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Original Article

Anti-Tumor Effect of *Marshallagia marshalli* Somatic Antigen on Inhibition Cell Growth of K562

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Abstract

Background: Today, the inhibitory effect of helminths on tumor cell growth has been proven. We investigated the anti-tumor activity of *Marshallagia marshalli* somatic against K562 cells.

Methods: Different concentrations of *M. marshalli* somatic antigen were incorporated in the culture medium of K652 cells, and the proliferation and apoptosis were measured after 24, 48, and 72 h, using MTT and Annexin V/PI staining assay.

Results: Treatment of cells with 1-2 mg/ml antigen for 24-72 h could suppress cell proliferation and increase apoptosis. While treating cells with 0.1 mg/ml antigen for 72 h could inhibit cell growth. There was no meaningful effect on treated cells in comparison with the control group ($P< 0.05$) after incubating cells for 24- 48 h with 0.1 mg/ml antigen.

Conclusion: *M. marshalli* somatic antigen had an anti-cancer property, and its role in cancer treatment could be considered as an effective therapeutic method.

Introduction

Cancer is one of the most feared diseases of the 20th century and spreading further with the continuation and growing occurrence in the 21st century. The condition is so disturbing that every fourth person is having a lifetime risk of cancer. Cancer is a group of diseases in which cells grow out of control, and they have the potential invading different organs. Among different types of cancers, leukemia is one of the most common forms of cancer worldwide (1). Scientific investigation has been started for decades to discover an effective anti-cancer drug, and it has become critical to find novel and effective chemotherapeutics.

Chemotherapy is followed by less therapeutic success, especially for those aged above 65 years. Furthermore, some of the most in use drugs, such

as doxorubicin and arsenic trioxide, have insufficient selectivity and many side effects (2, 3).

To reduce detrimental effects of chemotherapy, a range of novel therapies such as mitochondrial inhibitors, are introduced. These therapies include apoptosis therapies, therapies targeting specific oncogenic proteins and cellular immunotherapies (4). Helminthic therapy is another approach that could have anti-tumor properties. Helminths play a significant role in the protection of the infected host against autoimmune diseases (5-7), allergic inflammation (8-10), and even tumors (11, 12).

In Turkey (13), a low incidence of hydatid disease was incidentally found in patients with solid tumors. *E. granulosus* could be employed as an immunotherapeutic strategy against cancer (14).

Darani et al. proved anti-cancer activity of hydatid cyst protoscolices on mouse fibrosarcoma cells and baby hamster kidney fibroblast cells (15). Other parasites with anti-tumor effects are *Toxoplasma gondii* (16), *Trypanosoma cruzi* (17), *Plasmodium yoelli* (18) and *Trichinella spiralis* (19, 20). Concerning the mechanism, extracts of helminths modify signal transduction, which influences cell proliferation and cell death (21- 23). Nematode parasites such as *Trichostrongylus colubriformis*, have inhibitory effects on the proliferation of ovarian epithelial cells and fibroblast cells, and may protect the host against tumor (24).

To recognize cell health and viability, flow cytometry technique, as a powerful tool to analyse cells, was used. In this technique, cells are suspended in a fluid injected into the flow cytometer instrument. These cells, which are labeled with fluorescent markers, pass through a focused laser beam. Then, the scattered light captures from each individual cell to measure chemical, biological and physical characteristics of them (25-27). Flow cytometry test provides us large-sized datasets that should be processed by a computer (28-30).

In this study, we investigated the anti-tumor effect of *M. marshalli* somatic antigens on inhibition K562 cells growth. Apoptosis and, or necrosis might be the probable mechanisms that this parasite applies to inhibit cell growth.

Materials and Methods

Preparation of Marshallagia marshalli antigen

First, a large number of contaminated abomasums were transferred from the industrial slaughterhouse of Mashhad to the Parasitology Laboratory, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Iran. The contents of the abomasums were cleared and poured into a plate containing Phosphate Buffered Saline (PBS). The mature male *M. marshalli* parasites were isolated and identified under microscope according to morphological features.

