Chapter 1. Introduction

1.1 Mesothelioma

1.1.1 Mesothelioma

Mesothelioma is a cancer of the mesothelial cells that make up the lining around the outside of the lungs and inside of the ribs (pleura), and around the abdominal organs (peritoneum). Clinical features include dyspnea, cough, non-specific chest pain, weight loss and night sweats. Eighty percent of mesotheliomas originate in the pleural space, and they represent the most common primary tumour of the pleura (Sterman et al., 1999). Rarely, pleural mesothelioma is localized, benign, and readily resectable for cure (Ismail-Khan et al., 2006). The more common variety is the diffuse malignant pleural mesothelioma (MPM), a mesothelial malignancy that is locally aggressive, invasive, and almost universally fatal. Because most mesotheliomas are cancerous, malignant mesothelioma (MM) is often simply called mesothelioma.

Mesothelioma, which is not related to cigarette smoking, is usually associated with history of chronic asbestos exposure. Malignant mesothelioma was first recognized in 1870 (Wagner et al., 1960). However, the link between asbestos and malignant mesothelioma was not discovered until 1960 in South Africa, when the first convincing evidence of a link between malignant mesothelioma and both occupational and incidental asbestos exposure was reported (Cugell et al., 2004; Robinson et al., 2005). The incidence of malignant pleural mesothelioma has risen for some decades and is

expected to peak sometime between 2010 and 2020 (Robinson et al., 2005). This increase has been attributed to the widespread use of asbestos in the period from World War II until the end of 1970s (Mcdonald et al., 1987). Although therapeutic approaches range from single-modality therapy (i.e. surgery, chemotherapy or radiotherapy) to multimodal treatment, there is no approach that has achieved adequate objective responses in patients. Thus, malignant mesothelioma remains a difficult therapeutic challenge.

1.1.2 Incidence and Mortality

Malignant pleural mesothelioma is not rare, and it is dramatically increasing worldwide (Tsiouris et al., 2007). The highest prevalence is seen in the UK, South Africa, Western Europe and Australia (Figure 1-1)(Tsiouris et al., 2007). In Japan and other non-Western countries, heavy use of asbestos in industry occurred late in the twentieth century. There will be a corresponding delay in the peak incidence of this disease in these countries. Prior to the 1950s, malignant mesotheliomas were extremely rare. From the last half century, the incidence of mesothelioma has significantly increased; for example, currently 2500 cases are diagnosed per year in the United States alone (Ismail-Khan et al., 2006). In Western Europe, 5000 patients die of the disease each year. It was reported that Australia had the highest reported national incidence of mesothelioma in the world, and rates were increasing (Leigh et al., 2002). Up to 600 mesothelioma cases are diagnosed each year in Australia. The high incidence of the disease is not expected to decline in the short term. This is due to the high rate of asbestos use and mining in Australia over many years and the long latency between exposure and the development
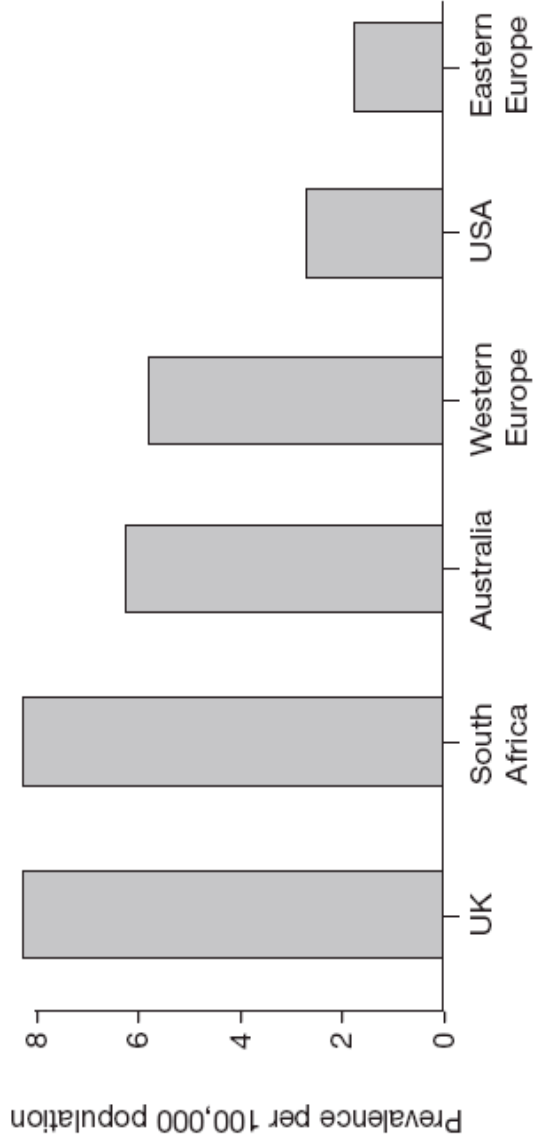
Figure 1-1 Regional difference in prevalence of malignant pleural mesothelioma.

Adapted from Tsiouris A, et al. Nature Clinical Practice Oncology, 2007, 4(6):344-352

of disease. The expected total number of cases from 1945 to 2020 is estimated to be about 18,000, based on models by Berry (Berry 1991) and De Klerk *et al* (De Klerk et al., 1989) (Figure 1-2)(De Klerk et al., 2002).

Mesothelioma remains a universally fatal disease of increasing incidence worldwide. The clinical outcome for patients with this disease is extremely poor, with median survival of about 1 year (Rizzo et al., 2001; Carbone et al., 2002). Based on World Health Organization reports (De Klerk et al., 1996; De Klerk et al., 2002; Berry et al., 2004), mesothelioma rates from different countries show large differences by sex and country. Industrialised countries have much higher rates than non-industrialised countries, reflecting the past production and use of asbestos in industry (De Klerk et al., 2002). Male rates are much higher than female rates in virtually all countries. Eighty percent of patients with pleural malignant mesothelioma are male (Robinson et al., 2005). Malignant mesothelioma has occurred in three principle cohorts of asbestos-exposed people (Leigh et al., 2002). The initial group consists of miners who were directly exposed to asbestos, especially blue asbestos. Possibly one of the worst industrial disasters in history occurred as a result of exposure at the blue-asbestos mine in Wittenoom, Australia (Leigh et al., 2002). Subsequently, a second wave of asbestos-related diseases occurred in people who worked in the process of manufacture and use of asbestos products. 20 to 30 percent of current cases of malignant mesothelioma were noted in the third group of affected people who were exposed to asbestos unknowingly and incidentally as a result of asbestos fibres being released into the atmosphere in industrialized countries (De Klerk et al., 2002). Other factors may contribute to the susceptibility of individuals to contract mesothelioma, for example a possible autosomal
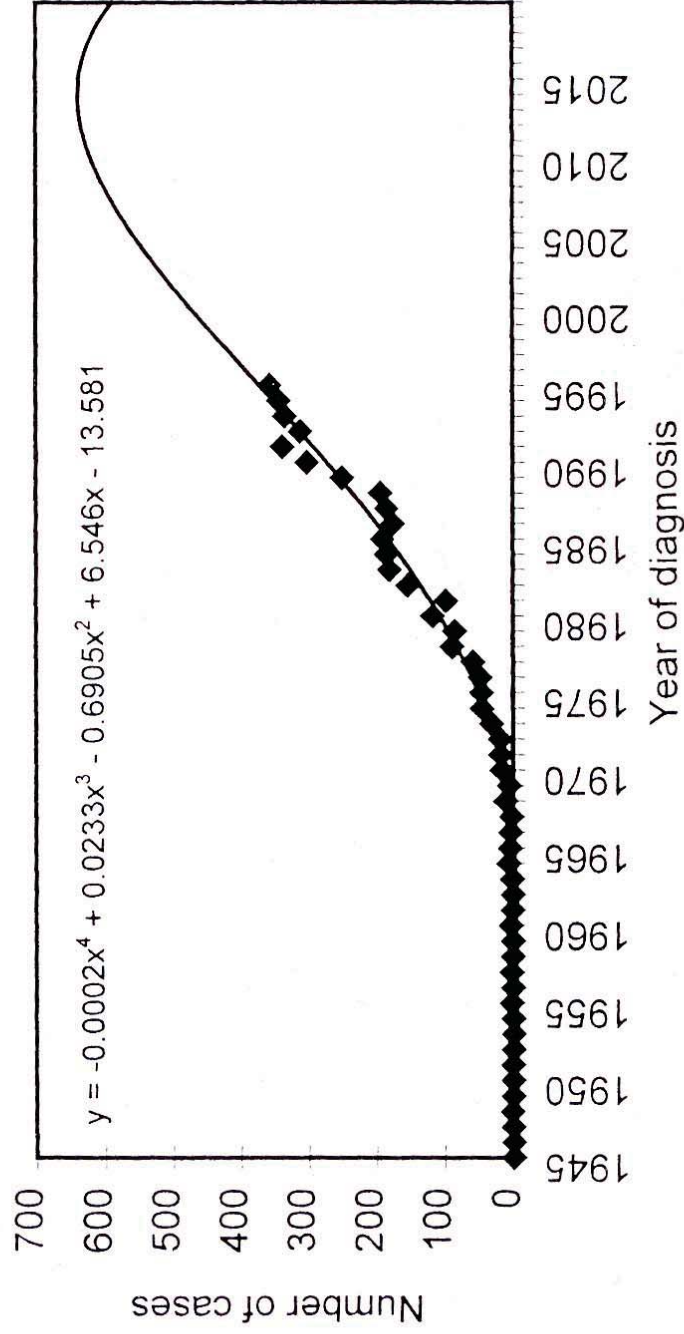
Figure 1-2 *Incident cases of malignant mesothelioma in Australia 1945–96 and extrapolation to 2020 assuming maximum at 2010.*

Adapted from De Klerk NH, et al. Mesothelioma, edited by Bruce W. S. Robinson and A. Philippe Chahinian, 2002, 55-86

The equation shown on the figure:

$$y = -0.0002x^4 + 0.0233x^3 - 0.6905x^2 + 6.546x - 13.581$$

dominant pattern in subjects has been studied in Cappadocia, Turkey (Roushdy-Hammady et al., 2001).

