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ORIGINAL PAPER

Neutralization of matrix metalloproteinase-9 potentially enhances oncolytic efficacy of tanapox virus for melanoma therapy

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Abstract Matrix metalloproteinases (MMPs), which are involved in degradation of extracellular matrix, are critical regulators in tumor progression, metastasis and angiogenesis. Although induction of MMPs is frequently observed during the viral infection, the effect of MMPs on viral replication varies between viruses. MMP-9, for instance, is upregulated and promotes the replication of some viruses, such as herpes simplex virus, but inhibits the replication of others. Here, we report that infection with tanapox virus (TPV) promotes the expression of MMP-9 in the melanoma cells. In addition, we show that MMP-9 exerts an anti-viral effect on TPV replication and plays a protective role in TPV-infected melanoma cells in vitro. Moreover, the neutralization of MMP-9 in melanoma cells remarkably enhances the TPV infection and leads to a significant reduction in cell survival. In summary, this study contributes to understanding of the role played by MMP-9 in TPV infectivity and provides more insights for using TPV as cancer virotherapy in future studies. Since TPV has shown substantial oncolytic efficacy in promoting melanoma tumor regression in animal models, identifying mechanisms that suppress MMP-9 expression upon TPV infection can potentially improve its use as a melanoma virotherapy.

Keywords Tanapox virus · Metalloproteinase-9 · Oncolytic virus · Melanoma

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Introduction

Melanoma is one of the most aggressive skin cancers, with respect to tumor cell invasion and metastasis. If not detected early, metastatic melanoma has approximately a 15% 5-year survival rate [1]. As a complex of genetic and epigenetic abnormalities, melanoma poorly responds to conventional cytotoxic therapies. For instance, the response rate in malignant melanoma patients with the treatment of dacarbazine and vemurafenib was less than 20% [2].

Oncolytic viruses (OVs) have appeared as a promising melanoma therapy. They possess the ability to infect and lyse the tumor cells and are capable of inducing anti-tumor immune responses. JX-594 and talimogene laherparepvec (T-Vec), the modified vaccinia virus (VV) and herpes simplex virus (HSV) both expressing granulocyte–monocyte colony-stimulating factor (GM-CSF), have been approved, respectively, for head and neck cancer and melanoma therapies [3]. Belonging to the family *Poxviridae* (genus *Yatapoxvirus*), tanapox virus (TPV) is a double-stranded (ds) DNA virus containing a genome of approximately 144 kbp [4]. TPV causes a self-limiting febrile illness in men [5–7]. With attenuated virulence, a large genome, inability to spread from man to man as well as the absence of anti-viral immunity in most of the global population, TPV appears to be an excellent OV candidate. In our previous studies, the abnormal characteristics of tumors have been manipulated for generating TPV recombinants and increasing the oncolytic efficacy of TPVs. Being essential for DNA synthesis, thymidine kinase (TK) is overly expressed in cancerous cells in comparison with normal cells [8]. Deletion of viral TK gene in OVs has commonly been used to increase the oncospecificity of viruses. By ablating *TPV-66R* gene encoding TK, we have

generated TPVΔ66R and demonstrated its efficacy in retarding melanoma tumor growth [9]. In addition, we have shown that TPV-15L gene product, a mimetic of neuregulin (NRG) [10], stimulated melanoma cell proliferation. Recombinant TPV lacking TPV-15L gene (TPVΔ15L) significantly regressed melanoma tumors in animal models [9].

Metastasis is the primary cause of the mortality of patients and is a complex process. It includes the invasion of the tumor cells to the surrounding tissues and basement membranes, the penetration into the blood and lymphatic vessels, and the re-penetration in other organs to form detectable tumors [11]. Different proteolytic enzymes, which degrade the extracellular matrix (ECM) proteins and basement membranes, have been shown to promote the liberation and seeding of the tumor cells, thereby increasing melanoma cell migration and metastasis [12]. Matrix metalloproteinases (MMPs) are a family of calcium-dependent zinc-containing endopeptidases that include collagenases, gelatinases, stromelysins and others [13]. Several MMPs, such as MMP-1, MMP-2, MMP-9 and MMP-13, are directly involved in the melanoma progression and are indicative of a poor prognosis [11, 14, 15]. In addition, tissue inhibitors of metalloproteinase (TIMPs), which inhibit the activation of latent enzymes or the proteolytic ability of active MMPs, decrease experimental and spontaneous tumor growth and metastasis, and possess an anti-metastatic effect on a number of cancer model systems. Approximately 22 MMPs have been characterized and cloned to date, and each targets specific protein substrates [11]. MMP-9, also known as gelatinase B, degrades type IV and V collagens and gelatins, which are essential components of the ECM [16]. Increased MMP-9 expression is correlated with many diseases and pathological abnormalities including melanoma [11]. Expression of MMP-9 has been shown to enhance melanoma growth and lung colonization [17]. In addition, in transgenic mice MMP-9 contributed to both keratinocyte proliferation and skin carcinogenesis [18]. Studies have also demonstrated that MMP-9 is expressed in tumor and stromal cells as well as in tumor-infiltrating immune cells, such as neutrophils and macrophages. The expression of MMP-9 by the bone marrow-derived inflammatory cells has been shown to induce the angiogenesis and enhance the tumor growth in animal models [18]. Therefore, host MMP-9 may contribute to cancer cell metastasis.

