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Myxoma virus-mediated oncolysis of ascites-derived human ovarian cancer cells and spheroids is impacted by differential AKT activity

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Abstract

Objective—We propose that metastatic epithelial ovarian cancer (EOC) is a potential therapeutic target for the oncolytic agent, Myxoma virus (MYXV).

Methods—Primary EOC cells were isolated from patient ascites and cultured as adherent cells or in suspension using Ultra Low-Attachment dishes. MYXV expressing green fluorescent protein was used to infect cells and spheroids. Infection was monitored by fluorescence microscopy, viral titrating and immunoblotting for M-T7 and M130 virus protein expression, and cell viability by alamarBlue assay. Akti-1/2 (5 μ M) and rapamycin (20 nM) were used to assay the role of PI3K–AKT signaling in mediating MYXV infection.

Results—Ascites-derived EOC cells grown in adherent culture are effectively killed by MYXV infection. EOC cells grown in suspension to form three-dimensional EOC spheroids readily permit

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Conflict of interest statement

G. McFadden holds inventor's intellectual property for Myxoma virus as an oncolytic virotherapeutic. The other authors declare they have no conflicts of interest.

MYXV entry into cells, yet are protected from the cytopathic effects of late MYXV infection. Upon reattachment (to model secondary metastasis), EOC spheroids are re sensitized to MYXV-mediated oncolysis. The critical determinant that facilitates efficient MYXV infection is the presence of an activated PI3K-AKT signaling pathway. Treatment with the specific AKT inhibitor Akti-1/2 reduces infection of monolayer EOC cells and spheroids. Direct infection of freshly collected ascites demonstrated that 54.5% of patient samples were sensitive to MYXV-mediated oncolytic cell killing. We also demonstrate that factor(s) present in ascites may negatively impact MYXV infection and oncolysis of EOC cells, which may be due to a down-regulation in endogenous AKT activity.

Conclusions—Differential activity of AKT serves as the mechanistic basis for regulating MYXV-mediated oncolysis of EOC spheroids during key steps of the metastatic program. In addition, we provide the first evidence that MYXV oncolytic therapy may be efficacious for a significant proportion of ovarian cancer patients with metastatic disease.

Keywords

Ovarian cancer; Ascites; Spheroid; Oncolytic virus; Myxoma virus; AKT kinase

Introduction

Ovarian cancer is the sixth most prevalent cancer in women and the most lethal of the gynecologic malignancies [1]. Epithelial ovarian cancer (EOC) constitutes approximately 90% of ovarian tumors, and is believed to originate from the ovarian surface epithelium (OSE) [2] although evidence for alternative origins is emerging [3]. Metastasis of EOC is unique in that it typically spreads by direct dissemination or shedding of cancer cells from the primary tumor site into the peritoneal space and secondary tumor formation on serosal surfaces of the peritoneal cavity [4]. Although about three-quarters of EOC patients treated with chemotherapy are initially responsive, most of these women will ultimately relapse with chemo-resistant disease. Thus, it is critical that we develop new therapeutics to better eradicate meta-static EOC cells.

Clinical application of viruses for cancer treatment has been attempted for nearly a century with sporadic success, but there has been a resurgence of virotherapy for cancer over the last two decades [5]. The premise of viral oncolysis is that naturally-occurring or genetically engineered viruses will undergo their replicative lytic cycle preferentially within cancer cells. This selective tropism of oncolytic viruses for cancer cells is in large part due to deficient anti viral responses, genetic mutations, and aberrant signaling pathways which are inherent to cancer cells, yet function properly in normal cells [6]. Thus, cancer cells, including those from EOC, have the potential to be targeted and eliminated by oncolytic viruses while leaving normal cells unscathed. For example, conditionally replicating recombinant human adenovirus strategies were the most extensively studied in EOC [7]. Other viruses have been applied to EOC including reovirus [8], measles and mumps virus [9] and vesicular stomatitis virus [10,11]; and some have progressed to clinical trials for EOC [12-15]. Thus, there remains continued interest in identifying and developing potent oncolytic viral therapies for EOC [16].

A recent player in the viral therapy arena is the rabbit specific poxvirus, Myxoma virus (MYXV) [17]. Like all poxviruses, MYXV is able to bind and enter a wide variety of mammalian cells [18]. Current data indicates that productive MYXV infection is highly dependent upon the ability of the virus to take control of specific signaling molecules in the host cell [19]. Specifically, MYXV encodes multiple host range factors, including M T5, which regulates tropism to cancer cells based on the presence of activated PI3K AKT signaling, a pathway commonly mutated or up regulated in human cancers [19,20]. MYXV infectivity profiles in human cancer cells can be divided into three distinct categories, designated as Types I (permissive), II (semi permissive, i.e. requires M T5 host range factor) or III (restrictive) [19,21]. Targeted inhibition of certain phosphatidylinositol 3 kinase (PI3K) AKT pathway components can boost AKT activity through feedback mechanisms, thus enhancing MYXV infectivity and improving oncolytic efficacy in both in vitro and in vivo cancer models [22-25].

