Myxoma Virus Induces Ligand Independent Extrinsic Apoptosis in Human Myeloma Cells

Mee Y. Bartee, Katherine M. Dunlap, Eric Bartee

Abstract

We have previously shown that the myxoma virus kills human myeloma cells. Here we show that this killing occurs because of the ligand-independent activation of caspase-8. We hypothesize that this activation is the result of viral host protein shutoff depleting a variety of cellular inhibitors of apoptosis.

Introduction: Multiple myeloma is a clonal malignancy of plasma B cells. Although recent advances have improved overall prognosis, virtually all myeloma patients still succumb to relapsing disease. Therefore, novel therapies to treat this disease remain urgently needed. We have recently shown that treatment of human multiple myeloma cells with an oncolytic virus known as myxoma results in rapid cell death even in the absence of viral replication; however, the specific mechanisms and pathways involved remain unknown. **Materials and Methods:** To determine how myxoma virus eliminates human multiple myeloma cells, we queried the apoptotic pathways that were activated after viral infection using immunoblot analysis and other cell biology approaches. **Results:** Our results indicate that myxoma virus infection initiates apoptosis in multiple myeloma cells through activation of the extrinsic initiator caspase-8. Caspase-8 activation subsequently results in cleavage of BH3 interacting-domain death agonist and loss of mitochondrial membrane potential causing secondary activation of caspase-9. Activation of caspase-8 appears to be independent of extrinsic death ligands and instead correlates with depletion of cellular inhibitors of apoptosis. We hypothesize that this depletion results from virally mediated host-protein shutoff because a myxoma construct that overexpresses the viral decapping enzymes displays improved oncolytic potential. **Conclusion:** Taken together, these results suggest that myxoma virus eliminates human multiple myeloma cells through a pathway unique to oncolytic poxviruses, making it an excellent therapeutic option for the treatment of relapsed or refractory patients.

Clinical Lymphoma, Myeloma & Leukemia, Vol. 16, No. 4, 203-12
Keywords: Caspase-8, cIAPs, Host protein shutoff, Oncolytics, Resistance

Introduction

Multiple myeloma (MM) is a clonal malignancy of plasma cells that is diagnosed in approximately 24,000 new patients each year.[1,2] Recent advances in the treatment of MM, including the development of novel chemotherapeutic agents such as bortezomib,[3-5] has significantly improved initial prognosis; however, the disease displays a high degree of genetic heterogeneity and virtually all patients eventually succumb to relapse. Therefore, even with the best available treatments, the 5-year survival rate for patients with a new diagnosis of MM remains only 45%, indicating that novel treatment modalities are urgently needed.[2]

Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, SC

Submitted: Aug 12, 2015; Revised: Dec 14, 2015; Accepted: Dec 15, 2015; Epub: Dec 23, 2015

Address for correspondence: Eric Bartee, PhD, Department of Microbiology and Immunology, Medical University of South Carolina, 173 Ashley Ave, Charleston, SC 29425
Fax: 843-792-4882; e-mail contact: bartee@musc.edu

One treatment option that has recently shown promise is the use of oncolytic viruses.[6-10] Treatment with these viruses, including: reovirus,[11,12] recombinant measles,[13-15] vesicular stomatitis virus,[16-18] and adenovirus[19,20] is highly cancer-specific, and reduces the chances of off-target toxicities. Additionally, oncolytic viruses often eliminate infected cancer cells through virally distinct mechanisms, which makes them particularly attractive for the treatment of relapsed and/or refractory MM patients. Indeed, a recent, well cited human trial showed that recombinant oncolytic measles virus could induce complete remission even in late-stage, chemotherapy-refractive relapsed MM patients.[21]

Our laboratory is interested in the clinical potential of an oncolytic poxvirus known as myxoma (MYXV). MYXV has several advantages as an oncolytic agent. First, infection is highly restricted to lagomorphs (rabbits).[22] No instance of MYXV infection of viral disease has ever been noted in any nonrabbit species. This provides MYXV with an excellent safety profile as well as eliminating the possibility of patients presenting with preexisting humoral immunity to the virus. Second, the virus is

2152-2650/$ - see frontmatter
http://dx.doi.org/10.1016/j.clml.2015.12.005

highly lytic and generally replicates to excellent titers in vitro, suggesting feasible clinical translation.

We have previously shown that treatment with MYXV can effectively kill human MM cell lines as well as primary MM cells found in patient bone marrow samples. Elimination of MM cells was highly efficient and treatment of MM-contaminated autologous transplant samples with MYXV before transplantation was sufficient to prevent disease relapse in animal models.[23] Interestingly, although most oncolytic viruses eliminate infected cells through direct viral lysis, elimination of malignant MM cells by MYXV was independent of viral replication and instead appeared to occur through virally mediated induction of programmed cell death.[23] The specific pathways involved in the induction of this programmed cell death, however, remained unknown.

Because resistance to treatment is a major clinical challenge in MM, and the development of this resistance depends on the specific pathways through which a treatment eliminates malignant cells, we sought to characterize the molecular pathways through which MYXV induces programmed cell death in infected MM cells with the goal of identifying how these pathways might influence treatment of relapsed or refractory patients.

