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Original article

Antitumor and immune-modulatory efficacy of dual-treatment based on levamisole and/or taurine in Ehrlich ascites carcinoma-bearing mice

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ABSTRACT

Many alternative and complementary therapies for cancer have been reported. The objective of the present work is to examine antitumor and immune-modulatory properties of dual-treatment based on levamisole (Lms) and/or taurine (Tau) in Ehrlich ascites carcinoma-bearing mice. In the current study, Lms (10 mg/kg; subcutaneously) and Tau (640 mg/kg; intragastrically) was administered alone or as a dual-treatment. Lms or Tau was administered in combination with cyclophosphamide (CTX) (100 mg/kg; intraperitoneal) in mice bearing Ehrlich ascites carcinoma. Treatment with CTX or (Lms plus Tau) significantly reduced the ascitic tumor cell count, percentage of tumor cell viability while elevated the tumor inhibition rate and apoptosis percentage compared to non-treated animals. Dual-treatment (Lms and CTX) or (Tau and CTX) significantly potentiated the reduction of the ascitic tumor cell count, viability and augmented the tumor inhibition rate and apoptosis percentage compared to CTX-treated mice. Dual-treatment of (Lms plus Tau), (Lms plus CTX) or (Tau plus CTX) altered splenocytes immunological profile of $CD3^+CD4^+$, $CD3^+CD8^+$, $CD4^+CD25^+$ and $CD11b^+Ly6G^+$ cells in order to achieve better immune surveillance against tumor cells. In conclusion, dual-treatments based on Lms and/or Tau are promising therapies for cancer, not only due to its abilities to induce apoptosis in the tumor cells and modulate the immune response against them, but also due to its capabilities to potentiate the chemotherapy anticancer efficacy and minimize its adverse effects.

1. Introduction

Several million death cases due to cancer have been registered by worldwide agencies and organizations in the period between 2008 and 2012 [1,2]. In general, cancer treatment was primarily depending on chemotherapy and radiation. Treatment of cancer by these ways is accompanied by various disadvantages such as lack of killing specificity which badly affect the normal cells (including the immune cells), high risk of cancer cells recurrence and low success rates. Therefore, many alternative and complementary approaches, including immuno-modulatory agents and anti-tumor immune response strategies have been reported [3–7].

Studies on the immuno-modulatory mechanisms of synthetic and natural agents have been encouraged not only due to the increased interest in fighting cancer, but also in order to meet the considerable need for therapeutic substances that could modulate the different immunodeficiency patterns [8,9]. Many reports have been studied the possible use of immuno-potentiators/modulator in the treatment of malignant tumors [10–12].

The immune-modulatory roles of levamisole (Lms) and taurine (Tau) have been revealed previously [13–19]. Lms, an anti-helminthic drug, is exhibiting immuno-stimulant properties and antitumor effects [20–23]. Ramanadham and Nageshwari [7] demonstrated the Lms anti-proliferative effect on human myeloma cell lines in vitro. Tau, 2-aminoethane sulfonic acid, is a β amino acid that present in different mammalian cell types including neutrophils, lymphocytes and monocytes [24]. As an antioxidant, Tau plays a critical role in scavenging free radicals, reducing formation of malondialdehyde, lipid peroxidation and stabilizing membranes [25–27]. Several reports have revealed that Tau exerts certain inhibitory effects on tumors and promotes immunological functions [15,16,28–30]. Tau resulted in the induction of apoptosis and anti-proliferation properties in human colon cancer cells and HepG2 cells [31,32].

A spontaneous murine mammary adenocarcinoma, Ehrlich ascites carcinoma (EAC), is a common model to study the antitumor efficacy of different agents as it has high transplantable capability, shorter life

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span, rapid proliferation, no-regression and 100% malignancy [33,34]. Cyclophosphamide (CTX) is an alkylating agent widely used in the treatment of many kinds of tumors, including breast cancer [35–37]. However, its clinical uses are restricted by its detrimental adverse effects, including, immunosuppression, hepatotoxicity and nephrotoxicity. Seeking for efficient and safe alternative therapy is a concern of many researchers around the world. In this regard, the current study investigated the potential antitumor role of dual-treatment based on Lms plus Tau in EAC-bearing mice. Moreover, this study examined the potential immune-modulatory roles of Lms or Tau after combination with CTX in EAC-bearing mice.

2. Materials and methods

2.1. Experimental animals

Female Swiss Albino mice (weighting approximately 20–22 g) were obtained from the holding company for biological products and vaccines (VACSERA), Cairo, Egypt. All animals were kept under planned laboratory conditions with a 12 h dark/light cycle. Standard rodent food and clean water were supplied *ad libitum*. The animals were acclimatized to laboratory condition for at least twelve days before the beginning of the experiments. The animals were used after consent of the Institutional Animal Ethical Committee, Menoufia University (approval ID: MUFS/F/HE/1/16).

