Chapter 6. Attenuated Measles Virus as a Therapy for Mesothelioma

6.1 Introduction

Measles Virus (MV) causes a common, acute infectious disease characterised by fever, cough, conjunctivitis and a generalized maculopapular rash (Griffin 2001; Rima et al., 2006). MV is transmitted via aerosol droplets. Initial infection is established in the respiratory tract, and then virus enters local lymphoid tissue. Monocytes and lymphocytes are the primary infected cells in the blood (Osunkoya et al., 1990; Esolen et al., 1993). Amplification of the virus in lymph nodes produces a primary viremia that results in the spread of virus to multiple lymphoid tissues and other organs, including the skin, kidney, gastrointestinal tact and liver (Hall et al., 1971; Sakaguchi et al., 1986; Kobune et al., 1996; Mcchesney et al., 1997). MV also infects the nervous system and causes various types of neurological diseases, including acute encephalitis and subacute sclerosing panencephalitis (SSPE) (Griffin 2001; Rima et al., 2006). MV was first isolated in 1954 from primary human kidney cells inoculated with the blood and throat washings from a child with measles (Enders et al., 1954). This first isolate, the Edmonston strain, is the progenitor of the currently used live vaccines with no pathogenicity. Despite the availability of effective live vaccines; measles remains the leading vaccine-preventable cause of childhood disease worldwide (Bryce et al., 2005). Deaths from measles are due largely to an increased susceptibility to secondary bacterial and viral infections.

6.1.1 MV Structure

MV belongs to the *Morbillivirus* genus of the *Paramyxoviridae* family. It is an enveloped virus with a non-segmented, negative-strand RNA genome (Griffin 2001). The genome encodes six structural and three non-structural proteins (Fig. 6-1)(Kerdiles et al., 2006). Six structural proteins are the nucleocapsid (N), phospho- (P), matrix (M), fusion (F), hemagglutinin (H), and large (L) proteins. The virus envelope consists of a lipid bilayer, derived from the cellular plasma membrane, with two inserted virus-encoded glycoproteins, the H and F proteins. The H and F proteins are responsible for receptor binding and membrane fusion (Wild et al., 1995). The M protein that lines the inner surface of the envelope plays a role in virus budding (Cathomen et al., 1998) and transcription regulation (Suryanarayana et al., 1994). The genomic RNA is encapsidated with N protein and, together with RNA-dependent RNA polymerase composed of the L and P proteins, form a ribonucleoprotein complex (Horikami et al., 1995). The P gene encodes additional proteins, the V and C proteins. The P and C proteins are translated from overlapping open reading frames, and the V protein is expressed from an edited mRNA. The functions of the V and C proteins are not understood completely, but some of their functions are concerned with their activities as IFN antagonists (Palosaari et al., 2003; Shaffer et al., 2003; Takeuchi et al., 2003; Yokota et al., 2003).

6.1.2 MV Receptors

MV enters cells by binding its hemagglutinin (H) attachment protein to one of two cellular receptors, CD46 (also called membrane cofactor protein) and signalling lymphocyte activation molecule (SLAM, also called CD150). For the vaccine strains of

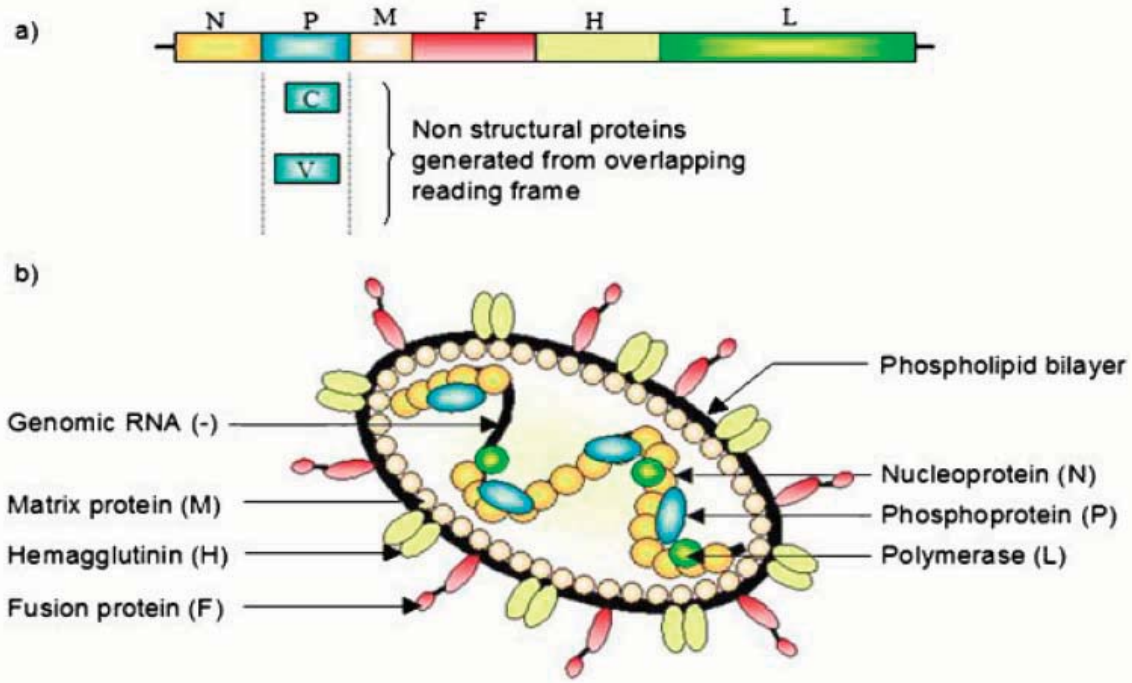


Figure 1. Schematic representation of measles virus genome with the position of two non-structural proteins, V and C (a) and the structure of measles virus particle (b)

Adapted from Kerdiles YM, et al. Rev Med Virol, 2006;16(1):49-63

MV, both receptors are relevant. However, the relevance of CD46 use is not clear for wild type MV (which is not selectively oncolytic); many wild type strains cannot enter cells via CD46. CD46 is a cell surface type1 transmembrane glycoprotein of 57-67KD, and is expressed on all nucleated human cells except red blood cells (RBCs) (Liszewski et al., 1991). CD46 is required to mediate intercellular fusion. Virally infected cells expressing the MV envelope glycoproteins H and F on their cell surfaces fuse with neighbouring cells through CD46 to form multinucleated syncytia, the characteristic CPE of MV infection. CD46 is the receptor not only for MV but also for some serotypes of adenovirus, human herpes virus 6 and bovine viral diarrhoea virus, as well as two types of bacteria (Cattaneo 2004). The relevance of signalling through CD46 to MV-mediated pathology remains unclear (Fielding 2005). However, it has been reported that high expression of CD46 on tumour cells may account for the selectivity of oncolytic MV in transformed cells (Anderson et al., 2004). The other MV receptor, SLAM (CD150), is a 70KD cell surface glycoprotein and is located on human chromosome 1q22-q23 (Cocks et al., 1995; Tangye et al., 2000; Veillette et al., 2003). Initially, SLAM was identified as a human T cell activation receptor in 1995 (Cocks et al., 1995). Till 2000, SLAM was confirmed as a cellular receptor for B cell isolated measles virus strains that cannot use CD46 as a receptor (Tatsuo et al., 2000). SLAM is expressed on immature thymocytes, memory T cells, a proportion of B cells, macrophages and mature DCs. After stimulation with antigens or mitogens, all T and B cells express SLAM (Cocks et al., 1995; Aversa et al., 1997). In addition, SLAM confers virus uptake and syncytium formation in various measles virus strains such as vaccine strains, wild type strains and various recombinant viruses. The distribution of SLAM is in accord with the lymphotropism and immunosuppressive nature of MV. SLAM is also a

cellular receptor for other members of the morbillivirus family such as canine distemper virus and rinderpest (Tatsuo et al., 2001).

However, SLAM and CD46 alone as receptors for MV do not readily explain the pathogenesis of MV in all tissues type. CD46 is ubiquitously expressed, but it is not a receptor for wild type MV strain. Compared to CD46, the distribution of SLAM is more consistent with the pathology of MV infection in humans and monkeys. This might lead to a conclusion that SLAM is the principal MV receptor *in vivo*. Some studies have shown that MV can infect SLAM-negative cells at low efficiency, via the SLAM- and CD46-independent pathway, which may account for MV infection of epithelial, endothelial and neuronal cells *in vivo* (Hashimoto et al., 2002; Yanagi et al., 2006). Therefore, other potential MV receptors might exist for MV *in vivo* (Dhiman et al., 2004). This area of research requires further investigation.

6.1.3 Pathogenesis of MV Infection

Measles infection is characterized by immunosuppression and lymphopenia. Deaths from measles are due largely to an increased susceptibility to secondary bacterial and viral infections, attributed to a prolonged state of immune suppression. Multiple mechanisms are likely responsible for the immune suppression following MV infection (Schneider-Schaulies et al., 2002) (Fig. 6-2)(Moss et al., 2004). Several abnormalities of the immune system have been described, including changes in lymphocytes number and function, shifts in cytokines responses, immunodulatory effects of IL-10, down regulation of IL-12, impaired antigen presentation, and altered interferon α/β signalling pathways.

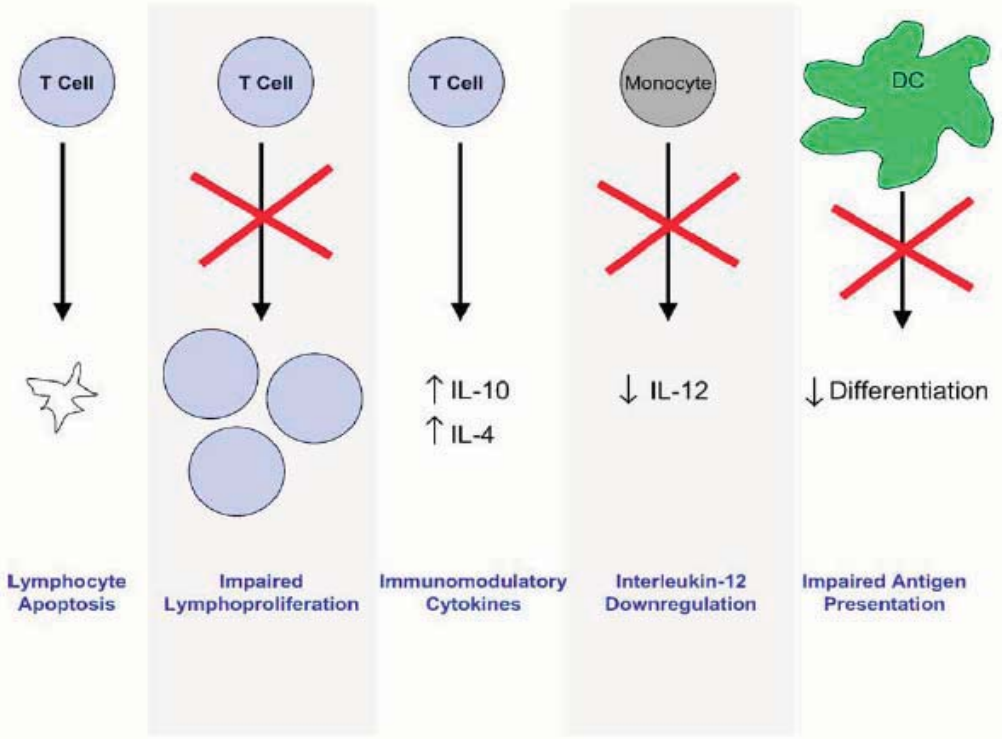


Fig. 6-2 Potential mechanisms of immune suppression following measles virus infection. Multiple mechanisms are likely responsible for the immune suppression following measles virus infection. Potential mechanisms of immune suppression include: (1) lymphocyte apoptosis; (2) impaired lymphoproliferative responses; (3) induction of immunomodulatory cytokines, including interleukin (IL)-10 and IL-4; (4) down-regulation of IL-12 production; and (5) impaired terminal differentiation and antigen presentation by dendritic cells (DC).

