

ORIGINAL ARTICLE

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Mixture of trypsin, chymotrypsin and papain reduces formation of metastases and extends survival time of C₅₇Bl₆ mice with syngeneic melanoma B16

Abstract Purpose: The aim of the present study was to investigate the effect of a mixture of proteolytic enzymes (comprising trypsin, chymotrypsin and papain) on the metastatic model of syngeneic melanoma B16. **Methods:** 140 C₅₇Bl₆ mice were divided into two control and two “treated” groups. Control groups received saline rectally, twice a day starting 24 h after intracutaneous transplantation (C1) or from the time point of the primary B16 melanoma extirpation (C2), respectively. “Treated” groups were rectally administered a mixture of 0.2 mg trypsin, 0.5 mg papain, and 0.2 mg chymotrypsin twice daily starting 24 h after transplantation (E1) or after extirpation of the tumor (E2), respectively. Survival of mice and B16 melanoma generalization were observed for a period of 100 days. Immunological evaluation of B16 melanoma cells in the ascites was accomplished. CD44, CD54 and CD106 cells were measured by flow cytometry. **Results:** Administration of proteolytic enzymes to mice inhibited the growth of primary tumors, and tumor recurrences were less numerous. Importantly, metastasis was considerably curtailed both in the vicinity of the primary tumor and at distant locales. These findings correlated with a

decreased expression of CD44 and CD54 molecules in tumors exposed to proteolytic enzymes in vivo. **Conclusions:** Our data suggest that serine and cysteine proteinases suppress B16 melanoma, and restrict its metastatic dissemination in C₅₇Bl₆ mice.

Key words Trypsin · Papain · B16 melanoma · Metastasis

Introduction

Lack of requisite immune surveillance in tissue differentiation is an important mechanism that gives rise to a clone of malignant cells, which eventuates in tumorigenesis. Thus, a hallmark of malignant tumors is uncontrolled, invasive growth and its spread. Tumors metastasize when targeted surgical intervention or systemic cytostatic treatments are unsuccessful. The prevalence of remote metastases is largely determined by the type of primary malignancy; the tissue(s) of its origin, such as lymph nodes, lung, liver, brain and bone marrow, etc., are central to metastatic growth. For example, CD44 plays a contributory role in the progression of tumors, being crucial in adhesion and motility; it putatively activates genes necessary for invasion and metastasis [15, 30–32, 35].

Ideally, a biological response modifier regimen is directed toward strengthening the defense response with a concomitant goal of suppressing tumorigenesis. Serine and cysteine proteases from both animal and plant sources have been shown to modulate immune response in several systems [20, 40]. Thus, enzymatic, radiochromatographic and immunological techniques underscore the fact that upon oral and rectal administration, enzymes are absorbed intact, and, therefore, are assimilated in their biologically active form [2, 9, 22]. After absorption, proteases are bound to circulating anti-proteases, such as α 2-macroglobulin and α 1-protease inhibitor, leading to a dynamic equilibrium between the free and bound molecules in the bloodstream. The

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proteases maintain their hydrolytic activity upon complexing with α 2-macroglobulin; likewise, after binding to sundry other circulating anti-proteases, they have the ability to modulate the cytokine network [13, 17, 24, 25].

It is known that bromelain, papain and amylase, which are generic proteases, induce cytokine production in vitro in peripheral blood mononuclear cells [4]. Ingestion of multienzyme mixtures, comprising bromelain, trypsin, chymotrypsin and papain, induce mononuclear cells to express, de novo, tumor necrosis factor alpha (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) when incubated ex vivo with interferon-gamma (IFN- γ) [5, 6]. It has also been reported that proteases induce a respiratory burst in granulocytes with concomitant production of reactive oxygen species. Additionally, proteases are known to induce cytotoxicity in peripheral neutrophils in vitro [49].