1.1.3 Pathogenesis

1.1.3.1 Asbestos

As noted previously, the pathogenesis of malignant mesothelioma is associated with asbestos exposure and several studies have demonstrated that exposure to asbestos fibres is the major cause of malignant mesothelioma (Mossman et al., 1990; Nicholson 1991; Sluis-Cremer 1991; Mossman et al., 1998; Robledo et al., 1999). Approximately 80% of mesotheliomas are associated with known asbestos exposure, and about 5% of asbestos workers develop mesothelioma (Sluis-Cremer 1991; Testa et al., 2001). Women are less likely to be affected than men, probably due to women's different employment patterns.

Lanphear and Buncher (Lanphear et al., 1992; Carbone et al., 2002) found that there was a long time delay between asbestos exposure and the development of malignancy. The latency period, which is the time between exposure to asbestos fibres and the clinical diagnosis of malignant mesothelioma, ranged from 15 to 50 years. The mean latency was 32 years. Two major theories have been proposed to explain the delay: The first theory supposes that malignant transformation occurs relatively soon after asbestos exposure, but that it takes a long time (years) for the tumour to grow. During the process of tumour growth, however, mesothelioma cannot be detected easily in its early stages. The second theory postulates that genetic damages induced by asbestos

accumulate over 30 years and lead to malignant cells. The finding of the latency supports the projection that the incidence of this disease will increase over the next two decades due to the use of asbestos over the last 50 years (Peto et al., 1995).

There are four principal processes by which asbestos fibres affect the pleura. First, asbestos fibres may irritate the pleura. The fibre shape and length-to-width ratio is an important physical attribute that determines how deeply into the lung they penetrate and their likelihood of inducing cancer (Sebastien et al., 1980; Pott et al., 1987; Boutin et al., 1993). Second, asbestos fibres may interfere with mitosis. Fibres can sever or pierce the mitotic spindle, disrupting mitosis, resulting in aneuploidy and other forms of chromosomal damage (Ault et al., 1995). Third, asbestos induces the generation of iron-related reactive oxygen species that cause DNA damage (Kamp et al., 1995). Fourth, asbestos induces kinase-mediated signalling, including phosphorylation of the mitogen-activated protein (MAP) kinases and of extracellular signal-regulated kinases (ERK) 1 and 2. Phosphorylation of these kinases increases the expression of early-response proto-oncogenes that encode members of the Fos-Jun and activator protein 1 families (Zanella et al., 1996).

1.1.3.2 SV40

Asbestos is considered the primary agent of malignant mesothelioma. However, 20% or more mesotheliomas occur in non-exposed individuals (with no known history of exposure), and only about 5% of people heavily exposed to asbestos develop mesotheliomas (Roggli et al., 1992). For instance, there are 400-600 cases in the US each year unrelated to asbestos exposure (Testa et al., 2001). Moreover, rare cases of

mesotheliomas in children and infants also suggest that additional factors may cause mesothelioma, alone or in conjunction with asbestos (Testa et al., 2001). Other possible agents, such as Simian virus 40 (SV40), have been implicated as a cofactor in the causation of malignant mesothelioma (Carbone et al., 1994). SV40 is classified as an oncogenic virus, and the large T antigen (Tag) is thought to mediate its oncogenicity (Fanning et al., 1992). This effect is mediated by binding and inactivating two tumour suppressor genes, *p53* and retinoblastoma gene, Rb, as well as their products, the p107 and p130 proteins. SV40 sequences have been found in brain and bone tumours, lymphomas, and malignant mesothelioma, as well as in non-invasive lesions of the mesothelium (Shivapurkar et al., 2002). SV40 Tag expression was also detected by both immunohistochemistry and immunoprecipitation experiments in mesothelioma samples (Carbone et al., 1994). However, data from two well-designed studies suggest that detection of SV40 in malignant mesothelioma tissue is a false-positive result because of technical factors (Lopez-Rios et al., 2004; Manfredi et al., 2005). Epidemiologic evidence also does not support a relationship between SV40 exposure and subsequent mesothelioma development. Overall, the link between SV40, human mesothelioma and asbestos needs to be examined further in detail, and any role for SV40 in the pathogenesis of mesothelioma remains unclear and unproven (Robinson et al., 2005). Asbestos remains the major causative agent.

1.1.3.3 Molecular Pathology

Molecular studies in animals and people suggest a genetic susceptibility to asbestos-induced tumour formation (Huncharek et al., 1996; Lichtenstein et al., 2000; Peto et al., 2000; Roushdy-Hammady et al., 2001; Saracci et al., 2001; Musti et al., 2002; Demant

2003). However, the role of genetics appears much stronger in terms of acquired somatic mutations rather than inherited factors. The majority of malignant mesotheliomas that have been cytogenetically examined show karyotypic changes with abnormalities involving both chromosome structure and number (Murthy et al., 1999). Loss of chromosome 22 is the most common gross change, but structural rearrangement of 1p, 3p, 9p, and 6q are often noted (Bjorkqvist et al., 1997; Balsara et al., 1999). The most common pattern of tumour suppressor gene loss in mesothelioma development involves p161INK4A, p14ARK and neurofibromatosis type 2 (NF2) (Gazdar et al., 2003).

Six features are common to most cancer cells, and there is evidence that all six are found in malignant mesothelioma (Hanahan et al., 2000)(Figure 1-3)(Robinson et al., 2005).

Growth Advantage: Mesothelioma cells show increased or dysregulated growth. A large number of growth factors, including platelet-derived growth factors A and B (Versnel et al., 1991), epidermal growth factor (Dazzi et al., 1990), and transforming growth factor β (Fitzpatrick et al., 1994; Marzo et al., 1997), could potentially act as autocrine or paracrine stimulators of malignant mesothelioma proliferation.

Immortalisation by the Action of Telomerase: The lifespan of replicating cells is controlled by gradual shortening of telomeres. The majority of malignant mesothelioma has been shown to express telomerases that prevent this shortening and allow cell immortalisation (Lee et al., 2004).

Figure 1-3 Biologic Features of Malignant Mesothelioma.

Mesothelioma-cell growth requires angiogenesis to provide nutrients and matrix interactions to provide a supportive environment. Growth signals and loss of tumor-suppressor genes provide the growth advantage that leads to tumor-cell proliferation. The host responds to mesothelioma with inflammation and immunity. Antiapoptosis and immortalization render such cells resistant to cell death. Although the overall events are likely to be similar, the particular molecular lesions that are acquired probably vary from patient to patient. Each patient probably has many mesothelial cells that carry different patterns of mutations. Only one of these cells may undergo the final crucial molecular changes that produce malignant mesothelioma, and this cell may then seed the rest of the pleural cavity. TGF-β denotes transforming growth factor β, PDGF platelet-derived growth factor, and NF2 the gene encoding neurofibromatosis type 2.

Adapted from Robinson BWS, et al. N Engl J Med, 2005;353(15):1591-603

Absence of Tumour Suppressor Genes: Tumour suppressor genes operate in different ways to block tumour growth. *Rb* and *p53*, the two principal tumour suppressor genes, are not commonly inactived in malignant mesothelioma. However, the commonest abnormalities seen are loss of p16 and p14 (Yang et al., 2000; Wong et al., 2002), and NF2 (Schipper et al., 2003), which are important in the *Rb* and *p53* pathways. This implies a particular pattern of tumour suppressor gene loss is necessary for mesothelioma.

Induction of Anti-apoptotic Processes: Cells can die when cell death receptors are activated by ligands, such as tumour necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL), and Fas ligand, or when growth factors are blockaded which leads to activation of the caspase death cascade. The apoptosis blocking protein Bcl-xl is associated with mesothelioma cell resistance to apoptosis (Riedl et al., 2004).

Increased Angiogenesis: In order to grow, tumours require the continuous formation of new blood vessels. Malignant mesothelioma cells produce angiogenic factor, such as vascular endothelial growth factor (VEGF) (Masood et al., 2003). Moreover, VEGF blockade reduces mesothelioma growth in animal models (Merritt et al., 2004). Both VEGF and VEGF receptors are upregulated in mesothelioma.

Matrix Interactions: Malignant mesothelioma cells make collagen, and it is likely tumour growth is related to matrix interactions. The prognosis of mesothelioma appears to be related to the expression of matrix metalloproteinases (Edwards et al., 2003; Mutsaers 2004).