Adapted from Moss WJ, et al. Int J Biochem & Cell Biol, 2004;36(8):1380-85

Measles is accompanied by a transient lymphopenia, with a reduction in the number of CD4+ and CD8+ T-lymphocytes (Ryon et al., 2002). Increased surface expression of Fas (CD95) and annexin V staining on both CD4+ and CD8+ T-lymphocytes during acute measles suggest that apoptosis of uninfected lymphocytes may explain some of the lymphopenia (Ryon et al., 2002). However, the rate of decline and return of the peripheral lymphocyte pool is so rapid that redistribution of lymphocytes within the lymphoid tissue is likely to account for much of the peripheral lymphopenia (Moss et al., 2004). Lymphopenia also could result from impaired thymic output. Measles virus infects epithelial cells and monocytes in the thymus, and can induce apoptosis in thymocytes. However, impaired thymic output does not appear to be responsible for the lymphopenia observed during acute MV infection. Another mechanism of MV-induced immunosuppression may result from a shift in cytokine responses from a T-helper type 1 (Th1) immune response in the acute phase of measles to a predominant Th2 response during period of recovery (Moss et al., 2002). Th1 response accounts for viral clearance while the Th2 response supports the development of MV-specific antibody. IL-10 down-regulates the synthesis of cytokines, suppresses macrophage activation and T cell proliferation, and inhibits delayed-type hypersensitivity responses. Cross-linking of monocyte CD46 may also contribute to suppression of cell-mediated immunity by down-regulation IL-12 production (Karp et al., 1996). Prolonged suppression of IL-12 production has been observed in children with measles (Atabani et al., 2001). Measles virus impairs antigen presentation *in vitro*. Dendritic cells (DCs) are critical for the presentation of antigen to naïve T-lymphocytes. MV infection induces formation of DC syncytia, and DCs lose the ability to stimulate lymphoproliferative responses in co-cultured T cells and undergo Fas-mediated apoptosis (Servet-Delprat et al., 2000). Wild type MV induces significantly less interferon α/β *in vitro* than tissue culture adapted,

attenuated measles virus (Naniche et al., 2000). The non-structural protein V and C of MV may interfere with interferon α/β signalling pathways. Inhibition of interferon α/β production or interferon signalling pathways by MV may be important in early evasion of host immune responses, but not likely to play an important role in long-term immune suppression.

6.1.4 Attenuated Measles Virus (MV-Edm) for Cancer Therapy

Since the first published demonstration of MV oncolysis in 2001 (Grote et al., 2001), substantial progress has been made towards engineering attenuated MV with many of the desired characteristic of the ideal virotherapy agent. Live attenuated Edmonston B strain of measles virus (MV-Edm) has potent and specific oncolytic activity against a variety of human tumours, including lymphoma, multiple myeloma, epithelial ovarian cancer and glioma (Grote et al., 2001; Peng et al., 2001; Peng et al., 2002; Grote et al., 2003; Phuong et al., 2003; Dingli et al., 2004). MV-Edm is selectively oncolytic, causing extensive syncytium formation and cell killing in a variety of human tumour cells but minimal cytopathic damage in non-transformed cells such as normal dermal fibroblasts, ovarian surface epithelium, mesothelial cells from peritoneal cavity, and peripheral blood lymphocytes (Peng et al., 2001; Peng et al., 2002). However, the mechanism underlying its oncolytic specificity has not been understood.

Replicating attenuated measles virus has been shown to be oncolytic in several murine models of lymphoma, myeloma, ovarian carcinoma and glioma (Grote et al., 2001; Peng et al., 2001; Peng et al., 2002; Phuong et al., 2003). All the models employ xenografts of human tumour cell lines implanted into immune deficient mice. Injections

of MV have resulted in slowing of growth of established tumours or in regression of established tumours. These studies suggest the considerable potential of MV to lyse human tumours. However, the limitations of studies in murine models are clear. Potential toxicities of MV cannot be assessed in this way, since MV does not infect murine cells. Mice transgenic for human CD46 (Mrkic et al., 1998; Mrkic et al., 2000) have been employed to assess toxicity in these animals. The human CD46 transgenic mice have been demonstrated the pathways for MV-infection following intraperitoneal injection elegantly by using MV expressing GFP (Peng et al., 2003). This model has been accepted as a suitable model for the conduct of toxicity studies in support of a human phase I study.

Although MV appears to have some selectivity of replication within transformed cells, there remain distinct theoretical advantages to engineering targeted replication to cancer cells. Excellent progress has been made in targeting MV entry to specific cell-types. Viral attachment H protein is the major protein to be responsible for MV tropism (Tatsuo et al., 2000). Displaying ligands, such as epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) (Schneider et al., 2000), on the C-terminal of MV H protein can modify MV tropism. Several single-chain antibodies, carcinoembryonic antigen (CEA), CD20 and CD38 have been displayed on H protein in order to target MV entry to epithelial malignancies, lymphomatous B cells and myeloma cells (Hammond et al., 2001; Bucheit et al., 2003; Peng et al., 2003). MV-CEA is currently been tested in patients with ovarian cancer.

Enhancing the oncolytic activity of MV is another important goal of current research. The approach has been proposed to address both tracking of MV replication and

increasing its therapeutic efficacy. MV-NIS is an oncolytic measles virus coding for the expression of thyroidal sodium iodide symporter (NIS). When MV is induced to express NIS, it retains the oncolytic potential of the parent virus as well as inducing NIS expression in infected cells. The distribution and expression of MV-NIS can be detected by gamma camera imaging in myeloma animal models (Dingli et al., 2004). Furthermore, the expression of NIS enhanced the therapeutic effect of MV, so that tumours normally resistant to MV-mediated lysis were eradicated at low dose of MV. Dual therapy with MV-CEA and MV-NIS is an attractive option for therapy of ovarian cancer and warrants testing in a phase I clinical trial (Hasegawa et al., 2006). It may be possible to enhance the oncolytic potential of MV when growth factors are expressed by MV. For example, a replication competent attenuated MV expressing murine GM-CSF (MV GM-CSF) showed no toxicity and enhanced efficacy in CD46 transgenic mice (Grote et al., 2003). Recombinant MV encoding suicide genes, such as HSV-TK and *E.coli* cytosine deaminase has also been shown to be feasible and studies of oncolytic efficacy *in vivo* are currently underway (Fielding 2005).

The great advantage of MV is that the tumour cells have no defence against the mechanism through which it destroys cells, and that the MV can be easily engineered to the target tumour. The major obstacles for clinical use of the measles viral vectors include neutralizing antibodies to the vector in human subjects and difficulties in production for gene therapy trials. The Russell group at the Mayo Clinic has overcome these production problems and may have found a way to circumvent the neutralizing antibodies through engineering new targeting ligands into the virus. They developed an Echistatin-targeted measles virus vector (MV-ERV), which binds the $\alpha_v\beta_3$ integrin receptor with a high affinity, which is displayed on the C terminal of the viral

attachment H protein. The MV-ERV vector can target to activate endothelial cells in tumour tissue by Echistatin binding to the $\alpha_v\beta_3$ integrin, and it can also target to tumour cells by the H protein binding to the native CD46 receptor. The MV-ERV vector has potential use in gene therapy for targeting tumour-associated vasculature for the treatment of solid tumours (Hallak et al., 2005). In phase-I clinical trials, two studies are being undertaken: intratumoural injection of attenuated MV in patients with relapsed lymphoma and intraperitoneal administration of MV-CEA with advanced ovarian cancer.

Our interest in measles is based upon several factors, both as a candidate viral agent *per se*, and for its potential as a synergistic agent for immuno-based therapy. Over the last 5-6 years our collaborators at the Mayo Clinic have conducted extensive pre-clinical development and evaluation of measles virus as an anti-tumour agent (Nakamura et al., 2004; Hasegawa et al., 2006). Pre-clinical studies included analysis of toxicity for normal peritoneal mesothelium, and the agent was found to be non-toxic. The major basis of our interest in measles however, is that the virus induces death via the formation of large syncytia which then undergo apoptosis (Hasegawa et al., 2006). This contrasts to adenovirus that induces death via lysis with rupture of the cell membrane. We are thus interested to evaluate the capacity for this agent to induce an anti-tumour immune response and to compare this with adenovirus. Because there is some evidence that induction of cell death by apoptosis may be a more potent stimulus of anti-tumour immunity than necrosis, MV therapy might ultimately prove an excellent adjunct to immunotherapy. As noted above however, pre-clinical evaluation is difficult because mice lack CD46, the principal receptor for the attenuated MV-Edm used as a therapy. Thus, the studies conducted here were designed to determine the oncolytic potential of

MV-Edm for mesothelioma cells, to determine whether death is induced by syncytia formation and apoptosis induction as seen in other cells and to assess the selectivity of killing for mesothelial tumour cells versus relevant normal cell types.

