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Antitumor and immunomodulatory effects of thymosin against tumor growth in mice

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Abstract: The current study determines the possible antitumor and immunomodulatory effects of thymosin against the *in vivo* and *in vitro* growth of tumor-derived cell line in mice. Peritoneal phagocytes count, Ehrlich ascites tumor (EAT) cells, T- lymphocytes, and B- lymphocytes activities were determined. In addition, serum level of interleukin 2 (IL-2) and liver functions were measured. In animal testing, thymosin at doses of 0.50 and 1mg activated the phagocytic function of macrophages, as well as T- and B- cell function. Thymosin caused a marked shortage in the proliferation of EAT cells in the peritoneal fluid with dose 0.50g as compared with that of the corresponding control group. Furthermore, treatment with thymosin caused effectively elevate in serum level of IL-2, on the contrary reduce in serum levels of ALT, AST and total proteins. The size of solid Ehrlich tumor was significantly decreased, as measured morphologically with the doses 0.50 and 1 mg ($P<0.01$). These results confirmed that many biological activities attributed to thymosin and is as an adjuvant for immune enhancement.

Keywords: Thymosin, Ehrlich ascite tumor, macrophages, T-lymphocytes, B-lymphocytes, IL- 2, hepatic function.

INTRODUCTION

Cancer is one of the most common causes of death in the world. At present, many researches are being carried out in the field of cancer in order to discover different therapeutic methods than usual methods. The immune system has a major role in both the positive and negative regulation of tumor development and progression. Crosstalk between tumor cells and immune cells has been incorporated into the list of main hallmarks of cancer (Bosch *et al*., 2018). There are many drugs that have proven to be anti-tumor. Recently, thymosin is considered to be one of the most important anti-tumor drugs, which has recorded impressive successes in the field of cancer treatments (Lipson *et al*., 1979; Raez *et al*., 2005; Lao *et al*., 2013). Thymosins Known as a small proteins occur in many animal tissues. They are named thymosins because they were originally isolated from the thymus, but most are now known to be occurred in many other tissues (Hannappel and Huff, 2003). Thymosin has many immunological effects. It acts as an anti-cancer, antiviral, neuroprotective and heart disease prevention. Thymosin fraction V is the first biologically active thymic extract. Fractionation of thymosin fraction V led to the isolation of a series of immunoactive polypeptide, including prothymosin alpha (Pro T α) (Samara *et al*., 2016). Thymosins have diverse biological activities and two in particular, thymosins α1 and β4, have potentially important uses in medicine field, some of which have already progressed from the laboratory to the clinic. In relation to diseases, thymosins have been classified as biological response modifiers (Low and Goldstein, 1982; Wang *et al*., 2018). thymosin α1 (Tα1) is a potent immune modulator, which has effects on immunodeficiency, cancer, and viral infectious diseases (Zhang *et al*., 2015). Currently, thymosin α is widely used in different countries for the treatment of several viral infections and as an adjuvant for immune enhancement. It has also been recognized as a treatment for non-small cell lung cancer (NSCLC), hepatocellular carcinoma, acquired immune deficience syndrome (AIDS) and malignant melanoma (Mandaliti *et al*., 2017). Thymosin has been shown to enhance both cell- mediated and humoral immune responses (Attia, 1995; Gabry *et al*., 2004; Tuthill and King, 2013). During attempts to determine the role of thymosin in T-cell ontogeny, thymosin fraction V has been shown to influence the development of cytotoxic T lymphocytes in congenitally athymic nude mice (Jr, 2018).

MATERIALS AND METHODS

Animals

Female Swiss albino mice (10- 12 weeks old, weighing about 30 g each) were used in this study. Mice were obtained from Helwan Research Animal Center, Cairo. The animals were preserved in aquite room at 28°C. Mice received laboratory chow and water *ad libitum* and were allowed a period of 14 days, prior to the initiation of experiments, to acclimatize to the laboratory conditions. The international protocol about ethics was followed in this research work.

Thymosin extraction

Thymosin was extracted from fresh calf thymus as the technique carried out Lowry *et al*. (1951).

