Implication of caspase-3 and granzyme B expression and activity in spleenocytes of ehrlich ascites carcinoma mice subjected to immunotherapy

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Abstract
Background Caspase-3 and granzyme B were claimed as apoptotic manipulative enzymes. Aims The present study was to determine the enzymes expression and activity in cancer and cancer immune therapeutic status and the possible association to cancer common pathological signs targeting the improvement of therapeutic conditions. Material and Methods Mice were immunized with cell lysate or cell lysate + CKI in the right thigh and challenged with live cells of ehrlich ascites carcinoma (EAC) in the left thigh. The expression and activity of both enzymes in the spleenocytes derived from different subjects (normal, EAC and cell lysate or cell lysate + CKI immunized mice) after cultured with EAC viable cells were determined by colorimetric assay and western blot analysis. In addition, the subjects DNA ladder and serum metalloproteases (MMP 2 and 9) zymography were observed. Results The experimental data revealed over expression of caspase3 and granzyme B in the groups of cell lysate or cell lysate + CKI immunized mice compared to control while down expression were recorded in the EAC subject. The over expression of the 2 enzymes were accompanied with increases in the activities of caspase3 and granzyme B, changes in DNA fragmentation and inhibition of metalloproteases. Conclusion It could be suggested that, the parameter estimation within the present experimental framework could identify the efficiency of therapeutic vaccine protocols and elucidate the impact of CKI adjuvant with vaccines therapy.

Keywords: Enzyme expression, MMPs, DNA ladder, apoptosis, CKI.

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Introduction
A key focus of biochemistry is determining the activity and function of enzymes, since every biochemical pathway depends on enzymes for catalysis. Enzymes are also important in drug discovery and development. Many of cancer therapeutic protocols include the work via triggering apoptosis (programmed cell death) of cancer cells. The therapeutic vaccination is one of the recent promising approaches in cancer treatments that evoked apoptotic process during clearing malignancy. The immune system may be particularly well suited to clear small number of residual tumor cells that may be poorly eradicated by radiotherapy and chemotherapy [1]. Caspases and granzymes are enzymes that often have critical roles in mammalian apoptosis or proteolytic activation of cytokines [2]. Caspases (cysteine aspartates) are specialized proteases that have cyteine at their active site and cleave on the C-terminal side of aspartate residues [3]. Granzymes, a family of serine proteases are expressed exclusively by cytotoxic T-lymphocytes and NK cells, which are components of immune system that protect higher organisms against viral infection and cellular transformation [4]. Granzyme B is a potent cytotoxic protein that is released from mammalian NK and CTLs following noxious stimuli, including foreign invaders [5]. Nonetheless, the non apoptotic cell death previously
labeled necrotic, and thus assumed to be passive cell death. Some forms of necrotic cells with semantic issues, could twist to be programmatic (necrosis like or programmed necrosis) [6].

The study of the multistep process of tumorigenesis demonstrated a sustained, successive accumulation of genetic, biochemical, and immune abnormalities during the disease progression before the appearance of clinically determinable tumors [7]. The conditions of lymphocytes membrane and cytoplasm differ structurally and functionally between individuals with and without malignancies [8]. The specific tumor-associated antigens induce mitogenic stimulation of peripheral lymphocytes resulting in intracellular microviscosity changes, which are manifested in a characteristic kinetic behavior. Moreover, these changes in microviscosity precede other alterations due to activation, such as gene, receptor, and protein expression [9]. The present study was to articulate the spleenocytes capase3 and granzyme B kinetic behaviors in response to therapeutic vaccination with/without cancer therapeutic adjuvant (creatine analog). The correlation of enzymes kinetic behaviors to cancer common signs will be intended in this work aiming to establish a valuable additional criterion for monitoring and prognosis individuals with cancer and high cancer risk.

Materials and Methods

Experimental animals
Female outbred Swiss albino mice (20-25g) were used as experimental animals. Animals were kept under optimized conditions along the experimental period. Animal experimentations were consistent with the guidelines of ethics by Public Health Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) in accordance with the recommendations for the proper care and use of laboratory animals approved by Animal Care Committee of the National Center for Radiation Research and Technology (NCRRT), Cairo, Egypt.

