfaces of these tumours were usually concave. We observed no further change in groups TG + CHG + A, and TG + A, while group CHG + A exhibited a peripheral spread from the tumour edges. No surface lesions were found in the control group. Remarkable differences were observed in tumour morphology. While most tumours in the control group had the shape of an oblate spheroid, spreading in direction to the backbone, all tumours in group TG + CHG + A were spherical.

Upon dissection (immediately after the death of each mouse), the biggest difference in size of the extracted tumours was found between group TG + CHG + A ($4026 \pm 2514$ mm$^3$) and the control group ($6792 \pm 2200$ mm$^3$). This difference was not statistically significant ($P = 0.06$) due to different times of dissection. As shown previously, treated mice survived longer than the controls and their tumours had more time to develop.

Histology did not reveal any significant differences between the proenzyme treated and the control group.

The experiment was repeated 5-times with similar results.

Proenzyme and enzyme therapy of melanoma B16-F10 bearing mice

C57BL/6 mice were injected with melanoma B16-F10 cells. Fig. 3 shows the development of tumours during experiment. The proenzyme-treated mice (group P) showed the largest reduction of tumour growth (to 45.2% of control group volume) during the evaluated period of cancer treatment (14–32 day,

![Fig. 3 - Development of melanoma B16-F10 tumours in mice treated by proenzyme and enzyme therapy. Mice were inoculated with $4 \times 10^5$ B16-F10 cells s.c., the treatment started on day 11 after tumour cell transplantation. Mice were injected daily (0.1 ml i.m.) with following solutions. Group P “proenzyme therapy” (14 mice): 7500 BAEE units of trypsinogen (after activation) + 31 BTEE units of alpha-chymotrypsinogen A (after activation) + 820 maltose units of alpha-amylase per ml in saline. Group E = P “enzyme therapy” (14 mice): 7500 BAEE units of trypsin + 31 BTEE units of alpha-chymotrypsinogen A + 820 maltose units of alpha-amylase per ml in saline. Group E = 1/10P “enzyme therapy, low concentrations” (14 mice): 750 BAEE units of trypsin + 3.1 BTEE units of alpha-chymotrypsinogen A + 820 maltose units of alpha-amylase per ml in saline. Group Control (13 mice): saline. Symbols as in Fig. 1.](image)
mean of all measurements in this period). The difference between group P and the Control became statistically significant at the significance level \( \alpha = 0.05 \) on the eighteenth day of experiment. Enzymes ten times less concentrated (group \( E = 1/10P \)) reduced the volume of tumours to 51.3% in comparison with the control (mean value in the same period).

We monitored the emergence of metastases (Table 1). Metastases were found in lungs only as melanoma B16-F10 is a lung metastases model. Group \( E = 1/10P \) had the largest number of mice with metastases (58.3%). Group P had the lowest incidence (20%).

Fig. 4 shows the survival of mice. Mice in the group \( E = 1/10P \) survived the longest, but the difference between this group and the control was not statistically significant. The difference between the group \( E = 1/10P \) and \( E = P \) (low versus high dose of enzymes) approached statistical significance (\( P = 0.058 \)), indicating the need of optimization of dosage and precise dispensing in the case of enzyme therapy.

The melanoma B16-F10 experiment (proenzyme therapy) was repeated six times with similar results.

One experiment (proenzyme therapy of melanoma B16-F10) was focused on measurement of total TGF-\( \beta \) in serum. Both the proenzyme treated and the control group each contained 9 melanoma bearing mice. Treatment started on day 11 after tumour cell transplantation. Mice were injected daily (0.1 ml i.p.) with following solutions. Group \( P \) (9 mice): 7500 BAEE units of trypsinogen (after activation) + 31 BTEE units of alpha-chymotrypsinogen A (after activation) + 820 maltose units of alpha-amylase per ml in saline. Group control (9 mice): saline. Symbols as in Fig. 1.

Proenzyme therapy of tumour bearing immunodeficient mice

To elucidate the mechanisms of proenzyme therapy, we compared its effect on BALB/c and SCID mice, both bearing tumour volumes, which was statistically significant at the end of the experiment. Fig. 6 shows total TGF-\( \beta \) determined in serum samples (each value is based on analysis of 3 serum samples). A significant reduction of TGF-\( \beta \) was detected at the end of the experiment.

In both the sarcoma and the melanoma experiments we observed time dependent changes of effectivity. Fig. 7 shows a typical course of effectivity of therapy dependent on its duration. At the beginning of the therapy both proenzymes and enzymes effectively hinder tumour growth. Then there is a period of time when the effect is reduced so much, that the tumours in the treated group grow faster then in the control group. The effect of therapy stabilizes after that.

### Table 1 – Incidence of metastases in lungs of mice bearing melanoma B16-F10 and treated by proenzyme and enzyme therapy.