All the same, evidence suggests that proteases inhibit tumors. For instance, peripheral lymphocytes, when incubated with trypsin in vitro, show a selective decrease in the density of CD4, CD44 and CD80 [26, 36]. Bromelain and trypsin, either alone or in combination, decrease the expression of CD44 in SMMU-2 and SK-MEL 28 melanoma, MOLT 4/8, leukemia cell line, and those of mammary carcinomas [12, 14, 34].

Materials and methods

Animals

A total of 148 female inbred C₅₇Bl₆ mice (average body weight: 18–20 g) were used (AnLab, Charles River, Czech Republic) in this study. The animal colony was maintained in a barrier facility with sterilized bedding (SAW1 Research Bedding), and fed radiation-sterilized diet ST-1 (Bergmann) with sterile water supplied *ad libitum*.

Tumor cells

B16 tumor cells were grown in the abdominal cavity of inbred mice. On day 10 after intraperitoneal transplantation of 2×10^6 tumor cells, ascites were harvested and adjusted to a concentration of 4×10^6 cells/0.2 ml of suspension. This suspension was injected individually into a total of 140 C₅₇Bl₆ mice intracutaneously, and in vivo incubation allowed to proceed for 10 days, at which time growing tumors were excised.

Tumor growth

Ten days after transplantation, the growing tumors were extirpated, and the tumor volume measured according to $V = 1/2 \cdot A \cdot B^2$, where A denotes the largest dimension of the tumor mass and B stands for the smallest dimension [16]. Malignance in the tumor mass was ascertained histologically.

Enzyme treatment-survival

To assess the anti-proliferative and anti-metastatic activities of the proteinase mixture used in this study, we divided the animals into control and “treated” groups.

In the control group (C1), 0.1 ml saline was rectally administered to 20 mice twice a day starting 24 h after intracutaneous transplantation, until the termination of the study (100 days). In the “treated” group (E1), a mixture (0.1 ml) of 0.2 mg trypsin, 0.5 mg papain and 0.2 mg chymotrypsin was rectally administered to 50 mice twice daily starting 24 h after transplantation, and was continued throughout the duration of the study. This regimen amounted to 0.9 mg of enzyme mixture per 20 g of body weight. This dose, in turn, corresponded to 45 mg/kg of the composite enzyme preparation Wobe-Mugos E (MUCOS Pharma, Germany). The enzyme mixture was prepared freshly before each administration.

In the control group (C2), again, saline (0.1 ml) was administered rectally to 20 mice twice daily from the time point of extirpation of the primary B16 melanoma, and continued for the duration of the study (100 days). A total of 50 mice pooled in a “treated” group (E2) were rectally administered 0.2 mg trypsin, 0.5 mg papain and 0.2 mg chymotrypsin (in 0.1 ml) after extirpation of the tumor, and the regimen continued for the duration of the study.

Effect of enzymes on adhesion molecules

Immunological evaluation of B16 melanoma cells in the ascites was accomplished in two groups, each comprising of six animals as outlined above. Mice in the control group (C3) received saline rectally from the time of B16 transplantation in the abdominal cavity, twice daily for a period of 7 days, whereas the “treated” group (E3) received enzyme mixture in exactly the same ratios as described above. After 7 days, ascites cells were harvested from each group of animals by lavage. Moreover, the density of adhesion molecules in lung metastases were determined in each group (C1, E1, C2, E2). Depending upon the time of sampling, metastasized and abdominal ascitic cells were homogenized in glutamine-supplemented RPMI medium, and the cell numbers determined according to Turk. Cells were diluted to the 2×10^6 cells/ml. The suspension was washed twice with PBS, and 2 μ l of antibodies specific to the adhesion molecules were added (CD44, CD54, CD106, fluorescein for negative control) (Pharmingen, San Diego, California). The mixture was incubated on ice for 30 min, washed three times with 1–2 ml PBS with a tissue medium, centrifuged at 1,000 rpm for 10 min at 4 °C, supernatant was removed and the precipitate was gently agitated. Tumor cells with bound antibody were measured on a Becton-Dickinson FACS analyzer.

