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Evaluation of the Effect of HSV-tk/GCV Adenoviral Vector Vaccine Candidate in Inhibiting the Growth of Myeloma Tumors by Inducing Autophagy in BALB/c Mice

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ABSTRACT

Introduction: Multiple myeloma is the second most common blood malignancy which has remained incurable with current therapies. However, gene therapy using suicide viral vectors such as adenoviral vectors appear more promising than other treatments. The aim of this study was to evaluate the effects of an adenoviral vector vaccine candidate containing HSV-TK gene on tumor reduction and autophagy mechanism in animal model. **Methods:** Myeloma tumor was created in mouse models using myeloma SP2/0 cell line. Three groups of negative control, positive control and target group of BALB/c mice were formed. Candidates for the Ad-HSV-tk /GCV vaccine at high titer (10^8) were then injected three times every 72 hours at target mice and metformin was injected into the control group for 12 consecutive days. Tumor size was measured in all 15 mice studied every three days, and finally, three days after the last dose of the vaccine, the tumors were removed for Western blotting and LC3B expression. **Results:** Examination of tumor size showed that injection of the vaccine and autophagy-inducing drug reduced tumor size compared to the negative control group. Western blotting indicated that LC3B expression was significantly higher in the target and positive control groups than in the negative control group. (Mean Diff: -0.4921; *P* value < 0.05; q: 7.911). **Conclusion:** The results suggest that Ad-HSV-tk/GCV vaccine candidate was able to induce autophagy and reduce the growth of tumor cells in the animal model studied due to the ability of adenovirus to induce the immune system response, the anti-myeloma nature of adenovirus and the function of HSV-tk suicide gene.

INTRODUCTION

Myeloma is the most common type of primary bone cancer, accounting for 10% of all blood cancers and 1% of all new cancers [1]. The pathology is caused when malignant B cells invade the bone marrow and produce large amounts of immunoglobulin. Traditional treatment protocols for this type of cancer include chemotherapy with bone marrow transplantation and drug treatments. Enhancing the body's spontaneous immune response to malignant cells has been the focus of recent cancer research, and one of the new tools for this purpose is the use of oncolytic viruses. These viruses can destroy cancer cells naturally or by modification, or elicit a reaction in the immune system to fight the tumors [2].

Among the various viral vectors, adenoviral vectors are the most commonly used vectors for the treatment of human cancer, particularly for gene therapy based on the injection of vectors into the tumor [3]. This virus has the ability to resume antitumor immune system activity in patients with myeloma [4]. According to the studies, one strategy for using adenoviruses as therapeutics in human cancer is to insert a sequence of a desired gene that encodes an enzyme into the viral genome which upon expression, activates a specific drug [5]. Using adenoviruses has many advantages such as having a well-known genome, genetic stability, high cloning capacity, high efficiency for human in-vivo studies, and minimal side-effects compared to chemotherapy and even other immunotherapy methods [3].

Adenoviruses induce cell death by apoptosis, necroptosis, necrosis, and autophagy. With the exception of apoptosis, other types of cell death are inflammatory and immunogenic [5]. The treatment of cancer based on the use of suicidal genes, was started by Molten *et al*, more than 25 years ago, and the original method has changed little over time [6]. Recent statistics have reported about 1,550 clinical trials, 7% of which are gene therapy with suicide genes [7, 8]. HSV-TK is one of the most well-known suicide genes used to treat cancer and other diseases without causing significant toxicity (7.11). The TK enzyme expressed by HSV-tk is able to convert the antiviral drug ganciclovir into a toxic monophosphate ganciclovir, which

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disrupts DNA replication and leads to apoptotic cell death. This gene has already been tested for pancreatic cancer [9]. Autophagy, as a protective mechanism in response to a variety of cellular stresses including malnutrition, hypoxia, and infection, is able to maintain cellular homeostasis by removing damaged organs and excess proteins [10].

Studies have shown that autophagy plays different roles in different cancer models. This cell death mechanism inhibits tumor growth in some cancers; however, in some cases it promotes cancer by helping tumor cells to grow [11].