To date only two reports indicate that MYXV can infect immortalized human EOC cell lines [21,26], but there is no data indicating that this virus can infect primary human EOC cells isolated directly from patients. Thus, we sought to test the efficacy of MYXV mediated cell killing of ascites derived EOC cells using suspension culture and three dimensional multicellular spheroids as a model for metastatic disease. Herein, we describe novel data regarding differential MYXV oncolysis in adherent EOC cells compared with spheroids, which impinges on the underlying level of PI3K AKT pathway activity. In addition, we provide the first evidence of the therapeutic potential for MYXV oncolytic action on malignant ascites directly isolated from EOC patients.

Materials and methods

Cell culture

The human ovarian cancer cell lines SKOV3 and OVCAR3 were purchased from the American Type Culture Collection (ATCC; Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Wisent, St. Bruno, Canada) supplemented with 5% fetal bovine serum (FBS; Wisent). OVCA429 cells (gift of Dr. Barbara Vanderhyden, OHRI) were maintained in alpha MEM with 5% FBS and 1 × non essential amino acids. OVCAR3 cells were purchased from ATCC and cultured in RPMI medium containing 5% FBS. The 4306 mouse ovarian cancer cell line (gift of Dr. Daniela Dinulescu, Brigham & Women's Hospital) and baby green monkey kidney (BGMK) cells were cultured in DMEM supplemented with 5% FBS. Cells were maintained in a 37 °C humidified atmosphere of 95% air and 5% CO₂.

Ascitic fluid collected from chemotherapy naïve patients at time of paracentesis or debulking surgery was used to generate primary human EOC cell cultures as described previously [27]. Primary cultures of ascites cells or spheroids isolated from ascites were maintained in a 1:1 mixture of MCDB 105 medium: medium 199 medium (Sigma, St. Lewis, MO) supplemented with 10% FBS (Wisent) and 50 µg/mL penicillin streptomycin (Wisent). Clarified ascites fluid was prepared by centrifugation at 1500 rpm for 5 min to remove cells and used immediately, or stored at – 80 °C for subsequent use. All work with patient materials has been reviewed and approved by The University of Western Ontario

Human Subjects Research Ethics Board. Adherent cells were maintained on tissue culture treated polystyrene (Sarstedt, Newton, NC). Non adherent cells and spheroids were maintained on Ultra Low Attachment (ULA®) cultureware (Corning, Corning, NY), which is coated with a hydrophilic, neutrally charged hydrogel to prevent cell attachment. Single cell suspensions of 1×10^5 cells/mL were seeded to ULA® plates to establish suspension cultures and to form spheroids. Spheroids were reattached to standard tissue culture plastic by transferring spheroids in a volume of b 200 μ L from individual wells of 24 well ULA® cluster dishes to individual wells of 6 well tissue culture treated plates containing 2 mL of complete growth medium.

Myxoma virus infection

Preparation and titering of MYXV (i.e. vMyxGFP [28]) using BGMK cells were performed as described previously [29]. For preparation of UV inactivated MYXV, virus stock was UV irradiated for 5 min using a Stratalinker UV crosslinker (Stratagene); this incubation time was determined empirically to inactivate vMyxGFP to undetectable fluorescence in BGMK cells at MOI of 10.

Primary EOC cells and cell lines were infected with MYXV by adding serially diluted virus (diluted in growth media) to cells to achieve an MOI of 0.1, 1, or 10, as indicated in each experiment. For infection of adherent monolayer EOC cells, cells were seeded 24 h prior to MYXV infection. For infection of EOC cells in suspension culture, MYXV was added immediately upon seeding cells to wells of ULA® plates. For infection of EOC spheroids, cells were seeded to wells of ULA® plates, allowed to form spheroids for 3 days, and then treated with MYXV. Virus infection was monitored over 24–96 h by phase contrast and fluorescence microscopy using an Olympus IX70 inverted microscope and photos were acquired using ImagePro image capture software.

Direct infection of patient derived ascites with MYXV was performed by transferring 2 mL of fluid into each well of a 6 well tissue culture treated dish, followed by addition of 10^5 , 10^6 or 10^7 ffu, as determined by titering in BGMK cells, or the equivalent of 10^7 ffu of UV irradiated control virus. Two wells remained as negative controls without any virus treatment. After 3–4 days, ascites fluid was replaced with MCDB 105:M199 medium containing 10% FBS and 50 μ g/mL penicillin/streptomycin, as per standard primary culture of EOC cells, and replaced every 3–4 days as needed. The experiment terminated when cells in the negative control wells reached 100% confluence, at which point cells were stained using the Hema 3 Stain kit.

Viability assays

Cell viability of MYXV infected cells and spheroids was assessed using alamarBlue® (Invitrogen, Burlington, ON) as per manufacturer's instructions. For adherent monolayer cells, medium was replaced with alamarBlue® reagent diluted 1:20 in complete medium, incubated for 4 h at 37 °C in a humidified atmosphere of 95% air and 5% CO₂, and then fluorescence was quantified with a Wallac plate reader using 560/590 nm excitation/emission filter settings. For suspension and spheroid cultures, 50% of the culture medium (500 μ L per well of 24 well plate) was removed carefully and an equal volume of alamar

Blue® reagent diluted 1:10 in complete medium was added; analysis was performed as described above.