Ehrlich ascites carcinoma (EAC)
The Ehrlich cell line was acquired as a gift from the National Cancer Institute, Cancer Cell Biology Department. Inoculation of ehrlich cells into the intraperitoneal cavity was carried out to develop the ehrlich ascites carcinoma in mice.

Tumor vaccine protocol
Ehrlich from mice with well developed ascites was subjected to cell lysing process [10]. Cells were incubated with 0.01 % EDTA solution for 10 minutes followed by washing twice in phosphate buffer saline; PBS (2.17g Na₂HPO₄, 0.1 g KH₂PO₄, and 7.1 g NaCl were dissolved in distilled H₂O and the volume was made to 1L, pH 7.3). The washed cells were re-suspended in serum-free medium (0.8 ml Na₂HCO₃ and 1 mL of streptomycin–penicillin antibiotics were contained in 100 ml of RPMI media) at a density of 5x₁₀⁶/mL. The cell suspensions were frozen at -80°C and disrupted by four freeze-thaw cycles. For the removal of crude debris, the lysate was centrifuged for 10 minutes at 300xg. Mice were injected weekly in the right thigh by 0.2 mL of cell lysate supernatant (subcutaneously; S.C. for three successive weeks). Mice were challenged with live cells 2x₁₀⁶ in the left thigh. Control mice were injected with EAC cells parallel with the challenge process.

Creatine kinase inhibitor (creatine analog)
The compound was purchased from Sigma Company. The β-guanidinopropionic acid (a creatine analog (Cr) or creatine kinase inhibitor) was used as adjuvant in human cancer treatment in a concentration of 0.5mg/kg body weight [11]. The human dose was extrapolated to mice. The immunized mice with creatine adjuvant received 0.002g CKI contained in 0.5 ml distilled water via i.p. injection for every 20g of body weight.

Experimental procedures
Mice were divided into 4 groups (n=10) with different vaccine treatments. Normal mice (normal), EAC injected mice (+ve control), cell lysate immunized mice (lysate) and cell lysate + CKI immunized mice (cell lysate + CKI). Mice of each group were examined using the classical methodology of measurements and analysis of caspase3 and granzyme B. Spleenocytes from different immunized groups were cultured with EAC viable cells for 24 hours before measuring the specific activities of the caspase3 and granzyme B [12, 13] at different substrate concentrations.

Western blotting analysis of caspase-3 and granzyme B
Protein concentration in each cell lysate sample was determined using Bradford reagent for protein quantification [14]. Immunoblot analysis was used to determine protein production of caspase3 and granzyme B. The cell lysates were electrophoresed on 12 and 8% SDS-PAGE for detection of granzyme B and caspase3 (20ug protein per lane). The proteins were transferred to nitrocellulose membranes (Amersham, UK). The membranes were subsequently pre blocked in T-TBS (TBS containing 0.1% Tween 20) containing 5% nonfat milk powder (blocking buffer) and then incubated with the primary antibody (goat polyclonal anti-caspase3 antibody and goat polyclonal anti-granzyme B antibody were purchased from Santa Cruz Biotechnology, Inc.) in blocking buffer at dilution of 1:100 overnight at 4°C, followed by incubation with rabbit anti-goat IgG antibody conjugated to peroxidase. The antigen–antibody complex was visualized with Western blotting luminol reagent (ECL, Santa Cruz Biotechnology, Inc.). The bands were quantified with Gel Doc 2000 system and Quantity One software (BIO-RAD).

The DNA fragmentation (DNA Ladder) as a key feature of programmed cell death was identified in different subjects’ spleenocytes EAC cultures [15]. The concentration of MMP-2 and MMP-9 was estimated by using polyacrylamide slab gel electrophoresis [15]. All of the values are presented as average ± SE. The comparisons between means were performed by one way ANOVA.
Results
The average specific activities of the two apoptotic manipulating enzymes in spleenocytes EAC culture samples of different immunized mice are presented in Table 1. The experimental data revealed that the activity of caspase-3 and granzyme B in spleenocytes EAC culture of EAC mice were significantly increased (P<0.05) as compared with their equivalent in normal spleenocytes EAC culture.