1.1.4 Diagnosis

1.1.4.1 Imaging

At presentation, the conventional chest radiography typically shows pleural effusion or irregular pleural thickening, often with evidence of pleural plaques. In the presence of a supportive exposure history these appearances should suggest mesothelioma.

Computer tomographic (CT) scanning often shows nothing other than the pleural effusion or pleural-based masses with or without thickening of interlobular septa at presentation (Figure 1-4)(Lee et al., 2002; Evans et al., 2004). CT scanning also is used to identify signs of asbestos exposure, such as plaques. However, CT scanning is not able to differentiate between malignant mesothelioma and benign pleural changes (Leung et al., 1990), nor can it aid in the differentiation between mesothelioma and metastatic adenocarcinoma. Therefore imaging cannot be regarded as a substitute for cytology or biopsy in establishing the diagnosis.

Magnetic resonance imaging (MRI) and positron emission tomography (PET) scanning are useful in determining the extent of tumour extension and chest wall invasion, especially on coronal images. It has been suggested that the results of PET combined with CT scanning more accurately reflect the likely response to chemotherapy than do the results of either PET or CT scanning alone; however, this suggestion requires further evaluation in random trials (Goerres et al., 2004).
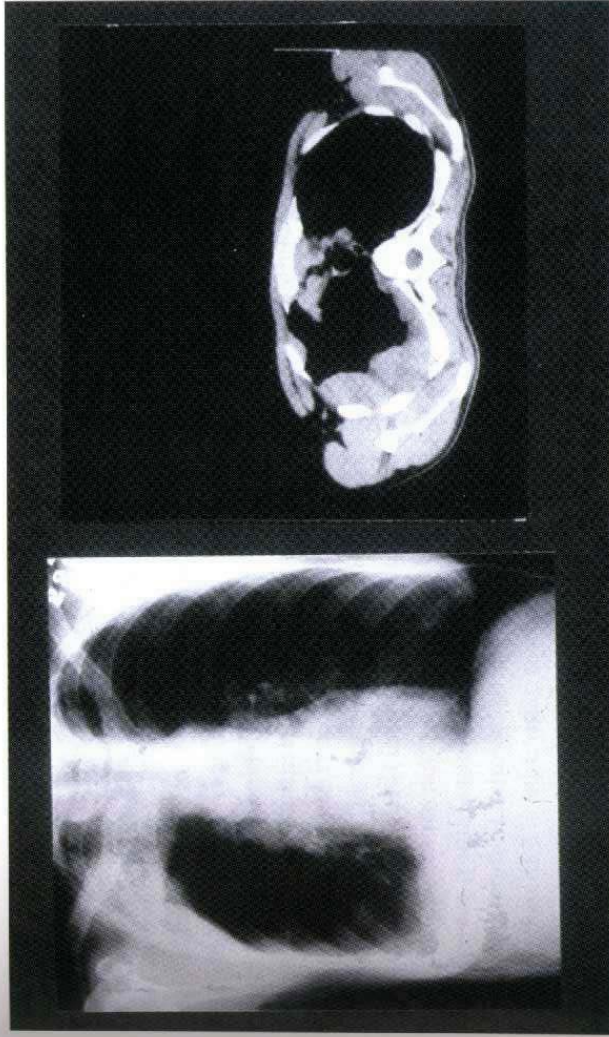
Figure 1-4 *Radiological appearance of a patient presenting with mesothelioma. Shown are the chest x-ray (left) and thoracic CT scan (right), clearly demonstrating a large mass in the right upper zone with some pleural fluid on that side.*

Adapted from Lee YC, et al. Mesothelioma, edited by Bruce W. S. Robinson and A. Philippe Chahinian, 2002, 111-126

1.1.4.2 Cytopathology

Cytological examination of pleural fluid plays a major role in the primary diagnosis of malignant mesothelioma, although in general, a diagnosis of malignant mesothelioma is made with histopathological confirmation (Tsiouris et al., 2007). A group of immunohistochemical markers is important in the differential diagnosis of malignant mesothelioma. A marker such as calretinin or the Wilms' tumour 1 antigen (WT1) is used to determine whether the tissue is mesothelial (Figure 1-5A and 5B)(Robinson et al., 2005). A marker such as epithelial membrane antigen (EMA) helps to determine whether the tissue is malignant (Figure 1-5C)(Robinson et al., 2005). Staining for EMA in a thick peripheral distribution is highly suggestive of malignant mesothelioma (Wolanski et al., 1998; Saad et al., 2005). Of the two anti-EMA antibodies, E29 has significantly greater specificity than MC-5 (Saad et al., 2005). Cytological findings can sometimes be inconclusive or pleural fluid might be absent, so a tumour biopsy is necessary which is usually obtained thoracoscopically.

1.1.4.3 Histopathology

When cytological examination of pleural effusion to distinguish adenocarcinoma of lung and pleural malignant mesothelioma is inconclusive (Nguyen et al., 1999), tumour biopsy is often needed for histopathological analysis. A suite of immunohistochemical markers has been developed to aid differential diagnosis of mesothelioma. Immunohistochemical staining is important for determining that the tissue of origin is mesothelial (e.g., calretinin) and malignant mesothelioma, especially epithelial membrane antigen expression on the luminal aspects of the tumour (Segal et al., 2002).

Fig. 1- 5 **Cytopathological Features of Malignant Mesothelioma.**
Cytologic cell-block samples of pleural fluid show positive staining for calretinin in a mesothelioma sample (Panel A) and negative staining in an adenocarcinoma sample (Panel B), except for a few benign mesothelial cells, which stain positive. In experienced hands, staining for epithelial membrane antigen in a peripheral distribution (Panel C) can establish the diagnosis of malignant mesothelioma. Images courtesy of Drs. A. Segal and D. Whitaker.

Adapted from Robinson BWS, et al. N Engl J Med, 2005;353(15):1591-603

Cytokeratin staining helps to confirm invasion and distinguish mesothelioma from sarcomas and melanoma. Malignant mesothelioma is distinguished from adenocarcinoma by the use of specific antibodies. Malignant mesothelioma is positive with staining for EMA, calretinin, WT1, cytokeratin5/6, human mesothelial cell 1 (HBME-1), or mesothelin. Carcinoembryonic antigen (CEA), CD15, thyroid transcription factor-1 (TTF-1), and the tumour glycoprotein B72.3 are almost never expressed in malignant mesothelioma and thus their presences suggest an alternate malignancy. Electron microscopy is also a useful method to distinguish desmoplastic or sarcomatoid mesothelioma from fibrous pleuritis (Segal et al., 2002). Histologically, mesothelioma *in situ* (atypical mesothelial proliferation) is characterized by a solid, linear, or more often papillary, surface growth of atypical mesothelial cells, which are EMA positive. It has not been proven that mesothelioma in situ is the earliest lesion in mesothelioma development, as has been shown for similar cellular atypia in cervical dysplastic lesions (Whitaker et al., 1992).

1.1.4.4 Blood Test

Patients with malignant mesothelioma, especially those with progressive disease, often have non-specific features of malignancy (e.g., anaemia, thrombocytosis, raised erythrocyte sedimentation, raised gamma globulins) (Lee et al., 2000). Abnormal results of liver function testes are common, and hypoalbuminemia often occurs with advancing disease and contributes to marked peripheral edema (Lee et al., 2002).

A new serum marker called soluble mesothelin-related protein (SMRP) has been discovered in up to 84% of patients with mesothelioma and in less than 2% of patients

with other pulmonary or pleural diseases (Robinson et al., 2003). SMRP levels can be detected with a simple blood test and increase with the progression of mesothelioma and decrease with its regression or with resection of the tumour, so it may be useful to help in diagnosis, monitoring treatment responses, and screening at risk individuals for malignant mesothelioma.

Other potentially useful serum markers, including CA125, CA15-3, and hyaluronic acid, are being analysed (Pass et al., 2005). These markers may have a role in paired analyses to improve the specificity or sensitivity of SMRP measurement. Recently, serum osteopontin levels were found to be significantly higher in patients with malignant mesothelioma than in patients with exposure to asbestos or those patients who have fibrosis alone (Pass et al., 2005). It may be useful in early diagnoses of patients who have a known history of asbestos exposure.

The role of serological tests such as these in clinical practice is still being defined. The current challenge is to improve the sensitivity of the testing and to establish how early in the development of malignancy that the tests become positive. This latter point is critical if early diagnosis can improve chance of cure – although for mesothelioma, that has not yet been clearly shown. Nevertheless, experience gained in other malignancies implies that early diagnosis should offer improved outcomes if effective therapy can be discovered.

1.1.4.5 Pulmonary Function Tests

A restrictive pattern with increased maximal expiratory flow rates is typical in patients with malignant mesothelioma. Changes in forced vital capacity (FVC) are an accurate guide to disease progression or regression provided there are no changes in the amount of pleural fluid (Byrne et al., 1999).

