RESEARCH ARTICLE

DOI: 10.4274/tjh.galenos.2024.2023.0403
Turk J Hematol 2024;41:16-25

Oncolytic *Myxoma virus* Increases Autophagy in Multiple Myeloma

Onkolitik *Miksoma virüsü* Multipl Miyelomda Otofajiyi Artırıyor

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Abstract

Objective: Multiple myeloma, which affects plasma cells, is the second most common hematological malignancy. Despite the development of new drugs and treatment protocols, patient survival has not reached the desired level. In this study, we investigated the effects of *Myxoma virus* (MYXV), an oncolytic virus, on autophagy in myeloma cells.

Materials and Methods: We analyzed protein expressions of ATG-5, p62, Beclin-1, LC3B, and the apoptosis marker Bcl-2 as autophagy markers in human U-266 and mouse MOPC-315 myeloma cell lines subjected to different doses of MYXV. In addition, autophagic images of myeloma cells were investigated using transmission electron microscopy (TEM).

Results: In the first 24 h, which is the early stage of autophagy, ATG-5 and Beclin-1 expression levels were increased in the U-266 and MOPC-315 cell lines in the groups that had received MYXV at a multiplicity of infection of 15. At 48 h, a significant increase was detected in the expression of LC3B, which is a late indicator. Autophagosomes were observed in myeloma cells by TEM.

Conclusion: MYXV shows an antimyeloma effect by increasing autophagy in myeloma cells.

Keywords: *Myxoma virus*, Multiple myeloma, Autophagy, Autophagosome

Öz

Amaç: Plazma hücrelerini etkileyen multipl miyelom, en sık görülen ikinci hematolojik kanserdir. Yeni ilaçların ve tedavi protokollerinin geliştirilmesine rağmen, hasta sağkalımı istenilen düzeye ulaşmamıştır. Bu çalışmada, onkoltik bir virüs olan *Miksoma virüsü*'nün (MYXV), miyelom hücrelerinde otofaji üzerindeki etkilerini araştırdık.

Gereç ve Yöntemler: İnsan U-266 ve fare MOPC-315 miyelom hücre hatlarına farklı dozlarda MYXV uygulanarak ve otofaji belirteçleri olarak ATG-5, p62, Beclin-1, LC3B ve apoptoz belirteci Bcl-2 protein ekspresyonları analiz edilmiştir. Ayrıca, miyelom hücrelerinin otofajik görüntüleri, transmisyon elektron mikroskobu (TEM) kullanılarak incelenmiştir.

Bulgular: İlk 24 saatte, yani otofajinin erken aşamasında, U-266 ve MOPC-315 hücre hatlarında MYXV'nin moi 15 enfektif dozda alındığı gruplarda ATG-5 ve Beclin-1 ekspresyon düzeyleri artmıştır. Kırk sekiz saatte, geç bir belirteç olan LC3B'nin ekspresyonunda önemli bir artış tespit edilmiştir. Miyelom hücrelerinde otofagozomlar TEM ile gözlemlenmiştir.

Sonuç: MYXV, miyelom hücrelerinde otofajiyi artırarak antimiyelom etkisi göstermektedir.

Anahtar Sözcükler: *Miksoma virüsü*, Multipl miyelom, Otofaji, Otofagozom

Introduction

Multiple myeloma (MM), which is most commonly diagnosed between the ages of 60 and 70 years, is the second most common blood cancer, accounting for 10% of all hematological malignancies [1]. MM, caused by clonal plasma cell (PC) proliferation, progresses with bone destruction, anemia, frequent infections, and decreased kidney function. The treatment of MM involves a combination of various therapies including corticosteroids, anthracyclines, alkylating agents, immunomodulatory drugs (IMiDs), proteasome inhibitors (PIs), histone deacetylase inhibitors, monoclonal antibodies, and autologous stem cell transplantation [2,3]. In recent years, median overall survival has increased from 2-3 years to 8-10 years due to a better understanding of the pathophysiology and heterogeneity of MM and with the help of new therapeutic approaches [4,5]. However, due to the development of resistance to both IMiDs and PIs, the desired patient survival could not be

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Received/Geliş tarihi: October 30, 2023
Accepted/Kabul tarihi: January 23, 2024

achieved in the treatment of MM patients [6]. Therefore, patients with MM continue to exhibit recurrent patterns of remission and relapse. Moreover, as the disease progresses, tumor cells become more aggressive, the remission period becomes shorter, and patients die from refractory disease [7].

As treatment-free periods are reduced in patients with MM and treatment-resistant disease persists, therapeutics with new mechanisms of action are needed to completely control the disease. Researchers have focused on effective new strategies for cancer treatment in recent years. Since the 1900s, when natural viral infection was observed to cause tumor regression, the idea that viruses could be used for cancer treatment has become widespread [8]. However, due to toxicity and viral pathogenicity, studies on this subject could not be conducted for many years. Thanks to advances in genetic engineering, a new generation of viruses called oncolytic viruses (OVs) that can be used effectively in therapy have been developed. OVs can selectively kill tumor cells without affecting healthy cells [9]. OVs used therapeutically can induce inflammatory responses [10,11], serve as in situ vaccine agents [12], act as adjuvant therapeutics [13,14], and cause DNA damage in cancer cells [15]. Furthermore, OVs can trigger various forms of programmed cell death, including apoptosis, pyroptosis, necroptosis, and autophagy [16,17].