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Incidence of metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – proenzyme therapy (P)</td>
<td>20%</td>
</tr>
<tr>
<td>2 – enzyme therapy (E = P)</td>
<td>25%</td>
</tr>
<tr>
<td>3 – enzyme therapy, low concentrations (E = 1/10P)</td>
<td>58.3%</td>
</tr>
<tr>
<td>4 – control group (Control)</td>
<td>44.4%</td>
</tr>
</tbody>
</table>

Fig. 6 – Proenzyme therapy of melanoma B16-F10 bearing mice – determination of total serum TGF-\( \beta \). The treatment was the same as in Fig. 5. TGF-\( \beta \) was determined by ELISA in three serum samples per time point from each group. Symbols as in Fig. 1.
sarcoma S-180. We used a well-tried dosage (7500 BAEE units of trypsinogen after activation + 31 BTEE units of alpha-chymotrypsinogen A after activation + 820 maltose units of alpha-amylase per ml in saline). Group E = 1/10P means enzyme therapy based on the use of active proteases in ten times lower molar concentration than that of proenzymes (750 BAEE units of trypsin + 3.1 BTEE units of alpha-chymotrypsinogen A + 820 maltose units of alpha-amylase per ml in saline). Tumour volumes in treated groups are expressed as a percentage of tumour volumes in control untreated group in particular time points.

We started the therapy after tumours appeared (eleventh day after transplantation). Mice were divided into four groups: SCID + P (SCID mice with sarcoma, proenzyme treated, 10 mice), SCID (SCID mice with sarcoma, saline treated, control, 9 mice), BALB/c + P (10 BALB/c mice: 7500 BAEE units of trypsinogen (after activation) + 31 BTEE units of alpha-chymotrypsinogen A (after activation) + 820 maltose units of alpha-amylase per ml in saline). Group SCID (10 SCID mice) and BALB/c (9 BALB/c mice): saline. * Statistically significant as compared with control (BALB/c).

Determination of alpha-2 macroglobulin, contraspins and alpha-1 antitrypsin in serum of melanoma B16-F10 bearing C57BL/6 mice treated by proenzyme therapy

To elucidate the mechanism of the effect of proteases on tumours, we measured protease-activated alpha-2 macroglobulin levels during the proenzyme therapy of the transplanted melanoma B16-F10 versus untreated melanoma. Since other serum inhibitors (especially contraspins and alpha-1 antitrypsin) compete in binding proteases, we determined their levels as well. Melanoma B16-F10 cells were transplanted to 28 female C57BL/6 mice. After 9 days tumour volumes were measured. Mice were randomly divided into two groups – treatment of group 1 started immediately, group 2 served as a control. The treatment caused a large reduction of tumour volume (to 42.1% versus control on average) during the evaluated period (15–29 day).

The difference between treated and untreated mice was statistically significant at the significance level alpha = 0.05 on day 25 of the experiment (data not shown). Blood from the tail vein was drawn in following intervals: before experiment, on day 17 of experiment (8 days of treatment), on day 29 of experiment (20 days of treatment). We performed MS analysis of the sera. Fig. 9 shows the dynamics of particular inhibitors. Mice treated by proenzyme therapy showed some statistically not significant increase of all inhibitors.

Table 2 summarizes all significant effects observed during the whole study.

Discussion

Proenzyme therapy significantly reduces growth of both sarcoma S-180 and of melanoma B16-F10. In case of sarcoma, the therapy prolongs survival time, causes tumours to retain a spherical tumour shape and hinders tumour penetration. In the case of melanoma B16-F10 the prevalence of metastases was reduced.

In melanoma, a single concentration of proenzymes is optimal for both effective reduction of tumour growth and prevalence of metastases, while two different concentrations (by order of magnitude) are needed for active enzymes. This makes the proenzyme therapy more versatile.
We observed a strong synergy of trypsinogen and chymotrypsinogen. We optimized the amount of amylase. The supportive role of amylase in protease-based cancer treatment was proposed by Beard (1911) and confirmed by Novak and Trnka (2005). Based on our experience from preliminary experiments, we used four times higher concentrations of amylase than used by Novak and Trnka (2005). This way we significantly reduced the lethargy and decreased mobility of the cured mice. What the mechanism of action of amylase is remains to be elucidated. One possibility is that amylase splits tumour glycogen (Rousset et al., 1980; Takahashi et al., 1999) released from tumours damaged by the therapy.

The concentration of trypsinogen used in experiments on both models corresponds to the study of Novak and Trnka (2005), who derived this concentration from a reconstruction of the original paper (Beard, 1911). Although Beard did not write about chymotrypsin and chymotrypsinogen (both were discovered many years latter), Novak and Trnka (2005) correctly realized, that Beard’s preparations had to contain chymotrypsinogen, but they did not use them in vivo experiments.

Both proenzyme and especially enzyme therapy highlighted the importance of optimal therapeutic doses. The amount of enzymes and especially the means of their application are important. Enzyme preparations currently offered as supportive therapy (Wobe-Mugos E, Wobenzym® N) not only contain active proteases (a misunderstanding of Beard’s papers), but their producers also recommend oral application. The question of absorbancy of proteases after oral administration is quite debatable. Although proteases are usually administered in large quantities and the daily dose reaches grams, some authors believe that they are not absorbed at all (Gewert et al., 2004), or only a small fraction of 0.002–0.0025% (Ziv et al., 1987), respectively 0.01–0.001% (Šťastný et al., 2002) passes the intestine wall. There is no way to guarantee the exact dosage, which can be risky for patients, as too high or too low dosages might not work.