2.2. Reagents

Tau and CTX was obtained from Sigma (Sigma, St Louis, CA, USA). Lms hydrochloride was purchased from ADWIA Company, Egypt. Concanavalin A (ConA) from *Canavalia ensiformis* [5 mg/ml stock solution in RPMI 1640 medium] were purchased from Sigma (Sigma, St Louis, MO, USA). Fluorescein isothiocyanate (FITC)-labelled anti-mouse CD3 mAb (clone: 17A2), FITC-labelled anti-mouse Ly6G mAb (clone: RB6-8C5), Phycoerythrin (PE)-labelled anti-mouse CD25 (clone: PC61), Allophycocyanin (APC)-labelled anti-mouse CD4 mAb (clone: RM4-5), APC-labelled anti-mouse CD11b mAb (clone: M1/70), PE.Cy5-labelled anti-mouse CD8 mAb (clone: 53-6.7), (Fc Block™) were obtained from BD Bioscience company (BD Bioscience CO, USA). All other chemicals and reagents were of the highest purity available.

2.3. Ehrlich ascites carcinoma and experimental design

EAC cells were purchased from the National Cancer Institute, Cairo University, Egypt, and maintained by means of bi-weekly serial intraperitoneal (i.p.) transplantation of 0.25×10^6 viable tumor cells in 200 µl of saline into female Swiss albino mice (9–10 weeks old). Tumor cell viability was evaluated using Trypan blue test and then counted by hemocytometer (cell viability was > 97%) before injection into naïve mice. Viable EAC cells (0.25×10^6) were injected i.p. in 49 Swiss female mice selected for the experiment on day 0 according to Abdel Salam et al. [38]. Eight groups contained 7 animals per each were divided as follows:

Group I: Normal control received 200 µl of saline; i.p.,

Group II: EAC-bearing mice without treatment received EAC cells 0.25×10^6 cells in 200 µl saline/mouse on day 0 as previously mentioned,

Group III: Positive control CTX (100 mg/kg; i.p.) injected on day 1 [39],

Group IV: Lms (10 mg/kg; subcutaneously) injected two days before and after EAC injection day -2 and then day 2 [20],

Group V: Lms (10 mg/kg; subcutaneously) plus CTX (100 mg/kg; i.p.) injected as the same as groups III and IV,

Group VI: Tau (640 mg/kg; intragastrically) administrated consecutively from day 1 to 8 [40],

Group VII: Tau (640 mg/kg; intragastrically) plus CTX (100 mg/kg; i.p.) injected as the same as groups III and VI,

Group VIII: Lms (10 mg/kg; subcutaneously) plus Tau (640 mg/kg; intragastrically) administrated as the same as groups IV and VI,

On day 9, blood was collected using orbital bleeding, mixed with EDTA and animals were sacrificed and ascitic fluid was collected from the peritoneal cavity of each mouse for the evaluation of tumor cell number/status. Tumor growth inhibition percentage was calculated by comparing the total number of tumor cells present in the peritoneal cavity of treated groups and the control group as on day 9 of the experiment. Tumor cell growth in saline-treated group (normal control) was taken as 100 percent cell growth [41].

2.4. Haematological analysis

White blood cells (WBCs), total and relative differential counts, were detected manually using blood samples mixed with EDTA as described previously [42].

2.5. Spleen cells phenotypes

Spleen cell suspensions were prepared and counted using a hemocytometer as described previously [43], then the cell viability were examined by trypan blue dye exclusion test. T-helper $CD3^+CD4^+$, T-cytotoxic $CD3^+CD8^+$, T-regulatory $CD4^+CD25^+$, natural killer $CD3^-CD8^+$, and myeloid-derived suppressor $CD11b^+Ly6G^+$ cells were quantified by flow cytometry. After incubation with the indicated conjugated mAbs for 30 min on ice, cells were post-fixed with 300 µl of Cell Fix 1× (BD, Biosciences) and kept at 4 °C in the dark. Surface marker expression was analyzed with BD FACS Canto II flow cytometer using BD FACS DIVA™ software (BD Biosciences).

2.6. Proliferation assay

Tumor and spleen cells proliferative responses to mitogen Con A were determined by a micro-tissue culture system as described by Ibrahim et al. [43] and modified by Hassouna et al. [44]. Cells were suspended in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma). Cells (5×10^5) from treated or control mice were cultured for 24 h with Con A. Then 10 µl of Cell Counting Kit-8 (Sigma, St Louis, MO, USA) reagent was added. After 3 h incubation at 37 °C in 5% CO_2, the optical density was determined at 450 nm (Seac, Radim Company, Italy).

2.7. Apoptosis detection

In order to discriminate viable, early apoptotic and late apoptotic cells, the tumor cells were washed and incubated in PBS (137 mM sodium chloride, 10 mM phosphate, 2.7 mM potassium chloride; pH is 7.4) containing 30% human AB serum, at 4 °C for 30 min, prior to staining with Annexin V-FITC and propidium iodide (PI) (at 25 °C for 15 min) using a commercial kit according to the manufacturer's instructions (Abcam, Canada). The cells were analyzed by flow cytometry within one hour of staining, and the percentage of cells undergoing apoptosis was determined [45].

2.8. Statistical analysis

Data are expressed as the mean ± standard deviation. For statistical analysis, the SPSS (IBM SPSS statistics for Windows Version 22, Armonk, NY) computer program was used. Data were evaluated by ANOVA test followed by post hoc analysis of group differences that was accomplished by the least significant differences (LSD) test; $P < 0.05$ was considered to be statistically significant.