OVs such as reoviruses, the measles virus, *Vaccinia virus*, and the vesicular stomatitis virus have been shown to have therapeutic potential for the treatment of MM [18,19,20,21,22]. *Myxoma virus* (MYXV), which is classified in the genus *Leporipoxvirus* of the family *Poxviridae*, has a double-stranded DNA genome. Having a strict tropism for rabbits and hares, MYXV causes no obvious pathology in either humans or mice [23,24]. The therapeutic effect of MYXV, an OV whose therapeutic potential has recently been recognized, has been investigated in pancreatic cancer, melanoma, glioma, and rhabdoid tumors [25,26,27,28,29,30,31,32]. MYXV has been reported to target leukemia cells in AML tumor xenografts without harming normal hematopoietic stem cells [33]. MYXV has also been shown to induce oncolysis by increasing apoptosis in myeloma cells [34]. Moreover, it has been observed that intravenous injection of MYXV causes reductions of 70%-90% in tumor volume [35].

Autophagy is a natural cleaning and recycling program that breaks down damaged or unnecessary structures within the cell and, if necessary, puts them back into use. There are three subtypes of autophagy in eukaryotic cells: macroautophagy, microautophagy, and chaperone-mediated autophagy [36]. In an unstressed cell, the basal level of autophagy is usually low, removing damaged proteins and organelles to maintain homeostasis. Under stressful conditions, autophagy is induced to restore homeostasis, produce essential amino acids, and maintain cellular life [37]. However, autophagic genomic damage or autophagic imbalance can occur in a wide variety of diseases and disorders, including aging, neurodegeneration, autoimmune diseases, and cancer [38].

Autophagy has a complex relationship with cancer, as it can have both positive and negative effects on tumor growth [39]. In cases of certain types of cancer, such as hepatocellular, pancreatic, and colorectal tumors, as well as lymphoma, leukemia, and myeloma, autophagy is impaired and can lead to resistance to chemotherapy treatments [40]. However, the absence of genes required for autophagy in tumors suggests that it may also act as a tumor suppressor. In breast cancer, for instance, there is a decrease in the expression of the autophagy protein Beclin-1 [41].

After the demonstration of the effectiveness of autophagy in cancer cells, autophagy became the new target of treatment in cancer cells. The oncolytic mammalian reovirus has been proven to trigger autophagy in colorectal cancer [42], while an oncolytic adenovirus equipped with Beclin-1, a key player in autophagy, causes autophagic cell death [43]. The Beclin-1-armed oncolytic *Vaccinia virus* (OVV) was determined to increase the efficacy of chemotherapeutics against lymphoma in vivo and in vitro [44]. Similarly, OVV armed with Beclin-1 has increased therapeutic efficacy in myeloma and leukemia [45].

It has been suggested that PCs have high levels of autophagic activity and autophagy has an important role in PC oncogenesis [46]. Furthermore, autophagy is a survival mechanism in long-lived human PCs [47]. MM cells, like PCs, have both an enlarged endoplasmic reticulum (ER) network and secrete immunoglobulin (Ig). For these reasons, there are misfolded or unfolded proteins that can be toxic in the cytoplasm of MM cells. Thus, MM cells use molecular pathways such as the ubiquitin-proteasome system (UPS), proteasomal degradation, and autophagy to protect against damage [36]. Autophagy in myeloma cells both removes UPS-ubiquitinated proteins [48] and plays a role in determining sensitivity to PIs, which are among the important drugs in MM therapy [49]. MM cells treated with emodin/carfilzomib overexpressed the autophagic protein p62 and LC3B compared to control cells [50]. Patients with high immunoreactivity of autophagic markers such as Beclin-1 and LC3 have been found to have longer survival [51]. In addition, increased Beclin-1 and LC3 expression in MM cells led to median overall survival results of 1171 and 934 days, respectively. Recently, it has been reported that inhibiting the late autophagy phase with elaiophylin inhibits autophagic flux, activates ER stress-mediated apoptosis, and consequently leads to anti-MM cell activity [52].

To our knowledge, there has been no research to date investigating the effect of MYXV on autophagy in MM cells. In this study, we aimed to investigate the expression levels of

autophagic proteins and damage to cells by applying different concentrations of MYXV to myeloma cells.

Materials and Methods

Cells, Reagents, and Virus

MYXV was kindly provided by the University of South Carolina School of Medicine (USA). The U-266 (*Homo sapiens* B lymphocyte cells), MOPC-315 (*Mus musculus* B lymphocyte cells), and Vero (African green monkey kidney-CCL81) cell lines were purchased from ATCC (USA). Fetal bovine serum (FBS), trypsin-EDTA, Dulbecco's modified eagle medium (DMEM), phosphate-buffered saline (PBS), L-glutamine, amino acids, and vitamins were purchased from Pan Biotech (Germany). RPMI 1640 medium and streptomycin were purchased from Sigma-Aldrich (USA), rapamycin was purchased from Biosynth (Switzerland), and WST-1 solution was purchased from Roche (Germany).