Autopsy and histological observation

The progression of tumorigenesis was monitored in each group for a period of 100 days, at which point all surviving animals were killed, and the tissue sectioned from lungs, liver and any recurring primary tumors and metastases in axillary lymph nodes was histologically examined. The tissue sections were fixed with formalin and stained with hematoxylin-eosin (HE) by the usual procedure. The lung tissue was embedded in toto, and transversally sectioned up to the heart ventricles.

Statistical analyses

The Gehan-Wilcoxon test was used for statistical analysis of the survival data. Volumes of primary tumors were statistically evaluated by the analysis of variants.

Results

On day 10, volumes of primary tumors were measured in all animals (Table 1). The results in groups E2, C1 and C2 are considered to be control data, since no specific

Table 1 Mean volume of primary tumor, tumor recurrence, occurrence of pulmonary metastases, and tumor cachexia in the individual groups

Parameter	Groups			
	C1 <i>n</i> = 20	E1 <i>n</i> = 50	C2 <i>n</i> = 20	E2 <i>n</i> = 50
Mean volume of B16 melanoma 10 days after tumor cell transplantation (mean in mm ³ ± SD)	310.4 ± 121.8	47.2 ± 54.4	282.3 ± 107.7	302.4 ± 108.6
Tumor recurrence (%)	30	6	30	2
Pulmonary metastases (%)	95	46	90	68
Tumor cachexia (%)	100	46	95	68

“treatment” regimen was implemented in these groups within this time period. In group E1, the “treatment” group, which was administered enzyme mixture however, no tumor formation was discernible in 36% of the cohort. The mean tumor size in the E1 group was statistically significantly smaller (47.2 mm³) than in all other groups ($P < 0.00001$) (Table 1).

Since tumorigenesis in groups E1, E2, C1 and C2 arose from a dissociated tumor, histologically the growth was irregularly oval shaped with frayed basophilic plasma membrane. In addition, the experimental tumors were pleomorphic, containing pleiochromic nuclei, with one to two nucleoli that were locally multinucleated, and showed signs of multiple mitoses. The tumor likely grew by infiltration into the epidermis layer, and extended in depth only to subcutaneous muscle layer. Melanin pigmentation could be observed in tumor and interstitial cells, suggesting that the tumors did, in fact, arise from B16 melanoma. As noted above, 36% percent of the animals in group E1 did not show any signs of tumor growth.

The cumulative survival rate in the study is summarized in Fig. 1. Briefly, the average survival time in group E2 was 54.92 days, whereas in control group C2 it was 28.65 days ($P < 0.01$). In contrast, for animals in group E1, the mean survival time was 72.06 days, and in group C1 it was 27.25 days ($P < 0.01$). It should be

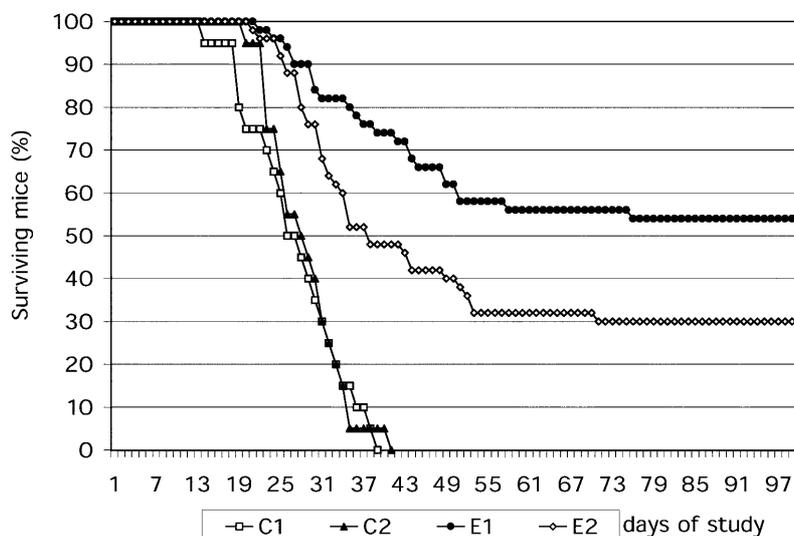
noted that 30% of the animals in group E2 survived through the duration of the study, and 46% survived in group E1 until the end of the study (100 days).