6.2 Materials and Methods

6.2.1 Materials

6.2.1.1 Cell Lines and Cell Culture

Vero (African green monkey kidney) cell line was obtained from Mayo Clinic, Minnesota, USA. They were grown in DMEM supplemented with 5% foetal bovine serum (FBS) (Trace Biosciences, Melbourne, Vic, Australia), with supplemental antibiotics, penicillin G (100u/ml) and gentamycin (50µg/ml) (Gibco BRL Life Technologies, Grand Island, NY, USA). Human mesothelioma cell lines included H226, H2052, Ju77, MSTO, H2596 and mesothelioma cells from patient fluid were cultured and maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine, penicillin G (100u/ml) and gentamycin (50µg/ml), and 10% FBS. Clonetics® Lung-derived normal human microvascular blood vessel endothelial cells (HMVEC-LB1) and pulmonary artery smooth muscle cells (PASMC) were cultured in EGM-2MV or SmGM-2 as recommended by the supplier (Cambrex Bio Science Walkersville, Inc., MD, USA) supplemented with 5% FBS and used between passages 4 and 9. Primary cultures of normal pericardial mesothelial cells (obtained from patients undergoing cardiac surgery, kindly provided by Dr Steve Mutsaers, Lung Institute of Western Australia) were maintained in Dulbeco's modified Eagle's medium (DMEM, Gibco BRL Life Technologies, Grand Island, NY, USA) supplemented with 15% FBS, 4 mM L-glutamine, 0.4 ug/ml hydrocortisone, 15mM HEPES, and penicillin G (100u/ml) and gentamycin (50µg/ml). All cells were maintained in a humidified atmosphere at 37°C in 5% CO_2.

6.2.1.2 Antibodies

Table 6-1 Antibodies used in this study

Antibodies	Purpose	Company
Mouse anti-CAR antibody (RmcB 05-644)	Flow Cytometry	Upstate Cell Signal Solutions, Charlottesville, VA, USA
Mouse anti-human CD46 antibody (555948)	Flow Cytometry	BD Biosciences, San Jose, CA, USA
PE-conjugated Rat anti-mouse (340270)	Flow Cytometry	BD Biosciences, San Jose, CA, USA
Rabbit anti-GFP, IgG fraction (A11122)	Immunohistochemistry	Invitrogen Australia Pty Limited, Mt Waverley, VIC, Australia
Alexa Fluor ® 488 Goat Anti-Rabbit IgG (H+L) (A11034)	Immunohistochemistry	Molecular Probes, Eugene, OR, USA

All antibodies were used at concentrations recommended by the manufacturer or at optimised concentrations determined in preliminary experiments.

6.2.1.3 Animals

All animal studies were approved by the Institute of Medical and Veterinary Science / Central Northern Adelaide Health Service Animal Ethics Committee. BALB/C nude mice were obtained from the IMVS animal care facility (Adelaide, Australia) and maintained under standard conditions. All mice used in these studies were female and between 4-6 weeks of age.

6.2.2 Methods

6.2.2.1 Viral Amplification

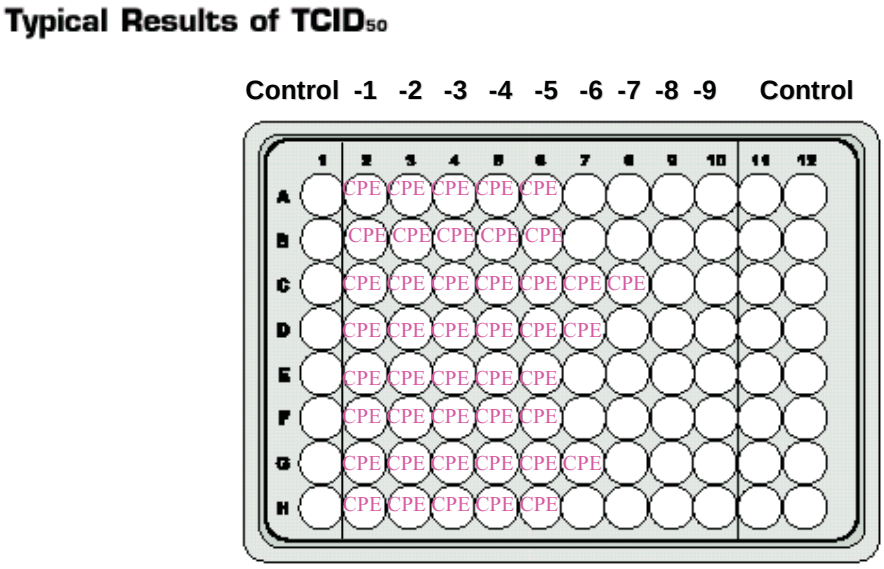
MV-CEA was a recombinant Edmonston strain of MV that expressed the soluble extracellular N-terminal domain of human carcinoembryonic antigen (CEA). MV-CEA propagation could be monitored by measuring blood CEA level. MV-NIS was an oncolytic measles virus coding for the thyroidal sodium iodide symporter (NIS) whose propagation could be mapped by serial radioiodine imaging. MV-GFP and MV-Luc were the recombinants MV-Edm expressing green fluorescent protein (GFP) or firefly luciferase (Luc). MV-NSE carrying the anti-genomic MV tag Edmonston B (MV-tag-Edm) sequence was slightly modified from MV. Measles stocks were generated by infecting Vero cells with MV at a multiplicity of infection (MOI) of 0.02 for 2 h at 37°C, after which the virus was removed, and cells were maintained in 5% foetal bovine serum-DMEM at 37°C. When 80-90% of the cells were in syncytia, the medium were removed, the cells were harvested into reduced serum Opti-MEM medium (Invitrogen Australia Pty Limited, VIC, Australia). Cell-associated virions were released by three freeze-thaw cycles, and cell lysates were clarified by a brief centrifugation step (2000 rpm for 5 min) and frozen in liquid nitrogen before storage at -70°C.

6.2.2.2 Viral Particle Titration

Virus titres were obtained by titration on Vero cells and expressed as 50% tissue culture infectious dose ($TCID_{50}$)/ml. Vero cells in 5% DMEM were plated in 96-well plates to give a final concentration of 7000 cells per well in 50 ul volume. Serial dilutions of the

viral stock (-1 to-9) were prepared in Opti-MEM medium and added 50 ul/well to each well. Virus was added to the titre plate as follows: one concentration/8 well leaving the ends as uninfected control. Kept plates in humidified 37°C incubator, and read titre 6 days after infection. Each well with syncytia was marked positive.

Figure 6-3: Example of calculating $TCID_{50}$



Virus titre calculation:

$$\text{Log10 } (TCID_{50}/ml) = L + d\,(s-0.5) + \log\,(1/v)$$

L= negative log10 of the most concentrated virus dilution tested in which all wells are positive

d = log10 of dilution factor

s = sum of individual proportions pi

pi = calculated proportion of an individual dilution

(amount of positive wells/ total amount of wells per dilution)

v= volume of inoculum (ml/well)

Example:

log10 virus dilution	ratio of infection	proportion
-1	8/8	1.0
-2	8/8	1.0
-3	8/8	1.0
-4	8/8	1.0
-5	7/8	0.88
-6	3/8	0.375
-7	1/8	0.125
-8	0/8	0
-9	0/8	0

$\log10\ (TCID_{50}/ml) = L+d\ (s-0.5)+\log(1/v) = 4+1(s-0.5)+\log10\ (1/0.05)$

$s = 1.0+0.88+0.375+0.125+0$

$\log10\ (TCID_{50}/ml) = 4+1(2.38-0.5)+1.3$

$\log10\ (TCID_{50}/ml) = 7.18$

$10^{7.18} = 1.5x10^{7}$

$TCID_{50}$ $= 1.5x10^{7}$

6.2.2.3 Viral Infection

The panel of human mesothelioma cells was infected with MV at the MOI of 0.01, 0.1, 0.5, 1 pfu per cell in Opti-MEM medium for 2 h at 37°C, after which the virus inoculum was removed, and the cells were maintained in standard medium for the required length of time prior to analysis.

6.2.2.4 Flow Cytometry

Flow cytometry was employed to detect the presence of Coxsackie Adenoviral Receptor (CAR) and CD46 on mesothelioma cell lines. Methods have been described previously in section 2.2.13.

Flow cytometry was also employed to detect apoptosis in uninfected and measles virus-infected cells. H226 cells, uninfected or infected with MV-CEA (MOI 0.5) for 72 h, were suspended by a brief trypsinization and washed twice in HEPES buffer. The cell membrane of apoptotic cells is permeable to 7-amino-actinomycin (7-AAD, Sigma, Saint Louis, Missouri, USA) due to loss of membrane pump action (Hodge et al., 2000). Briefly, cells were washed once with wash buffer, then centrifuged 4000 rpm for 1 min and the supernatant discarded. The cells were stained with 25 µl of 7-AAD, vortexed and then analysed using Cell Quest software (BD Biosciences, San Jose, CA, USA) on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

6.2.2.5 Luciferase Assay

MV containing the gene for firefly luciferase (Luc) was used to assess the transduction capability of specific vectors in the cell lines of interest. Cells were plated in 24-well plates as previously described. The cells were infected at 0.5 pfu per cell ratios. The infected cells were washed with PBS, and then treated with 100µl of cell lysis buffer 2 days after infection. A luciferase assay (Luciferase Assay System kit, Promega, WI, USA) and a luminometer (FB12 Luminometer, Berthold Detection Systems, Germany) were used for the evaluation of luciferase activities of Ad-infected cells. Briefly, 5µl of cell lysate was added to 50µl of luciferase reagent and analysed immediately using a luminometer. Luciferase activities were normalised by the protein concentration in cell lysate (Bio-Rad DC Assay Protein Kit, Bio-Rad Life Sciences Group, Hercules, CA, USA).

6.2.2.6 Cell Counting Assay

Initially cell counts were carried out using a standard haemocytometer and trypan blue exclusion. This provided the basis for the subsequent experiments, which assessed a variety of time points. Human mesothelioma cells, H226, MSTO, Ju77, H2052, H2596, mesothelioma cells from patient fluid, normal human microvascular blood vessel endothelial cells (HMVEC-LB1), pulmonary artery smooth muscle cells (PASMC) and primary cultures of normal pericardial mesothelial cells were plated in 6-well plate at a density of $2x10^5$ cells/well. Cells were infected with MV-CEA, MV-NIS, MV-NSE or MV-GFP at a MOI of 0.5 (or MOI of 1, 0.1, 0.01) the following day. The mediumwas removed and the cells washed with PBS at different time points. The cells were

trypsinised, detached from the plate and resuspended in 1 ml complete media. Using 0.01% trypan blue and standard haemocytometer the number of live cells were calculated in each well and a time line established. The percentage of surviving cells was calculated by dividing the number of viable cells in the infected well by the number of viable cells in the uninfected well corresponding to the same time point. In addition, cells in a separate parallel plate were fixed with 0.5% glutaraldehyde and stained with 0.2% crystal violet solution, and the CPE was photographed.