Several virus infections and their accompanying pathogenesis are associated with the induction of MMP-9. For instance, respiratory syncytial virus (RSV) infection is a common cause of bronchiolitis and pneumonia in premature infants, resulting in substantial morbidity and mortality. It has been shown that RSV infection increases MMP-9 expression in human epithelial cells [19]. Increased MMP-9 expression is also observed in the influenza virus-infected kidney cells in vitro [20], as well as in flu-infected human patients [21]. Moreover, infection of macrophages with human immunodeficiency virus (HIV) also results in increased MMP-9 production [22]. Although induction of MMP-9 is frequently observed during virus infections and elevated MMP-9 levels are often correlated with disease severity, the functional role of MMP-9 in viral replication and infection still remains controversial. For example, neuroblastoma cells transduced with MMP-9 showed significantly enhanced distribution and replication of HSV as a viral vector [23], but an anti-viral effect of MMP-9 has been demonstrated in RSV infection in the airway epithelial cells [24].

In this study, we aimed to investigate the interplay between MMP-9 expression and TPV replication in melanoma cells, and to determine whether oncolytic efficacy of TPV could potentially be enhanced by manipulation of MMP-9 expression. We show that TPV replication in melanoma cells is significantly decreased with the addition of MMP-9. In addition, the infection of human melanoma SK-MEL-3 cells with wtTPV and TPV recombinants remarkably induced the expression of the MMP-9, while blocking of MMP-9 significantly increased the TPV replication in human melanoma SK-MEL-3 cells and decreased the cell survival. Taken together, our results suggest that suppressing MMP-9 expression upon TPV infection will potentially improve the use of TPV as an oncolytic virotherapy.

Materials and methods

Cell lines, virus and reagents

Owl monkey kidney (OMK) cells and human melanoma cell line SK-MEL-3 were purchased from the American Type Culture Collection (ATCC product numbers CRL-1556 and HTB-69, respectively). OMK cells were cultured in complete growth medium containing Earle's Minimum Essential Medium (EMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Atlanta Biologicals), 2 mM L-glutamine (Sigma-Aldrich) and 50 μg/ml gentamicin sulfate (AMRESCO). SK-MEL-3 were cultured in a growth medium consisting of McCoy's 5A medium (Sigma-Aldrich) and 15% (vol/vol) FBS. Macoy's 5A medium without FBS was used as starving medium for melanoma growth. All cell monolayers infected with virus were maintained in a medium that is identical to the growth medium except that it contained 2% (vol/vol) FBS. All cell lines were incubated at 37 °C in a 5% CO_2 atmosphere. All cell counting and viability assays were conducted in a normal saline solution containing 0.4% (wt/vol) trypan

blue using an improved Neubauer hemacytometer. The wtTPV (Kenya strain) was originally a gift from Dr. Joseph Esposito (Centers for Disease Control, Atlanta, GA, USA). TPVGFP is a recombinant TPV that was modified to express green fluorescent protein (GFP) and provided by Dr. Grant McFadden. MMP-9 purified protein was purchased from Sino-Biological, China, and polyclonal goat anti-hMMP-9 antibody was purchased from R&D Systems, MN.

Generation of the recombinant TPVs

The p66R-mCherry plasmid was derived from a commercially available cloning vector pBluescript II KS (+) and was engineered to produce a virus in which the *TPV-66R* gene was knocked out through homologous recombination in the process of transfection and infection. The genomic sequences flanking the left and right sides of 66R open reading frame (ORF) were inserted in the plasmid. The left flanking sequence was amplified using PCR and primers containing SacI or NotI restriction endonuclease (RE) cut sites: 5′-AATGGATCACATAAAGGAGCTCTTAACG-3′ (forward) and 5′-CAGAAAACATGCGGCCGCATATAA TCT-3′ (reverse). The right flanking sequence was isolated using primers containing EcoRI or HindIII RE cut sites: 5′-GGAGATGAACAAGAAATAGAATTCATAGG-3′ (forward) and 5′-CTGTTCTTTATCACAAGCTTCTATCG GGTG-3′ (reverse). An early/late (E/L) synthetic poxvirus promoter [25] and a mCherry gene for expression were inserted in between the two flanking sequences. OMK cell monolayers plated in 35-mm tissue culture dishes were transfected (Superfect transfection reagent; Qiagen) with p66R-mCherry plasmid and infected with wtTPV at a multiplicity of infection (MOI) of 5. Transfected and infected OMK cells were incubated at 37 °C with 5% CO_2 until mCherry fluorescence was observed. The virus was harvested, and recombinant virus TPVΔ66R was plaque purified until all plaques displayed mCherry fluorescence and no *66R* gene was detected by confirmation PCR. Plaque assays were performed as described earlier [8].

The p15L-GFP plasmid was also derived from pBluescript II KS (+) and engineered to produce a virus in which the *TPV-15L* gene was knocked out (TPVΔ15L) by homologous recombination in transfection and infection. The procedures described for p66R-mCherry plasmid construction were used. The left flanking sequence of TPV-15L ORF was generated using primers that contained XhoI or ClaI RE cut sites: 5′-TAGGTACTCGAGAAAAACA CCAATA-3′ (forward) and 5′-GTTTAAATCGATGGA CCTG-3′ (reverse). The right flanking sequence was isolated using primers containing NotI or SacI RE cut sites: 5′-CATATTTTGCGGCCGCGGTAAACAATT-3′ (forward) and 5′-GTTAAAAATGGAAAAGAGCTCTAATTTTAAC AACAG-3′ (reverse). A synthetic poxvirus early/late (E/L) promoter and a GFP gene were in between left and right flanking sequences. All plasmids were confirmed by DNA sequencing.