The parasites were washed with PBS, cut, sonicated on ice, and centrifuged at 100,000× g for 40 min, then, the supernatants were pooled and filtered. The protein concentration of the supernatant was determined by the Bradford method using bovine serum albumin as the standard. Samples were stored in aliquots at -20 °C.

Culture condition and cell proliferation

The human chronic myeloid leukemia (K562) cell lines were obtained from the Pasteur *Institute* of Iran. The cryotube was thawed by moving in a water bath at 37 °C. As soon as all ice was melted, the sample was removed and diluted with RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/ streptomycin (10000 U penicillin/ ml and 10 mg/ml streptomycin). Then, the cell suspension was transferred to tissue culture flasks and incubated for 24h in a humidified atmosphere of 5% CO2 at 37 °C.

To calculate viability, 100 µL of cells were taken into an Eppendorf tube and added 400 µL 0.4% Trypan Blue. 100 µL of Trypan Blue-treated cell suspension was taken and filled both chambers underneath the coverslip. To calculate the number of viable cells/mL, the following formula was applied:

The number of viable cells/mL= The average number of cells counted from 4sets of 16corners squares $x\ 10^4\ x\ 2$ (to correct for the 1:2 dilution from the Trypan Blue addition).

In vitro cytotoxicity assays using worm somatic antigens

The cytotoxicity effect of *M. marshalli* somatic antigen was evaluated using a spectrophotometric assay. 10^5 cells/well were seeded in 96-well plates in medium (containing PRMI 1640 and 10% FBS) in the presence of different concentrations of crude *M. marshalli* somatic extract (0.1, 1, and 2 mg/ml). Control wells, also, received an equivalent amount of target cells without *M. marshalli* somatic

extract. Then, wells were incubated for 24-72 h, at 37 °C and 5% CO2.

Determination of cell survival using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT)

The test for determination of cell survival was conducted by adding 100µl MTT (5 mg/ml in PBS containing 10% FCS) to each well and the cells were incubated for another 4 h. To dissolve the resultant formazan, 100 µl dimethyl sulfoxide was added, and the absorbance values were measured by spectrophotometer at wavelength at 540 nm. To calculate cell survival, the ratio of the absorbance of the treated group divided by the absorbance of the untreated sample, multiplied by 100.

Additionally, to evaluate the cytotoxic effect of *M. marshalli* somatic extract on the normal cell lines, some wells were filled with HFF3 (10^5 cells per well) cultured in PRMI 1640 and 10% FBS, treated with 2 mg/ml *M. marshalli* somatic extract and incubated for 72 h. The addition of MTT and the following steps were taken as mentioned above.

A method for determination of cell survival using Annexin V-FITC & PI staining kit

When 1×10^6 cells/ml were incubated for 24-72h at 37 °C with the 50% inhibitory concentration (IC50) of *M. marshalli* somatic extract (that was calculated 7.54 mg/ml), cells were washed with PBS. Apoptotic cell deaths were determined using FITC Annexin V Apoptosis Detection kit with PI. Briefly, the cells were washed twice with FBS and suspended in 200 µl Annexin V binding buffer at a concentration of 10^5 cells/ml. Cell suspension (195 µl) was transferred to a 5 ml test tube, and 5 µl of FITC Annexin V added to the suspension.

Next, 10 µl of Propidium Iodide Solution was added, and after vortex, it was incubated for 15min in a dark place, at 25°C. Finally, 400 µl of Annexin V Binding Buffer was added to each tube, and the stained cells were analyzed by flow cytometry.

Statistical analysis

Statistical analysis was performed with SPSS 19.0 software (IBM Corp., Armonk, NY, USA). The reported data were expressed as mean ± standard deviation (SD). Statistical comparisons were carried out using one-way analysis of variance (ANOVA). The results were considered statistically significant for $P < 0.05$.