1.1.4.6 DNA Microarray Studies

Microarray studies have revealed expression patterns associated with the genesis and progression of some cancers (Raetz et al., 2004). One study has shown a coordinated up-regulation of the expression of genes associated with energy, protein translation, and cytoskeletal remodelling pathways in mesothelioma tumours compared to normal pleural samples (Singhal et al., 2003). Microarray techniques can be used to address the difficult pathological distinction between adenocarinoma of lung and pleural malignant mesothelioma by measuring the expression levels of genes including those encoding for calretinin and thyroid transcription factor-1 (TTF-1) (Gordon et al., 2002). Microarray studies also have revealed novel genes (eg, *Fra-1*) that are differentially regulated in mesothelioma. These genes may be helpful to enhance knowledge of the pathogenesis of mesothelioma and may present new diagnosis or therapeutic targets in the future (Gordon et al., 2002; Gordon et al., 2003; Ramos-Nino et al., 2003; Singhal et al., 2003; Gordon et al., 2005). The microarray measurements were extensively verified by quantitative polymerase-chain-reaction (PCR) and immunohistochemical analysis. Current bio-informatics tools applied to microarray data have shown utility in predicting both cancer diagnosis and outcome (Golub et al., 1999; Pomeroy et al., 2002). However,

these approaches have not yet become established in regular clinical practice. The potential benefits include improved diagnosis and prognosis, as well as identifying pathways that may be used to develop new treatments. Thus this field is an area of intense ongoing work.

1.1.5 Staging and Monitoring

Determining the stage of a patient's disease is an important step in determining prognosis, defining best therapies, and in the context of clinical trials provides an important basis upon which to decide the efficacy (or otherwise) of the agent or strategy under evaluation. In this latter situation, stage and extent of disease are not the only important parameters but are assessed along with quality of life and other performance indicators. In addition to the staging system outlined below, formal systems for assessing the extent of tumour around the pleural cavity have been devised so that treatment responses can be objectively determined. This system involves a number of measurements using CT scans and the current most commonly used system is the modified RECIST approach. FGD-PET scanning is also increasing being used (Ceresoli et al., 2007).

The current staging system for mesothelioma has been developed by the International Mesothelioma Interest Group, and is quoted directly from the American Cancer Society Website. An outline of the system is shown in Table 1-1, 1-2, and 1-3.

Table 1-1 T stages

Status	Region Involved
T1	Mesothelioma involves either the right or left pleura lining the chest. It has only spread to the pleura covering the lung, with the exception of possibly a few other small spots.
T2	Mesothelioma involves either the right or left pleura lining the chest and has spread from the lining of the chest into 1) the outer lining of the lung, 2) the diaphragm, or 3) into the lung itself.
T3	Mesothelioma involves either the right or left pleura lining the chest and has spread into 1) the first layer of the chest wall, 2) the fatty part of the mediastinum, 3) a single place in the chest wall, or 4) the outer covering layer of the heart
T4	Mesothelioma involves either the right or left pleura lining the chest and has spread 1) into the chest wall, either muscle or ribs, 2) through the diaphragm, 3) into any organ contained in the mediastinum (esophageus, trachea, thymus, blood vessels), 4) into the spine, 5) across to the pleura on the other side of the chest, 6) through the heart lining or into the heart itself, or 7) into the brachial plexus (nerves leading to the arm)

Table 1-2 N and M stages

Designation	Description
NX	Regional lymp nodes can not be assessed
NO	No spread to lymph nodes
N1	Spread to lymph nodes on the same side of the chest as the mesothelioma
N2	Spread to lymph nodes around the point where the windpipe branches into the left and right bronchi or to lymph nodes in the space behind the chest bone and in front of the heart (mediastinum). Affected lymph nodes are on the same side of the cancerous lung
N3	Spread to lymph nodes near the collarbone on either side, to hilar or mediastinal lymph nodes on the side opposite the cancerous lung
MX	Presence of distant metastases can not be assessed
MO	No spread to distant organs or areas
M1	The cancer has spread distantly

Once the T, N, and M categories have been assigned, this information is combined (stage grouping) to assign an overall stage of I, II, III, or IV. Patients with lower stage numbers have a better prognosis.

Table 1-3 Stage grouping for pleural mesothelioma

Stage	Status	Lymph Node	Metastases
I	T1	NO	MO
II	T2	NO	MO
III	T1 or T2	N1 or N2	MO
	T3	NO-2	MO
IV	T4	Any N	MO
	Any T	N3	MO
	Any T	Any N	M1

1.1.6 Treatments

1.1.6.1 Surgery

Surgery for malignant mesothelioma aims to eradicate the tumour, palliate and relieve dyspnea, and increase the efficacy of adjuvant therapies. Surgery has proved most useful for palliation — for example, for local control of recurrent effusions. Debulking surgery is used in some centres. Three surgical procedures may be used with malignant pleural mesothelioma for palliation and/or treatment: (1) video-assisted thoracoscopy (VATS) talc pleurodesis, (2) pleurectomy/decortication (P/D), or (3) extrapleural pneumonectomy (EPP). VATS plays an important role in malignant mesothelioma by permitting directed biopsy to obtain diagnostic tissue. During the same procedure, the effusion is drained, loculations are lysed, and pleurodesis is accomplished usually with aerosolised talc. Recent experience has shown that video-assisted thoracoscopic pleurectomy is also possible (Waller 2004). VATS pleurodesis by itself does not

prolong survival, but it is reasonable and often done in patients who may undergo systemic chemotherapy (Ismail-Khan et al., 2006). EPP is a radical and aggressive approach. It involves the *en bloc* resection of the visceral and parietal pleura, lung, pericardium, and ipsilateral diaphragm (Sugarbaker et al., 1997). EPP is performed on localized tumours, and in patients with adequate Karnofsky performance status. When patients are not fit for EPP or standard treatment options have failed to palliate breathlessness caused by malignant pleural effusions, P/D is an alternative cytoreductive option. P/D involves the removal of the visceral, parietal, and pericardial pleura from the apex of the lung to diaphragm. Study data for both EPP and P/D are relatively poor. In a series of 111 patients, the following results were reported: 4% postoperative morbidity, 0% postoperative mortality and a 14-month median survival after P/D (47 patients), and 21% postoperative morbidity, 9.1% postoperative mortality and a 13-month median survival following EPP (23 patients) (Aziz et al., 2002). Since neither EPP nor P/D appear to offer a significant improvement in survival (Grondin et al., 1999; Zellos et al., 2002), efforts have focused on multimodality approach. The largest study evaluating EPP with chemotherapy in 183 patients achieved a 5-year survival rate of 15%, a postoperative mortality of 3.8%, and postoperative morbidity of 50% (Sugarbaker et al., 1999). The consensus among centres is that surgery, whether debulking surgery or radical resection (EPP), is best performed in combination with adjuvant chemotherapy, radiotherapy, immunotherapy, or other treatment (Pass et al., 1997; Stewart et al., 2004; Sugarbaker et al., 2004; Waller 2004).

1.1.6.2 Chemotherapy

The aims of using chemotherapy are to improve overall survival, quality of life (QOL) and provide symptomatic relief. Despite numerous single-agent and combination chemotherapy trials in patients with mesothelioma in the past, response rates have generally not exceeded 20% with earlier regimens (Ryan et al., 1998; Byrne et al., 1999; Van Meerbeeck et al., 1999; White et al., 2000; Kindler et al., 2001; Van Haarst et al., 2002). These treatments may have limited effect on symptoms of mesothelioma such as pain, breathlessness, and chest wall masses. Comprehensive reviews of chemotherapy for mesothelioma were unable to recommend a standard of care because of the poor response rates although opinions vary in different countries (Janne 2003). However, several therapeutic regimens appear to be useful.

Pemetrexed plus cisplatin represents a combination of a multitargeted antifolate and a platinum compound. Cisplatin cross links DNA and prevents rapidly dividing cells from duplicating their DNA. Pemetrexed is a potent inhibitor of thymidylate synthase, which is required for DNA synthesis (Robinson et al., 2005). Pemetrexed is a major focus of ongoing clinical trials around the world, and this drug might present an advance in the chemotherapeutic treatment of malignant mesothelioma (Tsiouris et al., 2007). Vogelzang and colleagues reported results of a randomised phase III trial of pemetrexed and cisplatin versus cisplatin alone in 448 patients with mesothelioma. The trial showed that the combination had a significantly superior response rate (41.3% versus 16.7%), progression-free survival (5.7 months versus 3.9 months), and overall survival (12.1 months versus 9.3 months) than cisplatin alone (Vogelzang et al., 2003). However, the unfortunate reality is that few patients survive two years.

Gemcitabine plus cisplatin has also been shown to offer similar levels of palliation. Gemcitabine is a false nucleotide that inhibits DNA synthesis and, when incorporated into DNA, terminates DNA polymerization and inhibits repair. Two trials of this drug combination have yielded objective response rates of 48% and 33%, as well as symptomatic improvement and quality-of-life benefits (Nowak et al., 2002). However, further studies in several centres have shown much lower response rates, some as low as 9%. A direct comparison of gemcitabine and pemetexed has not been conducted and is probably unlikely to occur. Despite the high expense of pemetrexed and the marginal benefits this regimen has now become the "standard of care" in many places including Australia and the USA.