Table 1 The specific activity of apoptotic manipulating enzymes (U/mg protein) in different sample groups when 2 µmol substrate (acetyl-asp-glu-val-asp-pnitro anilide) concentration.

| Enzyme     | Different Subjects | EAC       | CL       | CL + CKI |
|------------|--------------------|-----------|----------|----------|
| Caspase-3  | N                  | 901±38    | 1234±52  | 1388±63  |
| Granzyme B | N                  | 11.2±0.56 | 26.3±1.72 | 35.7±2.8 |

Each value represents the mean of 6 records ± S.E. Means with dissimilar superscript letters within the same row are significantly different at p<0.05. N: spleenocytes of normal mice cultured with EAC viable cells, EAC: Spleenocytes of EAC bearing mice cultured with EAC viable cells, CL: spleenocytes of cell lysate immunized mice cultured with EAC viable cells, CL+CKI: spleenocytes of mice immunized by cell lysate and injected with creatine kinase inhibitor cultured with EAC viable cell.

Table 1.

Fig. 1 Western blotting analysis for caspase-3
Caspase 3 expression of control subject (1), EAC subject (2) cell lysate immunized subject (3) and cell ystate + CKI (4). Caspase 3 was over expressed (detection of active caspase-3; cleaved form) in cell lysate immunized subjects especially when cell lysate immunization was associated with CKI adjuvant whereas, down regulation of the enzyme expression was observed in EAC subject. Data were normalized with B actin expression.

However, the activities of caspase-3 and granzyme were significantly increased in spleenocytes EAC culture obtained from cell lysate immunized mice and cell lysate+CKI immunized mice as compared with the subject of EAC mice. The highest increases of enzyme activities were recorded in cell lysate + CKI immunized mice spleenocytes. Western blotting analysis for caspase-3 expression revealed enzyme over expression (detection of active caspase-3; cleaved form) in cell lysate immunized subjects especially when cell lysate immunization was associated with CKI adjuvant. The enzyme expression was down regulated in EAC subject (Fig.1). In addition, the expression of Granzyme B was up regulated in cell lysate and cell lysate+CKI subjects compared to EAC subjects (Fig. 2).

Fig. 2 Western blotting analysis for Granzyme B
Granzyme b expression of control subject (1), EAC subject (2) cell lysate immunized subject (3) and cell ystate + CKI (4). Granzyme B was over-expressed in cell lysate immunized subjects especially when cell lysate immunization was associated with CKI adjuvant, whereas down regulation of the enzyme expression was observed in EAC subject. Data were normalized with B actin expression.

Fig. 3 Apoptosis DNA fragmentation (DNA ladder)
Supernatants of normal and immunized spleenocytes EAC cultures analyzed by 1% agarose gel electrophoresis following lysis and centrifugation at 16000 g. DNA in gel after electrophoresis was visualized by ethidium bromide staining. DNA Marker (50:400bp; lane 1), Normal DNA ladder (lane 2), DNA ladder of EAC mice spleenocytes cultured with EAC viable cells of (lan3), DNA ladder of cell lysate immunized mice spleenocytes cultured with EAC viable cells (lane 4). DNA ladder of cell lysate + CKI mice spleenocytes cultured with EAC viable cells (lane5).
The DNA fragmentation assessment demonstrated the presence of DNA ladder in normal and cell lysate and cell lysate creatine kinase inhibitor subject (Fig. 3). Absence or slight DNA fragmentation was noticed in EAC subject.

**Discussion**

Apoptosis is a highly regulated process of cell death. Loss of apoptotic regulation is closely linked to human diseases including various forms of cancer, ischemic damage, neurodegenerative diseases and immune disorders [16]. Caspases and granzymes are enzymes that often have critical roles in mammalian apoptosis or proteolytic activation of cytokines [2]. Mammalian cell death is induced through chromosomal DNA damage by ionizing radiation, ultraviolet (UV) radiation, anticancer drugs and various triggers of apoptosis. The caspase-3 and granzyme B are thensensors of apoptosis. The results obtained reveals a significant increase in caspase-3 and granzyme B activities in culture of EAC spleenocytes and EAC viable cells associated with expression upgrading of the 2 enzyme (Figs. 1, 2). In addition, the DNA ladder of EAC subject demonstrated absent of DNA fragmentation.