MOPC-315 and U-266 cells were used to investigate the apoptotic and autophagic effects of the virus in MM cells. RPMI 1640 supplemented with 10% FBS, 1X penicillin/streptomycin, 2 mM L-glutamine, 1X amino acids, and 1X vitamins was used for culturing U-266 and MOPC-315 cells in suspension in tissue culture flasks at 37 °C with 5% CO_2. To prepare the subculture, cells were centrifuged at 1000 rpm for 10 min. The supernatant was then removed, viability was checked with trypan blue staining, and cells were counted on Thomas slides and suspended in tissue culture flasks.

Virus Titration and Purification

Vero cells were used to produce and titrate MYXV. The Vero cells were grown in DMEM with 10% FBS and 1X penicillin/streptomycin at 37 °C with 5% CO_2. Subculturing was carried out using trypsin-EDTA and PBS solutions. MYXV was purified and titrated as previously described [53]. Vero cells prepared at 1-1.5x10^5/mL were infected with green fluorescent protein-labeled MYXV and evaluated under a fluorescence microscope (see Figure 1). To determine the titer of MYXV, a microtitration test using a focal assay was conducted as previously reported [53]. The process involved preparing logarithmic 10-fold dilutions of MYXV and applying quadruple repetitions for each dilution. After a 48-h incubation period, the tissue culture infective dose ratio was calculated by counting the growth foci of MYXV. Virus purification was performed in an ultracentrifuge (Optima XPN-100 and Max-XP, Beckman Coulter, USA) with 24%-40% sucrose solutions with double and triple gradients created. The virus layer and two consecutive centrifugation protocols were collected at 50,000 x g for 40 min and at 33,000 x g for 40 min. Finally, the pellet obtained by centrifugation at 18,000 rpm was resuspended in PBS.

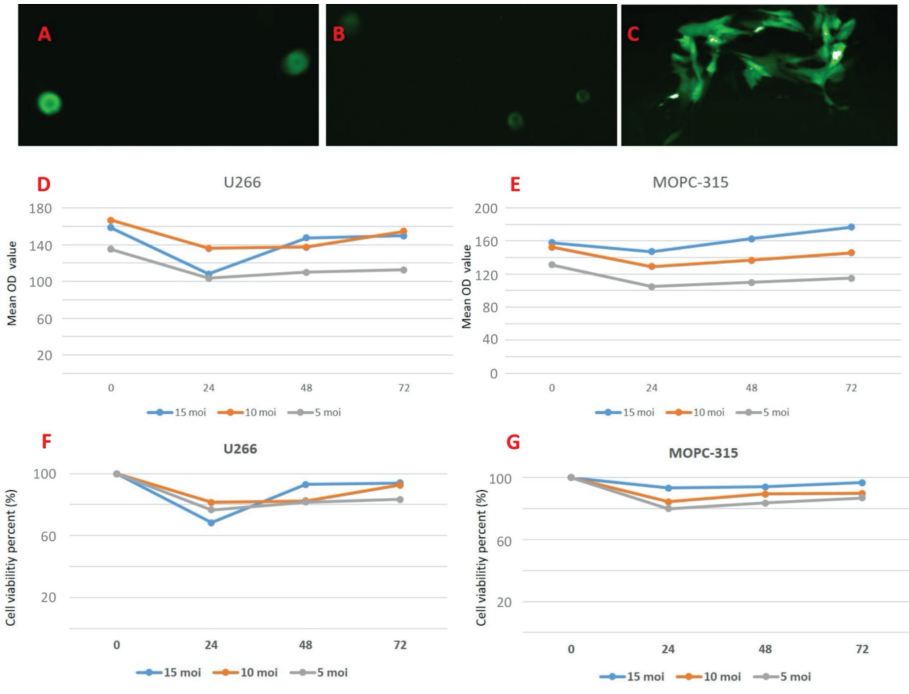

Figure 1. *Myxoma virus* (MYXV) infection in different cells and WST-1 results. MYXV expresses green fluorescent protein, and virus-infected cells were visualized as green under a fluorescent microscope. A) Virus-infected MOPC-315 cells, 10^x. B) Virus-infected U-266 cells, 10^x. C) Virus-infected Vero cells, 10^x. D) WST-1 results of MYXV-infected U-266 cells. E) WST-1 results of MYXV-infected MOPC-315 cells. F) MYXV-infected U-266 cell viability percentage for each incubation period. G) MYXV-infected MOPC-315 cell viability percentage for each incubation period. WST-1 results are shown as 15 multiplicity of infection (moi), 10 moi, and 5 moi virus-infected U-266 and MOPC-315 cells for 0, 24, 48, and 72 h of incubation.

WST-1 Assay

In this study, a set of cell groups was prepared to investigate the cytotoxicity, apoptosis, and autophagy caused by MYXV infection. The U-266 and MOPC-315 cells were prepared in 96-well microplates at a density of 3-4x10^4 cells per well. The cells were infected by diluting MYXV to a multiplicity of infection (moi) of 0.1, 1, 5, 10, or 15. All applications were performed in four repetitions. PBS was added to the wells for the control group. A rapamycin trial group was prepared in separate wells with MYXV-uninfected cells to induce autophagy. Cell viability rates after infection were tested at 24, 48, 72, 96, and 120 h. Cell viability was measured with 420-nm, 450-nm, 480-nm, and 630-nm filters using a spectrophotometer. The ideal incubation period for the cells was determined as 48 h. At the end of the 24, 48, and 72 h of incubation, cell supernatants were collected and analyzed.