A randomised trial in malignant mesothelioma of early versus delayed chemotherapy has been completed in 43 symptomatically stable patients (O'brien et al., 2006). The effect of early versus delayed chemotherapy on survival, progression and QOL has been assessed by the European Organization for Research and Treatment of Cancer (EORTC) questionnaire. In this trial, 21 patients were randomised to the early treatment group and 22 patients to be the delayed treatment group in which patients initially received best supportive care with steroids and opiate analgesia. Cytotoxic therapy was given to patients in the delay group at the time of symptomatic progression. Patients in the early treatment group demonstrated a median survival time of 14 months versus 10 months for those in the delay treatment group. Median time to symptomatic progression in early treatment group was 25 weeks comparing with 11 weeks for the delayed group. Moreover, the QOL was improved for patients in the early treatment group (O'brien et al., 2006).

The combination of Cisplatin and raltitrexed has been evaluated in 250 patients with unresectable malignant mesothelioma in a phase III trial (Bottomley et al., 2006). Overall survival, progression and QOL were evaluated by using the EORTC 30-question tool as well. Median survival time for patients who received cisplatin alone was 8.8 months versus 11.4 months for those received the combination of cisplatin plus raltitrexed. Both QOL and time to progression were comparable between the two regimens. The effects of neoadjuvant cisplatin and gemcitabine before EPP have been assessed in 19 patients (Weder et al., 2004). Response rate was 32% with a mean survival time of 23 months. The role of neoadjuvant chemotherapy before radical surgery is worthy of further investigation.

1.1.6.3 Radiotherapy

The radiosensitivity of malignant mesothelial cells is modest and the overall efficacy of radiotherapy for malignant mesothelioma is disappointing. Local radiotherapy directed to surgical sites may prevent seeding of tumour (but this is controversial and studies have given conflictimg results), and radiotherapy can provide palliative relief of somatic chest-wall pains (Baldini 2004). The biggest limitation to radical radiotherapy is the large field required and the consequent toxicity (Spugnini et al., 2006).

The most successful fractionation method is intensity-modulated radiation therapy (IMRT) (Ahamad et al., 2003). This technique has been suggested to improve overall survival and reduce recurrence, and is generally used after radical surgical resection of malignant mesothelioma. The combined use of extrapleural pneumonectomy and intensity-modulated radiotherapy has been largely successful at controlling local

disease, although these patients then tend to die of metastatic disease (Ahamad et al., 2003). A recent study has reported that standard-dose IMRT administered after EPP and adjuvant chemotherapy resulted in a high rate of fatal pneumonitis (Allen et al., 2006). Researchers established a new system for calculating thoracic IMRT doses (Jang et al., 2006). They found beam modelling was the most important factor to calculate IMRT doses accurately. Optimal beam models secure a safer and more accurate delivery of IMRT. More research is necessary to establish the role of IMRT in the treatment of malignant mesothelioma. Overall, radiotherapy seems to have a role in disease palliation but has no real impact on survival. There have been studies of intensity-modulated radiotherapy as part of multi-modality therapy and this technique needs to be evaluated further.

1.1.6.4 Immunotherapy

Mesothelioma seems to be sensitive to destruction by tumour immunotherapies in certain restricted settings (Mukherjee et al., 2002). This finding has been confirmed in animal studies and clinical trials. The antitumour action of immunotherapy is complex and remains poorly understood. For several reasons, mouse models of mesothelioma have been studied extensively from the immunotherapy perspective and a large body of preclinical information is available.

The goal of immunotherapy is to boost weak anti-tumour immune responses and induce tumour regression. Numerous cytokines have been studied, including interleukin-2 (IL-2), interferons-alpha (IFN-α), beta (IFN-β) and gamma (IFN-γ) as well as granulocyte-

macrophage colony-stimulating factor (GM-CSF) and more recently TNF. Other cytokines that may show promise include the co-stimulatory molecule B7-1 and IL-12.

IL-2 and IFN-γ have been tested in patients with malignant mesothelioma and have shown several objective antitumour responses (Boutin et al., 1994; Goey et al., 1995; Castagneto et al., 2001). The rationale for using IL-2 is based on the fact that it activated lymphokine-activated killer (LAK) cells and induces a cytolytic response (Yasumoto et al., 1987). *In vitro* studies have shown that human natural killer (NK) cell activity is suppressed by asbestos fibres, but restored by IL-2 (Robinson 1989), and NK and LAK cells could lyse human malignant mesothelioma cell (Manning et al., 1989). IL-2 could induce a complete or partial response in 50% of the patients with a median survival of 28 ± 12 months (Astoul et al., 1998). IFN-γ is a lymphokine produced by T-lymphocytes in response to specific antigenic or mitogenic stimuli. It shares the antiproliferative effects of other IFNs and can activate macrophage antitumour cytotoxicity (West et al., 2006). One property of interferon is its ability to facilitate cell differentiation (Ijzermans et al., 1989). This mechanism could explain the efficacy of interferon on pleural mesothelial cell cultures (Phan-Bich et al., 1997). Boutin and colleagues performed the most impressive study over a decade ago. In 86 patients with early stage malignant mesothelioma (Butchart Stage I or II), an overall response rate of 20% was seen after 46 months with repeated doses of intrapleural IFN-γ (Boutin et al., 1994). Importantly, patients with stage I disease treated with intrapleural IFN-γ had an overall response rate of 45%.

Other investigators have focused their attention on the use of colony-stimulating factors to initiate an antitumour immune response. Granulocyte-macrophage colony-stimulating

factor (GM-CSF) activates mature white blood cell effector function and augments antigen presentation (Metcalf 1989). It is able to enhance anti-tumour activity by increasing phagocytic activity of granulocytes and to stimulate antigen presentation by cells such as macrophages and dendritic cells (Morrissey et al., 1987). Robinson's group reported some local reduction in tumour mass associated with an intense intratumoural lymphocytic infiltrate in two patients by direct intratumoural injection of the recombinant cytokine GM-CSF (Davidson et al., 1998). This group has also demonstrated significant therapeutic effects with intraperitoneal delivery of cytokines genes for IFN-γ, IL-2, and antisense TGF-β in a murine model of mesothelioma (Astoul et al., 1998).

Numerous models have been established in animals to enable better understanding of the biology of malignant mesothelioma (Davis et al., 1992). The murine malignant mesothelioma cells behave similarly to human MM tumours in many respects (Christmas et al., 1991; Davis et al., 1992). Murine malignant mesothelioma cell lines have been transfected with a number of immunologically relevant molecules, such as B7-1, IL-2, IL-12, GM-CSF and others (Leong et al., 1994; Leong et al., 1997; Leong et al., 1997; Caminschi et al., 1998). IL-12 is one of the most active immunomodulatory cytokines with antitumour effects. It is a heterodimeric glycoprotein comprising M_r 35,000 and M_r 40,000 subunits (p35, p40) linked by a disulfide bond. Biological activity requires coexpression of the subunits. IL-12 is produced by macrophages and dendritic cells in response to various stimuli (D'andrea et al., 1992; Heufler et al., 1996). IL-12 has a number of immunostimulatory effects, including enhancement of natural killer (NK) and cytotoxic T-lymphocyte (CTL) function (Kobayashi et al., 1989; Gately et al., 1992; Zeh et al., 1993), promotion of the development T-helper (Th1) type cells

(Manetti et al., 1993), and induction of IFN-γ and TNF (Chan et al., 1991; Aste-Amezaga et al., 1994). Caminschi and colleagues have shown that the injection of recombination IL-12 into malignant mesothelioma has profound antitumour effects (Caminschi et al., 1998). In this study, however, IL-12 had to be present continuously and cessation of treatment leads to continued growth of the tumour. In order to sustain local production of an antitumour cytokine rather than the need for continuous dosing, murine mesothelioma cells transfected *ex vivo* with IL-12 gene (AB1-IL-12) were engineered (Caminschi et al., 1999). Injection of AB1-IL-12 induced systemic immunity that could inhibit growth of the parental tumour. In addition, CD8$^+$ effectors were generated which exhibited antitumour effects against a second, untransfected tumour growing at a distal site.

There is some evidence that immunotherapy for malignant mesothelioma is possible. However, new immunotherapy approaches will probably continue to be studied in this disease, almost certainly in combination with other therapeutic modalities (for example combination of apoptosis – inducing chemotherapies with immunotherapy – see below). The animal models suggest that other agents such as IL-12 are likely to be important and may hold promise. It is possible that in the future some form of immunotherapy will enter standard practice for treatment of mesothelioma.

1.1.6.5 Gene Therapy

Gene therapy for cancer generally involves the administration of engineered viruses as gene delivery vectors to patients. Malignant pleural mesothelioma is an attractive target for gene therapy based on the following: (1) it is an aggressive neoplasm with poor

prognosis (median survival of 1-2 years after initial diagnosis), (2) tumour is localised within the thoracic cavity, and the vector can be easily administered simply via a drainage tube, which also makes it easy to monitor the status of the lesion treated, (3) morbidity and mortality are primarily related to regional disease extension (Sterman 2005). Mesothelioma has been the target of two separate gene therapy approaches: suicide gene therapy and immuno-modulatory gene therapy. These approaches are discussed later in the Gene Therapy section.

1.1.6.6 Other Therapies

Photodynamic therapy is a surface-orientated, photochemical-induced cytotoxic therapy (West et al., 2006). It involves generation of toxic oxygen radicals when light converts a sensitising drug in the presence of oxygen. These radicals damage the cells and induce cellular necrosis. This treatment is labour intensive. It induces cytoreduction in malignant mesothelioma, although it has not been associated with impressive long-term responses (Robinson et al., 2005).