Immunoblotting

Cell lysates were obtained using a modified radioimmunoprecipitation assay (RIPA) buffer [50 mM HEPES pH7.4, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 mM NaF, 1% Triton X 100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1× protease inhibitor cocktail (Roche, Laval, QC)] as described previously[30]. To ensure complete dissociation of spheroids, lysates were vortexed several times during lysis on ice prior to high speed centrifugation. Protein concentration of each lysate was determined by Bradford assay using Protein Assay Dye Reagent (BioRad, Mississauga, ON). Total protein (10–30 µg) was resolved by 8% or 14% SDS PAGE, transferred to a polyvinylidene difluoride membrane (PVDF; Roche), blocked with 5% skim milk in Tris buffered saline with Tween 20 [TBST; 10 mM Tris HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20]. Membranes were washed in TBST and incubated overnight at 4 °C with specific antibodies (1:1000 in 5% skim milk/TBST or 5% BSA/TBST as per manufacturer's recommendations). Immunoreactive bands were visualized by incubating for 1 h at room temperature with a peroxidase conjugated anti rabbit or anti mouse IgG (1:10 000 in 1% skim milk/TBST; GE Healthcare) followed by enhanced chemiluminescence reagent (ECL Plus; GE Healthcare) and exposure to Kodak Biomax X ray film (GE Healthcare).

Antibodies and other reagents

Antibodies against phospho AKT Ser473 (#9271) and phospho p70S6K (#9234) were obtained from Cell Signaling Technology (Danvers, MA). Antibodies against total Akt1/2/3 (H 136; sc 8312) and p70S6K (S 04; sc100423) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against M T7 and M130 have been described previously [20,31]. Anti actin antibody (A2066; Sigma) served as a protein loading control. AKT inhibitor VIII (Akti 1/2; EMD/Calbiochem) and rapamycin (R0395; Sigma) were prepared according to manufacturer's instructions.

Spheroid dispersion assay

Spheroids were transferred in a volume of 200 µL from individual wells of 24 well ULA® cluster dishes to individual wells of 6 well tissue culture treated plates containing 2 mL of complete growth medium. Reattachment was allowed to proceed for up to three days with images captured during this time by phase contrast and fluorescence microscopy using an Olympus inverted microscope and ImagePro image capture software. After 3 days, dispersing spheroids were fixed and stained using Hema 3 Stain kit (Fisher Scientific) and images were captured of independent reattached spheroids using an Olympus stereomicroscope and ImagePro image capture software. The dispersion area was quantified for each image using NIH ImageJ (NIH, Bethesda, MD) area measurement tool.

Results

MYXV infection of EOC cells and spheroids

There is limited evidence in the literature that MYXV is capable of infecting established human ovarian cancer cell lines indicating that they are either permissive or semi permissive to complete oncolysis [21,26]. To significantly add to these data, we performed direct analysis of primary EOC cells derived from patient ascites and have also broadened this approach by applying MYXV infection studies to both adherent as well as suspension and spheroid culture systems to mimic EOC dissemination in ascites fluid in vitro. We have determined empirically that seeding 50,000 cells per milliliter using Ultra Low Attachment (ULA®) tissue culture plastic results in multicellular spheroids that closely resemble those observed in patient derived ascites. Ovarian cancer cell lines, such as SkOV3, OVCA429, OVCAR3 and 4306 cells used in this study, can also be used to generate spheroids using the same approach.

Adherent primary EOC cells are readily infected by MYXV within 24 h at an MOI of 1, as evidenced by visualization of green fluorescent protein (GFP) expression from the vMyxGFP recombinant virus, and eventually undergo oncolysis as demonstrated by cytopathic effect by 72 h post infection (Fig. 1A). Entry of MYXV into suspension cells is also efficient and comparable to adherent cells, with all visible cells and small aggregates exhibiting GFP positive fluorescence (MOI of 1 at 24 h post infection). Interestingly, under these same infection conditions, established EOC spheroids have a markedly reduced ability to be infected by MYXV. This phenomenon is diminished over time with EOC cells comprising large spheroids efficiently infected by MYXV ($91.8\% \pm 8.4\%$) as quantified by disaggregating multicellular spheroids using trypsin digestion 72 h post infection and visualizing fluorescent single cells that had formerly comprised the interior of spheroids (Supplementary Fig. S1).

We used Western blot detection of two virus specific proteins, M T7, an early protein [20] and M130, a late protein [31] to further confirm that MYXV was infecting primary EOC cells in monolayer, suspension and spheroids and to examine the apparent retarded kinetics of infection in established spheroids. Both adherent and suspension EOC cells expressed M T7 and M130 proteins within hours after infection, with a slightly faster rate of early protein synthesis after infection in adherent cells as evidenced by detectable M T7 expression (Fig. 1B). By 16 h, however, both adherent and suspension cells exhibit detectable M T7 expression and robust M130 protein levels. In contrast, expression of both M T7 and M130 proteins is largely undetectable 16 h after infection of established EOC spheroids. This reduced expression was observed in primary EOC cells ($n = 2$), the SkOV3 cell line, and in the 4306 mouse ovarian tumor cell line (data not shown).