Enzyme-Linked Immunosorbent Assay (ELISA)

For autophagy and apoptosis tests, cells were prepared as described above, and 500 nM rapamycin (Biosynth) was added to wells as a positive control. All experimental cell groups were incubated at 37 °C with 5% CO_2. Cell supernatants were collected after 24, 48, and 72 h of incubation and ELISA tests were performed.

In MM cell lines, the levels of apoptotic protein Bcl-2 (catalog no: E1832Hu, BT LAB, China) and the autophagic proteins Beclin-1 (catalog no: E2011Hu, BT LAB), human sequestosome-1 (p62) (catalog no: E6779 Hu, BT LAB), human LC3B (catalog no: MBS1603826, MyBioSource, USA), and human ATG-5 (catalog no: EH1729, FineTest, USA) were measured by ELISA according to the manufacturer's instructions.

ELISA tests were performed in triplicate, with each marker being tested twice. Thus, six measurements were made in each group for comparisons. The utilized kit contains 96-well microplates that are coated with capture antibodies, and biotinylated antibodies are used as detection antibodies. The standards in the kit are diluted in a twofold logarithmic manner. Standards, samples, and biotin antibodies are added to the wells, followed by the addition of HRP-streptavidin conjugate after washing. After another washing step, a substrate solution is added, and the target protein concentration is calculated by reading the optical density absorbance at 450-nm on a microplate reader (Epoch 2, BioTek, USA) and then adding the acidic stopping solution.

Transmission Electron Microscopy (TEM) Analysis

Cells were fixed with Karnovsky fixative (2.5% glutaraldehyde, 2% paraformaldehyde in cacodylate buffer) for 24 h. After fixation, the cells were pelleted by centrifugation at 300x g for 5 min. Cells embedded in 1% agar were then washed with distilled water and postfixed with 2% osmium tetroxide for 30 min. After washing with distilled water, cells were kept in 1% uranyl acetate at 4 °C overnight. The next day, after washing with distilled water, cells were kept in a lead aspartate solution at 65 °C for 1 h, then rinsed with distilled water and passed through an increasing alcohol series (30%, 50%, 70%, 80%, 90%, 96%, and 100%) for 1 h. Cells were dehydrated by putting them into pure acetone after alcohol and were kept in 1:1 acetone-Epon and 1:2 acetone-Epon mixtures for 2 h. After being kept in pure Epon for 2 h, cells were placed into an embedding mold and kept at 60 °C for 24 h to form a block. Thin sections of 60-100 nm in thickness were taken from the obtained cell blocks using an ultramicrotome (UC7, Leica, Germany) and a diamond blade, and the sections were dried on formvar-coated gold grids. The prepared sections were visualized at 30 kV using a GeminiSEM 500 electron microscope and a STEM detector (ZEISS, Germany). At least fifty cells were evaluated in each group.

Statistical Analysis

IBM SPSS Statistics (IBM Corp., USA) was used for statistical analysis. In group comparisons, the Kruskal-Wallis analysis of variance test was used for three or more groups. The Bonferroni-corrected Mann-Whitney U test was used for subgroup comparisons. For all statistics, $p<0.05$ was accepted as significant.

Results

WST-1 Assay Results

The WST-1 test results revealed the cell viability/toxicity in all experiments with viral infections of 5 moi, 10 moi, and 15 moi at 24, 48, and 72 h of incubation. It was determined for both cell types that cell viability decreased in the first 24 h following viral infection. There was no statistical difference in cell viability according to virus amounts ($p>0.05$). No significant increase in cell toxicity was detected in other incubation periods ($p>0.05$) (Figure 1).

ELISA Results

The results of autophagic proteins in MYXV-infected cell lines are given in Table 1 and Figures 2-5. The highest LC3B expression in the U-266 cell line was observed in the rapamycin group at 24 and 72 h, and in the group that received 5 moi MYXV at 48 h. At 24 h, the LC3B expression of the 15 moi MYXV group was significantly lower than the LC3B expression of the control group ($p=0.009$). The LC3B expression of the 5 moi MYXV group at 48 h was significantly higher than the LC3B expression of the control group at 48 h ($p=0.009$). The LC3B expression of the rapamycin group was significantly higher at 72 h compared to the LC3B expression of the control group ($p=0.009$). The highest expression of LC3B in the MOPC-315 cell line was observed in the control group at 24 and 72 h, and in the group that received 10 moi

Table 1. ELISA results.