Several clinical trials of other novel agents are underway (Kindler 2000; Kindler 2004). The antiangiogenic agents that target the vascular endothelial growth factor (VEGF) pathway, such as bevacizumab, thalidomide, BAY43-9006, and PTK787 have been or are being tested. Studies are being conducted on other agents that block specific mesothelioma pathways, including the histone deacetylase inhibitor superoylanilide and hydroxamic acid (Kindler 2004). Proteosome inhibitors (PS-341), other histone deacetylase (PXD101), other vascular endothelial growth factor antagonists (AZD2171), as well as antimesothelin monoclonal antibodies labelled with toxins, are

also being investigated for the treatment of malignant mesothelioma (Hassan et al., 2004).

Recently, studies in animal models indicate that malignant mesothelioma can be cured in the majority of cases in which an apoptosis-inducing agent (e.g., Gemcitabine) is combined with an immunotherapeutic approach that targets the antigen-presenting cell (e.g., the use of antibodies directed at CD40 molecule) (Nowak et al., 2003). Gemcitabine increases tumour antigen cross-presentation, T lymphocyte expansion, and infiltration of the tumour (Nowak et al., 2003). The increase in cross-presentation does not lead to functional or deletional tolerance. CD40 is a M_r 40,000 type I glycoprotein and member of the tumour necrosis factor receptor superfamily. Initially, CD40 was identified on bladder carcinoma cells and later on normal and malignant B cells (Paulie et al., 1985). It is expressed on DCs, monocytes, epithelial cells, endothelial cells, carcinomas of the lung, colon and breast, and leukaemia (Banchereau et al., 1994; Karmann et al., 1995). The interactions between CD40 and CD40 ligand (CD154) have an important role in CTL priming (Bennett et al., 1998; Schoenberger et al., 1998). When CD8 cells recognise antigen on DCs without the presence of CD4 T cells and CD40 ligation, tolerance may occur (Guerder et al., 1992; Kurts et al., 1997). Exogenous CD40 can be substitute for CD4 T cells. It has been found CD40-activated DCs can also restore antigen-specific CTL responses in CD4-depleted mice (Ridge et al., 1998). Therefore, the activating anti-CD40 antibody, FGK45, can replace or augment CD4 help in priming DCs to activate CD8 T cells. Nowak *et al* combined FGK45 with gemcitabine to treat established solid tumours, and showed the synergy between the drug and immunotherapy in the context of tumour cell death. This study proves that chemotherapy has the capacity to augment cellular antitumour immunity.

Chemotherapeutic agents are also synergistic with TRAIL agonists in mesothelioma cells (Broaddus et al., 2005).

1.1.7 Palliation

Recurrent pleural effusions are best controlled by pleurodesis, usually involving talc instillation, and occasionally surgery. Most patients with mesothelioma eventually experience pain and dyspnea. Pain is a major issue and there are a variety of different pain types in patients with malignant mesothelioma (Lee et al., 2002). Local involvement of the chest wall causes somatic pain. Intercostal nerve or vertebral invasion causes neuropathic pain. Organ invasion causes more diffuse visceral pain. Pain control can be difficult, and the early use of opioids and laxatives often are required (Robinson et al., 2005). Somatic pain often responds to paracetamol together with a non-steroidal anti-inflammatory medication. Neuropathic pain is best treated with anticonvulsants. Interventional pain relief, such as intrathecal analgesia or nerve block can be useful for some patients (Robinson et al., 2005).

Dyspnea due to pleural effusion or more often tumour spread is also common and usually progressive in tandem with the disease process (Lee et al., 2002). Effusions can be managed by drainage, talc pleurodesis and surgery if required. Pleurodesis with talc offers good efficacy. The diagnostic and therapeutic procedure can be combined if talc pleurodesis is performed as part of thoracoscopy (West et al., 2006). Chemotherapy can improve symptoms in some cases irrespective of their marginal effect on tumour size. Radiation has shown modest palliative benefit in pain control and symptoms of dyspnea. The basis of effective palliation in malignant mesothelioma is to combine

these treatments with adequate pain control and attention to respiratory function, along

with attention to social and emotional needs.

1.2 Gene Therapy

Classic gene therapy is the direct use of genetic material in the treatment of disease. This usually involves inserting a functional gene or DNA fragment into key cells to mitigate, or cure a disease. A broader definition of gene therapy includes all applications of DNA or RNA technology to treat disease. Gene therapy has been made possible by rapid advances in molecular biology. Advances in the understanding of growth factors and tumour immunobiology have provided the rationale for developing cancer gene therapy.

1.2.1 Vectors

The ideal vectors for gene therapy should be able to (1) deliver the appropriate genetic material into the target cells, (2) provide protection from gene degradation, and (3) achieve gene expression in the target cells (Gardlik et al., 2005). The vectors would be able to select the target population of cells to infect and deliver its gene of interest. This process should also have a limited effect on the immune system and thus avoid detection and destruction. The ideal vectors should also be suitable for clinical application. Many viral and non-viral systems are employed in gene therapy approaches to various diseases including cancer. A variety of viral vectors, including retroviruses, adenoviruses, adeno-associated viruses, herpes simplex viruses, and poxviruses, have been employed to deliver genes to cells. The non-viral agents that are being assessed are plasmids and liposomes.

1.2.1.1 Retroviral Vectors

Early gene therapy studies utilised retroviral vectors due to their high gene transfer efficiency and stable therapeutic gene expression. Retroviruses are a group of viruses whose RNA genome is converted to DNA in the infected cell. The retroviral genome consists of few genes, including those essential for replication, and long terminal repeats (LTRs). To improve retroviral vector safety, all genes other than the signals in or around the LTRs required for RNA genome packaging into the capsid, reverse transcription and integration are usually removed. These viruses can carry transgenes up to 8 kilobases (kb) in length (Seth 2005). A transgene is inserted into the deleted virus, which can then infect its target cell. The transgene is integrated into the host genome and confers expression of both viral genes and the transgene to the infected cell (Young et al., 2006). Retroviruses use a receptor-mediated entry into cells and this receptor is ubiquitously expressed on human cells. This leads to widespread expression of the transgene where more selective expression is often required. Efforts to engineer the outer coat of the virus to improve cell-selectivity and efficacy have had modest success in model systems (eg the use of integrin binding motifs to bind to vascular cells).

Since retrovirus is capable of integrating into the host genome, it is a suitable vector when the long-term expression of the foreign gene is needed (Kohn et al., 2003). The main concern in using recombinant retrovirus is the random integration into the host chromosome which can have unforeseen effects. It could lead to activation of oncogenes or inactivation of tumour-suppressor genes (Verma et al., 1997). Another limitation is the requirement of cell division for the provirus integration. Thus, in the context of a tumour, only those small percentages of cells that are actively undergoing

replication are susceptible. Retrovirus cannot invade blood cells, skin cells, and many other tissues (Miller et al., 1990). This problem can in some circumstances be overcome by using lentivirus, which is capable of integrating into non-dividing cells.

Members of this class of RNA viruses are the murine leukaemia viruses (MuLV) and the lentiviruses, which are extensively used for virus vector engineering. The retroviral vectors currently used for gene therapy are predominantly derived from the Moloney murine leukaemia virus (MoMuLV) (Walther et al., 2000). This amphotropic virus is able to infect murine cells and cells of a variety of other species including human cells (Battini et al., 1995). Lentivirus can infect both dividing and non-dividing cells (Lewis et al., 1992). The best-known lentivirus is the human immunodeficiency virus (HIV), which has been disabled and developed as a vector for *in vivo* gene delivery. It has been demonstrated that lentiviral vector can transduce macrophages and airway epithelial cells (AECs)(Goldman et al., 1997). The virus integrates into the genome of transfected cells and expression is therefore likely to last for the lifetime of the cell (100 days for AECs). However, lentivirus can only enter AECs via the basolateral surfaces (Borok et al., 2001). Unless lentivirus is able to come in contact with airway stem cells efficiently it will need to be re-administered and therefore will encounter the same problems that the immune system poses for the other viral vectors. This is especially limiting for applications such as the correction of genetic disorders such as cystic fibrosis. Concerns about long-term expression are generally less of an issue in cancer applications, where short term, high-level expression of a candidate therapeutic gene is generally the goal.

1.2.1.2 Adenoviral Vectors

Adenovirus is one of the most commonly used viral vectors in gene therapy. It is the best described and most used system for gene transfer, originally prepared to overcome the limitations of retroviral vectors. It is described further in the Adenoviral Vectors section below.

1.2.1.3 Adeno-Associated Virus Vectors

Adeno-associated virus (AAV) is a small non-enveloped, single-stranded linear DNA virus, which belongs to the Parvoviridae family (Seth 2005). The virus is non-pathogenic and by itself non-replicating. For productive virus replication, AAV needs a helper virus such as adenovirus or herpes virus. However, in the absence of a helper virus, wild type AAV can integrate into the host cell genome, thus making it an attractive vector for gene therapy for long-term correction of genetic disorders. AAV vectors have a good safety profile, broad tissue tropism, and long duration of expression. They may have a superior ability to escape from immune system surveillance compared with other viruses because they do not infect antigen-presenting dendritic cells (Zaiss et al., 2002). Unlike retroviruses, which are of murine origin, the adeno-associated viral transfer systems are derived from human viruses and therefore maybe more feasible for human gene therapy.