These results indicate that, compared to adherent and suspension cells, EOC spheroids exhibit early resistance to MYXV infection. After prolonged infection, however, the entire EOC spheroid became GFP positive. In order to determine whether there was virus replication and progeny virus production, infectious particles were quantified after complete infection. Lysates and culture supernatant were collected from adherent, suspension and spheroid cultures 96 h after MYXV infection and titered using permissive baby green

monkey kidney (BGMK) cells. Although the kinetics of MYXV infection progression appeared delayed in EOC spheroids, there was no difference in final MYXV production among the three culture conditions (Fig. 1C).

Given that single EOC cells were readily infected in adherent and suspension culture, yet large spheroids showed delayed progression of infection, we postulated that MYXV penetration into spheroids was largely determined by the overall size of the multicellular aggregates. By using three different amounts of MYXV (MOI of 0.1, 1 and 10), we infected established EOC spheroids of various sizes ranging from 10 to 100 μm in diameter, and observed an inverse correlation between the rate of MYXV infection and increasing spheroid size (Supplementary Fig. S2).

EOC spheroids are resistant to MYXV mediated cell killing

Primary human EOC cells and ovarian cancer cell lines are readily infected and exhibit cytopathic effects when grown as adherent cultures. To determine that this results in reduced viability and oncolytic cell death, we performed alamarBlue® cell viability assays over 3 days post infection. Adherent primary EOC cells displayed a marked reduction in cell viability over the 3 days post infection, compared to the continued growth and viability of UV irradiated MYXV treated cells (Fig. 2A). This reduced viability was observed in MYXV infected SkOV3 cell line and the 4306 mouse ovarian tumor cell line, as well (Fig. 2B, and data not shown). To determine if EOC cells in suspension and as spheroids are also sensitive to MYXV mediated cell killing, we performed alamarBlue® assays on cells seeded to ULA® plates after 3 days of infection. In fact, we did not observe any further reduction in cell viability due to MYXV infection (Fig. 2B). This indicates that, although MYXV can enter and produce virus in EOC suspension cells and spheroids, it is unable to reduce the overall bulk viability (i.e. induce oncolysis) of cells under these conditions.

Reattached EOC spheroids regain sensitivity to MYXV oncolysis

The ability of EOC cells and spheroids to establish and grow as secondary metastases in the peritoneal cavity requires successful attachment to the surface of abdominal organs. To determine whether MYXV infected EOC spheroids are capable of performing this step of the metastatic process, we reseeded infected EOC spheroids to standard tissue culture plastic. EOC spheroids derived from the SkOV3 cell line and primary EOC cells when treated with UV irradiated MYXV readily attached and cells efficiently dispersed from the central spheroid after 3 days (Fig. 3A). In contrast, EOC spheroids that were infected with live MYXV while in suspension had a marked reduction in their ability to both adhere and disperse upon reattachment (Fig. 3A,B). SkOV3 spheroid cell dispersion was reduced by 80.7% by MYXV infection, and this was even more pronounced in primary EOC cells with a 93.9 98.0% reduction compared to mock infected controls (Fig. 3B).

We then sought to determine whether EOC spheroids that have already attached and are actively growing and dispersing are now capable of being infected by MYXV, as well. Established EOC spheroids were seeded to standard tissue culture plastic and allowed to reattach and disperse for 72 h before initiation of infection with MYXV or control virus. Both the spheroid (still present in most cases) as well as dispersing EOC cells were readily

infected by MYXV even at a low MOI of infection. However, only the adherent cells emanating from the spheroid were rendered susceptible to MYXV mediated cell killing as evidenced by cytopathic effect (Fig. 3C).

MYXV mediated oncolysis requires active AKT signaling

Wang and colleagues [21] originally showed that activated AKT kinase activity is required for efficient MYXV mediated oncolysis of human cancer cells. Interestingly, our laboratory has demonstrated recently that endogenous AKT activity is reduced in EOC cells upon spheroid formation [32], thus providing a potential explanation for the reduced kinetics of MYXV infection in spheroids and the attenuated oncolytic response. To directly determine whether MYXV infection influences AKT activity in EOC cells, we prepared total protein lysates from control and MYXV infected cells for immunoblot analysis. AKT phosphorylation at serine 473, a modification indicative of activation of this pathway, is induced within 2 h post infection (Supplementary Fig. S3a); this is observed in both adherent and suspension EOC cells, but delayed in EOC spheroids (Supplementary Fig. S3b) MYXV infection was confirmed by detection of early M T7 and late M130 viral protein expression. The increased level of AKT phosphorylation in adherent primary EOC cells in response to MYXV infection was blocked by treatment with the specific AKT kinase inhibitor Akti 1/2 (5 μ M; Fig. 4A). This was further reflected by reduced M T7 and M130 protein expression levels. However, we observed that even in the presence of the AKT inhibitor, MYXV infection was still capable of increasing p70 S6 kinase phosphorylation. This suggests that MYXV can utilize an alternative, AKT independent mechanism for activation of p70 S6 kinase during infection.