Results

There was a dose-dependent growth inhibition effect on cells treated with *M. marshalli* somatic antigens for 24 h. While 1-2 mg/ml of antigens significantly reduced cell viability, 0.1 mg/ml of antigens had no meaningful effect on the treated cells in comparison with the control group ($P< 0.05$). Incubating cells for 24 h with 0.1, 1 and 2 mg/ml antigens reduced mean of cell viability. Similarly, after 48-72h incubation, cell viability in the treated group decreased progressively. Results for treated cells with 0.1 mg/ml antigens showed a meaningful decrease after 72 h compared with those treated with 1 and 2 mg/ml antigens ($P< 0.05$). The results of the treatment with different concentrations of *M. marshalli* somatic antigens during 24-72 h incubation revealed that the cell growth inhibition was both dose and time-dependent (Table 1).

Table 1: Anti-proliferative effect of different concentration of crude *M. marshalli* somatic extract on the proliferation of K562 cells after 24- 72 h

Antigen concentration (mg/ ml)	Antigen concentration (mg/ ml) after 24h	Antigen concentration (mg/ ml) after 48h	Antigen concentration (mg/ ml) after 72h
0.1	99.2333 ± 0.73711	98.1667 ± 0.70946	96.5333 ± 0.75056*
1	95.8667 ± 0.63509*	93.2333 ± 1.15036*	90.5667 ± 0.47258*
2	89.4333 ± 0.90738*	88.2667 ± 0.90738*	87.7000 ± 0.51962*

To investigate cell toxicity of *M. marshalli* somatic antigen on healthy cells, HFF3 cell lines were treated with 2 mg/ml crude *M. marshalli* somatic extract for 72 h. Cell viability of HFF3 cells incubated for three days with the 2 mg/ml of antigens was 98.40 ± 1.34, which was 1.59±1.34 less than the control group.

The results of flow cytometry revealed that IC50 depended on the incubation time of the experiment. Incubation of treated K562 cells with *M. marshalli* somatic antigens for 72 h increased the number of apoptotic bodies, and it was confirmed by Annexin V/PI staining assay. The apoptosis rate in the treated group (69.49%) was significantly ($P< 0.05$) more than that of the control group (0.17%). While apoptosis was the leading cause of cell death in the treated group, most of the cells in the control group were necrosed (Fig. 1). The comparison between the number of apoptotic cells in the treated group and the untreated one showed a significant ($P< 0.05$) rise in the proportion of apoptotic cells in the treated group (Fig. 2). Fig. 3 gives information about the percentage of apoptotic, non-apoptotic, and necrotic K562 cells after incubating 72h with *M. marshalli* somatic antigens.

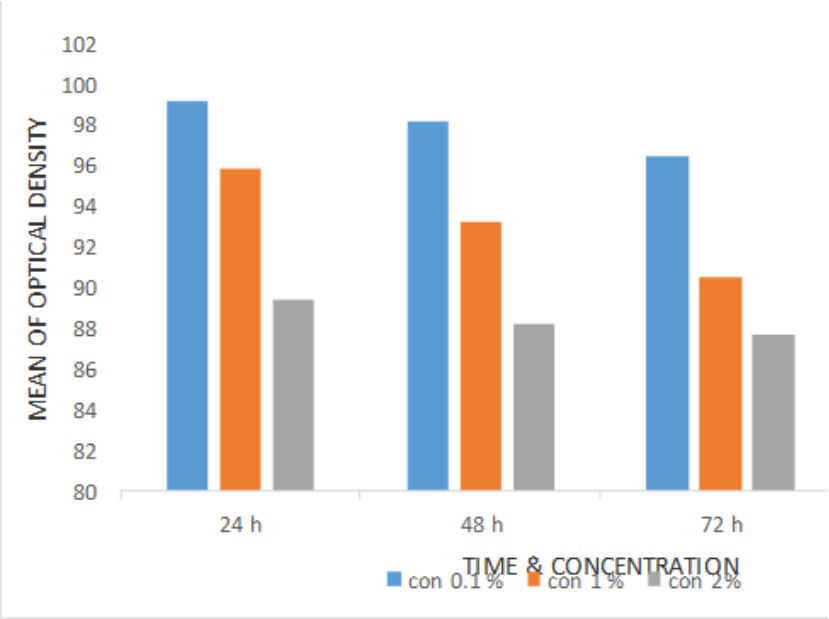

Fig. 1: Viability rate of HFF3 cells treated with 2 mg/ml crude *M. marshalli* somatic extract after 72 h incubation