During treatment with viruses, there is exposure to the mechanism of autophagic cell death, which, like the conflicting role of autophagy in cancer, can act like a double-edged sword. Autophagy can act both as an adjunct to oncolytic viruses and to manage ICD (Immunogenic cell death), as a mechanism for tumor cell survival, and as a factor in creating resistance to viral therapy [10].

LC3 includes a soluble form of LC3I (molecular weight 18 kDa) and a lipid form called LC3II (molecular weight 16 kDa), expressed as 3 isoforms (LC3A, LC3B, LC3C) in mammalian tissues while LC3B is associated with autophagy. Various types of stressors activate LC3 and transform it into a cytosolic form and enhance the interaction of LC3I with phosphatidylethanolamine to form LC3II which is specifically found in the autophagosome. LC3II is also considered as the most reliable autophagy marker [12].

In malignant blood disorders, autophagy attenuates the effects of drug-induced cell death by inducing resistance. In myeloma, the production of significant amounts of immunoglobulins causes the accumulation of misfolded proteins and one of the important factors to eliminate the presence of these proteins in cells is autophagy [11]. Therefore, in this study, by inserting the HSV-tk suicide gene in adenovirus vector to make a viral vaccine, we investigated the effect of this vaccine on tumor cell growth and autophagic cell death.

MATERIALS AND METHODS

Adenovirus Production

HSV-tk gene was cloned into the pAdTrack CMV shuttle vector. The shuttle vector with AdEasy-1 was transferred to BJ5183 by electroporation. Homologous recombination was performed in BJ5183 electrocompetent Escherichia coli cells. The recombination was confirmed by restriction endonuclease analysis. Co-transfection of pAdTrack CMV shuttle containing HSV-tk gene and AdEasy-1 were performed by the Lipofectamine according to the protocol into the HEK-AD cells. Virus production were examined by GFP expression under an immunofluorescence (IF) microscope and CPE virus was observed [13]. The virus titer was determined by 50% Tissue Culture Infectious Dose ($TCID_{50}$) assay. In a 96-well plate, cells were cultured. 24 h after cell growth analysis Serial dilution was made. The plate was incubated for 2-5 days and the percentage of cytopathic effect (CPE) was examined under a microscope each day and the results recorded. Finally, the calculation was performed using the formula $TCID_{50}$ =10 log total dilution over 50% - (I log h) [14].

In-vivo Study

Myeloma cell line SP2/0 was cultured in RPMI 1640 supplemented with 10% FBS, 1% penicillin–streptomycin to induce tumor in mouse model. Four to six-week-old female BALB/c mice were purchased from the Department of Laboratory Animals, Pasteur Institute of Iran and 2×10^6 SP2/0 myeloma cells/mouse were subcutaneously inoculated into right flank of all 15 mice. A subcutaneous tumor developed 7 days after cell injection, which is considered day zero. Then the mice were randomly divided into three groups of 5: positive control, negative control and autophagy target group. The target mice were injected with 50 µl of Ad-HSV / TK candidate vaccine which was injected intratumorally three times at a dose of 3.0×10^8 every 72 h. Ganciclovir (CYMEVEN, England; 75 mg/kg) was also injected daily at the tumor site for 12 days. In control mice, Metformin was injected as an autophagy stimulant for 12 consecutive days (Merck, PHR1084). Tumor growth was recorded every 3 days and tumor size was calculated based on the following formula:

$$V = ½ (\text{Length} \times \text{Width}^2)$$

Mice were sacrificed 3 days after the last Ad-HSV-tk/GCV injection and tumor tissues were collected.