Rapamycin has been shown to increase MYXV infection of human cancer cells, particularly those cells that are restrictive to MYXV oncolysis, due to compensatory feedback activation of AKT [22,33]. To determine whether rapamycin may enhance MYXV oncolysis, we treated primary EOC cells and cell lines with rapamycin (20 nM) and performed immunoblot assays to assess AKT activity. As expected, rapamycin treatment was effective at reducing phosphorylated p70 S6 kinase levels (both basal and MYXV induced), indicating that the drug was functional under our culture conditions (Fig. 4A). However, for the most part, rapamycin had little effect on AKT phosphorylation after MYXV infection as well as on the expression of MYXV proteins M T7 and M130.

To determine the requirement of functional AKT activity in EOC cells and spheroids during MYXV infection, adherent EOC cells or established spheroids were infected with MYXV in the presence of 5 μ M Akti 1/2 or DMSO vehicle control. We consistently observed that Akti 1/2 treatment reduced MYXV infection (as observed by GFP expression using fluorescence microscopy) of both adherent primary EOC cells and spheroids. This was tested in three different cell lines (SkOV3, OVCA429, OVCAR3) and with two different doses of MYXV (MOI of 0.1 and 10) (Fig. 4B, and data not shown). Contrary to the results of blocking AKT activity, inhibition of mTOR activity using rapamycin did not have a detectable effect on MYXV infection in terms of promoting or inhibiting infection (Fig. 4B). This correlates well with the immunoblotting results for M T7 and M130 expression, where rapamycin did not exhibit any effect (Fig. 4A). Taken together these data imply that MYXV infection of

primary EOC cells and spheroids requires AKT activation; however, the positive effect of rapamycin on this process observed in other classes of cancer cells does not appear operative in human EOC cells or spheroids.

Efficacy of MYXV oncolysis on direct EOC ascites samples

To function as a therapeutic MYXV must infect EOC tumor cells within the peritoneal cavity and likely within the environment of malignant ascites fluid. We assessed the effect of MYXV treatment on 22 independent patient ascites samples immediately upon collection. Within this cohort of patient samples, 16 represent women diagnosed and clinically confirmed to have EOC (Table 1). As expected, the majority of samples represent EOC of the most common histotype of serous adenocarcinoma. However, we received and tested ascites from other gynecologic malignancies ($n = 5$) within this sample set. Ascites fluid containing tumor cells was seeded to tissue culture dishes and immediately treated with three increasing doses of MYXV [10^5 , 10^6 and 10^7 foci forming units (ffu) per 35 mm well] or with the equivalent of a 10^7 dose of UV irradiated control virus. Note that the concentration and viability of EOC cells are unknown since we seeded ascites directly; therefore, we do not refer to MOI, but rather number of viral infectious units applied per well of ascites fluid (2 mL volume per well). From this experiment, we observed that 12 out of 22 samples demonstrated a qualitative and quantitative level of response to MYXV infection, as compared with controls (Table 1 and Supplementary Fig. S4). From this sample set, 3 were considered highly sensitive as illustrated by efficacy at all three MYXV doses, 3 were moderately sensitive, and 6 were weakly sensitive (Table 1). Among the 22 samples tested in this experiment, 10 appeared resistant to MYXV infection, at least to the doses that were used. With respect to clinical data, however, there does not appear to be a correlation between MYXV oncolytic sensitivity and tumor histotype, stage or grade.

To further investigate the mechanisms underlying differential sensitivity of primary EOC samples to MYXV infection, we performed western blotting against phosphorylated AKT on protein isolated from a subset of the clinical specimens. We selected four representing MYXV resistant samples (EOC61, EOC65, EOC81 and EOC100) and six that were sensitive to MYXV oncolysis to different degrees (low: EOC75 and EOC103; intermediate: EOC74 and EOC98; high: EOC67 and EOC101). From this selective analysis, however, we did not observe any correlation in endogenous expression levels of either phosphorylated AKT or total AKT protein and the inherent sensitivity to MYXV mediated oncolysis by direct infection of patient ascites (Supplementary Fig. S5).

We then postulated that acellular factors in the ascites fluid may impact the ability of MYXV to infect EOC cells. To test this experimentally, we selected two independent patient samples and their matched clarified ascites fluid (i.e. cellular constituents were removed by centrifugation) and performed a MYXV infection with increasing concentration of ascites fluid. Indeed, MYXV infection (MOI of 1 and 10) was dramatically reduced in EOC140 in the presence of clarified ascites at all concentrations tested (Fig. 5A). In EOC141, infection and MYXV mediated oncolysis were largely unaffected by the presence of clarified ascites, although a dose dependent increase in cell viability was observed at an MOI of 1 with increasing ascites concentration (Fig. 5A, B). Analysis of phosphorylated

AKT levels in response to ascites treatment indicated that down regulation of AKT activity may contribute, at least in part, to the decreased MYXV oncolysis observed in EOC140 as compared to EOC141 (Fig. 5C). MYXV infection was confirmed by detection of M T7 and M130 proteins in EOC141 lysates in both the presence and absence of ascites, whereas these viral proteins were only detected in EOC140 lysates in the absence of ascites treatment.