6.2.2.7 Immunodetection of MV-GFP Transgene Expression

BALB/c nude mice were implanted s.c in the right flank with H226 human mesothelioma cells. The tumours were allowed to achieve a volume of 50 mm^3, and then mice (n= 5/group) received one intratumoural injection of MV-GFP ($1x10^6$ pfu) or saline. Three days later, the mice were killed and tumours were removed, and fixed in 10% buffered-formalin (ACE Chemical Co., Camden Park, SA, Australia). After 24 h fixation, the tissue was processed into paraffin blocks. Sections were cut at 5µm and heat mounted (58°C for 1 h) on poly-L-lysine-coated slides (Polysine™, Menzel-Gläser, Germany). Tumour sections were deparaffinized and rehydrated through grades alcohols, and then immersed in preheated Target Retrieval Solution as described previously in section 2.2.16.1. Tissues were blocked with 10% goat serum for 1 h and incubated overnight at 4°C with a rabbit anti-GFP antibody (1:200; Invitrogen Australia Pty Limited, Mt Waverley,VIC, Australia). The next day, slides were washed and followed by detected using Alexa Fluor®488 Goat Anti-Rabbit antibody (1:1000; Molecular Probes, Eugene, OR, USA). Nuclei were counterstained with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI, Roche, Mannheim, Germany) for 2 min. Slides

were then mounted, and sections were examined using an Olympus BX51 fluorescent microscope system (Japan); images were captured using a Photometrics CoolSnap$_{fx}$ camera (Roper Scientific, Tucson, AZ) and recorded using V^{++} Precision Imaging system (Digital Optics, Auckland, New Zealand).

6.2.2.8 Caspase-3 Protease Activity Assay

Caspase-3 protease activity assay was performed in cell lysates as described (Koglin et al., 1999). Briefly, H226 cells infected with MV-CEA (MOI 0.5) and collected 24,48, 72 h post infection. The cells were lysed in cell lysis buffer shown in 2.1.8, and then fluorometrics caspase-3 protease activity assay was performed in 96-well plates by incubating 20 µl cell lysates (10µg) with 50µM Caspase-3 substrate II, Fluorogenic (Ac-DEVD-AMC) (Calbiochem, La Jolla, CA, USA) at room temperature for 6-24 h in the dark. Fluorescence was detected using a LS 50B Luminescence Spectrometer (Perkin Elmer Ltd, Beaconsfield, Buckinghamshire, UK) at an excitation wavelength of 380 nm and emission wavelength at 460 nm.

6.2.2.9 In Situ TUNEL Staining

Apoptosis is characterized by specific changes in cellular morphology. When observed under electron microscopy the cell is seen to undergo shrinkage, chromatin condensation, membrane blebbing and nuclear condensation. The cell then segments and divides into apoptotic bodies, which are usually phagocytosed. DNA fragmentation in these cells usually precedes cell death and removal from the tissues. The changes in the DNA structure are usually associated with the ultra structural changes in cellular

morphology in apoptosis. The TUNEL assay detects the DNA fragments by enzymatically labelling the free 3'-OH termini with modified nucleotides. Normal or proliferative cells, which have very low numbers of DNA 3' -OH ends, do not stain with this method. An ApopTag® Plus Peroxidase In Situ Apoptosis Detection kit (Chemicon International Inc, Temecula, CA) was used to detect DNA fragmentation in apoptotic cells *in vivo*. The DNA fragments are labelled with the digoxigenin-nucleotide, and then labelled with an anti-digoxigenin antibody that is conjugated to a peroxidase reporter molecule. This generates a permanent localised stain from chromogenic substrates.

Briefly, after deparaffinization, tissues were digested with proteinase K (20µg/ml) and endogenous peroxidase inactivated with 3% hydrogen peroxide. Subsequently, a mixture of Digoxenin-dUTP and terminal deoxynucleotidyl transferase enzyme was added, and samples incubated for 1 h at 37°C. Anti-digoxigenin conjugate was added. The colour was developed with peroxidase substrate under close observation and then counterstained with methyl green (0.5% w/v methyl green in 0.1M sodium Acetate, pH 4.0). Specimens were mounted under glass cover slips. TUNEL positive cells were visualised by direct microscopy.

6.3 Results

6.3.1 Mesothelioma Cells Express Abundant MV Receptor CD46

MV enters cells by binding via its hemagglutinin (H) attachment protein to one of two cellular receptors, CD46 or signalling lymphocyte activation molecule (SLAM). The pathogenic wild type MV (which is not selectively oncolytic) uses primarily SLAM. In contrast, attenuated vaccine stains such as MV-Edm use predominantly CD46. The use of MV-Edm in mesothelioma has not previously been investigated, but we identified that 13 mesothelioma cell lines express high levels of CD46, predicting MV-Edm susceptibility. In this study, we compared adenoviral receptor (CAR) versus measles virus receptors CD46 expression on human mesothelioma cell lines by flow cytometry. We found strong CD46 expression across all human mesothelioma lines tested (Fig.3-1). All lines demonstrated higher level of CD46 expression than level of CAR expression.

6.3.2 Marker Gene Expression in Mesothelioma Cells

Luciferase gene expression from MV-Edm was determined in 5 human mesothelioma lines (H226, H2052, Ju77, MSTO and H2596). Cells were infected with MV-Luc at an MOI of 0.5 and luciferase activity was measured 48 h later. Luciferase activities were normalised by the protein concentration in cell lysate. MSTO cell line showed the strongest luciferase gene expression in the 5 mesothelioma cell lines (Fig. 6-4), and Ju77 showed the lowest luciferase gene expression.

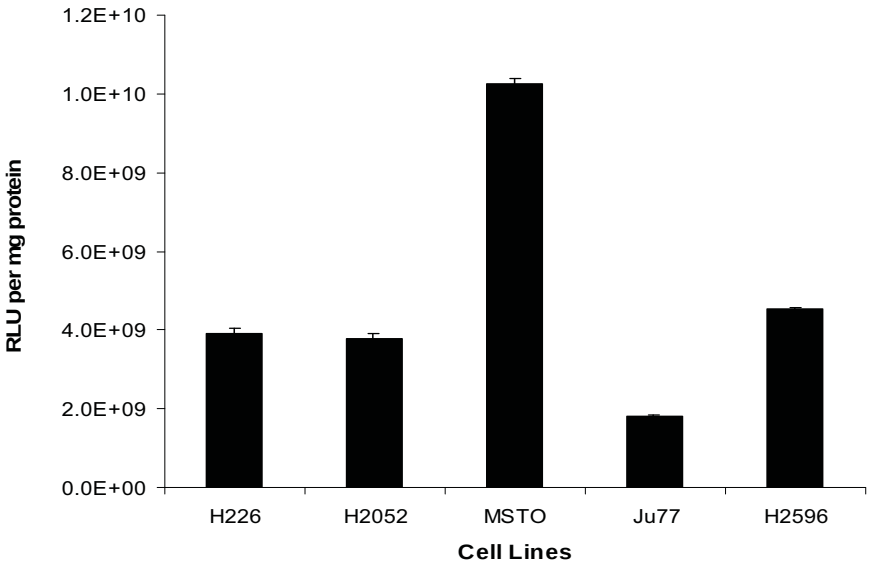


Fig. 6-4 Luciferase gene expression from MV-Edm in Human mesothelioma cells. Cells were infected with MV-Luc at an MOI of 0.5 and luciferase activity was measured 48 h later. MSTO cell line showed the strongest luciferase gene expression, and Ju77 showed the lowest luciferase gene expression in the 5 mesothelioma cell lines. Data was presented as the mean ± SD of triplicate wells.

GFP marker gene expression was also detected in these 5 human mesothelioma cell lines and mesothelioma cells from patient pleural fluid. Cells were infected with MV-GFP (MIO 0.5) and observed for GFP expression different times post infection (24, 48, 72, 96 h) using an inverted stage fluorescent microscope. Figure 6-5 showed GFP gene expression in these mesothelioma cells, compared 24 h with 48 h post infection. MV caused significant cytopathic effect with syncytia formation on all cells tested; this effect was more obvious at 48 h post infection. After MSTO cells were infected with MV-GFP, the cells showed the strongest GFP gene expression and cell death 48 h later. Ju77 showed the lowest GFP gene expression, since the syncytia formation just started 48 h post infection.

6.3.3 MV-Edm Replicate and Cause Significant CPE in Mesothelioma Cells

To demonstrate the cell-killing capacity of MV-CEA, MV-NIS, MV-NSE *in vitro*, I infected 5 human mesothelioma cell lines, H226, H2596, MSTO, H2052, Ju77 with small amounts of each virus (MOI 0.5). All lines tested here were susceptible to cell killing, including cell lines (H2596, H2052) that had previously proven resistant to killing with wild type adenovirus (Fig. 6-6). In all 5 of the mesothelioma cell lines, cell death was observed in 70% to 100% of cells by 96 h (death is relative to uninfected). Less than 1% of the cells were viable by 96 h after MV-CEA infection in H226, H2596, MSTO and H2052, or after MV-NIS infection in H226, H2596 and MSTO. MV-NSE showed less cell-killing effect than MV-CEA and MV-NIS in these cell lines. Toxicity was also assessed in primary cultures of pulmonary endothelium and pulmonary vascular smooth muscle cells (HMVEC).