MMP-9 anti-viral assay

Human melanoma cells (SK-MEL-3) were plated in a 48-well plate in McCoy's 5A medium with 15% FBS and incubated overnight at 37 °C with 5% CO_2 in humidified chambers. The next day, cells were infected with TPVGFP at 0.1 or 5 MOIs. After virus adsorption for 1 h at room temperature, the virus inocula were replaced with 250 µl serum-free McCoy's 5A medium containing 0.1, 1 or 10 µg/ml purified MMP-9 proteins. Infected cells with no MMP-9 treatment were used as mock. Virus replication and cell viability were determined in each group at day 4 post-treatment. The total number of viable cells was evaluated by counting cells on a hemocytometer chamber, and trypan blue stain was used to exclude non-viable cells. Viral replication was assessed using plaque assays. Each experiment was repeated three times independently, and standard deviations were calculated.

Reverse transcription-polymerase chain reaction (RT-PCR)

SK-MEL-3 cells plated in T75 flasks were infected with 1 MOI of wtTPV, TPVΔ66R or TPVΔ15L, respectively. A mock-infected culture served as a control. After 72 h, cells were harvested and total RNA was extracted using the Purelink RNA mini kit (Thermo Fisher Scientific). Each RNA sample was then dissolved in DEPC-treated water and adjusted to the same volume. MMP-9 cDNA was amplified using RT-PCR with the 5′-GAGACCGGT GAGCTGGATAG-3′ forward primer and the 5′-CAAA CTGGATGACGATGTCTGC-3′ reverse primer. The primers used to amplify GADPH cDNA were 5′-CTCTG ATTTGGTCGTATTGGG-3′ (forward) and 5′-TGATT TTGGAGGGATCTCGC-3′ (reverse). After 20, 25, 30, 35, 40 cycles, PCR products amplified from each RNA sample were separated on a 2% agarose gel containing ethidium bromide.

Western blot analysis

SK-MEL-3 cell monolayers were cultured on 35-mm petri dishes and infected with wtTPV, TPVΔ66R or TPVΔ15L at 5 MOI, respectively. Mock-infected cells served as a control. Infected SK-MEL-3 cells were incubated in serum-free medium at 37 °C for 72 h until the monolayer was destroyed. Cells were harvested and lysed with sterile ice-cold 1% NP-40, and proteins were collected. Samples were

mixed with 5 × SDS gel loading buffer (25% glycerol, 5% SDS, 0.002% bromophenol blue, 15% β-mercaptoethanol) and boiled for 3 min. Protein samples were separated on SDS–PAGE gels and transferred to Immobilon-P polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer apparatus (Bio-Rad Trans-Blot SD) at 14 V for 1.25 h. Membranes were blocked in TBST (20 mM Tris, 137 mM NaCl [pH 7.6], 0.1% Tween 20) containing 5% nonfat dry milk for 2 h at room temperature and subsequently incubated with 1: 1000 dilution of the goat anti-MMP-9 primary antibody (R&D systems) at 4 °C overnight. The membranes were washed five times for 10 min each with TBST before incubation with a 1:7500 dilution of anti-goat IgG-horseradish peroxidase (HRP) (Sigma) in TBST with 5% nonfat dry milk at room temperature for 1 h with gentle agitation. The membrane was washed five times for 10 min each with TBST, and the signal was detected by using enhanced chemiluminescence reagents (thermo scientific).

Anti-MMP-9 assay

Human melanoma cells (SK-MEL-3) were plated in a 48-well plate in McCoy's 5A medium with 15% FBS and incubated overnight at 37 °C. The following day, cells were switched to a serum-free medium containing 0.1 μg/ml, 1 μg/ml or 10 μg/ml anti-MMP-9 and incubated for 2 h at 37 °C. Cells incubated with medium containing no anti-MMP-9 served as mock. Following incubation, the cells were infected with TPVΔ15L at 0.1 or 5 MOI. Virus replication and cell viability were assessed at 2 and 4 days post-treatment. Total numbers of viable cells were evaluated by counting cells on a hemocytometer chamber using trypan blue stain to exclude non-viable cells. Viral replication was determined via using plaque assays. Each experiment was repeated three times independently, and standard deviations were calculated.

Statistical analysis

All the in vitro experiments were done in triplicates, and the measurements were presented as mean ± SD. The two-sample unequal variance Student's t test analysis was applied for testing the differences. The significance level used was $P < 0.05$.