What is the mechanism of proenzyme therapy? We suppose that proenzymes as nonactive molecules migrate

![Fig. 9](image-url)  
**Fig. 9** – Serum levels of alpha-2 macroglobulin (subunit – Mw 165 722), contrapsin (Mw 45 969) and alpha-1 antitrypsin (Mw 46 850) in melanoma B16-F10 bearing C57BL/6 mice treated with proenzyme therapy. Therapy (as in Fig. 8) started on day 9 after tumour cell transplantation. All protease inhibitors were determined by MS. Treated and control groups contained 14 mice each.

| Table 2 – Summary of all significant effects observed during whole study. |
| --- | --- |
| Effects | Demonstrated |
| synergy between trypsinogen and chymotrypsinogen leads to significant reduction of tumour growth | Fig. 1 |
| proenzyme therapy based on mixture of trypsinogen and chymotrypsinogen is effective in treatment of both sarcoma S-180 and melanoma B16-F10 | Fig. 1, Fig. 3, Fig. 5, Fig. 8 |
| proenzyme therapy reduces prevalence of metastases | Table 1 |
| tumour bearing SCID mice did not respond on proenzyme therapy | Fig. 8 |
| proenzyme therapy reduces level of serum TGF-β | Fig. 6 |
| proenzyme therapy of melanoma B16-F10 bearing mice slightly enhances the serum levels of alpha-2 macroglobulin, contrapsin and alpha-1 antitrypsin | Fig. 9a-c |
through blood circulation. They can pass into tumours on the basis of tumour vascular permeability. Their strong positive charge caused by a high isoelectric point can contribute to adherence on negatively charged tumour cells in solid tumours and metastases. Rapidly growing and metastasizing tumours typically secrete large amounts of active proteases which may, in turn, cause local activation of the proenzymes (Novak and Trnka, 2005). They comprise membrane-associated cathepsin B (Figarella et al., 1988; Kobayashi et al., 1993), tumour-derived trypsin (Koivunen et al., 1991; Nyberg et al., 2006), a urokinase-type plasminogen activator (Uchima et al., 2003) and enteroactinase (Miyata et al., 1999) or enterokinase-like enzymes (Nyberg et al., 2002). Autoactivation of trypsinogen by newly formed trypsin may also play a significant role in a ‘feed forward’ reaction (Kay and Kassell, 1971). Trypsin as a key enzyme initiates chymotrypsinogen activation. In the case of sarcoma S-180 we even observed an effect of chymotrypsinogen alone, perhaps being activated by trypsin-like proteases previously described in this sarcoma (Chu et al., 1997).

The above steps allow active trypsin and chymotrypsin to accumulate in the site of tumour growth. Trypsin and chymotrypsin are trapped by alpha-2 macroglobulin. It leads to transformation of alpha-2 macroglobulin to its “fast” form connected with enhanced ability to bind cytokines (and eliminate them as resulting complexes are removed from blood). At this point the mode of action of proenzyme therapy is identical to mechanisms considered in the case of systemic enzyme therapy based on active enzymes, where the possibility of binding TGF-β and IL-10 (cytokines, secreted by tumours to suppress immune system and establish tumour tolerance) is frequently discussed (Desser et al., 2001). We suppose that the main advantage of proenzyme therapy is activation of proenzymes mainly in the tumour area. Therefore the impairment of immunotolerance is mainly local, not systemic, as in the case of enzyme therapy.

The above mentioned mechanisms of (pro)enzyme therapy (binding of TGF-β and IL-10) remain to be proven. Our experiment with SCID mice clearly shows that the effect of proenzymes (and expected high trypsin and chymotrypsin activities in the place of the tumour) is fully dependent on the presence of completely developed acquired immunity. This experiment does not directly prove proposed mechanisms, nevertheless all observations are in accordance with this scheme. The binding and removing of TGF-β during proenzyme therapy of tumours was directly supported by our experiment with SCID mice clearly shows that the effect of proenzyme therapy (local activation of alpha-2 macroglobulin, augmentation of immune attack hindered by TGF-β) is valid for metastases as well. Since both the invading primary tumours and metastases destroy surrounding tissues, the conditions for triggering the local activation of proenzymes are fulfilled in both cases.

We can hardly explain the time fluctuations of proenzyme therapy. After the first strong effect of therapy (removal of TGF-β and IL-10) it is indeed possible to expect that activated alpha-2 macroglobulin will attack other cytokines important for development of the immune response. It contrasts with the observations of Harthun et al. (1998), describing preferential binding of TGF-β and partial selectivity of this effect. Detailed understanding of mechanism of proenzyme and enzyme effect on tumours and its changes in the course of therapy will require further studies.

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