Materials and Methods

Cells and Reagents

U266 and MM1.S cells were purchased from ATCC (Manassas, VA), RPMI-8226 cells were obtained from Dr Bei Lu at the Medical University of South Carolina. Cells were cultured in RPMI-1640 supplemented with 20% fetal bovine serum and penicillin/streptomycin/glutamine (Mediatech, Manassas, VA). Cells were maintained between 0.2 and 0.8 $\times$ 10^6 cells per mL with no more than 10 $\times$ 10^6 cells per T175 flask. z-VAD-fmk, z-DEVD-fmk, z-LEHD-fmk, and z-IETD-fmk (BD Biosciences, San Jose, CA) were used at a final concentration of 20 μM. GSK2606414 (Calbiochem, Billerica, MA) was used at a final concentration of 10 nM. The following antibodies were used in these studies: caspase (Casp)-8 (12F5; Enzo Life Sciences Inc, Farmingdale, NY); BH3 interacting-domain death agonist (BID) (2002), Casp-2 (2224), Casp-9 (9502), Casp-10 (9752), myeloid Cell Leukemia 1 (Mcl1) (5453), poly ADP ribose polymerase (PARP) (9542), survivin (2808), tumor necrosis factor receptor 1 (TNFR1) (3736), apoptosis stimulating fragment (FAS) (8023), death receptor 5 (DR5) (8074), and X-linked inhibitor of apoptosis protein (XIAP) (2045) (all from Cell Signaling Technology, Beverly, MA); cellular FADD-like IL-1β-converting enzyme)-inhibitory protein (cFLIP)$^{S/L}$ (sc5276), actin (sc1615), eukaryotic initiation factor 2alpha (eIF2α) (sc11386), and phospho-eIF2α (sc101670) (all from Santa Cruz Biotechnology, Dallas, TX).

Virus and Viral Infections

Recombinant myxoma virus expressing green fluorescent protein (vMYX-GFP) was a kind gift from Dr Grant McFadden and was grown and purified as previously described.[24] A viral construct overexpressing the viral decapping enzymes M084 and M085 (vMYX-M083) was constructed in our laboratory (unpublished). Unless otherwise noted, infections were done by concentrating cells to 10 $\times$ 10^6 cells per mL and then infecting at a multiplicity of infection (MOI) = 10 for 30 minutes at 37°C. After infection, cells were diluted to a concentration of <1 $\times$ 10^6 cells per mL with fresh media. All drug treatments were done by pretreating cells with drug for >30 minutes before infection and subsequently diluting cells in complete media containing additional fresh drug.

Multiple Myeloma Patient Samples

Bone marrow aspirates from patients with various stages of MM were obtained through the Medical University of South Carolina biorepository in accordance with institutional review board guidelines. Red blood cells were removed through ammonium-chloride-potassium lysis and then samples were infected with vMYXV-GFP at an MOI = 10 as previously described herein.

Western Blot Analyses

Cell lysates were generated at a concentration of 1 $\times$ 10^6 cells per 100 μL of Laemmli sample buffer. Samples were separated on sodium dodecyl sulfate poly-acrylamide gel electrophoresis gels, and subsequently transferred to polyvinylidene difluoride. Membranes were blocked for 30 minutes with 5% nonfat dry milk (Mix'n Drink; SACO, Middleton, WI) in TBS-T (25 mM Tris, 150 mM NaCl, 2 mM KCl, 0.1% Tween 20 pH 7.4), and then incubated overnight at 4°C with primary antibody diluted in 1% nonfat dry milk in TBS-T. Appropriate secondary antibody incubations were done at 4°C for 1 hour, and blots were treated with chemiluminescent substrate (Pierce Biotechnology, Rockford, IL) before exposure to film.

Immune Precipitations

Per condition, we used 2 $\times$ 10^7 cells. Cells were lysed with 1 mL cold precipitation buffer (20 mM Tris pH 7.4, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 5% glycerol, 1% NP40, Protease Inhibitor Cocktail) for 20 minutes at 4°C. Samples were then centrifuged at 15,000 rpm in a prechilled centrifuge for 10 minutes to remove large cell debris. The supernatant was placed into a fresh tube containing 30 μL of equilibrated Protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA), and rocked on ice for 30 minutes. Samples were centrifuged and the precleared supernatant was transferred to a fresh tube containing 10 μL of primary antibody. Samples were incubated for 45 minutes on ice. Equilibrated Protein A/G beads (30 μL) were then added and samples were rocked for an additional 45 minutes. Bead beds were washed 5 times with 500 μL cold precipitation buffer, and then eluted in Laemmli Sample Buffer.

Flow Cytometry

For analysis of mitochondrial membrane potential, U266 cells were labeled with 3 μM tetramethylrhodamine, methyl ester, perchlorate (TMRM; Life Technologies, Grand Island, NY) in complete media for 30 minutes at 37°C. Cells were then pelleted and resuspended in complete media containing 1 μM TMRM. At the indicated times samples were pelleted, resuspended in 2% paraformaldehyde in phosphate buffered saline, and analyzed using a flow cytometer. For analysis of cell surface phosphoserine exposure, U266 cells were infected with vMYX-GFP as previously indicated herein for 6 hours. Cells were then pelleted, resuspended in binding buffer (100 mM HEPES pH 7.4, 140 mM NaCl, 25 mM $CaCl_2$), and labeled with APC-Annexin V (BD Biosciences)