Results

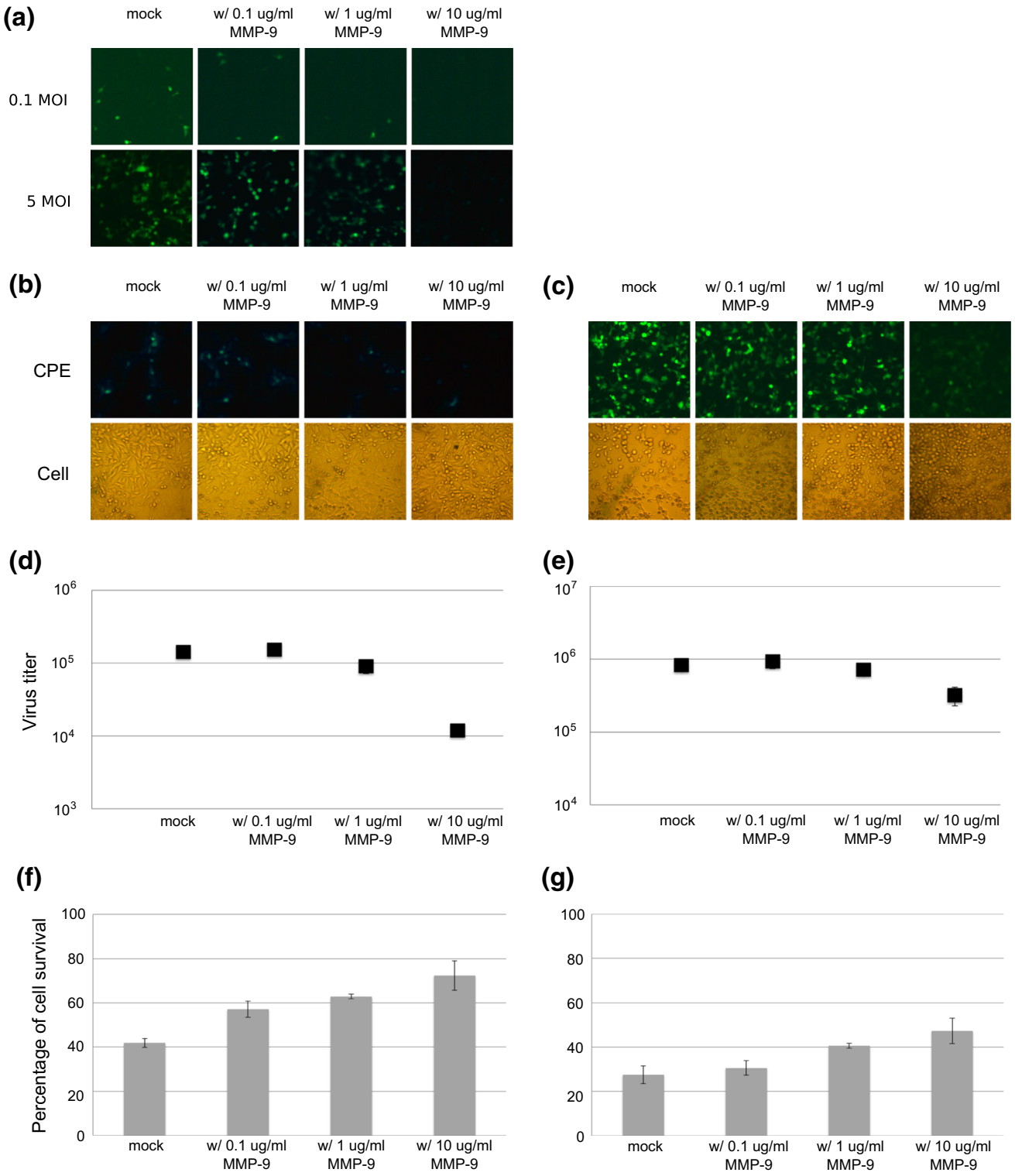
MMP-9 reduces the TPV replication in melanoma cells

The role of MMP-9 in virus replication varies between viruses. MMP-9 can have anti-viral activity and inhibit the replication of some viruses [24], while MMP-9 expression will enhance the infectivity of other viruses and increase the severity of the associated symptoms [19]. The effect of MMP-9 on TPV replication is unknown and requires further investigation as enhanced MMP-9 expression is observed in many cancers and TPV is a potential oncolytic therapy. To investigate the effect of MMP-9 on TPV replication, SK-MEL-3 cells were infected with TPVGFP at 0.1 and 5 MOI and incubated in the serum-free medium containing MMP-9 protein (0.1, 1, 10 μg/ml, respectively) for 96 h. The infected cells in the mock wells received no treatment of MMP-9. The yield of TPVGFP was significantly reduced upon treatment with MMP-9. Infection with a MOI of 0.1 was not significantly affected by 0.1 μg/mL MMP-9 treatment, but 1 and 10 μg/ml MMP-9 treatments did result in significant decrease in the number of plaques ($p < 0.05$) (Fig. 1). Compared to the replication of TPVGFP with no MMP-9 treatment, the 10 μg/ml MMP-9 treatment resulted in a ~tenfold decrease in virus titer. Incubation with MMP-9 also protects infected melanoma cells, since there is a significant increase in cell survival ($P < 0.05$). The percentage of infected cell survival upon treatment with 10 μg/ml MMP-9 was approximately two-fold greater than that of those in mock wells. At 5 MOI in SK-MEL-3 cell, TPVGFP replication was significantly decreased only with treatment of 10 μg/mL MMP-9 and was around threefold lower than that with no MMP-9 treatment ($P < 0.01$). Cell survival, however, was significantly increased with both 1 and 10 μg/mL treatments ($P < 0.05$). These results provide compelling evidence that MMP-9 exerts an anti-viral activity on TPV replication and protects TPV-infected cells.

Fig. 1 Effect of MMP-9 on the replication of TPVGFP and proliferation of human melanoma SK-MEL-3 cells. Each well in 48-well plate was planted with 5×10^4 SK-MEL-3 cells. Cells were infected with a mixture of TPVGFP at 0.1 MOI (**b**, **d** and **f**) or 5 MOI (**c**, **e** and **g**) and MMP-9 purified protein with final concentration being at 0.1, 1 or 10 ug/ml. The infected cells with no MMP-9 treatment serve as the mock. *Fluorescence* and *light images* were taken at 48 hpi (**a**) and 96 hpi (**b** and **c**). The titer of TPVGFP at 0.1 MOI (**d**) and 5 MOI (**e**) was evaluated at 96 hpi using plaque assay. The number of viable infected cells in each treatment group was counted in hemocytometer chamber using trypan *blue* stain at 96 hpi. The percentage of cell survival (**f** and **g**) was determined at 96 hpi and calculated as 100 × (number of viable cells in each treatment group/viable cell number in the wells with no infection and no MMP-9 treatment). The percentage of cell survival of cells infected with 0.1 MOI TPVGFP is shown in (**f**), while that of cells infected at 5 MOI is shown in (**g**). Each experiment was repeated three times independently, and standard deviations are shown