Several different serotypes of human AAV have been identified and further screening for new human and non-human primate forms are underway (Gao et al., 2003). The most widely used AAV serotype 2 (AAV-2) use heparin sulphate proteoglycans as the

primary receptor (Summerford et al., 1998) and co-receptors fibroblast growth factor 1 receptor (Qing et al., 1999) and $\alpha_v\beta_5$ integrin (Rabinowitz et al., 2002). Although AAV-2 displays a broad host range, it has been reported that certain cell types are resistant to AAV-2 infection, probably due to the lack of appropriate receptors. The use of other AAV serotypes has been explored to improve transduction of specific cell types (Young et al., 2006).

Two major limitations in the use of AAV as gene therapy vectors are the small transgene capacity and the effect of neutralising antibodies. Recent studies indicate that the main limitation of small packaging capacity will also be overcome (Wright et al., 2003). Many people have neutralizing antibodies to AAV due to prior infection (Chirmule et al., 1999). The administration of AAV also elicits a strong humoral response, which can interfere with re-administration of the vector (Brockstedt et al., 1999). Using different serotypes of AAV may circumvent this problem and allow effective long-term treatment.

One of the major attractions of the AAV has been its relative non-toxicity and general lack of pro-inflammatory effects and its ability to achieve log-term expression in animal models. AAV-2 has been used in human phase II clinical trials for cystic fibrosis (Wagner et al., 2002; Moss et al., 2004). However, in a phase I trial for haemophilia B (factor 1X), efficacy was poor due to the immune response mounted by the patients to the capsid proteins (Manno et al., 2003). This trial has highlighted the problem of extrapolating animal data to humans; the latter have pre-existing immunity to AAV. If this approach is to be used, transient immunosuppression at the time of vector administration may be needed. The difficulties encountered in the haemophilia trial,

despite the high degree of optimism for AAV as a vector, served to again highlight the major ongoing challenges faced by the gene therapy field in general.

1.2.1.4 Herpes Simplex Virus Vectors

Herpes simplex virus (HSV) is an enveloped, double-stranded DNA virus 150kb in size. It is a natural human pathogen, replicating in epithelial cells, but able to persist in a latent state in non-dividing cells (neurons). HSV attachment to cells is via binding of glycoproteins expressed at the viral envelope to cellular membrane receptors (Geraghty et al., 1998). HSV can infect lytically or can establish latency. HSV vectors have the advantages of being able to establish latency in some cell types, having a wide host range and a large capacity for insertion of transgene (Liu et al., 2006). The ability to establish latency in neuronal cells makes HSV an attractive vector for treating neurological disorders such as Parkinson's and Alzheimer's. In addition, the ability of HSV to infect efficiently a number of different cell types, such as muscle and liver, may make it an excellent vector for treating non-neurological disease.

Much of published work has been done using HSV type 1 (HSV-1) vectors. Two major technologies are followed for the generation of HSV-1 vectors: insertion of the therapeutic gene directly into the virus establishing the recombinant HSV-1 vector, or insertion of the foreign DNA into an amplicon plasmid vector (Oehmig et al., 2004). The major problem of recombinant HSV-1 vectors is their cytopathic effect and the induction of an immune response by viral gene expression. The development of amplicon vectors and a helper-free packaging system has overcome this problem to a great extent. HSV-1 amplicon vectors contain no viral genes, besides the origin of viral

DNA replication sequences, and require a super-infection with HSV helper virus for propagation. A helper-free packaging system markedly decreases the generation of replication competent virus and cytotoxicity; however, only low titres of amplicon vectors are generated (Cunningham et al., 1993). Some studies have been done to increase vector titres (Saeki et al., 1998; Stavropoulos et al., 1998). However, the inflammatory response is still one of the major obstacles to the clinical use of HSV therapeutically (Liu et al., 2006).

1.2.1.5 Non-viral Vectors

Gene delivery using non-viral approaches has been extensively studied as a basic tool for cellular gene transfer and gene therapy. Non-viral vectors include naked-DNA and liposomes (Zhou et al., 2004). They are based on plasmids that are closed, circular DNA strands. Therapeutic genes can be inserted into these plasmids, and then this recombinant plasmid can be transferred into cells in different ways. Naked-DNA can be injected directly into targeted tissues by several approaches including "gene gun" and electroporation. "Gene gun" can be used to transfer genes to non-dividing cells and the DNA-gold beads are cheaper and easier to prepare. In this technique, DNA is loaded onto microscopic gold beads and "shot" into the cells with a helium gun (Wells 2004). The efficiency of this method is variable, and the duration of expression is transient (Robbins et al., 1998). Of the non-viral gene delivery methods, liposomes are the most widely used. There are two types of liposomes, anionic and cationic, with the cationic type more frequently used for human gene therapy (Gao et al., 1995; Liu et al., 1996). Liposomes are non-pathogenic and can be used for multiple treatments. Compared to viral vectors, liposomes are cheaper and easier to produce, but have less efficiency of

transfection. In order to improve transfection efficiency, the liposomal vectors can be conjugated with defective viral particles, viral proteins, or virally derived peptides (Robbins et al., 1998). The key advantage of non-viral methods is that they are safer to administer and do not elicit major immune responses. However, the main problem is that compared to viral vectors efficiency of gene transfer is generally low. Although non-viral method is generally safe, unmethylated CpG motifs in bacterial and plasmid DNA are recognised by immune systems as a dangerous signal or immunostimulatory, thus careful attention to plasmid design is needed.

1.2.2 Adenoviral Vectors

1.2.2.1 General Properties of Adenovirus

Adenoviruses are non-enveloped DNA viruses carrying linear double-stranded DNA of about 36kb and were discovered in 1953 (Enders et al., 1956). The viral genome coding region is flanked by inverted terminal repeats (ITRs), and contains five early transcription regions (E1A, E1B, E2, E3, E4) and one late transcription region from which five families of late mRNAs are generated. The adenoviral genome is intimately associated with viral proteins (core) and is packaged in the viral capsid, which consists primarily of three proteins: hexon, penton base, and knobbed fibres (Shenk 2001). In general, the fibre knob functions as the major attachment site for cellular receptors, while the penton base is involved in secondary interactions that are required for virus entry into the cell. For a long time hexon was thought to play only a structural role as a coating protein, but more recently interactions between regions of the hexon protein and

blood coagulation factors have been identified that are involved in viral uptake by the liver (Waddington et al., 2008).

To date, 51 different serotypes of human adenoviruses have been identified, which are classified into 6 subgroups (A-F) based on their haemagglutination properties (Lukashok et al., 1998). The most frequently utilized for gene therapy are type 2 and type 5, which are members of the C group. Adenoviruses can cause acute upper respiratory tract infections, pharyngoconjunctival fever, conjunctivitis and gastroenteritis, but have low pathogenicity in humans (Majhen et al., 2006).

The primary cellular receptor responsible for attachment of all adenovirus serotypes except those from group B is the coxsackie-adenovirus receptor (CAR)(Bergelson et al., 1997; Tomko et al., 1997; Roelvink et al., 1999). The normal function of CAR is as part of the cell-cell adhesion complex. Recent research has shown that adenoviruses can use some other molecules as receptors as well, such as major histocompatibility complex I (MHCI) and heparan sulfate glycosaminoglycans (Hong et al., 1997; Dechecchi et al., 2001). The first step for infection of cells is the binding of the head domain (knob) of the viral fibre protein to a specific cell-surface receptor CAR followed by internalisation of the adenoviral particle (Bergelson et al., 1997). The cytoplasmic domain of CAR is not required for virus attachment or infection which means that cell signalling through this receptor is not involved in viral entry (Wang et al., 1999). Next, an exposed Arg-Gly-Asp (RGD) motif on the penton base of the virus interacts with the cellular integrin receptors ($\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_1$ integrins), which leads to virus internalisation and transport of the viral DNA into the cell nucleus (Wickham et al., 1993; Stewart et al., 1997; Nemerow et al., 1999; Meier et al., 2004). Integrins normally react with the

extracellular matrix to facilitate adhesion, differentiation and other cell-cell phenomena (Meredith et al., 1996).

The adenoviruses replication cycle can be divided in two phases, early and late. During the early phase of the replication cycle, the viral DNA is transported to the nucleus and transcription of early viral genes is initiated. Early gene products interfere with antiviral host cell defence mechanisms and direct the host cell to enter the cell cycle, supporting transcription and DNA replication. As soon as the E2 gene product initiates DNA replication, late events in the viral replication start. Gene expression of mRNA regulated by the late promoter increases, which results in high-level production of structural proteins that assemble together with viral genomes in the nucleus. The newly synthesized virions are released from the cell by the induction of cell lysis (Shenk 2001).

1.2.2.2 Adenoviral Vector Development

Accurate information about the structure and molecular biology of the adenovirus has allowed development of adenoviral vectors for gene delivery *in vivo* and in clinical trials for cancer and cystic fibrosis (CF) (Zabner et al., 1993). Adenoviral vectors are relatively easy to manipulate using recombinant DNA techniques (Vorburger et al., 2002). Recombinant adenoviruses can be produced at very high titres, have relatively high capacity for transgene insertion, and efficiently transduce both quiescent and actively dividing cells without incorporation of viral DNA into host genome.