Taken together, these data imply that a subset of EOC, and perhaps other gynecologic malignancies, are sensitive to MYXV oncolysis, with the important implication that intracellular AKT activity levels in concert with putative acellular mediators present in malignant ascites can impact its potential as an anticancer therapeutic.

Discussion

Our results provide the first evidence of MYXV mediated oncolytic effects using primary human EOC cells. Previously, a few reports have demonstrated that select established human ovarian cancer cell lines are infected by MYXV [21,26], however direct testing of MYXV using primary tumor material had been lacking. Access and application of viral oncolytics using patient derived tumor cells are a common hurdle of most studies both in fundamental and preclinical research. Even more importantly, we have tested MYXV efficacy using a relevant cell culture model system that mimics the mechanisms of metastasis, as well as tumor pathobiology in a three dimensional context. By using this novel system to perform MYXV oncolytic testing in cell culture, we have discovered unique characteristics that directly impact the life cycle of MYXV infection and its potential efficacy as an anti cancer therapeutic.

Metastasis of EOC by direct dissemination of tumor cells into the peritoneal cavity and subsequent formation of multicellular spheroids is thought to be a critical step in the spread of malignant cells [34]. We have recently observed that EOC cells present in multicellular spheroids undergo a state of dormancy where cell proliferation is markedly reduced [32], a phenotype which would render EOC spheroids relatively resistant to standard cytotoxic chemotherapeutics. Our results herein indicate that dormant EOC spheroids are effectively targeted by MYXV; however, this occurs with slower kinetics and does not result in efficient cell death until reattachment occurs. Interestingly, our data also points to the notion that AKT activity may be in part responsible for efficient establishment of infection or spread of MYXV, since EOC spheroids, which possess decreased endogenous levels of phospho AKT, experience reduced kinetics of MYXV infection. Furthermore, we have data clearly demonstrating that AKT activity is required during EOC spheroid reattachment particularly for re initiation of cell proliferation [32]. This result supports the notion that reactivation of AKT activity is likely the essential requirement for oncolytic efficacy of MYXV in EOC spheroids. Thus, it is not surprising that MYXV, already present in the infected yet dormant EOC cells, is able to efficiently complete the oncolytic program once AKT activity is re established upon spheroid reattachment. This phenomenon is also relevant to EOC pathogenesis, since reattachment of spheroids is a likely mechanism of EOC secondary metastasis [34]. Thus, these data clearly demonstrate the potential therapeutic efficacy of MYXV infection in targeting and killing secondary EOC metastases.

There is a well established significant role for the PI3K AKT mTOR pathway in human EOC [35-38]. Genes encoding several components of this pathway are commonly mutated in EOC tumors. For example, amplification of the genes PIK3CA, encoding the catalytic subunit of PI3K, and AKT2, its downstream kinase, are frequently observed [29-31]. Inactivating mutations in the PTEN tumor suppressor gene has also been documented, particularly in endometrioid ovarian cancers [39]. We postulate that the differential sensitivity of some ovarian tumors to MYXV infection is related to the underlying status of this pathway. Perhaps genetic analysis of PI3K AKT pathway genes within EOC samples, or determination of endogenous AKT activity levels, would predict whether malignant EOC cells are sensitive or restrictive to MYXV infection. Indeed, the classification of sensitive (Type I) and restrictive (Type III) cells in response to MYXV oncolytic infection has its mechanistic basis upon the activity level of AKT kinase [19,21]. Others have demonstrated that treatment with rapamycin can render Type III restrictive cancer cells sensitive to MYXV oncolysis due to upregulation of AKT kinase activity by feedback signaling [22,33]. Although we did not observe significant effects of rapamycin treatment on MYXV infection of EOC cells and spheroids, there is the potential to use mTOR inhibitors or similar drugs to modulate AKT activity and increase sensitivity of primary EOC cells and spheroids to MYXV infection.

Our results support the established connection between AKT activity and MYXV mediated oncolysis, however we have additional data from direct infection of patient ascites cells that there is a subset of EOC samples, which are apparently insensitive to MYXV. It is possible that some ascites samples harbor additional factors or characteristics that inhibit or reduce the ability of MYXV to infect the EOC cells and spheroids present in the ascites [16]. For example, a number of complement protein precursors have been identified in the ascites that may contribute to an innate antiviral immune response against MYXV [40]; this potential alternative complement pathway has been observed to affect adenovirus infection in other systems [41]. Alternatively, it has been observed that ascites treatment of EOC cells and ovarian cancer cell lines can regulate AKT activity to promote cell survival in the presence of pro apoptotic stimuli [42]. Our results suggest that some ascites may have the opposite effect of down regulating AKT activity in EOC cells, thereby rendering cells less sensitive to MYXV infection and oncolysis. An alternative explanation, however, is that underlying mutations in other dominant oncogenic pathways may be present in the unresponsive EOC samples. For example, recent data indicates that all high grade EOC of the serous histotype carry mutations in TP53 [43,44]. Alternatively, low grade EOC tumors often possess oncogenic mutations in KRAS and BRAF [45]. Therefore, it may be more effective to utilize other oncolytic viruses that target these types of mutations (e.g. reovirus for KRAS mutations). It is of interest to test a panel of different oncolytic vectors against primary human EOC samples derived from patient ascites, and correlate therapeutic efficacy with mutational analysis of putative underlying disease causing genes such as AKT2, PTEN, PIK3CA, TP53, BRAF and KRAS. This would provide a personalized cancer therapy approach to the use of oncolytic viruses in EOC, which could be amplified with combination therapy using multiple oncolytic viruses and chemotherapeutics in sequence [46,47].