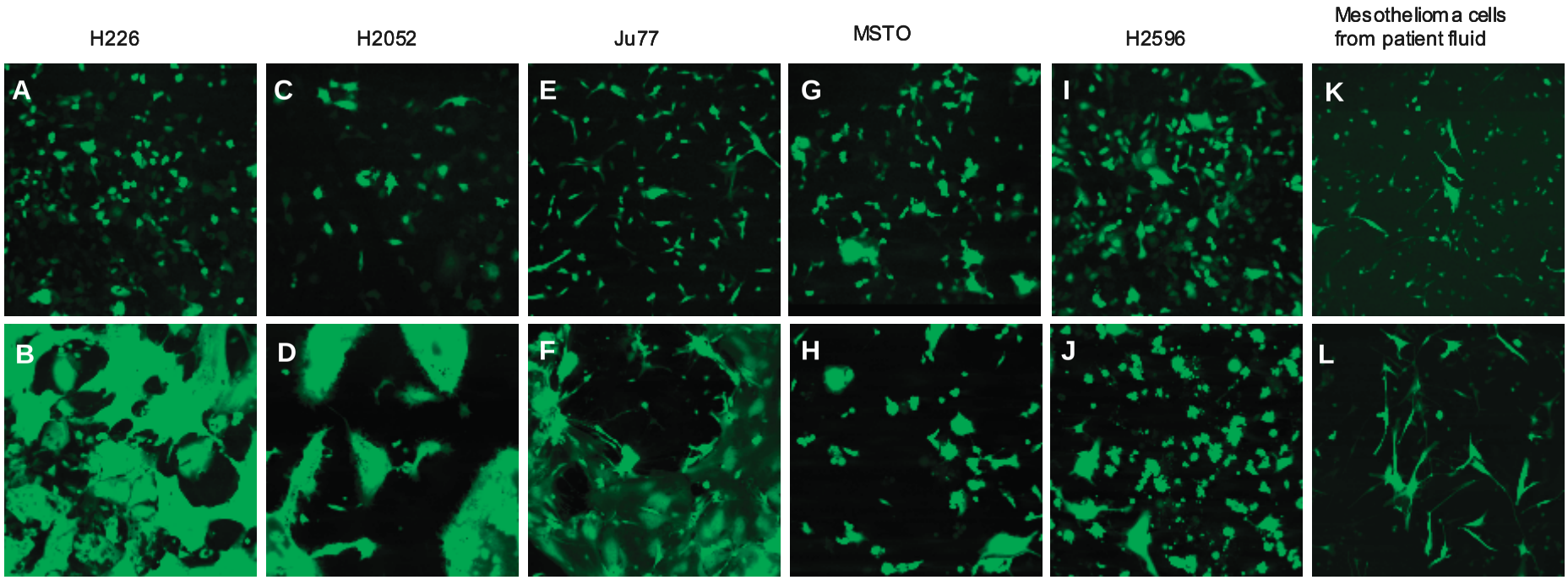
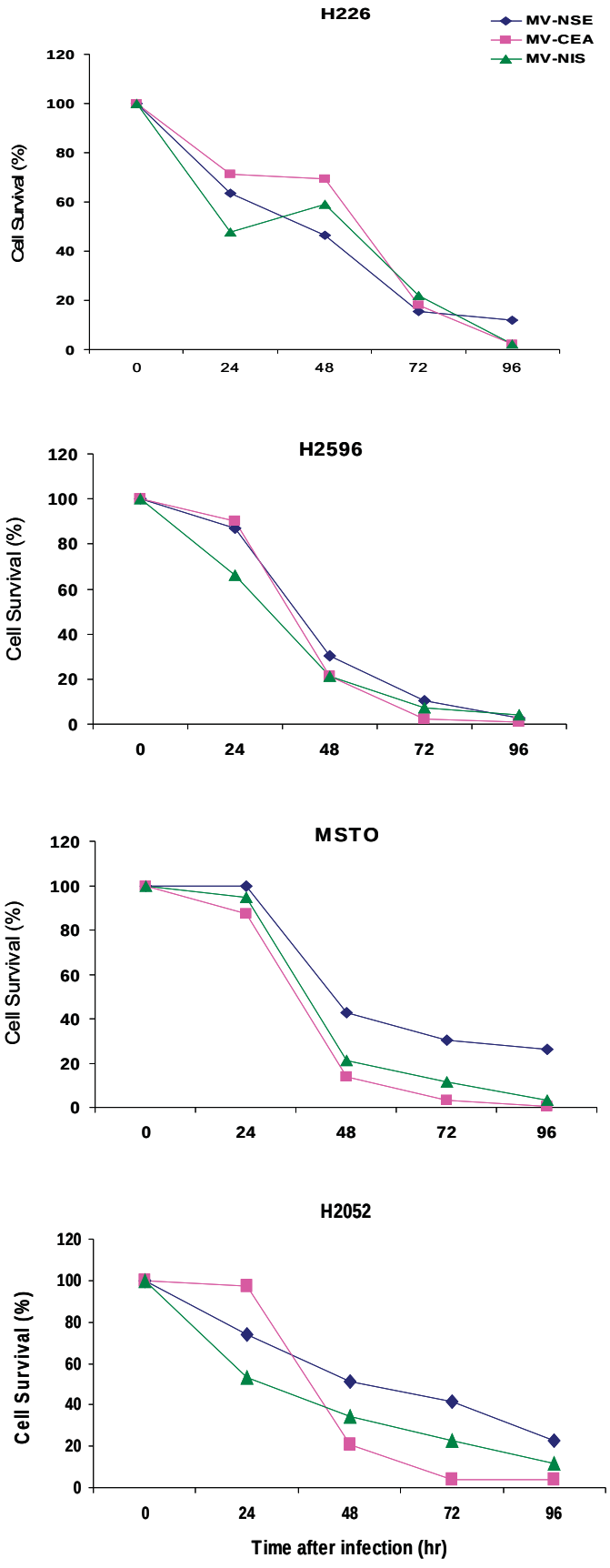


Fig. 6-5 GFP marker gene expression in human mesothelioma cell lines and mesothelioma cells from patient fluid. Cells were infected with MV-GFP (MIO 0.5) and observed for GFP expression different times post infection (24,48,72,96 h). A,C,E,G,I,K: 24 h post infection; B,D,F,H,J,L: 48 h post infection. MV caused significant cytopathic effect with syncytia formation on all cell lines and primary cells tested, this effect was more obvious at 48 h post infection.


H226
MV-NSE
MV-CEA
MV-NIS
Cell Survival (%)
120
100
80
60
40
20
0
0
24
48
72
96
H2596
Cell Survival (%)
MSTO
Cell Survival (%)
H2052
Cell Survival (%)
Time after infection (hr)

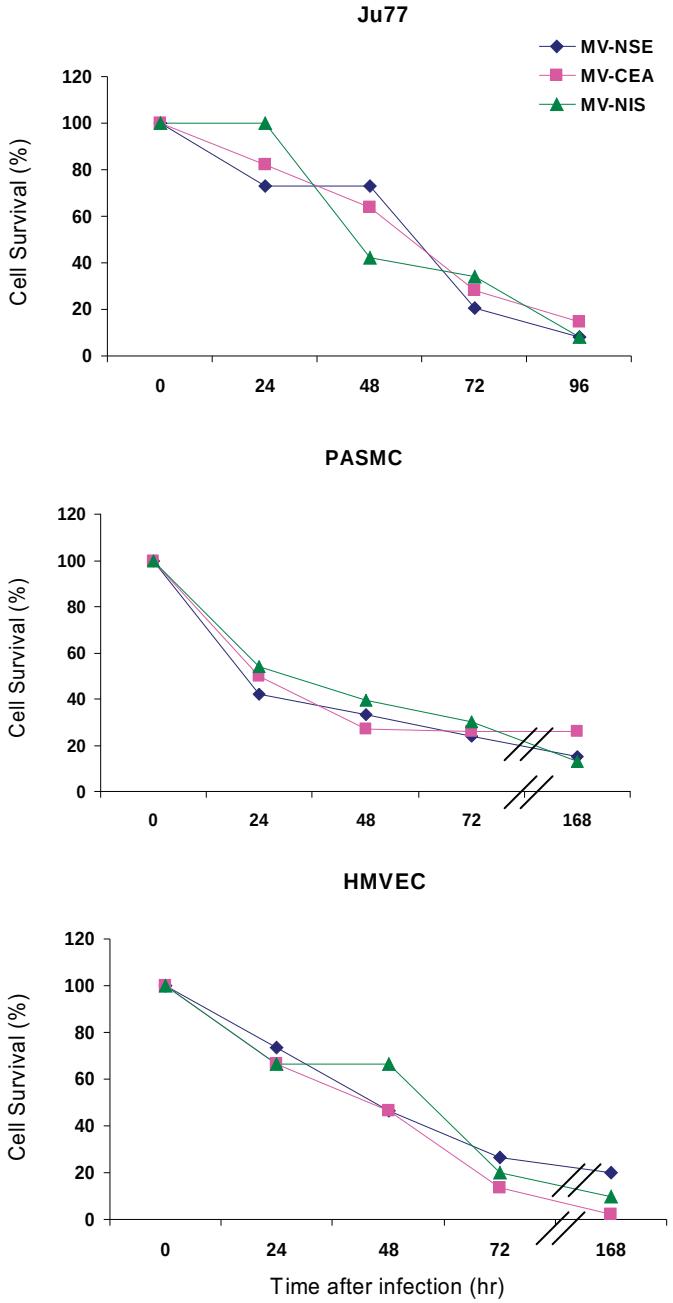


Fig. 6-6 Cell killing effect of MV in human mesothelioma cell lines. 5 human mesothelioma cell lines (H226, H2596, MSTO, H2052 and Ju77) and primary cultures of pulmonary endothelium and pulmonary vascular smooth muscle (PASMC, HMVEC) were infected with MV-NSE, MV-CEA and MV-NIS (MIO 0.5). Cell viability was determined by trypan blue exclusion. In all 5 of the mesothelioma cell lines, cell death was observed in 70% to 100% of cells by 96 h. At low doses there appears to be some selectivity of toxicity for tumour versus normal cells.

and PASMC). There were 10% to 30% of cells still alive even 168 h post infection in some cases although on balance the degree of selectivity of toxicity for normal cells versus tumour cells was not strong. An effort was made to evaluate cell killing in primary cultures of human mesothelioma cells obtained from pleural fluid. Unfortunately the cells available grew very slowly and did not develop a good confluent monolayer. Thus it appeared that in this setting syncytia formation was being compromised and the effectiveness of MV-Edm for cell killing was not well determined.

For the cell lines, the cells were stained with crystal violet solution to better illustrate the observed CPE. Figure.6-7 showed characteristic syncytia formation after 72 h infection with MV-CEA in H226 cell line. Similar effects were seen in all other mesothelioma lines. Efforts to try to discern any selectivity of toxicity for cancer versus normal cells were made by varying cell confluency and viral dose, but no improvement in the distinction compared to the above assays was identified. Primary cultures of normal pericardial mesothelial cells were infected with MV-GFP at a MIO of 1, 0.1, 0.01. Cell viability was determined by trypan blue exclusion 72 h post infection. A concurrent comparison was made with the cell toxicity on H226 cells. No convincing selectivity of killing for tumour line versus normal primaries was able to be demonstrated (Fig. 6-8).