Experimental mice were subject to necropsy analysis. Metastases in the lung were evaluated, and were found to contain melanin with a necrotic core (Fig. 2). Tumor progression was mainly through perivascular and peribronchial lymph vessels. The progression was also observed intravascularly and intrabronchially, however. The lung parenchyma showed hemorrhagic foci in several animals. Similar foci were also observed in the myocardium, brain, kidney, ovary and spleen (see below).

Some animals succumbed to transplanted tumor progression and its regional metastases or massive metastatic dissemination into the lung, resulting in cachexia. In group E1, cachexia induced the deaths of 46% of animals, whereas the entire cohort in group C1 showed massive metastases causing cachexia. On the other hand, in group E2, cachexia induced 68% cases of death, whereas in group C2, it was recorded at 95% (Table 1). In one mouse in group E2 (2%) and in one mouse in group C2 (5%), the cause of death was of cardiac origin without manifestation of the tumorous process progression.

In group E1, primary tumor recurrence was observed in 6% of the cases, and metastasis in the regional lymph

Fig. 1 Cumulative survival of mice bearing B16 melanoma in treated (E1, E2) and control (C1, C2) mice



node was 4%. On the other hand, in group C1, tumors recurred at a frequency of 30%, with a metastasis rate of 25% in the regional lymph nodes. Other relevant data are summarized in Table 1. In group E1, in addition to the metastasis in the lung, remote metastases were observed in ovary and retroperitoneal lymph nodes in 6% of the cases. In group C1, however, with the exception of

the lung, no remote metastasis was observed. In group C2, metastasis in the kidney was observed in only one case (5%). In contrast, in group E2, metastases in the myocardium brain, spleen and ovary were observed in addition to that in the lung (five cases, 10%).

Immunological examination showed no observable difference in the surface markers (CD44, CD54, CD106) on cells harvested from the lung metastasis (data not shown). However, in the ascitic cells (B16 melanoma cells made up 80% of the population), a decrease in the antigenic activity and, in certain cases, the population of elements reacting negatively with antibody against CD44 and CD54, were found in the treated group (E3) in comparison with the control group (C3). Figure 3 schematically depicts typical findings in the “treated” and control animals.

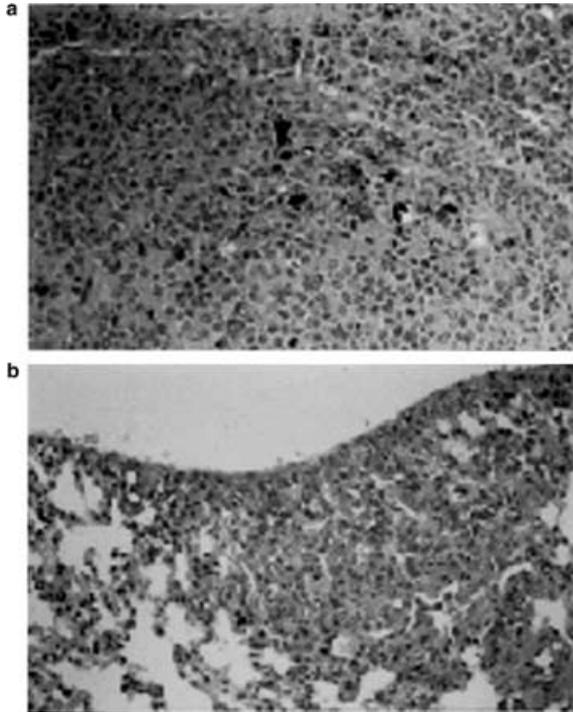
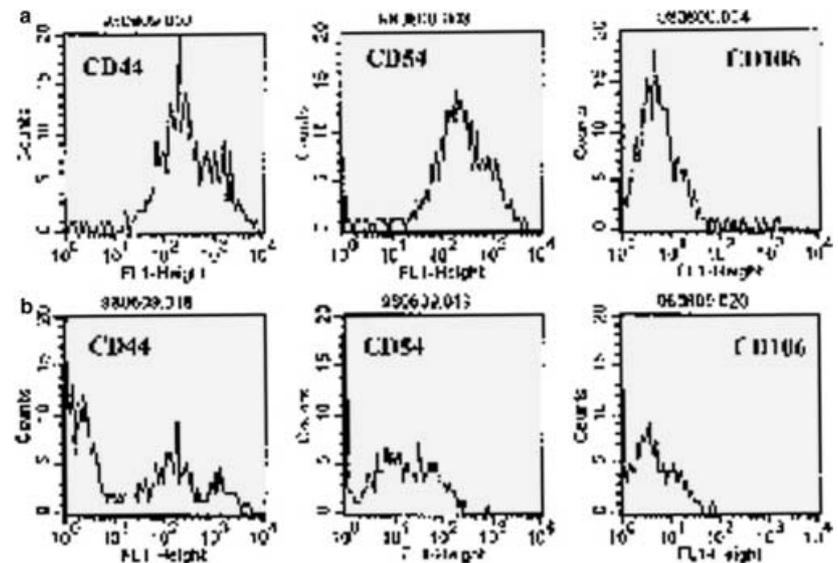