Western Blot for LC3B Analysis

Tissues were lysed in RIPA lysis buffer (DNAbiotech,Iran) with protease inhibitor for protein extraction. The protein concentration was evaluated by the Lowry method. Proteins were separated based on molecular weight using SDS-PAGE. The separated proteins were transferred to a PVDF membrane and placed on a shaker. Then washed with T-BST and refrigerated with LC3B antibody (Sigma-Aldrich, USA) overnight. The next day, it was washed three times with T-BST. The PVDF membrane was then placed in a secondary antibody solution on a shaker at room temperature for 2 h. Then it was washed again with T-BST. The bands were exposed on an X-ray film by the ECL method. Each sample was assayed in duplicate and GAPDH was used as an internal control.

Statistical Analysis

Western blot was performed with binary iterations and data were expressed as mean standard deviation (SD). The GraphPad Prism 8 software was used to evaluate the results. ANOVA and then Tukey's multiple comparison test were used to compare the study groups.

RESULTS

Induction of Autophagy and its Effect on Tumor Growth

The mean tumor size in all 3 groups before the first injection was 259-251 mm^3. Two days after the last injection, the tumors sizes decreased to 129-100 mm^3 in the positive and control groups; however, in the negative control group, the tumor growth continued and the average tumor size reached 916.5 mm3. Fig.1, shows a significant decrease in the tumor size in the autophagy positive control group and the target group. On the other hand, an exponential increase in tumor size in the negative control group was observed. Hence, Ad-HSV-tk/GCV may result in a decrease in tumor size and also may lead to the induction of autophagy.

HSV/tk-GCV Induces LC3B Expression and Inhibits Tumor Growth by Inducing Autophagy

Fig. 2 shows Western blot results in which LC3B expression increased significantly in Ad-HSV/tk-GCV group compared to the control group.

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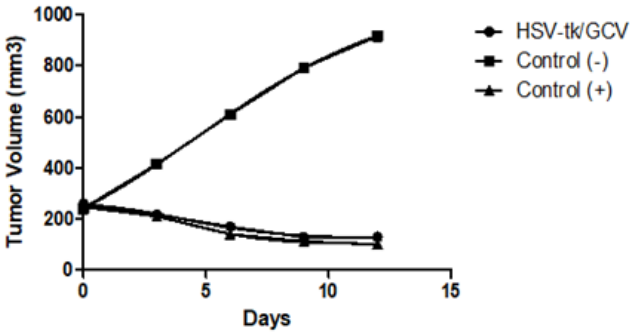

Fig. 1. Tumor volume sizes over 15 days.

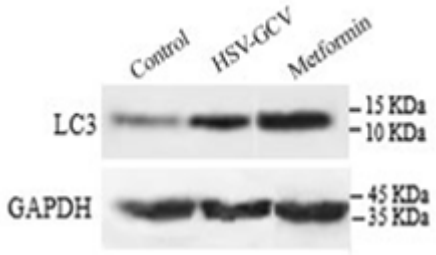

Fig. 2. LC3B expression compared to GAPDH control (Mean Diff: -0.4921; *P* value < 0.05; q: 7.911).

Also as shown in Fig. 3, in the autophagy positive control group, the expression of LC3B had a significant increase compared to the control group.

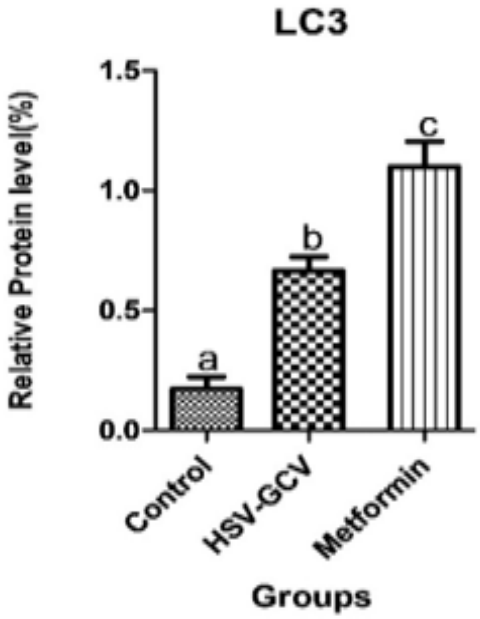

Fig. 3. LC3B protein expression levels (mean diff: -0.9250; *P*-value < 0.05; q: 14.87).