According to cancer features the anticipated results is the inhibition of apoptotic sensor enzymes. The increases in activities of the 2 enzymes could be due to the genuine cellular immune response that triggers the activation of existing inactive form of enzymes. Caspase-3, a cysteine-aspartate protease, is involved in the execution pathway of apoptosis [1] under normal cellular conditions caspase-3 exists in inactive zymogen form. Upon oxidative stress, procaspase-3 is autoproteolytically cleaved resulting in the formation of p20 and p10 domains [17] leading to assembly of a homodimer of heterodimers which represents the active form of the enzyme [18]. Whereas granzymes is a family of serine proteases is contained within the cytoplasmic granules of cytotoxic lymphocytes (CLs), and the pore-forming protein, perforin. According to the model of granule-mediated apoptosis, killing involves degranulation and subsequent transfer of these proteases into the cytoplasm of the target cell, where they rapidly induce apoptosis [19].

The apoptosis inducer granzyme B is released from the granules of cytotoxic lymphocytes and natural killer and it is the most abundant protease contained within cytoplasmic granules [20]. Once released from cells, granzyme B forms a macromolecular complex with perforin and serglycin [21], penetrates into the cytoplasm of target cells and triggers apoptosis by three different pathways: (i) activation of procaspases, (ii) direct cleavage of intracellular targets, and (iii) induction of the mitochondrial pathway of apoptosis [22]. Activation of procaspases by granzyme B results in the formation of active enzymes (caspases), which include initiator (caspase-8) and effectors' (executioner) caspases (3, 7 and 9). Effectors’ caspases cleave intracellular targets (cytoplasmic and nuclear proteins) leading to subsequent cleavage of DNA. These processes collectively lead to apoptotic cell death. The increases reported in caspase3 activity could be brightly considered a cascade of granzyme B activation. The purposed involvement of granzyme B-in induction of cell death via activation of caspases [17] could be interpreted in the view of parallel increase in the two enzymes expression (Figs. 1, 2). The
increased enzymes activities presumed the increase of active enzyme on the account of proenzymes in addition to enhancement of enzymes expression.

However, there is mounting evidence that granzyme B can also kill cells via a caspase-independent pathway [23]. The serine protease and the caspases appear to cleave some of the same cellular substrates, resulting in the demise of the cells [24]. This mechanism has presumably evolved to facilitate killing of cells infected with viruses that encode caspase inhibitors.

The increases in apoptosis in EAC subject was coupled with the absent of DNA ladder in spleenocytes EAC culture of EAC mice and the appearance of MMP 2 and 9 active bands (Figs.3, 4). The DNA ladder (DNA fragmentation) is a feature of programmed cell death. Two distinct patterns of cell death have been identified based on the morphology of dying cells, and on the DNA fragmentation or damage. These have been termed necrosis and apoptosis [25]. Probably, the present DNA fragmentation is associated with the increases in apoptotic enzymes activities. However, the absence of DNA ladder in spleenocytes EAC culture of EAC mice hypothesizes the impermanent act of caspase3 and granzyme B based on the down regulation of the two enzymes expression as observed in the present study (Figs. 1, 2).

In addition, the majority of EAC sera show stronger intensity of gelatinolytic bands for both the latent enzymatic forms of MMP-2 (66 kDa) and MMP-9 (86 kDa) when compared with normal sera (Fig. 4). The qualitative analysis of the zymographs showed that the majority sera samples from EAC bearing mice displayed sharp bands of lysis corresponding to the latent forms of MMP-2 and MMP-9, respectively, in contrast to the control samples which showed less pronounced lytic bands. In addition, the sera samples from EAC mice exhibited two other lytic activities of larger molecular size, similar to those previously identified as active MMP-9 dimers (92 kDa) as a complex active MMP-2 (72 kDa). Monitoring serum metalloproteinase levels has potential for forecasting tumor aggressiveness and response to therapy [26].