			Control	Rapamycin	5 moi MYXV	10 moi MYXV	15 moi MYXV	Post-hoc	p
			$\bar{X} \pm SD$	$\bar{X} \pm SD$	$\bar{X} \pm SD$	$\bar{X} \pm SD$	$\bar{X} \pm SD$		
U-266	LC3B (ng/L)	24 h	2377±0.577	3427±0.577	1487±0.577	1417±0.577	987±0.577	[7]*	**0.009**
		48 h	1677±0.577	2247±0.577	2502±0.577	2232±0.577	1902±0.577	[2]*	**0.009**
		72 h	1537±0.577	2587±0.577	1542±0.577	1872±0.577	1577±0.577	[1]*	**0.009**
	p62 (ng/mL)	24 h	2.033±0.057	2.033±0.057	3.033±0.057	3.033±0.057	3.033±0.057	ns	0.055
		48 h	3.033±0.057	3.033±0.057	3.033±0.057	2.033±0.057	1.533±0.057	ns	0.089
		72 h	3.533±0.057	3.033±0.057	3.033±0.057	3.033±0.057	3.033±0.057	ns	0.087
	Beclin-1 (ng/mL)	24 h	6.033±0.057	6.033±0.057	8.033±0.057	9.033±0.057	14.033±0.057	[4]*, [7]*	**0.011**
		48 h	6.033±0.057	6.033±0.057	6.033±0.057	8.033±0.057	7.033±0.057	ns	0.065
		72 h	5.033±0.057	8.033±0.057	8.033±0.057	8.033±0.057	8.033±0.057	ns	0.078
	ATG-5 (ng/mL)	24 h	0.650±0.043	0.650±0.043	8.033±0.057	16.033±0.057	20.033±0.057	[4]*, [7]*	**0.011**
		48 h	0.650±0.043	0.650±0.043	14.033±0.057	20.033±0.057	20.033±0.057	ns	0.067
		72 h	0.650±0.043	0.650±0.043	14.033±0.057	19.033±0.057	20.033±0.057	[4]*, [7]*	**0.011**
	Bcl-2 (U/mL)	24 h	5.033±0.057	5.033±0.057	6.033±0.057	5.033±0.057	5.033±0.057	ns	0.087
		48 h	4.033±0.577	4.033±0.577	4.033±0.577	5.033±0.577	5.033±0.577	ns	0.075
		72 h	5.033±0.057	6.033±0.057	5.033±0.057	4.033±0.057	5.033±0.057	[6]*	**0.020**
MOPC-315	LC3B (ng/L)	24 h	2632±0.577	2162±0.577	2562±0.577	1047±0.577	1687±0.577	[3]*	**0.009**
		48 h	1607±0.577	1732±0.577	1947±0.577	1987±0.577	1812±0.577	[3]*	**0.009**
		72 h	2547±0.577	1657±0.577	1297±0.577	1627±0.577	1492±0.577	[2]*	**0.009**
	p62 (ng/mL)	24 h	5592±0.577	4863±0.577	4706±0.577	4784±0.577	4916±0.577	[2]*	**0.009**
		48 h	9870±0.577	6333±0.577	6121±0.577	6280±0.577	6439±0.577	[2]*	**0.009**
		72 h	5658±0.577	5022±0.577	5101±0.577	3645±0.577	6227±0.577	[10]*	**0.009**
	Beclin-1 (ng/mL)	24 h	8.067±0.115	7.033±0.057	6.033±0.057	6.100±0.173	6.067±0.115	ns	0.101
		48 h	5.033±0.057	8.067±0.115	7.100±0.173	5.100±0.173	7.067±0.115	[1]*, [6]*	**0.013**
		72 h	5.100±0.173	7.067±0.115	8.067±0.115	8.100±0.173	6.033±0.057	[2]*, [3]*	**0.011**
	ATG-5 (ng/mL)	24 h	0.650±0.043	0.650±0.043	7.067±0.115	17.100±0.173	18.067±0.115	[4]*, [7]*	**0.011**
		48 h	0.650±0.043	0.650±0.043	14.033±0.057	18.100±0.173	19.067±0.115	[4]*, [7]*	**0.040**
		72 h	0.650±0.043	0.683±0.101	15.067±0.115	18.100±0.173	20.067±0.115	[4]*, [7]*	**0.011**
	Bcl-2 (U/mL)	24 h	5.033±0.057	5.067±0.115	8.033±0.057	6.033±0.057	5.067±0.115	ns	0.058
		48 h	4.100±0.173	5.100±0.173	4.067±0.115	6.100±0.173	4.033±0.057	ns	0.084
		72 h	3.033±0.057	5.033±0.057	5.067±0.115	6.033±0.057	5.100±0.173	[3]*	**0.021**

[1]: Control versus rapamycin; [2]: control versus 5 moi; [3]: control versus 10 moi; [4]: control versus 15 moi; [5]: rapamycin versus 5 moi; [6]: rapamycin versus 10 moi; [7]: rapamycin versus 15 moi; [8]: 5 moi versus 10 moi; [9]: 5 moi versus 15 moi; [10]: 10 moi versus 15 moi; *: $\alpha<0.05$, ns: nonsignificant; ELISA: enzyme-linked immunosorbent assay; moi: multiplicity of infection; MYXV: *Myxoma virus*; SD: standard deviation.

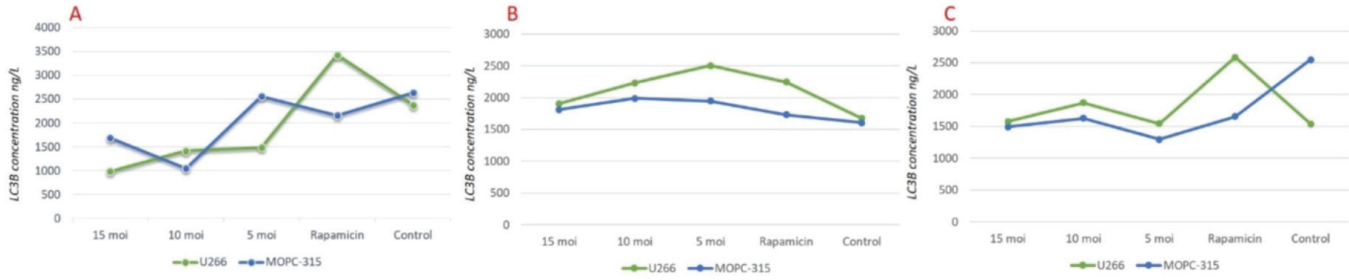

Figure 2. ELISA results of LC3B protein concentrations in MYXV-infected U-266 and MOPC-315 cells according to hours of incubation: A) 24 h of incubation period, B) 48 h of incubation, C) 72 h of incubation.