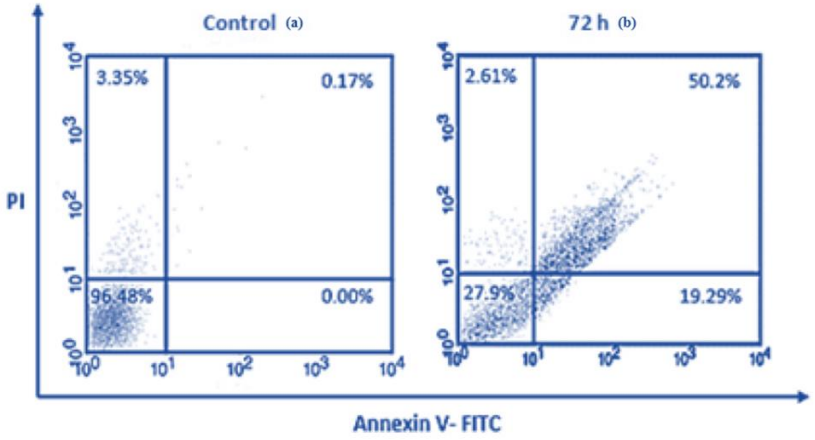

Fig. 2: Flow cytometry for the detection of apoptosis in K562 cells. As shown in the figure, there is a significant difference in the group a and b ($P<0.05$). Apoptosis in group b was higher than in groups a

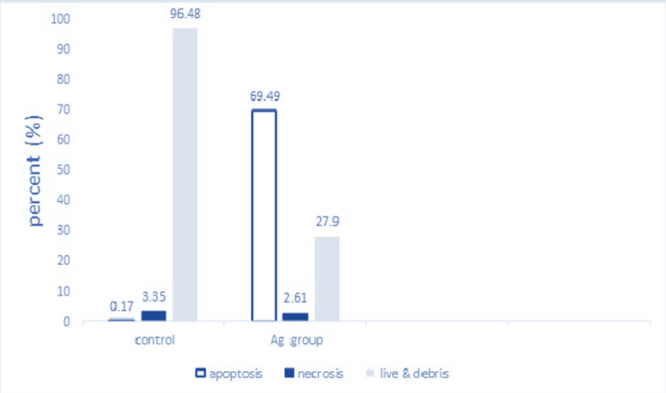

Fig. 3: The comparison between different types of cell death and non-apoptotic cells

Discussion

Our experiments have depicted that *M. marshalli* somatic antigen has anti-tumor activity against K562 cells. Administration of different doses of crude *M. marshalli* somatic extracts in different periods significantly inhibited the growth of K562 cell lines. Using 1-2 mg/ml of *M. marshalli* somatic extract over 24-72 h and 0.1 mg/ml of that for 72h demonstrated a more significant amount of apoptotic cells compared with the control group. Additionally, apoptosis was of importance in the cytopathogenic effect of *M. marshalli* somatic antigen on cells.