TPV infection induces the expression of MMP-9

In our previous studies, we have shown that wtTPV and TPV recombinants such as TPVΔ66R and TPVΔ15L


(a)
mock
w/ 0.1 ug/ml MMP-9
w/ 1 ug/ml MMP-9
w/ 10 ug/ml MMP-9
0.1 MOI
5 MOI
(b)
CPE
Cell
(c)
(d)
Virus titer
10^6
10^5
10^4
10^3
(e)
10^7
10^6
10^5
10^4
(f)
Percentage of cell survival
100
80
60
40
20
0
(g)

significantly retarded melanoma tumor growth in athymic nude mice models [9]. A variety of cytokines, such as interleukin (IL)-8, IL-6, transforming growth factor (TGF) and MMPs, are associated with melanoma progression and metastasis. Elevated expression of MMP-9 has been observed in the cells and organisms infected with different viruses including RSV [19], influenza virus [20] and coxsackievirus [16]. Therefore, we sought to test the effect of TPV infection on the expression of MMPs in melanoma cells. The expression of MMP-9 was monitored using RT-PCR and Western blot analyses. Both analyses reveal that MMP-9 expression was significantly enhanced in SK-MEL-3 cells infected with wtTPV, TPVΔ66R and TPVΔ15L, relative to control samples that were mock-infected. Moreover, the MMP-9 expression appeared even more remarkable in TPVΔ15L-infected cells, compared with that in wtTPV- and TPVΔ66R-infected cells (Fig. 2). While MMP-2, the other gelatinase, has been shown to degrade type IV collagen and promote cancer invasion and metastasis [26], we have been unable to detect MMP-2 in SK-MEL-3 cells, either infected or uninfected (data not shown). Moreover, expression of mRNAs of TGF-β, protein kinase R (PKR), interferon-stimulating gene (ISG)-15 and IL-8 has also been detected in the SK-MEL-3 cells and elevated TGF-β was also observed in the SK-MEL-3 cells infected with TPVs compared with that in uninfected cells (data not shown). MMP-9 expression is clearly enhanced by TPV infection, even when 15L and 66R genes are knocked out of the genome.

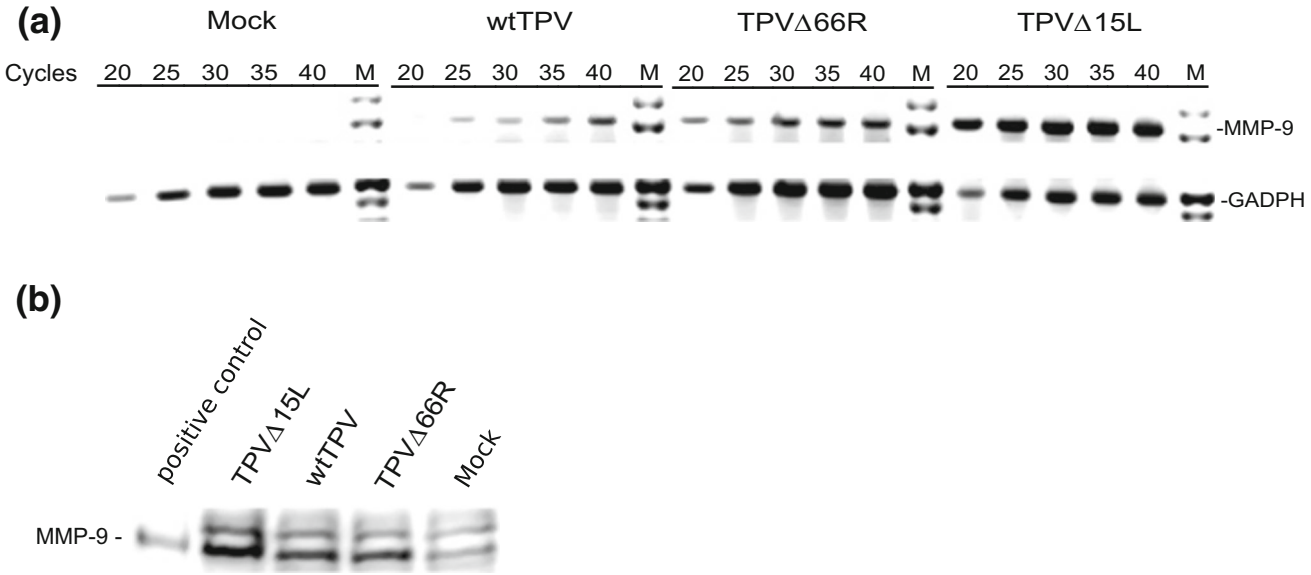
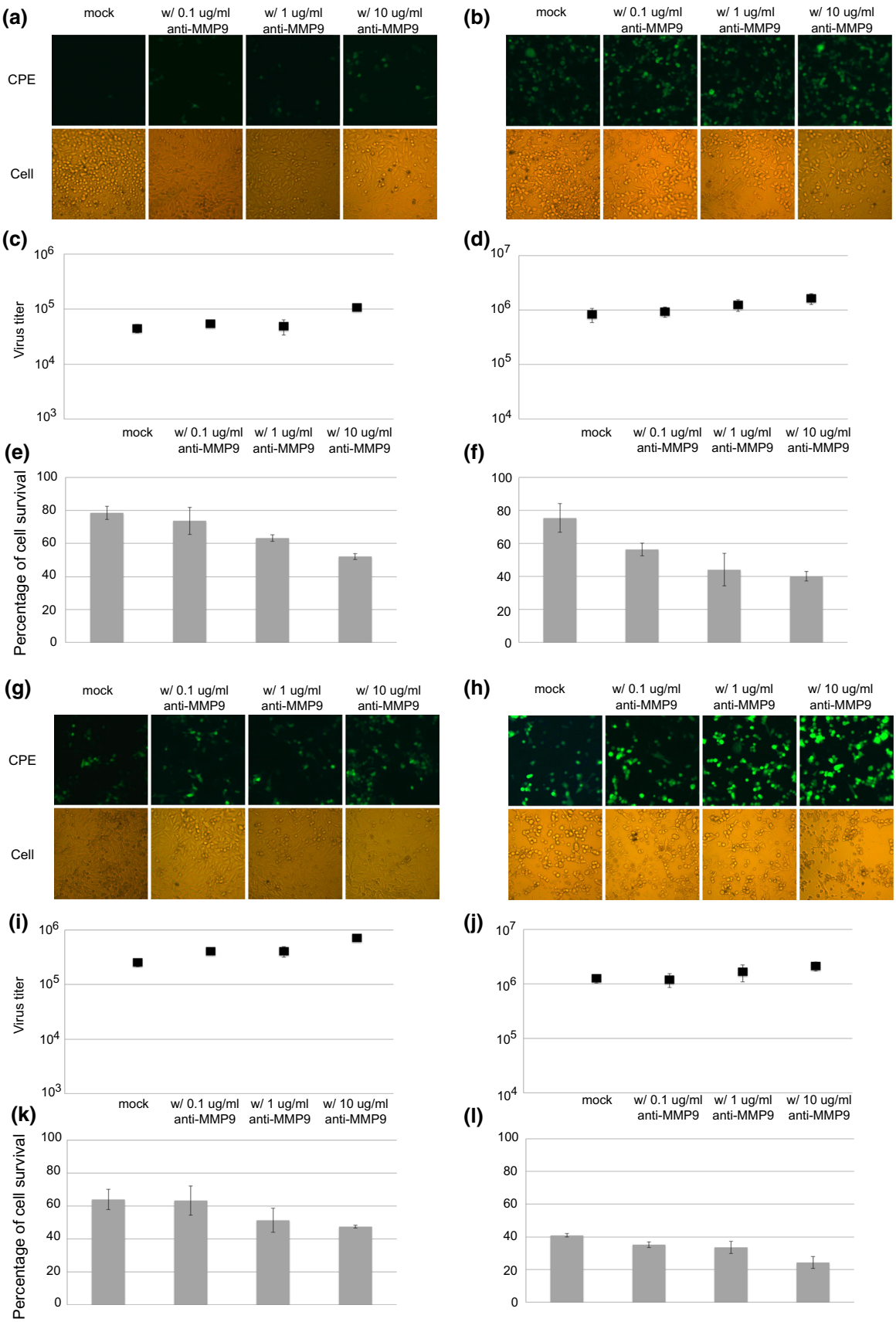