ELISA: Enzyme-linked immunosorbent assay; MYXV: *Myxoma virus.*

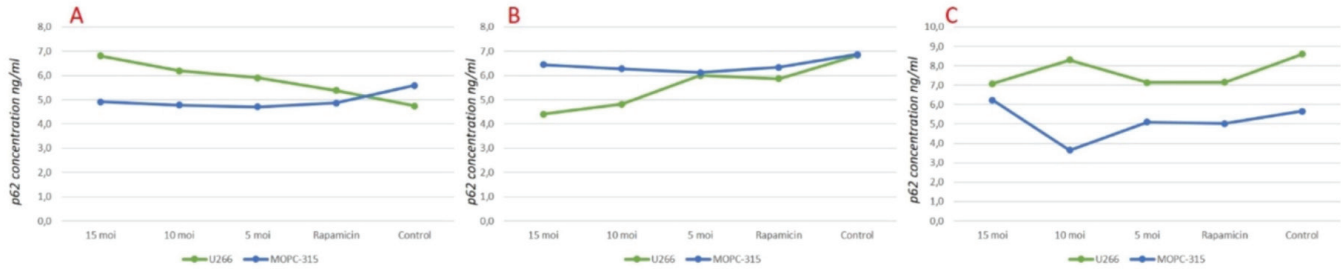

Figure 3. ELISA results of p62 protein concentrations in MYXV-infected U-266 and MOPC-315 cells according to hours of incubation: A) 24 h of incubation period, B) 48 h of incubation, C) 72 h of incubation.

ELISA: Enzyme-linked immunosorbent assay; MYXV: *Myxoma virus.*

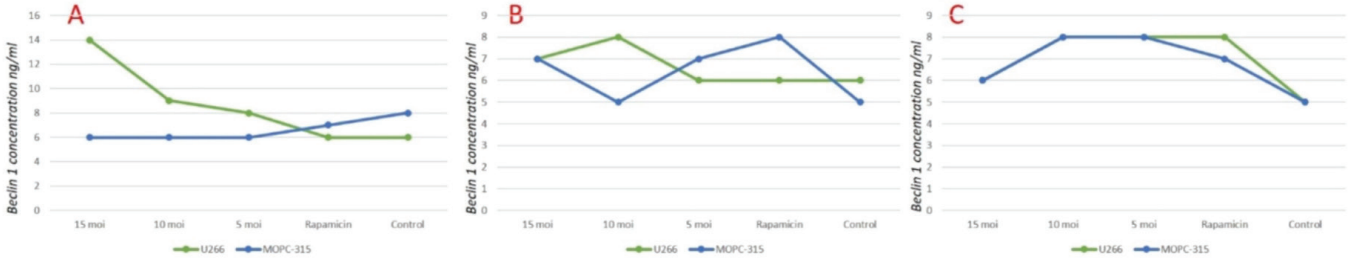

Figure 4. ELISA results of Beclin-1 protein concentrations in MYXV-infected U-266 and MOPC-315 cells according to hours of incubation: A) 24 h of incubation period, B) 48 h of incubation, C) 72 h of incubation.

ELISA: Enzyme-linked immunosorbent assay; MYXV: *Myxoma virus.*

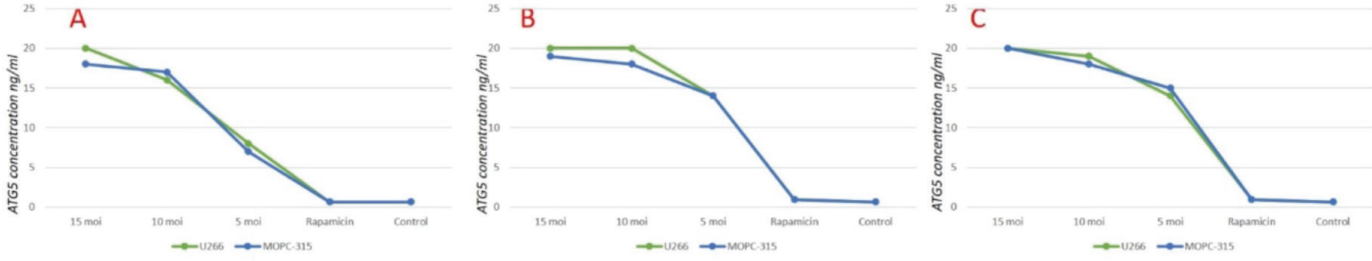

Figure 5. ELISA results of ATG-5 protein concentrations in MYXV-infected U-266 and MOPC-315 cells according to hours of incubation: A) 24 h of incubation period, B) 48 h of incubation, C) 72 h of incubation.