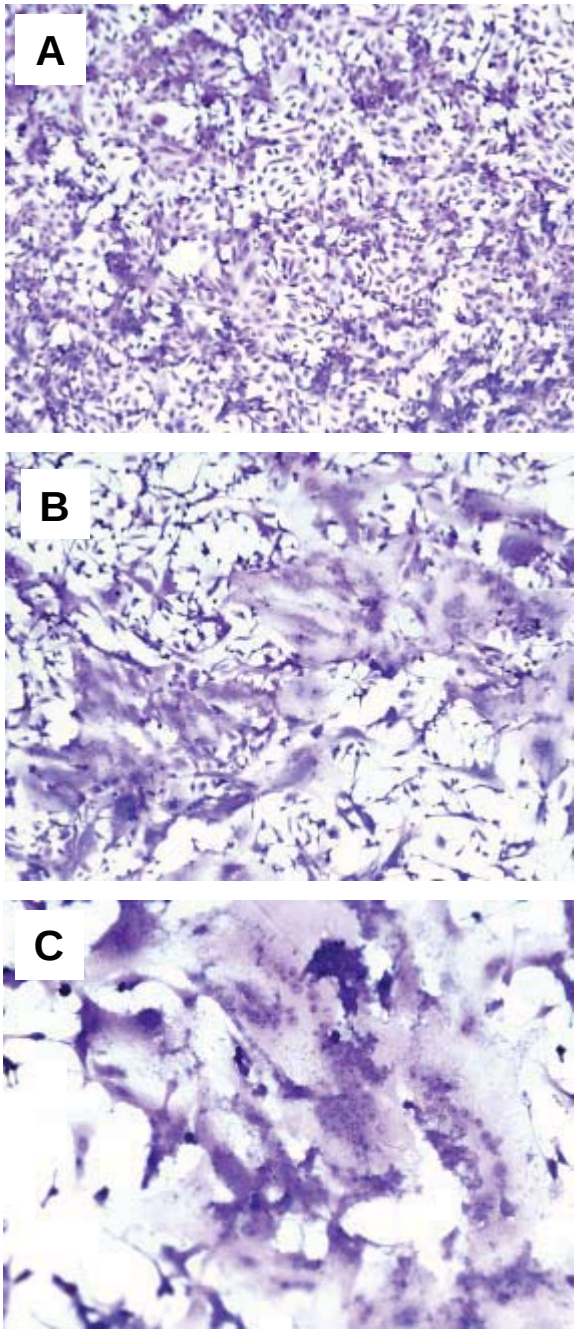


Fig. 6-7 MV-CEA infection results in prominent syncytia formation in H226 human mesothelioma cells. A: uninfected cells, x 40 magnification; B: 72 h after infection of H226 cells with MV-CEA (MOI 0.5), x 40 magnification; C: 72 h after infection of H226 cells with MV-CEA, x 100 magnification. Extensive syncytia formation is observed preceding cell death.

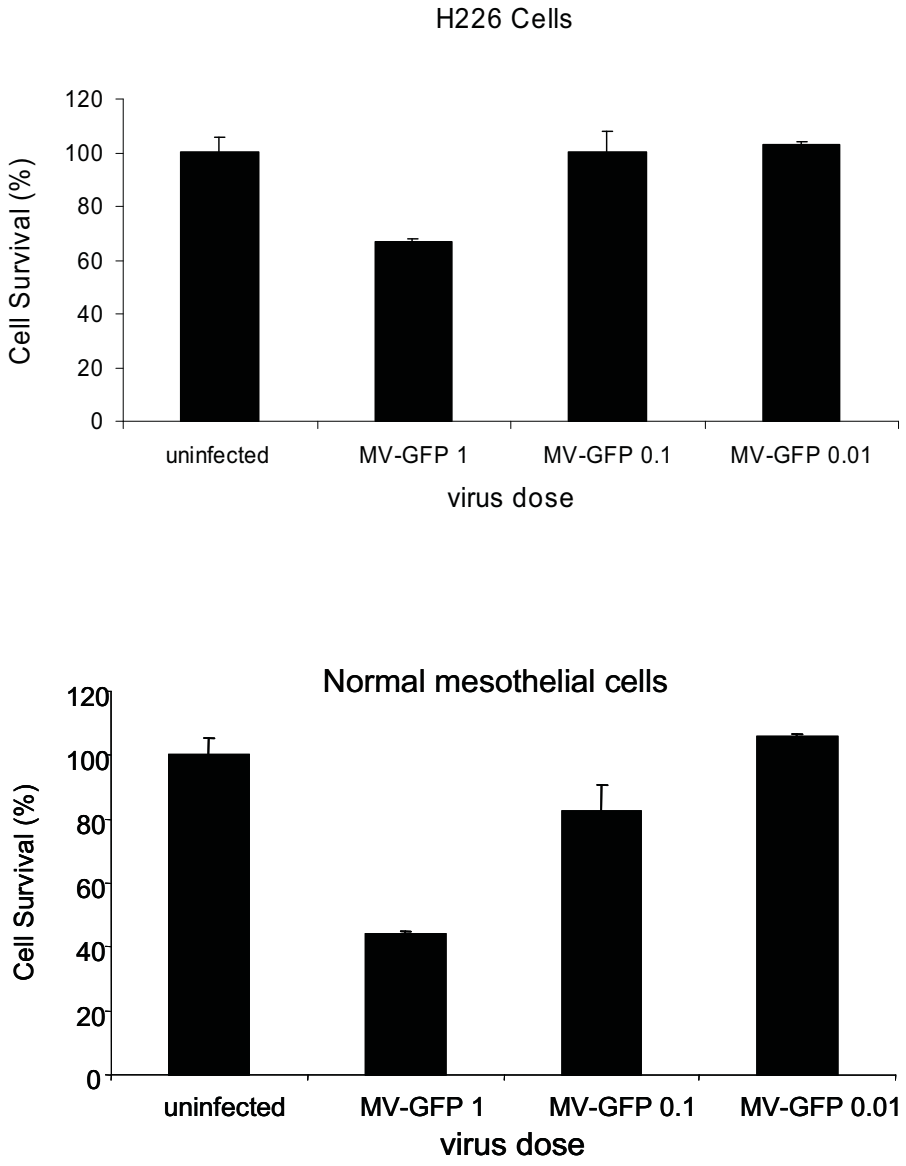


Fig. 6-8 Cell killing effect of MV in human mesothelioma H226 cells and normal pericardial mesothelial cells from patient. Human mesothelioma H226 cells and primary cultures of normal pericardial mesothelial cells were infected with MV-GFP (MIO 1, 0.1, 0.01). Cell viability was determined by trypan blue exclusion 72h post infection. No convincing selectivity of killing for tumour line versus normal primaries was able to be demonstrated. Data was presented as the mean ± SD of triplicate wells.

6.3.4 GFP Gene Expression of MV-Edm *in Vivo*

H226 cells were implanted subcutaneously in BALB/c nude mice. MV-GFP or PBS was delivered to the tumours when the tumours achieved a volume of 50 mm^3. Three days later, the mice were killed and tumours were removed and fixed in 10% buffered-formalin. The tumours were sectioned and stained for GFP expression on paraffin embedded specimens by immunohistochemistry. Immunohistochemical staining results (Fig. 6-9) showed GFP expression within MV-GFP injected tumours. The tumours injected with PBS showed negative staining of GFP.

6.3.5 MV-Edm Infection Induced Apoptosis

6.3.5.1 MV Induced Late Apoptosis

I assessed apoptosis in uninfected and measles virus-infected cells by flow cytometry. H226 cells were infected with MV-CEA (MOI 0.5) and collected 72 h post infection. The cells were stained with 7-AAD and caspase antibody. I found the MV-CEA led to increased apoptosis, compared with uninfected cells. This effect was evident by an increase in 7-AAD staining 72 h after infection (Fig. 6-10A). However, flow cytometry analysis of cells for caspase staining did not detect an increase in the MV-CEA infected cells (not shown).

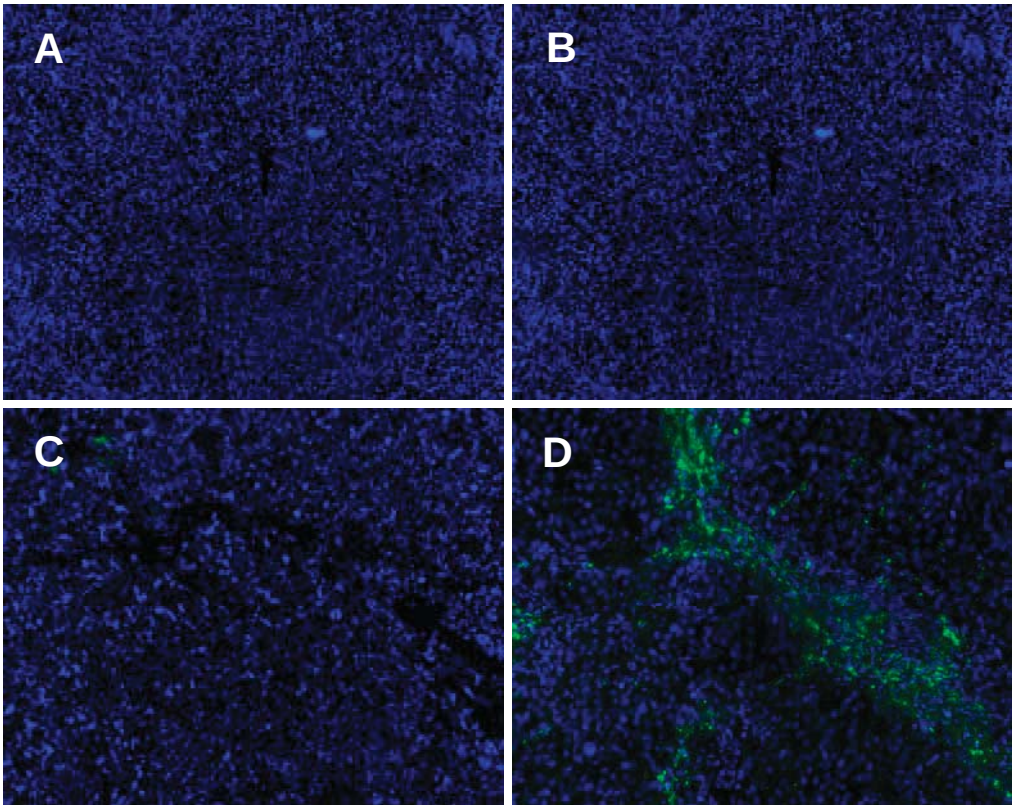


Fig. 6-9 Immunodetection of MV-GFP transgene expression in mesothelioma xenografts tumours. H226 mesotheliomas were established in the right flank of female BALB/c nude mice. The tumours were received one intratumoral injection of MV-GFP ($1x10^6$ pfu) or PBS. Three days later, the mice were killed and tumours were removed. The tumours were sectioned and stained for GFP expression. A: tumours were injected with PBS, no primary antibody; B: tumours were injected with PBS, anti-GFP antibody stained; C: tumours were injected with MV-GFP, no primary antibody; D: tumours were injected with MV-GFP, anti-GFP antibody stained. Immunohistochemical staining showed GFP expression within MV-GFP injected tumours (x100 magnification).

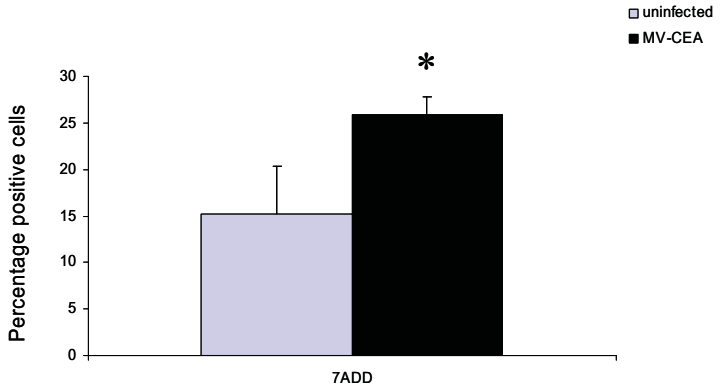


Fig. 6-10A Detection of apoptosis in uninfected and measles virus-infected cells by flow cytometry. H226 cells were infected with MV-CEA (MOI 0.5) and collected 72 h post infection. The cells were stained with 7-AAD. MV induced late apoptosis in H226 mesothelioma cells. Data was presented as the mean ± SD of triplicate wells. * $p<0.05$ compared with uninfected cells.