Fig. 2 Verification of MMP-9 expression in SK-MEL-3 cells **a** RT-PCR amplification of MMP-9. PCR products were amplified using the mRNAs extracted from the cells with mock infection, infection with wtTPV, TPVΔ66R and TPVΔ15L. The PCR products amplified after 20, 25, 30, 35 and 40 cycles were separated on a 2% agarose gel with ethidium bromide. Marker is the 500-bp DNA ladder. No PCR products were detected in the negative controls, in which either no RNA or reverse transcriptase was present (data not shown). **b** *Western blot* analysis of MMP-9 expression. SK-MEL-3 cells were infected with wtTPV, TPVΔ15L, TPVΔ66R and medium (mock) separately. The cells were harvested after 72 h and centrifuged. The cell pellets were subsequently frozen and thawed three cycles to release the MMP-9 from the cytoplasm, which were collected as the cell lysate samples. The protein samples were subjected to 12% SDS–PAGE gel and *Western blot* analysis using a goat antibody against MMP-9 and the secondary anti-goat HRP conjugated antibody. ECL was applied for visualization of the bands

Fig. 3 Effect of anti-MMP-9 on the replication of TPVΔ15L and the cell survival of infected SK-MEL-3 cells. Each well in 48-well plate was planted with 5×10^4 SK-MEL-3 cells. Following adhesion, cells were incubated in the serum-free medium containing anti-MMP-9 at 0.1, 1 and 10 ug/ml for 2 h in 37 °C incubators. Following incubation, the cells were infected with TPVΔ15L at 0.1 or 5 MOI. The infected cell incubated with medium containing no anti-MMP-9 served as mock. Fluorescence and light images were taken at 48 hpi (**a** and **b**) and 96 hpi (**g** and **h**). The titer of TPVΔ15L at 0.1 MOI and 5 MOI was evaluated at 48 and 96 hpi using plaque assay and shown as **c** 0.1 MOI at 48 hpi; **d** 5 MOI at 48 hpi; **i** 0.1 MOI at 96 hpi and **j** 5 MOI at 96 hpi. The number of viable infected cells in each treatment group was counted on hemocytometer chamber using trypan *blue* stain at 48 and 96 hpi. The percentage of cell survival was determined at 48 hpi and 96 hpi and calculated as 100 × (number of viable cells in each treatment group/viable cell number in the wells with no infection and no MMP-9 treatment). The percentage of cell survival of cells infected with 0.1 MOI TPVΔ15L is shown in **e** (48 hpi) and **k** (96 hpi), while that of cells infected at 5 MOI is shown in **f** (48 hpi) and **l** (96 hpi). Each experiment was repeated three times independently, and standard deviations are shown

Neutralization of MMP-9 enhances TPV replication in melanoma cells

In light of the anti-viral effect exerted by MMP-9 on TPV replication and the induced expression of MMP-9 by TPV infection, the effect of blocking MMP-9 on the replication