Another intriguing and emerging concept is that metastatic EOC cells and spheroids may represent a reservoir of ovarian cancer initiating cells [48-52]. Collection and isolation of

ovarian cancer initiating cells from patient ascites have been successful by a number of groups using cell surface markers, such as CD133⁺ [52], CD44⁺/CD24⁺/EpCAM⁺ [49], CD44⁺/CD117⁺ [48], and CD44⁺/MyD88⁺ [50], or by verapamil sensitive side population cells [51]. This has increased relevance with regard to targeted killing by MYXV, since the idea of oncolytic viral purging of pluripotent cancer stem cells using MYXV has been recently applied in the context of human myeloid leukemia [53]. We propose that MYXV application directly to malignant ascites may function in the same way, and provide an effective way to target ovarian cancer stem cells, perhaps those residing within dormant EOC spheroids and secondary metastases. Future studies would involve selection and culture of ovarian cancer initiating cells from EOC patient ascites and directly assaying their sensitivity to MYXV oncolysis.

Supplementary materials related to this article can be found on line at doi:10.1016/j.ygyno.2012.01.048.

Supplementary Material

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Fig. 1.

EOC spheroids have reduced MYXV infection kinetics compared to monolayer and suspension cultures. (A) Adherent monolayer, suspension cells, and 3-day EOC spheroids were infected with MYXV (MOI of 1), or UV-inactivated MYXV as a control. Phase contrast and fluorescence images were captured 72 h post-infection to assess MYXV infection. Cytopathic effect was noted in MYXV-infected adherent monolayer cultures as characterized by rounding up of cells. (B) Western blotting was performed to determine expression of MYXV-specific M-T7 (early) and M130 (late) proteins. Reduced expression of both M-T7 and M130 was observed in MYXV-infected EOC spheroids compared to monolayer and suspension cultures. Actin served as a loading control. (C) MYXV was collected at 96 h post-infection of monolayer, suspension and spheroid cultures and titered using BGMK cells. Comparable levels of infectious MYXV were collected in each culture condition.

Fig. 2.

MYXV infection does not affect viability of EOC cells in suspension or as spheroids. (A) Cell viability was assessed using alamarBlue® reagent after 24, 48 and 72 h of MYXV infection (MOI of 1) in primary EOC cells grown in monolayer culture. MYXV infection decreased cell viability as compared to cells treated with UV-irradiated MYXV control. (B) Cell viability was assessed after 72 h of MYXV infection (MOI of 1) in SkOV3 cells and two ascites-derived primary EOC samples (EOC14 and EOC11) grown in monolayer, suspension and spheroid cultures. MYXV infection decreased cell viability in all monolayer EOC cell samples, yet no effect was observed in suspension and spheroid cultures.

Fig. 3.

MYXV readily infects and kills attached EOC spheroids. (A) SkOV3, EOC14 and EOC11 spheroids were infected with MYXV (MOI of 1) or UV-irradiated control (ctrl) and 72 h post-infection were transferred to standard tissue culture plastic to facilitate reattachment. Three days post-reattachment attached spheroids were fixed and stained to visualize EOC cell dispersion. MYXV infection substantially reduced the number of cells migrating away from the attached spheroids compared to control infected spheroids. (B) Dispersion area was quantified from images of 12–20 attached spheroids per sample and treatment using ImageJ software. (C) Primary EOC spheroids were plated to standard tissue culture plastic to facilitate reattachment, and subsequently infected with MYXV (MOI of 0.1, 1 and 10) or UV-irradiated control (ctrl). Phase contrast images were captured prior to infection and at 96 h post-infection, along with fluorescence microscopy to visualize infected cells. Cytopathic effect was evident in the dispersing cells; the attached, intact spheroids permitted MYXV entry, as well.

Fig. 4.