A critical event, during progression of malignant carcinomas, is the invasive growth of neoplastic cells into the host tissues: this involves the onset of a number of complex interactions occurring at the tumor–host interface, including an extensive remodeling of the extracellular matrix (ECM). Degradation of the ECM requires the concerted action of a number of extracellular enzymes. Several enzyme families are known to be involved in extracellular proteolysis. These include serine proteases [27] matrix metalloproteinases [28] and disintegrin-metalloproteinase [29]. The enzyme activity is regulated extracellular and its regulation is mainly based on the balance between pro-enzyme activation and inhibition by tissue inhibitors of MMPs (TIMPs). MMPs, specifically MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are responsible for degradation of type IV and V collagen, elastin and fibronectin. MMP-2 is constitutively expressed and participates in the remodeling of the ECM, promoting the angiogenesis in the tumor tissue and facilitates the invasion and metastasis.

In the present work the evidence of developed tumor model was used to study the potential modulator effects of cell lysate immune vaccination and creatine analog adjuvant immune vaccination. This therapeutic strategy resulted in increased activities of caspase-3 and granzyme B associated with enzymes expression up regulation when compared with normal control or EAC subjects (Tables 1, Figs. 1, 2). Also, the gelatinase zymograph as well as activities measurements revealed the inhibition of MMP2 and MMP9 expression in the two therapeutic challenges (Table 2, Fig. 4).

The results demonstrated the reflex of enzyme expression and the category of enzymatic activity on the DNA fragmentation of the two challenge therapeutic models where the obvious appearance of DNA ladder compared to the EAC subject (Table1, Fig.3). It could be mentioned that, the injection of cell lysate or cell lysate + CKI has directed the cancer cells towards programmed cell death. The killing of tumor cells by anticancer therapies commonly used in the treatment of cancer, e.g. chemotherapy, gamma-irradiation, immunotherapy or suicide gene therapy, is predominantly mediated by triggering apoptosis, the cell’s intrinsic death program [30].

Activation of the proteolytic cascade involving caspase family members is a critical component of the execution of cell death in apoptotic cells. Active caspases cleave numerous intracellular substrates activating or deactivating them resulting in various nuclear and cytoplasmic alterations which culminates in DNA fragmentation and cell death [31]. The granzyme B not only activates pro-death functions within a target, but also has a previously unidentified role in inactivating pro-growth signals to cause cell death [32]. The zymographic analysis of the serum matrix metalloproteinase (MMP2 and MMP9) pointed out to the depletion in gelatienase activities of the cell lysate or cell lysate + CKI challenged mice as matched to their equivalent in EAC mice (Table 2, Fig4). This observation together with the apoptotic stimulation hypothesized the carcinoma immunotherapeutic action of cell lysate or cell lysate + CKI.

The apoptotic modulation observed could be correlated with the disturbances occur in the MMP system after vaccination. The activity of MMPs is specifically inhibited by tissue inhibitor of metalloproteinases (TIMP). The inhibitory effects of TIMP on tumor progression are not only due to their ability to inhibit MMP activity, but also because of their ability to directly modulate the cell growth and apoptosis of tumor cells, as well as host endothelial cells [33]. The invasive carcinomas of no special type, with the most increased aggressiveness, display the highest levels of MMP-9 expression and
activity, the lowest level of TIMP-1 and the highest ratio values for MMP-9 /TIMP-1. The MMP-9 and TIMP-imbalance could be involved in the configuration of invasive carcinoma of no special type [34]. The matrix degrading enzymes and their inhibitors regulate the integrity and remodeling of extracellular matrix components as well as the cleavage of non matrix proteins such as cell surface molecules and growth factors. Thus, these metalloproteinases and TIMPs indirectly regulate diverse cellular processes including cell proliferation, differentiation, migration and apoptosis [35].

Conclusions
The present data suggest that the measurement of apoptotic manipulating enzymes activation and expression together with the DNA ladder and MMPs activities and zymography may demonstrate a valuable discriminating ability for the cancer therapeutic competence and support the hypothesis that the increased production of gelatinases disputes the apoptosis of cancer cells.

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