DISCUSSION

Although multiple myeloma remains the second most common incurable blood malignancy, the advent of novel treatment tools and approaches may pave the path to combat against this cancer [15, 16]. Adenoviral vectors can be used as a new therapeutic tool as well as a multidimensionally vaccine to fight cancer. This is because they are able to specifically destroy malignant cells independently, without making changes or through modifications in normal cells. On the other hand, they control the host's immune system and stimulate cellular mechanisms against cancer cells by inducing immune system responses [3, 17]. Initially, Ad5 was not considered a candidate for treating myeloma; however, modifications in the structure of Ad5 allowed this strain to be used for treating blood malignancies. Of all the different adenovirus serotypes, the Ad5 serotype was used as an alternative therapy in 2007 [18].

A practical example of altering the structure of viruses as a cancer vaccine is the use of the HSV-TK suicide gene in combination with the viral drug ganciclovir. In this structure, TK function is removed and it is no longer able to synthesize DNA and repair the errors, which leads cancer cells to death [17]. Viruses through destroying tumor cells, cause the accumulation of dead cells and reactive oxygen species (ROS). In fact, viruses stimulate immune system responses by inducing cell death pathways [19]. As a result of the double and contrasting function of autophagy in cancers such as myeloma, this mechanism can be used to treat myeloma in two ways:

1. Inhibiting the role of autophagy in spreading of cancer via reducing the generation of misfolded proteins and reducing the energy levels.
2. By inducing autophagic cell death mechanism which can be used to kill tumor cells [20, 21].

In this study, adenoviral vector was used to prepare a viral vaccine candidate based on the proven anti-myeloma effect of some adenovirus serotypes. We then evaluated its effect on tumor cells suicide gene HSV-tk and its effect on the tumor growth and the induction of autophagy. The study of autophagy activation in myeloma model with the suicide gene Ad-HSV-tk is carried out for the first time. The results of a study conducted in 2018 by Jung *et al*. has shown that HSV-tk/GCV in retinal cells leads to a significant increase in the expression of autophagy-related proteins LC3B and p62; however, it cannot activate autophagy through the mTOR signaling pathway [22]. In this study, we observed a decrease in the size of myeloma tumors in Ad-HSV-tk / GCV target group mouse model.

Highashi *et al*. have reported that the antitumor activity of HSV-tk / GCV is activated when GCV is converted to its toxic triphosphate form and induces cell death in tumor cells [23]. Our Western blot analysis revealed that the Ad-HSV-tk/GCV vaccine candidate lead to LC3 expression as a main marker of autophagy activation pathway. Therefore, it may culminate in tumor growth reduction in myeloma mouse model. In the course of normal conditions and early stage of cancer, autophagy acts as a shield to guard cells from destructive stimuli and malignant transformation. Furthermore, autophagy via restraining the devastating effect of ROS, lead to inhibition of DNA damage and retains genome integrity [24]. Although not many studies have been published to examine the role of adenovirus vaccines in inhibiting the growth of myeloma tumors, the results of research on other malignancies such as Gliom and other blood malignancies such as Chronic Myeloid Leukemia CML and Acute Myeloid Leukemia (AML) have been similar to the results observed here. This confirms the

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antitumor role of the adenovirus vaccine and also the potent role of these viruses in activating autophagic death.

In conclusion, using Ad-HDV-tk/GCV vaccine candidate appeared to be effective in both tumor cell killing and stimulation and activation of host immunization in reducing the size of tumors in the mouse model. Moreover, it was able to kill tumor cells through autophagy. Due to the dual role of autophagy cell death mechanism in cancers, it is suggested that other cell death pathways such as apoptosis and unfolded protein response be investigated by designing virus-based vaccines and using immune system inducers in the structure of these vaccine candidates.

ETHICAL STATEMENT

Ethical approval for treatment of the mice was granted by the Faculty of Convergent Sciences and Technologies, University of Sciences and Research , Tehran, Iran.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interests.

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