ELISA: Enzyme-linked immunosorbent assay; MYXV: *Myxoma virus.*

MYXV at 48 h. The LC3B expression of the 10 moi MYXV-treated group was significantly lower at 24 h and significantly higher at 48 h compared to the LC3B expression of the control group (p=0.009). The LC3B expression of the 5 moi MYXV group was significantly lower than the LC3B expression of the control group at 72 h (p=0.009).

There was no significant difference observed in the levels of p62 expressed in the U-266 cells (p>0.05). However, in the MOPC-315 cell line, the control group had the highest levels of p62 expression at 24 and 48 h. At 72 h, the group administered 15 moi MYXV had the highest p62 expression. The 5 moi MYXV group had significantly lower p62 expression than the control group at both 24 and 48 h (p=0.009). Additionally, at 72 h, the group administered 15 moi MYXV had significantly higher p62 expression than the group administered 10 moi MYXV (p=0.009).

The highest expression of Beclin-1 in the U-266 cells was observed in the group administered 15 moi MYXV at 24 h, and this value was significantly higher than the expression of Beclin-1 in the control and rapamycin groups. There was no significant difference in Beclin-1 expression between the groups at 48 and 72 h (p>0.05). In the MOPC-315 cells, the highest Beclin-1 expression was observed in the control group at 24 h, in the rapamycin group at 48 h, and in the group administered 10 moi MYXV at 72 h. While no difference was observed between the groups at 24 h (p>0.05), Beclin-1 expression in the rapamycin group at 48 h was significantly higher than in the control and the

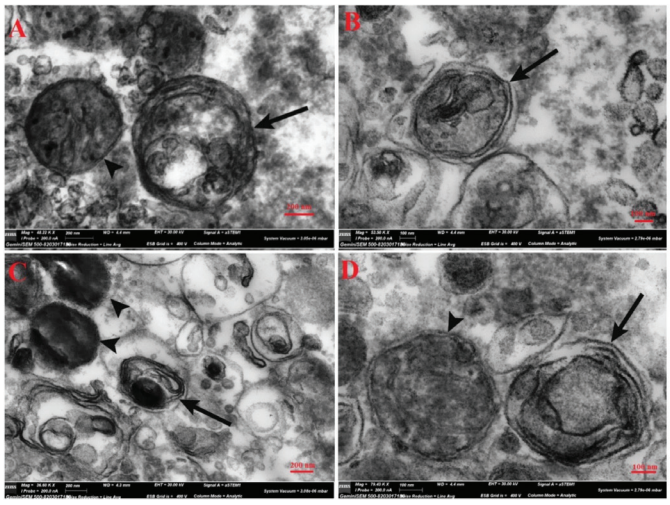

Figure 6. A) Electron microscopic image of cells from the MOPC-315 cell line. An autophagosome (arrow) characterized by a double-layered membrane structure containing various vesicles and degenerate structures and a mitochondrion (arrowhead) that maintains its normal structure are seen. B) Electron microscopic image of cells from the MOPC-315 cell line. An autophagosome (arrow) is seen, which is characterized by a double-layered membrane structure containing vesicles and membranes. C) Electron microscopic image of cells obtained from cell line U-266. An autolysosome (arrow) and larger lysosomes (arrowhead) adjacent to it are in the process of fusion of autophagosome membranes and lysosome. D) Electron microscopic image of cells obtained from cell line U-266. An autophagosome (arrow) and adjacent mitochondrion (arrowhead) are seen, characterized by a double-layered membrane structure containing membranes.

10 moi MYXV group (p=0.009). At 72 h, the Beclin-1 expression levels of the groups that received 5 moi MYXV and 10 moi MYXV were significantly higher than the Beclin-1 expression of the control group (p=0.009).

The highest expression levels of ATG-5 in the U-266 cells at 24, 48, and 72 h were determined in the 15 moi MYXV group (Figure 5). At 24 and 72 h, ATG-5 expression was significantly higher in the 15 moi MYXV group than in the control and rapamycin groups (p=0.011). There was no difference between the groups at 48 h (p>0.05). The highest expression levels of ATG-5 in the MOPC-315 cells at 24, 48, and 72 h were observed in the 15 moi MYXV group, being significantly higher than the ATG-5 expression in the control and rapamycin groups (p=0.011).

There was no significant variation in the expression of Bcl-2 in the U-266 cells after 24 or 48 h (p>0.05). However, after 72 h, the group that was administered rapamycin exhibited significantly higher expression of Bcl-2 compared to the 10 moi MYXV group (p=0.020). Similarly, no significant difference was observed in Bcl-2 expression in the MOPC-315 cell line after 24 or 48 h (p>0.05). Nevertheless, after 72 h, the group that was administered 10 moi MYXV exhibited significantly higher Bcl-2 expression compared to the control group (p=0.021).

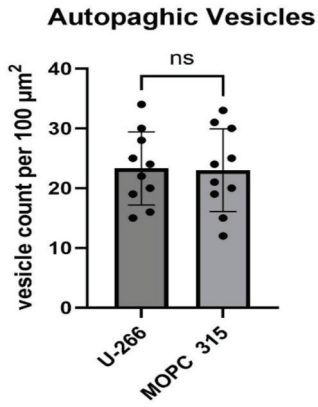

Figure 7. Autophagic vesicle counts for MYXV-infected U-266 and MOPC-315 cells.