The results of our investigation were similar to the results of Zhang et al. reporting the apoptotic effect of *Toxoplasma gondii* tachyzoite on K562 cell lines. In this study, cells were incubated for 42h with *Toxoplasma gondii* tachyzoite, and analysis of cell survival was performed by MTT method (31). The administration of different doses of *T. spiralis* extracts inhibited the growth of K562 cells (11). Cell cycle distribution analysis demonstrated that because of the expression of p53 genes during the cyst formation, the crude *T. spiralis* extract could stop the growth of K562 and H7402 cells at the G1 phase and S phase, respectively (11). In a study, the survival of mouse (B16) melanoma cells significantly reduced when they were incubated with 0.1 mg/ml and 0.2 mg/ml *T. spiralis* ES L1 antigen for 72h. The results of staining cells with annexin V-FITC/PI showed mild apoptosis in the treated cells (32). In another study, the treatment of human glioma U373MG and U87MG cells with different doses of *Toxoplasma* lysate antigen (TLA) could suppress the proliferation and invasion of these cells in a dose-dependent manner. The combination of TLA with Quil-A (synthetic polynucleotide complex) showed significant inhibition of cell proliferation and invasion, and the administration of TLA at a high concentration can induce glioma cell apoptosis (33)

Huby et al. examined the effect of excretory/secretory products of *Trichostrongylus colubriformis* on seven cell lines. Although the excretory/secretory products increased the number of epithelial intestinal cells (RIC, IEC-6, IRD-98) and epithelial kidney cells (MDCK), they inhibited the proliferation of ovarian epithelial cells (CHO) and fibroblasts (3T3). These products imposed no changes in the cell growth of hepatocytes (HepG2) (24).

In another study, to investigate the anti-cancer activity of hydatid cyst protoscolices, WEHI-164 fibrosarcoma cells and baby hamster kidney fibroblast cells were treated with 50-100 live hydatid cyst protoscolices for 48 h. Hydatid cyst protoscolices can inhibit cell proliferation of WEHI-164 fibrosarcoma cells and baby hamster kidney fibroblast cells, and also increase lysis of fibrosarcoma cells (15).

Tumor graft reduces cytokine expression to improve its growth (11, 20, 34-36). Nematode infection triggers the secretion of cytokines, as well as the production of antibodies and T- cells. Infection with *T. spiralis* could induce production of B cells, splenic CD4 and CD8 T- cells, stimulation of Th2 response, and subsequently, the release of cytokines, such as interleukin- 5 (IL-5) and inter-

leukin-10 (IL-10). Additionally, Interferon-Ɣ (IFN-Ɣ) could be produced as the result of migrating newborn larvae of *T. spiralis* (37). Cytokines that were released because of contamination by *T. spiralis* inhibit the proliferation of several tumor cells (11, 20). Regarding the anti-tumor mechanism of nematodes, they activate proinflammatory stimuli such as lipopolysaccharides, TNF, or IL1. Some of these cytokines, including IL4, play a key role in regulating humoral and adaptive immunity. Th1 cells control the production of IgG2a and promote cell-mediated immunity. However, Th2 cells produce IL-4, IL-5, and IL-10 and inhibit cell-mediated immunity (16).

Infection with *T. gondii* leads to the rapid induction of a strong cell-mediated immune response in the experimental murine host. These facts suggest the possibility that new immunostimulating molecules could be found in *Toxoplasma* organisms.

Conventional therapeutic strategies in cancer, such as chemotherapy, radiotherapy, and surgery; were followed by a bad prognosis. They had limited success especially in the advanced stage of the disease (38-40). Nematodes trigger new immunostimulating molecules when they confront with tumor cells (27). They could inhibit tumor growth with high efficiency and few side effects. Helminth therapy could be considered as a safe and effective therapeutic modality (11), especially for the treatment of some cancers (41, 42).

The empirical results reported herein should be considered in the light of some limitations, such as whether infections should be systemic or localized, whether the dose should be light or heavy, of acute or chronic duration, and the role of host genetics. In addition, the use of helminth-derived anti-cancer molecules is yet to be tested on a clinical scale.

Conclusion

M. marshalli somatic antigen had an anti-cancer property, and its role in cancer treatment could be considered as an effective therapeutic method. These results may offer some new insights into immunotherapy against cancer. However, further studies are needed to discover more helminth-derived products with anti-tumor properties and identify the biological and molecular mechanisms of helminths.

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Conflict of interest

The authors declare that there is no conflict of interest.

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