Experimental design and treatment regime

A line of Ehrlich ascites tumor has been provided through the courtesy of Dr. G. Klien, Amsterdam, Holland. The

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tumor line was preserved in the Cancer Institute (Cairo, Egypt) in female Swiss albino mice. Tumor cell suspensions were prepared in balanced salt solution. All experimental animals were inoculated with EAT cells intraperitoneally. The white powder of thymosin obtained by Lowry *et al*. (1951) was suspended in phosphate buffer saline (PBS). One week later, doses of 0.20, 0.50 and 1.00 mg of thymosin/100 g body weight were intraperitoneally (i.p.) injected to tumor-bearing mice daily for consecutive 2 Weeks (0.2 ml/ mouse). Mice i.p. injected with 0.2 ml (PBS) only were used as a control**.**

Peritoneal phagocytes count

To obtain inflammatory peritoneal phagocytes, normal, tumor-bearing mice and tumor-bearing mice treated with thymosin (0.20, 0.50 or 1mg/100g BW, daily for 2 weeks) were intraperitoneally injected with 2.0ml of starch suspension. Three days later, mice were anesthetized by ether and peritoneal macrophages were obtained by peritoneal lavage with 5.0 ml of PBS. Cells were resuspended in PBS. Peritoneal phagocytes counts were determined using hemocytometer, where phagocytes stained with neutral red (Othman *et al*., 2018).

Activity of T- lymphocytes

The determination of T- cell function by intraperitoneal injection of 1x10^8 SRBC in 0.2ml saline. After 7 days, spleens from normal, tumor-bearing mice and tumor-bearing mice treated with thymosin (0.20, 0.50 or 1 mg/100 g BW, daily for 2 weeks) were excised and cleaned. Single cell suspensions were prepared and mixed with an equal volume of 0.5% SRBC in test tube and incubated for 2-4 hours at 37°C. The rosettes were counted in a hemocytometer and calculated per million mononuclear cells (Hsu *et al*., 1975).

Activity of B- lymphocytes

The determination of B- cell function by intraperitoneal injection of 1x10^8 SRBC in 0.2 ml saline. After 5 days, spleens from normal, tumor-bearing mice and tumor-bearing mice treated with thymosin (0.20, 0.50 or 1 mg/100g BW, daily for 2 weeks) were excised and cleaned. The assay mixture was prepared by adding 50 µl of 25% SRBC and 50 µl of guinea pig complement to 100 µl of spleen cell suspension and plated to a slide chamber and incubated for 30- 45 minutes at 37°C. The plaques were counted microscopically and calculated per million mononuclear cells (Brousseau *et al*., 1999).

Spleen lymphocytes proliferation assay

The determination T-cell mitogenesis was detected by preparation single spleen cell suspension after teasing through a sterilized autoclaved mesh. The adjusted cells were placed in a 96-well microtiter plate and added with 200mL aliquots per well. Con A (5mg/mL) and RPMI 1640 were set as positive and negative control, respectively in the presence of thymosin (0.02, 0.05 and 0.1 mg/mL). After 68 h, cells were counted by MTT assay (Lao *et al*., 2013).

Detection of Ehrlich ascites tumor (EAT) cells

EAT cells were obtained by peritoneal lavage with 5 ml of HBSS as described by Attia *et al*. (2007).

Measurement of solid tumor

Palpable tumors were measured using Vernier calipers (Tricle Brand, Shanghai, China). Tumor volume was calculated as described by Attia *et al*. (2007).

Determination serum level of interleukin 2 by using enzyme-linked immunosorbent assay (ELISA)

The release of interleukin 2 (IL-2) in serum was measured by using the Duoset ELISA Development kit (R&D Systems Inc, MN, USA) (Wang *et al*., 2012).

Determination of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities

AST and ALT activities were determined using BIO ADWIC AST and ALT kits, Egypt (Varliy, 1974).

Determination of total lipids content

Total lipids content was detected using Biodiagnostic total lipids kit, Egypt (Zollner *et al*., 1966)

Determination of total protein content

Total protein content was detected using BIO ADWIC protein kit, Egypt (Domas, 1975).

STATISTICAL ANALYSIS

All *in vivo* results are expressed as the mean $\pm$ SD of groups consisting of 10 mice. The *in vitro* data are also expressed as the mean $\pm$ SD of groups consisting of four wells. Every experiment was performed independently at least four times. By using Student's *t*-test all data were analyzed for significance (*Significantly different from tumor- bearing group at $P < 0.05$ and **significantly different from tumor- bearing group at $P < 0.01$).