(a)
mock
w/ 0.1 ug/ml anti-MMP9
w/ 1 ug/ml anti-MMP9
w/ 10 ug/ml anti-MMP9
CPE
Cell
(b)
mock
w/ 0.1 ug/ml anti-MMP9
w/ 1 ug/ml anti-MMP9
w/ 10 ug/ml anti-MMP9
(c)
Virus titer
10^6
10^5
10^4
10^3
mock
w/ 0.1 ug/ml anti-MMP9
w/ 1 ug/ml anti-MMP9
w/ 10 ug/ml anti-MMP9
(d)
10^7
10^6
10^5
10^4
mock
w/ 0.1 ug/ml anti-MMP9
w/ 1 ug/ml anti-MMP9
w/ 10 ug/ml anti-MMP9
(e)
Percentage of cell survival
100
80
60
40
20
0
(f)
100
80
60
40
20
0
(g)
mock
w/ 0.1 ug/ml anti-MMP9
w/ 1 ug/ml anti-MMP9
w/ 10 ug/ml anti-MMP9
CPE
Cell
(h)
mock
w/ 0.1 ug/ml anti-MMP9
w/ 1 ug/ml anti-MMP9
w/ 10 ug/ml anti-MMP9
(i)
Virus titer
10^6
10^5
10^4
10^3
mock
w/ 0.1 ug/ml anti-MMP9
w/ 1 ug/ml anti-MMP9
w/ 10 ug/ml anti-MMP9
(j)
10^7
10^6
10^5
10^4
mock
w/ 0.1 ug/ml anti-MMP9
w/ 1 ug/ml anti-MMP9
w/ 10 ug/ml anti-MMP9
(k)
Percentage of cell survival
100
80
60
40
20
0
(l)
100
80
60
40
20
0

of TPV in melanoma cells was assessed in vitro. As TPVΔ15L appeared even more potent in regard to MMP-9 induction (Fig. 2), a neutralizing anti-MMP-9 antibody was used at different concentrations (0.1, 1, 10 μg/mL, respectively) to block MMP-9 activity in SK-MEL-3 cells infected with TPVΔ15L at MOIs of 0.1 and 5 (Fig. 3). The infected cells with no antibody treatment served as mock. The total virus titer and percentage of cell survival was assessed at 48 and 96 h post-infection (hpi). At 0.1 MOI of infection, the incubation of infected cells (0.1 MOI) with 10 μg/mL anti-MMP-9 significantly increased replication of TPVΔ15L at both 48 and 96 hpi ($P < 0.05$). Cell survival was significantly decreased at 48 hpi upon treatment with 1 μg/ml anti-MMP-9, while the 10 μg/ml anti-MMP-9 treatment significantly reduced cell survival at both 48 and 96 hpi, relative to the controls ($P < 0.05$). At a MOI of 5, TPVΔ15L virus replication at 48 and 96 hpi was enhanced by approximately twofold when treated with 10 μg/ml of anti-MMP-9 ($P < 0.05$). Infected cell survival at 48 hpi was significantly decreased upon treatment with 1 and 10 μg/ml anti-MMP-9 ($P < 0.05$), while a significant decrease in cell survival at the 96 hpi was only observed with the 10 μg/ml anti-MMP-9 treatment ($P < 0.05$). The results reveal that blocking MMP-9 expression in TPV-infected melanoma cells significantly increased virus replication and cell death.

Discussion

In this study, the interplay between MMP-9 expression and TPV replication is investigated in melanoma cells. TPV infection stimulates elevated expression of MMP-9 in melanoma SK-MEL-3 cells (Fig. 2), which is similar to the effect observed in the cells or organisms infected with other viruses, such as RSV [19], dengue virus [27] and coxsackievirus [16]. Nevertheless, some other viruses, such as human cytomegalovirus (HCMV), decrease the MMP-9 expression and instead increase the production of TIMPs [28]. The study also revealed that TPV replication is inhibited by the presence of MMP-9. The addition of MMP-9 to TPV-infected melanoma cells decreased the virus titer and increased melanoma cell survival (Fig. 1). Also, specifically inhibiting MMP-9 in TPV-infected melanoma cells resulted in a significant increase in virus replication and decreased cell survival (Fig. 3). The antiviral effect of MMP-9 on TPV is similar to what is observed for some other viruses. For example, MMP-9 has also been shown to counter the infectivity of RSV and regulate RSV replication and spread [24]. Although the involvement of MMP-9 in decreasing virus replication has been demonstrated, the exact mechanism remains obscure. Several possible implications have been suggested. First, MMP-9 could proteolytically cleave proteins on virus or cell surface to impede virus entry. Second, MMP-9 has been shown to interact with cell membrane proteins such as epidermal growth factor [29], which potentially interfere with virus attachment and subsequent replication. Third, it has been suggested that MMP-9 is one of the host defense mechanisms which regulate the virus replication through altering the signaling processes [30]. Nevertheless, while enhanced expression of MMP-9 is observed in TPV-infected SK-MEL-3 cells, no increased expression of IFNs and ISGs has been detected in this study, which suggests inactivation of IFN-signaling pathways (data not shown). Fourth, some in vivo studies indicated that the induced MMP-9 and accumulated proteolytic activity may contribute to the infiltration of lymphocytes, which potentially limit the virus infectivity [24].