Fig. 2 Typical appearance of **a** B16 pulmonary metastasis (high-power magnification). Anaplastic tumor composed of irregular oval cells with pleiomorphous and pleiochromatic nucleoli; many mitoses present high aggressivity of the tumor. Intracellular melanin storage is seen in the figure. **b** Subpleural pulmonary micrometastasis of B16 melanoma (high-power magnification)

Fig. 3 Typical immunological profile of **a** B16 melanoma after 7-day passage in abdominal cavity of $C_{57}Bl_6$ mice (control group), and **b** B16 melanoma after 7-day passage in abdominal cavity of $C_{57}Bl_6$ mice (treated group). In the population of cells after treatment with a mixture of trypsin, chymotrypsin, and papain, a certain population of cells non-conjugated with CD44 and CD54 antibody appears



Discussion

Serine and cysteine proteases have previously been shown to suppress tumorigenesis. Thus, Taussig and Batkin documented anti-proliferative activity of bromelain in several model systems, including V2 carcinosarcoma, Brown-Rearce carcinoma in rabbits, Walker carcinoma and Yoshida sarcoma in rats, and Lewis pulmonary carcinoma in mice [1, 37, 38]. Rectally dispensed mixture of proteases, such as trypsin, chymotrypsin and papain, inhibit metastasis of the Lewis pulmonary carcinoma in the $C_{57}Bl_6$ inbred strain of mice. Furthermore, this enzyme preparation also shows improvements in diagnostic parameters, and prolongs the survival time in spontaneous T-lymphoblastic leukemia rat model SD/Ipcv [47, 48]. In addition, anti-metastatic properties of proteases have been described previously [11, 46]. These findings can be explained by a non-specific increase in the toxicity of NK cells [11, 5, 8, 49]. The anti-metastatic potential of proteases has also been attributed

to their ability to modulate the expression of certain adhesion molecules on the tumor cell surface.

The effect of trypsin, chymotrypsin and papain on the functional expression of adhesion molecules has been published. Thus, Lehmann et al. investigated 21 types of molecules on T-lymphocytes using trypsin as a biological response modifier, and reported selective reduction in the density of CD4, CD44, and CD80 [26]. Likewise, Kleef et al. provided evidence that bromelain reduced CD44 and CD62L populations on T-lymphocytes and NK cells [21]. Harrach et al. studied the effect of bromelain on adhesion molecules, and found that it decreased CD44 density in MOLT 4/8 cells of the human leukemia and SK-Mel 28 melanoma cells [14]. The work of Grabowska et al. showed a decrease in vitro in CD44 on B16F10 mouse melanoma upon exposure to bromelain and papain [11].