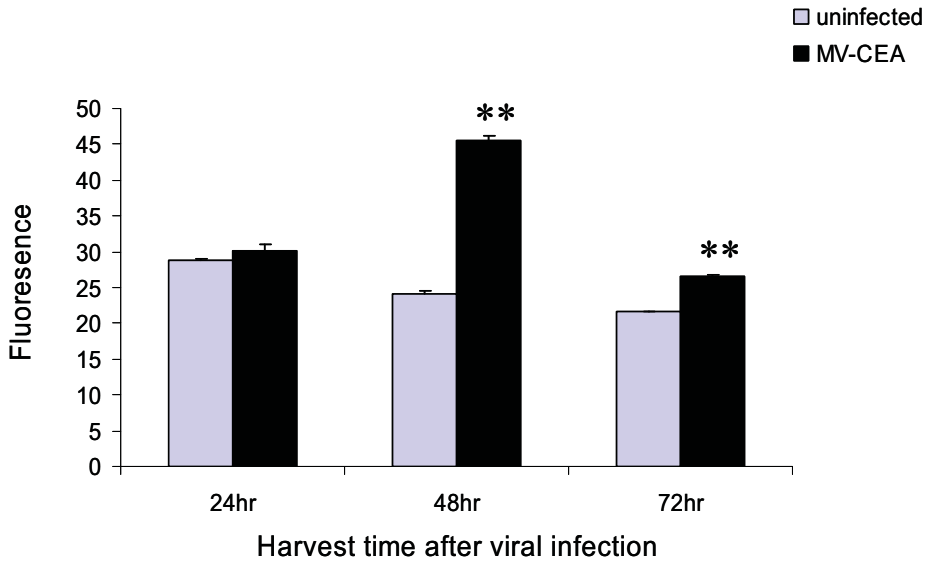


Fig. 6-10B Detection of apoptosis in uninfected and measles virus-infected cells by caspase-3 protease activity assay. H226 cells were infected with MV-CEA (MOI 0.5) and collected 24,48,72 h post infection. Fluorometrics caspase-3 protease activity assay was performed. Significant difference between uninfected and MV-CEA-infected cells at 48, 72 h post infection, Data was presented as the mean ± SD of triplicate wells. ** $p<0.001$ compared with uninfected cells.

6.3.5.2 MV Activates Caspase-3

To assess the role of caspase activity in uninfected and measles virus-infected cells in more detail I used a caspase-3 protease activity assay. H226 cells were infected with MV-CEA (MOI 0.5) and lysed 24, 48, 72 h post infection. Fluorometrics caspase-3 protease activity assay was performed. Ac-DEVD-AMC is a fluorogenic substrate for caspase-3. Cleavage of this substrate by caspase-3 can be monitored by excitation at 380nm and emission at 460. MV infection caused an increase in the amount of caspase-3 protein expression 48 and 72 h post infection (Fig. 6-10B). Early after infection (24 h), apoptosis could not be detected in MV-CEA-infected cells, but significant difference was found between uninfected and MV-CEA-infected cells at 48, 72 h post infection.

6.3.5.3 MV Induced Apoptosis *in Vivo*

TUNEL assay was used to detect DNA fragmentation in apoptotic cells *in vivo*. TUNEL assays were performed on the paraffin embedded sections from mice injected with MV-GFP or PBS. H226 mesotheliomas were established in the right flank of female BALB/c nude mice. Tumours received one intratumoural injection of MV-GFP ($1x10^6$ pfu) or PBS once the tumours achieved a volume of 50 mm^3. Three days later, the mice were killed and tumours were removed. The tumours were sectioned and TUNEL assays were performed. There were positive apoptotic cells shown in the tumours injected with MV-GFP (Fig. 6-11), and none in the tumours injected with PBS. The mechanism of tumour cell death was predominantly apoptotic.

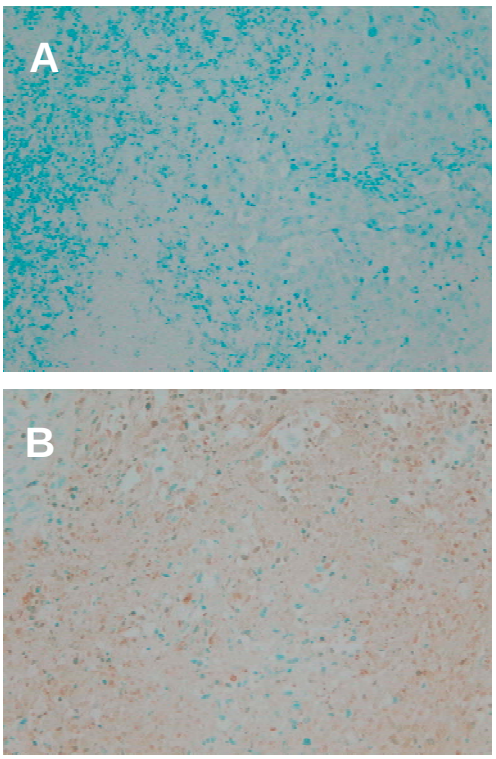


Fig. 6-11 Apoptosis in tumours shown by TUNEL assay. H226 mesothelioma were established in the right flank of female BALB/c nude mice. The tumours were received one intratumoral injection of MV-GFP ($1x10^6$ pfu) or PBS. Three days later, the mice were killed ad tumours were removed. The tumours were sectioned and TUNEL assay was performed. A : tumours were injected with PBS; B: tumours were injected with MV-GFP. There were positive apoptotic cells shown in the tumours injected with MV-GFP (x100 magnification).

6.4 Discussion

A panel of human mesothelioma cells (H226, H2596, MSTO, H2052 and Ju77) was infected with MV-GFP or MV-Luc to assess MV gene expression. In these 5 cell lines, MSTO cell line showed the strongest luciferase gene expression in the 5 mesothelioma cell lines, and Ju77 showed the lowest luciferase gene expression 48 h post infection. Similar effects were seen in detection of GFP expression. After MSTO cells were infected with MV-GFP, the cells showed the strongest GFP gene expression and cell death 48 h later. Ju77 showed the lowest GFP gene expression since the syncytia formation just started 48 h post infection. Therefore, the level of MV luciferase expression could be correlated to the level of GFP expression in the tested cell lines. However, a relationship between gene expression levels and CD46 receptor density was not observed. It might be caused by too high CD46 expression on each mesothelioma cell line (nearly 100%) (Fig.3-1).

Attenuated strains of the Edmonston vaccine lineage of MV have been used in vaccines for many years with an excellent safety record. MV-Edm has selectively oncolytic activity against a variety of human tumour cells, but minimal cytopathic damage in non-transformed cells such as mesothelial cells from the peritoneal cavity, normal dermal fibroblasts and peripheral blood lymphocytes (Peng et al., 2001; Peng et al., 2002). I infected primary cultures of pulmonary endothelium and pulmonary vascular smooth muscle (HMVEC and PASMC) with measles viruses at a MOI of 0.5 for safety evaluation. There were 10% to 30% of primary cells still alive even 168 h post infection compared to 99% of cell death 96 h after MV infection. Toxicity was relatively less than that seen in the tumour lines, but was nonetheless still apparent. I also used normal

primary mesothelial cells to assess toxicity, but could not convincingly demonstrate selectivity. The degree of selectivity I was able to demonstrate was somewhat less than that reported in the pre-clinical studies of ovarian cancer where peritoneal mesothelial cells were used. At this time the reason for the apparent difference remains unclear and warrants further clarification. One factor may be that the cells I had available were at a slightly higher passage number than those reported using the peritoneal cells (the latter were passage 2-3 versus approximately 4-6 for the cells I had). The other "normal" cells I used may also have been at a higher passage than ideal (again being greater than 4). Access to mesothelial cells at lower passage was not available to me at the time but is being pursued in future studies. Also, the peritoneal cells were obtained from renal failure patients undergoing peritoneal dialysis (Peng et al., 2001; Peng et al., 2002), but whether this is significant is uncertain.

The mechanisms of cell death associated with measles virus infection are poorly known. It has been proven apoptosis is a cause of death in measles virus-infected cells (Esolen et al., 1995). I assessed apoptosis induced by measles virus *in vitro* and *in vivo*. *In vitro*, I found MV induced late apoptosis (7-AAD) by flow cytometry. According to the result from caspase-3 protease activity assay, I detected apoptosis induced by caspase-3 in MV-infected cells 48, 72 h post infection. The result from TUNEL assay *in vivo* also confirmed the mechanism of tumour cell death caused by MV was predominantly apoptotic.

Adenoviral receptor (CAR) versus measles virus receptor (CD46) expression was compared on human mesothelioma cell lines by flow cytometry. We found strong CD46 expression across all human mesothelioma lines tested, and all lines demonstrated

higher level of CD46 expression than level of CAR expression. I also investigated the cell-killing capacity of MV-CEA, MV-NIS, MV-NSE *in vitro*, all cell lines tested were susceptible to cell killing with measles viruses, including cell lines (H2596, H2052) that had previously proven resistant to killing with wild type adenovirus. Therefore, MV-Edm achieves killing of human mesothelioma cell lines, and may have broader utility than adenovirus, subject to further safety evaluations. Based on several reports that apoptosis induction may be favourable combined with immunotherapy (Nowak et al., 2003), MV-Edm could have a role in future combined therapies.

Chapter 7. Discussion and Future Work

In view of the lack of adequate therapies for mesothelioma and many other malignancies, research into new treatment approaches is a major health care priority and this has been the subject of my thesis.

My contributions to the field encompassed within this work are:

1) Development of new CRAds using the Flt-1 promoter to control E1, and demonstrating that both these and CRAds using the VEGF promoter had good cell killing efficiency for a range of human mesothelioma cell lines and primary human mesothelioma cells.

2) Demonstration of the cell killing efficiency gains achieved by using Ad3 tropism in place of Ad5 tropism and providing some rationale for these improvements based on the relative expression of Ad3 versus Ad5 receptor on mesothelioma cells.

3) Demonstration that CRAds can be combined with an MMP-9 expressing vector without unduly compromising either viral replication or MMP-9 expression.

4) Demonstration that combined therapy with CRAd plus MMP-9 was more efficacious for tumour growth inhibition than CRAd alone.