AKT activity is required for efficient MYXV infection of EOC cells and spheroids. (A) Western blotting was performed to determine expression of phosphorylated AKT kinase and phosphorylated p70 S6 kinase at 24 h post-MYXV infection of primary EOC11 cells and SkOV3 cells grown in monolayer culture and treated with the AKT inhibitor Akti-1/2 (5 μ M), the mTOR inhibitor rapamycin (20 nM), or DMSO vehicle control. Phosphorylated AKT was completely absent in Akti-1/2 treated cells and this markedly decreased MYXV infection as determined by M-T7 and M130 expression. MYXV infection increased phospho-p70S6 kinase, which is unaltered by Akti-1/2 treatment. Rapamycin treatment did not affect MYXV infection, but was effective at reducing phospho-p70 S6 kinase levels to below detection. Actin served as a loading control. (B) SkOV3 and OVCA429 monolayer cells and spheroids were infected with MYXV (MOI of 0.1 and 10) treated with the AKT inhibitor Akti-1/2 (5 μ M), the mTOR inhibitor rapamycin (20 nM), or DMSO vehicle control. Images were captured by fluorescence microscopy at 96 h post-infection for MOI of 0.1 and 24 h post-infection at an MOI of 10. Treatment with Akti-1/2 decreased the efficiency of MYXV infection in both monolayer and spheroids when compared to rapamycin treatment and DMSO controls as determined by intensity and degree of fluorescence.

Fig. 5.

Ascites may reduce the efficiency of MYXV infection and oncolysis of primary EOC cells. Two independent primary EOC samples (EOC140 and EOC141) were infected with MYXV at an MOI of 1 and 10, or UV-inactivated control virus, while in the presence of clarified ascites (0, 20, 50 or 80% diluted in complete media) which was matched to the same patient. (A) Marked reduction of MYXV infection (MOI 10) was observed in EOC140 at all ascites concentrations tested when compared with complete media alone control (0 as- cites); ascites had little to no observable effect on infection in EOC141. (B) MYXV-mediated oncolysis of EOC140 cells was inhibited by the presence of ascites at all concentrations tested for both MYXV doses when compared with complete media alone control, as determined by alamarBlue® assay after 3 days of treatment. Reduction in EOC141 cell viability by MYXV oncolysis was largely unaffected at an MOI of 10, whereas oncolysis at the lower dose (MOI 1) was inhibited by increasing concentration of ascites. Note that ascites fluid alone (control, UV-inactivated virus) did not affect the overall viability of EOC cells over the 3-day treatment period at all concentrations tested. (C) Western blot analysis was per- formed using lysates from EOC140 and EOC141 cells to determine phospho-AKT (Ser473) and total AKT protein expression levels in the presence (+ A) or absence (- A) of ascites (20% in complete media) after 24-hour treatment and concomitant infection with MYXV at an MOI of 1. Detection of M-T7 and M130 proteins confirmed MYXV infection of EOC cells in the absence of ascites, whereas M-T7 and M130 were only present in EOC141 cells in the presence of ascites, which correlates with cell viability data.

Table 1

Summary of clinical data for ascites samples used in direct MYXV infection experiments.

Sample	Age	Site	Stage	Histotype and grade	Infectivity score ^a
EOC59	47	Ovarian	IIIc	Serous adenoca, grade 3	+
EOC61	78	Ovarian	IIIc	Serous adenoca, grade 3	–
EOC65	67	Ovarian	IV	Serous adenoca, grade 3	–
EOC67	51	Ovarian	IIIc	Serous adenoca, grade 2	+++
EOC72	77	Ovarian	IIIc	Serous adenoca, grade 3	+
EOC74	69	CMML, ITP ^b	n.a.	n.a.	++
EOC75	77	Ovarian	IIIc	High grade pap serous	+
EOC81	69	Ovarian	n.a.	Mucinous borderline	–
EOC83	69	Fallopian tube	IV	Poorly differentiated pap adenoca	++
EOC84	68	Endometrium	IV	Serous adenoca	–
EOC85	49	Ovarian	IIIc	Pap serous, grade 3	–
EOC95	79	Peritoneal carcinomatosis ^c	n.d.	n.d.	+
EOC96	52	Ovarian	IIIc	Serous adenoca, grade 3	–
EOC98	51	Ovarian	IIIc	Serous adenoca, grade 3	++
EOC100	65	Endometrium	IV	High-grade carcinoma	–
EOC101	43	Ovarian	IIIc	Serous adenoca, grade 3	+++
EOC103	64	Ovarian	IIIc	Carcinosarcoma	+
EOC106	61	Ovarian	IIIc	Serous adenoca, grade 1	–
EOC107	46	Cervix	II	Mucinous adenoca, intestinal type	+
EOC108	70	Ovarian	IIIc	Serous adenoca, grade 3	–
EOC109	56	Ovarian	IIIc	Endometrioid adenoca, grade 2	–
EOC110	45	Endometrium and ovarian ^d	IIb	Endometrioid adenocarcinoma, grade 1 ovarian adenoca, grade 1	+++

n.a.: not applicable

n.d.: not determined

^aInfectivity Score: –, no infection at all three doses; +, infection at one (highest) dose; ++, infection at two doses; +++, infection at all three doses; see Supplementary Fig. S3.

^bCMML, ITP: chronic myelomonocytic leukemia with idiopathic thrombocytopenia purpura.

^cPatient EOC95 diagnosed with peritoneal carcinomatosis, likely ovarian; chemotherapy only, therefore was not staged.

^dPatient EOC110 had synchronous primaries of the endometrium and right ovary; staging is based on endometrium.