RESULTS

Effect on the phagocytic cell count

As shown in table 1, the number of macrophage of tumor-bearing mice was reduced as compared to normal group. Tumor-bearing mice treated with thymosin (0.20, 0.50 or 1 mg/100 g BW, daily for 2 weeks) repaired this effect. Thymosin elicited a statistically significant increase in the number of phagocytic cells ($p< 0.01$) at a dose 0.50 mg/100 g BW as compared with the control group.

Influence on the activity of T and B lymphocytes

Table 2 showed that the number of activated T-Lymphocytes and activated B- Lymphocytes of tumor-bearing mice were reduced as compared to normal group.

Table 1: Number of macrophages in normal and in tumor-bearing mice treated i.p. with vehicle (0.2 ml PBS) or thymosin daily for 2 weeks.

Treatment	No. of macrophages (Mean ± SD x10^6)
Normal	3.95 ± 0.24
Tumor-bearing + Vehicle	1.60 ± 0.18
Tumor-bearing + Thymosin (0.20 mg)	1.92 ± 0.31
Tumor-bearing + Thymosin (0.50 mg)	2.99 ± 1.01**
Tumor-bearing + Thymosin (1 mg)	2.47 ± 0.25

Table 2: Number of activated T- Lymphocytes and activated B- Lymphocytes/ 10^6 nucleated spleen cells in normal and in tumor-bearing mice.

Treatment	No. of activated T- Lymphocytes /million nucleated spleen cells (Mean ± SDx10^3)	No. of activated B- Lymphocytes / million nucleated spleen cells (Mean ± SDx10^3)
Normal	2.46 ± 0.11	1.56 ± 0.11
Tumor-bearing + Vehicle	0.91 ± 0.07	0.74 ± 0.11
Tumor-bearing + Thymosin (0.20 mg)	1.38 ± 0.13	1.22 ± 0.10
Tumor-bearing + Thymosin (0.50 mg)	2.00 ± 0.24	1.41 ± 0.10
Tumor-bearing + Thymosin (1 mg)	1.97 ± 0.66**	1.49 ± 0.21

Table 3: T cell mitogenic response *in vitro*. Cultured splenocytes (2 x10^6 cells/ml) were exposed to Culture medium (Control), Con A (5µg/ml) in the absence or presence of Thymosin (0.02, 0.05 or 0.1mg/ml) for 72 hours.

Optical density (570 nm)		Treatment
Con A (5 µg/ ml)	Control (Culture medium)	
0.44 ± 0.08	0.30 ± 0.05	Vehicle
0.53 ± 0.10	0.38 ± 0.03	Thymosin (0.02 mg /ml)
0.49± 0.17**	0.36 ± 0.05	Thymosin (0.05 mg /ml)
0.68 ± 0.23**	0.34 ± 0.03	Thymosin (0.1 mg/ml)

Table 4: Number of tumor cells in the abdominal cavity of tumor-bearing mice treated i.p. with vehicle (0.2 ml PBS) or thymosin (0.20, 0.50 or 1 mg/100 g BW) daily for 2 weeks.

Treatment	No. of tumor cells (Mean ± SD x10^6)
Tumor-bearing + Vehicle	19.25 ± 1.31
Tumor-bearing + Thymosin (0.20 mg)	15.64 ± 1.53
Tumor-bearing + Thymosin (0.50 mg)	10.47 ± 4.82**
Tumor-bearing + Thymosin (1 mg)	10.58 ± 3.67**

Table 5: Volume of solid Ehrlich carcinoma growth of mice treated i.p. with vehicle (0.2 ml PBS) or thymosin daily for 2 weeks.

Treatment	Volume of tumor (mm^3)
Tumor-bearing + Vehicle	5.97 ± 0.30
Tumor-bearing + Thymosin (0.20 mg)	4.70 ± 0.47
Tumor-bearing + Thymosin (0.50 mg)	3.25 ± 1.50**
Tumor-bearing + Thymosin (1 mg)	2.68± 0.90**

Table 6: Serum levels of interleukin 2 in normal and in tumor-bearing mice treated i.p. with vehicle (0.2 ml PBS) or thymosin (0.20, 0.50 or 1 mg/100 g BW) daily for 2 weeks.