5) Identification of murine mesothelioma cell lines that were susceptible to cell killing by adenovirus.

6) Progression of one of the susceptible lines to *in vivo* evaluation, allowing the assessment of replicative Ad therapy in an immune-competent setting.

7) Demonstration that replicative Ad therapy can be improved by a combination with immune-based therapy via CD40 activation, and providing evidence that this was associated with greater influx of CD8 effector cells.

8) Demonstration that mesothelioma cells are susceptible to killing with a range of attenuated measles viruses, and that this cell killing was achieved via apoptosis induction.

There are several aspects of this work that warrant further discussion.

Selectivity of cell killing

I have attempted to evaluate the relative toxicity of the various agents used for cancer cells versus normal cells. The selectivity I have been able to demonstrate has been somewhat disappointing. This may be a reflection of shortcomings of the viral agents themselves, limitations of the *in vitro* test systems used or both. A careful review of the voluminous literature concerning replicative agents reveals that no agent, even those that have entered many human clinical trials such as the ONYX-015 E1B-55K deleted virus are tightly selective for normal versus cancer cells. Many published accounts strive to demonstrate some selectivity, but typically present data in only one or two "negative control" cell lines. The generalisability of these findings is unclear. The Δ24-RGD CRAd, which I have used in some studies here for example, is known to replicate

in replicating normal cells. This virus is now in Phase I human studies and is being administered by intraperitoneal injection to women with advanced ovarian cancer. Nine out of a planned 12 patients have received virus representing 3 out of 4 planned dose cohorts. No toxicity problems have been encountered to date (D. Curiel, personal communication). Numerous other CRAd clinical trials have been published, with minimal toxicity. As discussed in my introduction, the problem has really been poor efficacy. *In vitro* testing of specificity is problematic when using cultured "normal" cells because the relevance of this approach to the *in vivo* setting is uncertain, specifically as cultured cells will typically be replicating themselves at a greater rate than would be the case *in vivo*. Better animal model systems are needed to test these issues, and some researchers are developing the cotton rat as a test animal model. The standard mouse models used for much of the pre-clinical CRAd work are not ideal owing to the poor replication capacity of human Ad viruses in mouse cells.

Irrespective of the above comments, some attempts to improve selectivity of a replicating Ad therapy over wild type Ad is necessary and certainly a critical component of any regulatory approval process before a human trial can proceed. With respect to strategies to improve selectivity, the two major approaches – "tissue-specific" promoters driving E1, or mutation of E1 have been discussed in the introduction. With regard to the promoters themselves, these will have intrinsic variability in their tightness of regulation. Ideally one would strive for a promoter with low activity in the liver, because this organ has a high propensity to take up any virus released into the circulation and is thus at particular danger of toxicity. Techniques have been developed to use human liver slices (obtained from excess donor organ material) as an ex-vivo organ culture system for testing CRAd selectivity (Rots et al., 2006). This approach

appears promising but is limited by tissue availability. The Flt-1 promoter that I used in the current studies had previously been shown to have low activity in the liver, and is upregulated in mesothelioma primary tumours, so appeared to be a reasonable choice for evaluation. Nevertheless, even a small amount of leakage may be enough to allow replication and toxicity on *in vitro* testing. Besides the tightness of the promoter per se, there is the issue of the Ad genome itself potentially acting to undermine the promoter specificity. Read-through effects from the region of the left inverted terminal repeat may be a problem. To address this issue, some researchers have developed vectors with "blocking" sequences (e.g. polyA sequence) placed upstream of the tissue-specific promoter, and have shown selectivity gains. Concurrent with my work, Zhu et al (Zhu et al., 2006) used such a strategy with the survivin promoter although the absolute selectivity achieved is uncertain. For use as a mesothelioma therapy, in the first instance the idea would be to administer CRAds into the pleural space, thus some degree of "selectivity" will be achieved simply by the mode of administration, much as peritoneal administration is being used for ovarian cancer. For mesothelioma treatment, obliteration of the normal pleural mesothelium would be of no consequence and perhaps even an advantage. Current treatment practices often entail obliteration of the pleural space by instillation of talc as a palliative measure. It is hoped that the current ovarian studies underway will provide some very useful toxicity insights that may be extrapolated to the pleural situation. With the above comments in mind, I had considered whether further efforts to improve the Flt-1 CRAd should be made by inserting a poly-A sequence upstream, but felt that my time would be better spent addressing the real key problem with CRAds, which is the efficacy.

Efficacy of cell killing

After demonstrating good cell killing effect *in vitro* especially with the Ad3 tropism variants of the Flt-1 and VEGF CRAds, I progressed to *in vivo* evaluation. It was already well-understood in the field that translating *in vitro* cell killing efficacy to the *in vivo* setting was problematic, even when using tumour xenograft models using highly Ad-susceptible cell lines such as A549 and wild type virus. Some improvements had been seen with viral tropism-modification/infectivity enhancement (e.g. using the RGD modified AdΔ24)(Suzuki et al., 2001), but complete eradication of tumours was most unusual. Thus, my aim from the outset was to incorporate a complementary approach. Previous studies in our laboratory and this of others (Sauthoff et al., 2003) had shown that virus can remain "trapped" within a tumour mass, even some weeks after inoculation. I hypothesised that physical factors such as fibrotic barriers might play a part in this limitation, thus I co-administered the CRAds with a vector expressing MMP-9, with the idea being that this may improve efficacy by improving viral spread. The animal study I performed indeed showed that CRAds alone really did not have a very great effect, which was what I was concerned about. However, co-administration with an MMP-9 expressing vector showed a significant enhancement in response. This effect waned after three weeks, which was most likely due to falling expression levels of MMP-9 provided by the non-replicative vector carrying this gene. In principle, this suggests that matrix digestion may indeed improve efficacy. Whether MMP-9 is really the best choice of agent for this is however controversial, as MMP-9 is known to be associated with increased incidence of metastases. I checked for any evidence of this in the mice and did not find this in this model. An alternate matrix digestion strategy that does not have this association might be a more attractive proposition for ultimate use in

human trials, but as interim future studies to develop a CRAd containing the MMP-9 genome would be reasonable.

Although the MMP-9 strategy shows some promise, an even greater enhancement could possibly be achieved if CRAds could be combined with stimulation of anti-tumour immunity. If successful, this approach could ultimately achieve an anti-tumour impact throughout the body, to help eradicate metastatic disease. Immunotherapy alone has, in the past, shown occasional promise but generally has been not effective enough to enter clinical practice. Some additional stimulus to improve the influx of effector cells is desirable, and the inflammatory response induced by a replicating virus is an appealing way to achieve this. Unfortunately the available animal model systems usually used for CRAd evaluations are not suitable to address this question, because human Ad replicates very poorly in murine cells. This means that studies are done using human xenografts in immuno-compromised mice, which precludes assessment of immune interactions. Therefore I had to identify a model system that would be useful for my purpose. I was able to screen a range of mouse mesothelioma cells provided by collaborators at the University of Western Australia, and found two lines that were susceptible to Ad toxicity, and in which some degree of viral replication could be achieved as determined by output TCID50 assay. One of these lines, AE17, reliably formed subcutaneous tumours in immune-competent syngeneic C57 Black6 mice.

My subsequent studies using this system showed that (once again) virus alone had limited efficacy *in vivo* despite good killing effect *in vitro*. Importantly however, the combination of replicating Ad with immune activation via the CD40 activating FGK45 antibody resulted in greater therapeutic effect than either treatment alone. This was

associated with greater influx of CD8+ T-cells. Thus I have established the important principle that combined viral and immunotherapy is feasible. Further work needs to done on this system to optimise the components for greatest effect. It would also be rational to genetically incorporate CD40 ligand into the replicating virion. The AE17-C57 Black6 model may indeed prove useful for other combination of viral and immunotherapies. To extend the possibilities of this system I have now optimised intrapleural inoculation of the tumour cells to better replicate the human clinical situation. We have also developed an AE17 cell line that is stable transfected with luciferase to enable non-invasive sequential imaging (using a Xenogen system) of intrapleural tumours to monitor tumour growth and response to therapies.

The adenovirus is of course not the only replicative system being developed for cancer therapy. Another system which shows promise is the use of attenuated measles vaccine, Edmonston strain (MV-Edm). I was particularly interested in this system for a number of reasons. This virus has progressed to the stage of human clinical trial by peritoneal administration for ovarian cancer, as for the AdΔ24-CRAd, and again the anatomical parallels between peritoneal and pleural administration are of interest. The published pre-clinical studies had indicated a high degree of selectivity of cell killing for ovarian tumours cells versus normal mesothelium (Peng et al., 2001; Peng et al., 2002). Further, MV-Edm induces cell death by stimulating the membrane-membrane adhesion of cells to form large syncytia, which then undergo death by apoptosis. This syncytia formation might improve the spread of the virus through a tumour mass. Also a number of studies have shown that induction of cell death by apoptosis is a particularly powerful stimulus for the development of anti-tumour immune response. In fact, Nowak et al (Nowak et al., 2003) had shown that apoptosis induction by Gemcitabine chemotherapy could be

combined with FGK45 to achieved significant therapeutic benefits in a murine mesothelioma model. I therefore evaluated MV-Edm for its cell killing, syncytia formation and apoptosis induction in a range of human mesothelioma cell lines and primary human material. Cell killing and syncytia formation was highly efficient and induction of apoptosis was confirmed. Selectivity for tumour versus normal cells was however disappointing and still to be reconciled with the previously published results. More studies of earlier passage "normal" cells of relevance to the thoracic cavity are needed. Nevertheless, MV-Edm clearly has efficacy as a potential mesothelioma therapy. Toxicity results from the currently ongoing human trail may provide further insights into how this may be progressed. Unfortunately because MV-Edm relies upon CD46 expression for infection, and murine cells do not express this, I was unable to use my AE17 model to assess combined MV-Edm and FGK45 therapy. A transgenic C57Black6 model expressing CD46 (Mrkic et al., 1998; Mrkic et al., 2000) has however been described which could potentially be used to advance these studies.

In summary I have identified some novel strategies which may help to progress the use of replication competent viral agents towards useful therapies for mesothelioma. These approaches are not mutually exclusive, and some combination of viral therapy, matrix digestion and immune-stimulation might be feasible. In ongoing studies, in addition to the ideas discussed above, we are evaluating the impact of Treg depletion of viral replication and immune-stimulation in the intrapleural model. Further, we have been fortunate to acquire a human clinical grade (i.e. GMP grade) Ad5/3Δ24 virus through collaboration with colleagues in the USA and the NIH, which we are currently evaluating in pre-clinical studies with a view to progressing to a Phase I study in mesothelioma. This is the necessary first step in progressing the strategy to the clinic,

with the possibility of incorporating combination strategies as proposed in this thesis at a future date.