OVs have emerged as an appealing option for cancer therapies, as they can selectively replicate in the tumor cells, cause the cell lysis and stimulate the host anti-tumor immune responses. A variety of viruses, such as HSV [31], coxsackievirus [32], reovirus [33] and VV [34], have been demonstrated for their enormous oncolytic potential and tested in the clinical trails for cancer treatment. Our previous studies have clearly demonstrated the oncolytic efficacy of TPVs in treating melanoma, colorectal cancer and breast cancer [8, 9, 35, 36]. TPV and TPV variants, namely TPVΔ15L and TPVΔ66R, significantly reduced melanoma tumors xenografted into athymic nude mice using SK-MEL-3 cells [8, 9]. Since the presence of MMP-9 can reduce the efficacy of oncolytic viruses used as immunotherapies, studies into the interplay between MMP-9 and TPV are necessary, and prompted the current investigation. The results reveal that TPV infection significantly elevates MMP-9 expression and that MMP-9 protects SK-MEL-3 melanoma cells infected with TPV by reducing virus replication and promoting cell survival. In light of the anti-viral effect of MMP-9 on TPV replication, it is reasonable to evaluate a combined TPV and anti-MMP-9 therapy of melanoma in vivo.

MMPs have been demonstrated to protect the tumor cells and promote cancer cell survival in many ways. For example, MMP-7 has been shown to protect the sarcoma and colon cancer cells by cleaving Fas-ligand and reducing the Fas-mediated cell apoptosis [37]. It has also been shown that MMP-9 promotes the tumor cell survival and proliferation via coordination with TGF-β and CD44 [38]. Similarly, we show here that MMP-9 treatment protects SK-MEL-3 cells infected by TPV and increases the cell survival. Inhibition of MMP-9 decreased cell survival of the infected cells (Figs. 1 and 3). These observations further support the combination of TPV and MMP-9 neutralization in our future studies. In light of the contribution of MMPs to cancer aggressiveness and the corresponding

poor prognosis, a variety of MMP inhibitors have been designed to block the activity of MMPs [39]. However, the use of MMP inhibitors as an oncotherapy has led to unsatisfactory outcomes in clinical trials, likely due to several reasons. First, although MMPs are essential in promoting tumor progression at early stages, targeting MMPs for treatment is probably less effective for cancers that have metastasized. Second, since expression levels of different MMPs vary among cell types, the inhibitors used may not target the MMPs responsible for cancer progression. For example, SK-N-AS neuroblastoma and U373 glioma cells have been identified as lower expressers of MMP-9, compared to SNB-19 glioblastoma and U251 neuroblastoma cells [23]. Third, more and more studies have revealed that certain MMPs possess highly complicated functional activities. For example, although over-expression of MMP-9 is often associated with cancer progression, it induced tumor regression and generation of anti-angiogenesis components in breast cancers, thereby serving as tumor suppressors [40, 41]. Therefore, based on the functionally diverse roles of MMPs, it is necessary to determine the effect of a MMP on particular cancer types before administering MMP inhibitors. Moreover, the combinational therapy of TPV and MMP-9 inhibition might not exert similarly remarkable anti-tumor efficacy in some other types of cancer cells as in melanoma SK-MEL-3 cells.

While tumor microenvironment plays a critical role in tumor progression and metastasis and affects OVs' delivery and spread in the tumors, strategies have been designed to target tumor microenvironment in order to enhance the efficacy of OVs. Transgenes of collagenase, hyaluronidase and MMPs, which are enzymes that degrade ECM components, have been incorporated into OVs to assist the virus replication and spread [42]. Since MMPs have variable functions and effects on cancer cells and virus infectivity, careful consideration of the incorporated MMP is required. For example, while both MMP-8 and MMP-2 show the ECM-degrading activities, only MMP-8 has been shown to suppress melanoma growth [43]. Consequently, an OV that incorporates MMP-8 may be better as a melanoma therapeutic than one that incorporates MMP-2. Nevertheless, a comparison between MMP-8 and MMP-2 expressing viruses would be critical to determine their relative oncolytic efficacies. To identify MMP-expressing OVs that have maximal efficacy, the MMPs' efficacy in enhancing virus spread and the effects of the MMP on tumor progression and oncolytic virus replication must be considered.

While expression of cytokines and enzymes such as MMP-9, TGF-β, PKR and IL-8 has been confirmed in the melanoma SK-MEL-3 cells, elevated TGF-β expression following TPV infection was also observed in this work (data not shown). TGF-β regulates the expression of MMPs, through the TGF-β-inhibitory element (TIE) binding site found in the promoters of MMP-1, 7, 9 and 13 [44, 45]. MMP-9 proteolytically activates the latent TGF-β via cleaving the latency-associated peptide (LAP) from TGF-β precursor [38]. Therefore, TGF-β and MMP-9 function in a bidirectional manner to regulate each other in cancer progression. It would be interesting to postulate that the MMP-9 induced by the TPV infection subsequently stimulated the elevated expression of TGF-β. Similarly to MMP-9, TGF-β has been demonstrated as either tumor suppressor or tumor promoter. While TGF-β plays a critical role in inducing apoptosis and anti-tumor responses in the pretumoral stage, it is highly expressed and secreted in the advanced stages of a variety of cancers, such as colorectal cancer, breast cancer and prostate cancer [45]. Further studies are required to assess whether the elevated level of TGF-β in infected SK-MEL-3 cells is associated with the over-expression of MMP-9, and results in enhanced or reduced melanoma cell proliferation.

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Author's contributions Tiantian Zhang and Karim Essani conceived or designed the work; Tiantian Zhang took part in data collection; Tiantian Zhang was involved in data analysis and interpretation, and participated in drafting the article; Tiantian Zhang, Yogesh R. Suryawanshi, Blair R. Szymczyna, and Karim Essani performed critical revision of the article; and Tiantian Zhang, Yogesh R. Suryawanshi, Blair R. Szymczyna and Karim Essani approved the final version to be published.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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