Treatment	IL-2 (pg/mL)
Normal	27.12±0.70
Tumor-bearing + Vehicle	20.59 ± 1.46
Tumor-bearing + Thymosin (0.20 mg)	21.35 ± 2.69
Tumor-bearing + Thymosin (0.50 mg)	25.18 ± 0.40
Tumor-bearing + *Thymosin* (1 mg)	25.26 ± 1.56

Table 7: Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in normal and in tumor-bearing mice treated i.p. with vehicle (0.2 ml PBS) or thymosin (0.20, 0.50 or 1 mg/100 g BW) daily for 2 weeks.

Treatment	ALT Ref Range: up to 32 U/L	AST Ref Range: up to 31 U/L
Normal	14.50 ± 5.19	16 ± 2.65
Tumor-bearing + Vehicle	52.25 ± 8.18	146 ± 9.64
Tumor-bearing + Thymosin (0.20 mg)	36.42 ± 5.26	100.33 ± 10.41
Tumor-bearing + Thymosin (0.50 mg)	26.70 ± 4.06	78.67 ± 8.50
Tumor-bearing + *Thymosin* (1 mg)	23.67 ± 9.60*	64.33 ± 11.06**

Table 8: Serum levels of total lipids and total proteins in normal and in tumor-bearing mice treated i.p. with vehicle (0.2 ml PBS) or thymosin (0.20, 0.50 or 1 mg/100 g BW) daily for 2 weeks.

Treatment	Total lipids Ref Range: 0.4-1.0 g/dl	Total proteins Ref Range: 6.5-8.3 g/dl
Normal	0.76 ± 0.11	7.12 ± 0.93
Tumor-bearing + Vehicle	0.42 ± 0.06	9.41 ± 0.32
Tumor-bearing + Thymosin (0.20 mg)	0.51 ± 0.06	8.28 ± 1.18
Tumor-bearing + Thymosin (0.50 mg)	0.56 ± 0.10**	6.34 ± 1.10**
Tumor-bearing + *Thymosin* (1 mg)	0.52 ± 0.05	7.85 ± 0.61

Tumor-bearing mice treated with thymosin (0.20, 0.50 or 1 mg/100 g BW, daily for 2 weeks) repaired this effect, and caused a gradual increase in number of activated T-Lymphocytes and activated B- Lymphocytes as compared to the control group. This increase in the number of activated T- Lymphocytes was statistically significant ($p < 0.01$) with thymosin at a dose 1 mg/ 100 g as compared with the control group.

Effect on the mitogenic response of T- Lymphocytes

As described in table 3, in the absence of Con A mitogen, thymosin by itself had no mitogenic effect under the cultured conditions explained. However, in the presence of Con a (5µg/ ml), thymosin at doses 0.05 and 0.1 mg / ml significantly motivate the proliferation of cultured splenocyte ($p < 0.01$).

Effect on the number of tumor cells in the abdominal cavity

Table 4 explained that the number of tumor cells in the abdominal cavity of mice treated with thymosin (0.20, 0.50 or 1 mg/100 g BW, daily for 2 weeks) was gradually decrease as matched to the control group. This decrease was statistically significant ($p < 0.01$) with thymosin in doses 0.50 and 1 mg/100 g BW.

Influence on the volume of solid Ehrlich carcinoma growth

Table 5 showed that the volume of solid Ehrlich carcinoma growth of mice treated with thymosin (0.20, 0.50 or 1 mg/100 g BW, daily for 2 weeks) was gradually reduced as compared with the control group. This decrease was statistically significant ($p < 0.01$) with thymosin in doses 0.50 and 1 mg/100 g BW.

Effect on serum levels of Interleukin 2

As explained in table 6, the serum levels of Interleukin 2 of tumor-bearing mice were clearly reduced as compared to normal mice. Ttreatment of tumor-bearing mice with thymosin (0.20, 0.50 or 1mg/100g BW, daily for 2 weeks) improved this effect, and caused a marked raised in serum levels of Interleukin 2 as compared to the control group.