Among adhesion molecules, CD44 is considered to play a critical role in metastasis [7, 10, 15, 34]. Thus, reduction in the density of CD44 on the surface of tumor cells is expected to control metastasis [30, 31]. This can be achieved either by blocking CD44 activity with monoclonal antibodies, or by the action of proteases, as the present study attempts to demonstrate with B16 melanoma in C₅₇Bl₆ mice. As the results of this study show, a protease combination of trypsin, chymotrypsin and papain leads to a decreased density of CD44 on tumor cell surface.

One plausible mechanism of action of proteases may be the proteolytic cleavage on the cell surface proper to restrict the functional expression of adhesion molecules. This action, however, can be mediated indirectly as well. The proteinase-activated receptor 2 (PAR2) is a potent effector of certain signaling mechanisms [3, 27]. Activation of PAR2 with trypsin may induce the mobilization of arachidonic acid metabolism in the intestinal cells, and secretion of prostaglandins. By the same token, intracellular calcium is mobilized and IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) expression is induced [23, 45]. In MIA PaCa-2 cells, an in vitro model system for pancreatic carcinoma, trypsin and its agonist peptides mobilize the Ca²⁺ pool, resulting in a transient increase in IP₃ and translocation of protein kinase C (PKC). A concomitant decrease in DNA synthesis was also observed. Based on these findings, the authors assumed that PAR2 could potentially regulate and suppress tumor growth [19]. This is corroborated by the work from this laboratory, that revealed that the mixture of trypsin, chymotrypsin and papain showed antiproliferative potential for a well-defined period of time in human carcinoma and carcinoma of the tonsil (unpublished observations).

In light of these findings, it is reasonable to deduce that serine and cysteine protease activity could possibly regulate certain intracellular regulatory pathways. The caveat thereby is that the mode of action of these proteases has not been satisfactorily explained to-date. Thus, the role of such mechanisms in the plasma or in the intracellular milieu has yet to be fully elucidated. It is

quite conceivable that in cases of inflammation and/or in tumor pathology, protease inhibitors [e.g., tumor-associated trypsin inhibitor (TATI), pancreatic-secretory-trypsin inhibitor-1 (PSTI-1), tissue inhibitor of matrix proteinase-1 (TIMP-1)] may perturb the steady-state equilibrium and, consequently, contribute to pathology [29, 33, 41]. Metalloproteinases, on the other hand, reportedly play a rather different role in inflammation and tumorigenesis [18, 28, 29, 39, 42–44]. Specific matrix metalloproteinases (MMP), which are expressed in tumor cells, or in chemotactic response to inflammatory allergens, do impinge upon tumor progression, albeit at relatively high concentrations. Metalloproteases primarily affect invasive and infiltration processes in tumorigenesis, however. Since dissemination of malignancy from the primary focus to remote tissues is under immunological control, additional regulatory mechanisms must play a role, including substrate specificity of proteases. The protease activity, however, may be considerably inhibited in case of overproduction of protease inhibitors in frank tumorigenesis [41]. Thus, increasing proteolytic activity by administration of serine and cysteine proteases to stimulate the cell to jumpstart its anti-tumorigenic potential may well counteract regulatory networks.

Immune surveillance by proteases would most likely affect, first and foremost, tumor cells in the bloodstream, and those cells that have not yet been anchored by intracellular and stromal adhesion. This working hypothesis is based on the findings of this study that proteases play a preventive role in tumorigenesis by inhibiting primary tumor formation after transplantation of B16 melanoma. This study does not address the issue of remote micrometastases, which need to be demonstrated. Nonetheless, the data show rather convincingly that proteases limit tumor dissemination effectively.

Conclusion

Administration of a combined trypsin, chymotrypsin and papain regimen exerted anti-metastatic effect in C₅₇Bl₆ mice subsequent to intracutaneous inoculation of B16 melanoma cells. As such, this mixture of enzymes can be evaluated as a potential therapy for biological response modification in the treatment of cancer.

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