MYXV: *Myxoma virus;* ns: not significant.

Electron Microscopic Screening

Several cells in all examined groups showed deterioration in their cell membrane integrity and ultrastructural structure, while a small number of cells maintained their integrity. Electron microscopic images were obtained from the structurally intact cells, which revealed autophagic vesicles and autophagosome structures at different stages (early and late) in all groups, as shown in Figure 6.

Autophagosomes were counted in a unit area of cells (100 µm^2) in the autophagic particle count groups (as seen in Figure 7). There was no statistical difference between the cell groups based on unpaired t-tests and Mann-Whitney U tests (p>0.05).

Discussion

The targeted survival in MM has not been achieved although different treatment modalities have been applied for many years. The heterogeneity of MM cells and the emergence of drug-resistant clones preclude a complete cure. Recently, both experimental and clinical studies have suggested that OVs could be a potential therapeutic alternative to treat hematological malignancies [45,54]. OVs can be used for therapeutic purposes alone and/or in combination with standard chemotherapeutic agents [55].

Autophagy exerts an oncosuppressive effect in the early phase of tumorigenesis by preventing genome instability, inflammation, and chronic tissue damage [56]. Conversely, once the tumor has grown, autophagy promotes tumor growth, progression, and drug resistance [57]. Therefore, targeting autophagy in tumor treatments would be a good approach. Indeed, a link between OVs and autophagic cell death has also been demonstrated [58]. Various OVs such as the herpes simplex virus [59], adenovirus [60], paramyxovirus [61], and Newcastle disease virus [62] have been reported to induce autophagic cell death. In addition, OV-induced autophagy has been found to induce immunogenic

cell death in cancer cells, thereby inducing stronger antitumor immunity [63].

Survival has improved significantly with the use of new therapeutic agents such as PIs in the treatment of MM, but resistance to these drugs develops over time [64]. When PIs are administered to MM patients, PCs activate autophagy for protein degradation for survival [57]. Therefore, the co-targeting of proteasome and autophagy in the treatment of MM will have an enhanced antimyeloma effect. Vogl et al. [65] found high antitumor efficacy in MM with the concomitant use of bortezomib, a PI, and hydroxychloroquine, an autophagy inhibitor. Lei et al. [45] reported that OVV armed with Beclin-1 induced enhanced cell death by inducing autophagic cell death in leukemia and MM cell lines in vitro and in vivo. In the same study, they determined that OVV armed with Beclin-1 increased necrosis in tumor tissue and exhibited increased Beclin-1 expression and irregular p62 expression; autophagosomes and autolysosomes were observed by TEM study [45].

In our study, the expression of proteins involved in autophagy was evaluated. The levels of ATG-5 and Beclin-1, which are involved in the formation of phagophores, constituting the initial stage of autophagy, were found to be significantly higher in MM cell lines at 24 h in our study ($p<0.05$). The highest expression levels of both ATG-5 and Beclin-1 were observed in the group administered 15 moi MYXV. LC3B, which helps phagophore elongation by joining the late autophagosome ring of autophagy, was found to be significantly higher at 48 h in our study. The highest expression of LC3B was seen in the group treated with 5 moi MYXV in the U-266 cell line and in the group treated with 10 moi MYXV in the MOPC-315 cell line. In terms of p62 expression, no significant difference was observed in the U-266 cell line ($p>0.05$), while there was an irregular expression pattern in the MOPC-315 cell line. TEM analysis was performed on MYXV-treated cell lines to confirm the ELISA results and visualize autophagy. As a result of TEM images, autophagosomes were determined in both early and late autophagy processes. These findings show that MYXV causes the death of malignant PCs by autophagy in MM cell lines. Both the ELISA and TEM results of our study reaffirm the results of previous studies showing increased expression of autophagy proteins in MM cell lines [45].

Study Limitations

There are some limitations of our study. First, the results of the autophagy proteins whose expressions we examined in this study could also be confirmed by methods such as western blotting, immunohistochemistry, and/or flow cytometry. Experimental animal studies with MM cell lines or MM cell lines from clinical patients would also strengthen the results. It is suggested that autophagy is responsible for drug resistance, especially in MM patients. Therefore, adding a PI to the study would have been helpful to elucidate the cause of drug resistance.

Conclusion

The development of resistance to the drugs used in MM is the most important obstacle to improving the survival of these patients. Autophagy is one of the mechanisms that may be responsible for the development of drug resistance in MM. OVs with successful results in treating hematological malignancies can also induce autophagy. Our study determined that the expression of autophagy proteins ATG-5, Beclin-1, and LC3B increased in MM cell lines with the application of MYXV, an OV. Targeting autophagy with OVs is promising in the treatment of MM to prevent drug resistance and increase the effectiveness of MM treatment. More research is needed to understand the role of autophagy in MM fully and to develop targeted therapies.

Ethics

Ethics Committee Approval: The sample was not derived from patients. Commercial cell lines were utilized.

Informed Consent: The sample was not derived from patients. Commercial cell lines were utilized.

Authorship Contributions

Concept: A.Y., D.M., B.E.; Design: A.Y., D.M., B.E.; Data Collection or Processing: A.Y.; Analysis or Interpretation: A.Y.; Literature Search: A.Y., D.M., B.E.; Writing: A.Y., D.M., B.E.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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