Effect on serum levels of ALT and AST

As illustrated in table 7, serum enzyme levels of ALT and AST of tumor-bearing mice were obviously raised as compared to normal mice. Treatment of tumor-bearing mice with thymosin (0.20, 0.50 or 1 mg/100 g BW, daily for 2 weeks) improved this effect and caused a gradual reduce in serum enzyme levels of the liver as compared to the corresponding control group. Serum levels of ALT and AST were significantly reduced with dose 1 mg/100 g BW ($P<0.05$& $P<0.01$) as compared to the control mice.

Effect on serum levels of total lipids and total proteins

As shown in table 8, serum levels of total lipids were decreased, while serum levels of total proteins were increased in tumor-bearing mice as compared with those of normal mice. Treatment of tumor-bearing mice with thymosin (0.20, 0.50 or 1 mg/100 g BW, daily for 2 weeks) caused statistically significant increase in the serum levels of total lipids and a decrease in the serum levels of total proteins with dose 0.50 mg/ 100 g BW ($P<0.01$) when compared with those of control group.

DISCUSSION

The data of this study demonstrated that treatment of tumor-bearing mice with thymosin raised the number of peritoneal macrophages as compared to that of corresponding control group. These results are consistent with the results shown thymosins β4 is an important macrophage component involved in many immunological processes and acts as an antioxidant and antimicrobial

factor that prevent aggravation of inflammation (Rath *et al*., 2007; Hwang *et al*., 2019). In addition, previous studies have indicated that thymosin α1 activated tumor-associated macrophages by improving production of IFN-γ which is a potent macrophage activating cytokine and a decrease in the production of PGE2 which is inhibitory to macrophage activation (Shrivastava *et al*., 2004& 2005)

The results of this study revealed that treatment of tumor-bearing mice with thymosin elevated number of activated T- Lymphocytes and activated B-Lymphocytes, in addition a shortage in the number of tumor cells in the abdominal cavity as well as the volume of solid Ehrlich carcinoma growth as compared to the standard control group. These results are in agreement with the results of an *in vivo* studies which showed that Tα1-Fc restores NK activity and reconstructs cell immunity in immunosuppressed mice by stimulating cytokine production, such as IFN-γ and IL-2 (Wang *et al*., 2018). Tα1 stimulated the proliferation of splenic lymphocytes of mice. Tα1 increase lymphocytic infiltration to sites of diseases by enhancement the release of certain chemokines. Tα1 mediated maturation and differentiation of dendritic cells that synthesizes specific cytokines leading to an increase in anti-tumor T- cells (Li *et al*. 2011). Tα1 restored the T cell-mediated antibody production (Li *et al*., 2002).

In the current work, there is a reduce in serum levels of ALT, AST and total proteins and these results are agreed with the results reported in another study which showed decreased activities of serum ALT, AST of ethanol- and LPS-induced liver injury in mice that treated with Tβ4. Tβ4 prevented oxidative stress through preventing the loss of mitochondrial membrane potential of hepatic cells. Tβ4 also lowers the fibrosis as measured by the percentage of collagen fibers and the amount of hydroxyproline in the liver tissue, therefore finding that Tβ4 protects hepatic tissues from damage (Shah *et al*., 2018). In another previous study, TB4 has been shown to be prevent CCl4-induced acute liver injury and subsequent fibrosis by reducing inflammation oxidative stress and inflammation, and therefore improved functions for liver (Li *et al*., 2017). Free radicals derived from CCl-4 is one of the most important factors that lead to liver damage, causing damage to the cell membrane and thus leaking hepatotoxic marker enzyme (Weber *et al*., 2003). Cytochrome P450 enzyme is involved in the process of CCl4-induced liver damages (Marcolin, 2012). Tβ4 decrease nitro-tyrosine levels, but increased GSH levels and SOD activities in the liver tissues (Li *et al*., 2017). From all previous studies, complete compatibility with the present study is clear in the fact that thymosin exerted an anti-fibrotic effect and acts as free radicals scavenger, thus protects hepatic cells from damage and improves liver function in tumor-bearing mice (Kim *et al*., 2015).

CONCLUSION

This study demonstrated that thymosin is able to improve the functional performance of immune cells in tumor-bearing mice through elevate phagocytes count and enhancement of T- lymphocytes and B- lymphocytes activities and thereby increase the efficiency of the immune system. In addition, thymosin acts as a promising antitumor compound against different tumors.

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