Foreign DNA can be inserted in at least three regions of the adenoviral genome: E1, E3 and the short region between E4 and the end of genome. The first recombinant adenoviral vectors have been generated by deleting the E1 and/or E3 gene regions in the viral genome, allowing the introduction of promoter and transgene sequences up to 6.5-8.3 kb (Kootstra et al., 2003). However, removal of the E1 gene from the vector markedly hampers transcription of E2 genes and, consequently, DNA replication and production of structural viral proteins. This is of course ideal and intentional for a gene delivery vector when delivered into target cells. However, for production of vector stocks, helper cells, which provide the essential E1 protein in *trans* for efficient viral encapsidation, are needed for the generation of infectious viral particles. The widely used human embryonic kidney cell line 293 are stably transfected with Ad E1 genes (Graham et al., 1977). There are also several other cell lines available now that can be used (e.g., human embryonic retinoblast cell line PER.C6) that have been further engineered to have no overlapping E1 sequences within the vector genomes, thus greatly reducing the risk of inadvertently generating recombinant replication competent virus (Fallaux et al., 1998). Even in the absence of E1 genes first generation adenoviral vectors are still able to replicate at very low levels, thereby inducing a cellular immune response, which is the major barrier to the use of these vectors. The second generation of adenoviral vectors have deleted E1, E2, and E4 encoding sequences. They are less immunogenic than the first generation vectors and provide a larger capacity for transgene insertion (Amalfitano et al., 1998; Lusky et al., 1998; Moorhead et al., 1999). Third generation adenoviral vectors, also called "high capacity" or "gutless" vectors contain only ITR repeats and a packaging signal and can accommodate up to 37kb of foreign DNA (Hardy et al., 1997; Lieber et al., 1999). These vectors require a helper virus for their replication. Removal of viral genes from the vectors prevents the

induction of immune responses, and long-term gene expression can be obtained using the gutless vectors (Gao et al., 1996). However, separation of gutless vectors from helper virus is difficult (Gardlik et al., 2005).

Many strategies are being investigated to combine adenoviral vectors with reduced immunogenicity and long lasting, high level expression of therapeutic genes. To enable chromosomal integration of adenoviral vectors and prolong expression of transgenes, chimeric Ad-AVV or Ad- retrovirus vectors have been designed (Reynolds et al., 1999; Lundstrom 2003).

1.2.2.3 Adenoviral Vector Tropism and Targeting

Most often used as a vector in gene therapy is the Ad5 serotype because of its ability to mediate high levels of transgene expression, its ability to transduce dividing and non-dividing cells, and its broad tissue tropism. The efficiency of Ad5 gene transfer is related to the density of CAR on the surface of the target cells. The expression of CAR is highly variable, sometimes being low in tumour cells, resulting in relative resistance to Ad5 infection. Previous studies suggest that CAR may act as a tumour suppressor, which could be linked to the frequent down-regulation seen in highly tumorigenic cells (Okegawa et al., 2000). Therefore Ad5 gene therapy may be compromised as a cancer gene therapy strategy (Hemmi et al., 1998; Li et al., 1999; Cripe et al., 2001).

Ad3 has a distinct receptor and therefore different tissue tropism (Stevenson et al., 1995; Roelvink et al., 1998). Ad3 has been shown to have tropism for CD34 positive cells within human haematopoietic stem cells (Mei et al., 2004). It has also been shown

to bind to CD46 on human cells (Sirena et al., 2004). CD46 is a membrane bound co-factor for the complement cascade. It is widely expressed on many cells and may prove to be a more generally useful attachment receptor for cancer than CAR. Genetic re-targeting with the Ad3 may allow more efficient tumour cell transduction in the context of *in vivo* gene delivery and thus may offer the potential to improve Ad-based cancer gene therapy approaches (Kanerva et al., 2002).

Targeting of adenoviral vectors include transductional targeting and transcriptional targeting (Majhen et al., 2006). Transductional targeting of adenovirus aims at enhanced or specific transduction of the target cell. The goal is to delete the broad tropism of Ad5 in normal epithelial cells and /or enhance the virus infectivity toward CAR-deficient tumour cells (Douglas et al., 1999). There are two strategies to modify the virus tropism to avoid CAR deficiency, through genetic or physical modification. Genetic targeting strategies have focused on several methods, either by ablating CAR binding, introducing novel peptides into the fibre knob domain, or introducing a fibre or fibre knob domain from a different serotype such as B species viruses, which lack CAR binding capacity. Some studies have shown encouraging results *in vitro* or *in vivo*, such as the insertion of RGD containing peptides into HI-loop of fibre protein (Dmitriev et al., 1998) and L1-loop of hexon protein (Vigne et al., 1999), the insertion of poly-lysine into the C-terminus of capsid protein IX (Dmitriev et al., 2002), the insertion of RGD or heparan binding motifs into C-terminus of fibre protein (Wickham et al., 1997; Douglas et al., 1999)(Figure 1-6)(Noureddini et al., 2005). These latter vectors showed increased transduction of macrophages, and endothelial and smooth muscle cell types, but insertion of longer peptides disrupted functional fibre structure (Wickham et al., 1997). The physical targeting strategy has focused on conjugation of Ad with bi-specific

Figure 1. Cross section of native adenovirus serotype 5 capsid. A generalized graphical displaying the major structural proteins of the Ad5 capsid is shown. The locales that have previously been exploited for genetic modification have been indicated: fiber, protein IX, penton base, and hexon.

Adapted from Noureddini SC, et al. Molecular Pharmaceutics, 2005; 2 (5): 341-7

molecules. Bi-specific antibodies have been developed to bind penton base and fibre knob domain, to simultaneously ablate CAR interactions and to allow retargeting through the second functionality (Wickham et al., 1996; Rogers et al., 1997; Miller et al., 1998). An additional benefit of targeting is that some modifications of Ad vectors can reduce interaction with neutralizing antibodies (for example in a patient that may have had a prior natural Ad infection) thereby achieving further efficacy gains over unmodified Ad (O'riordan et al., 1999; Romanczuk et al., 1999; Croyle et al., 2000).

Transcriptional targeting does not change the tropism of the viruses but restricts the expression of the delivered "therapeutic" gene to target cells. Transcriptional targeting is achieved by placing this transgene under the control of a tissue- or tumour-specific promoter. The viruses are able to infect various cells but the transgene is expressed only in the cells types that actively express the transcription factors needed for the tumour specific promoter activity (Kanerva et al., 2004). This strategy has been introduced to overcome the obstacle in treatment of melanoma where an enhanced infectivity vector with an RGD motif in the HI loop still showed transgene expression in healthy normal tissue (Okada et al., 2005). In the AdRGD mediated HSV-TK (thymidine kinase)/ganciclovir system, the therapeutic HSV-TK gene was put under control of either melanoma-specific tyrosinase (Tyr) promoter or tumour-specific telomerase reverse transcriptase (TERT) promoter. Compared to vectors using traditional non-specific promoters (e.g. CMV), these targeted vectors; AdTyrRGD/HSV-TK or AdTERTRGD/HSV-TK had much reduced toxicity for non-melanoma cells.

1.2.3 Strategies for Cancer Gene Therapy

Traditional gene therapy approaches initially favoured a "gene replacement" strategy, which evolved from the initial concept of gene therapy for treating inherited genetic disorders. In view of recent advances in the understanding of growth factors, molecular oncology, and tumour immunology, strategies for cancer now include a range of gene therapy paradigms, including gene replacement gene delivery, suicide gene delivery, and the manipulation of immunomodulatory mechanisms (Albelda et al., 2000). Gene therapy will achieve maximum clinical benefit when the sum of the gene potency and the delivery efficiency can be maximised in all target cells (Verma et al., 1997).

1.2.3.1 Oncogene Inactivation

It is well known that development of many cancers is associated with the expression of dominant oncogenes, such as *ras*, HER2/neu and MYC genes (Balmain et al., 2003). Vectors expressing genes that will inactivate the dominant oncogenes can be used to inhibit tumour progression. This can be accomplished using the following strategies: (1) inhibition of oncogene transcription into mRNA; (2) reduction of mRNA translation into protein; and (3) interference with oncoprotein transportation and function (Hughes 2004). For example, vectors expressing anti-sense oligonucleotides, ribozymes or small interfering RNAs (siRNA) directed against *ras* and HER2/neu oncogenes have been extensively studied. This approach can be also targeted against the tumours *in vivo* or for killing cells *ex vivo*.

1.2.3.2 Tumour Suppressor Gene Replacement

Loss of tumour suppressor function is commonly associated with many human malignancies (Weir et al., 2004). Tumour suppressor genes encode a variety of proteins that regulate the cell cycle and mediate DNA repair pathways. The best-known tumour suppression genes are *p53* (regulates cell cycle and apoptosis), retinoblastoma gene *Rb* (regulates cell cycle and differentiation), *p16INK/CDKN2* (regulates cell cycle), and *PTEN* (regulates cell survival)(Hughes 2004). Cancer cells with DNA mutations can inhibit the expression of tumour suppressor genes, and become less differentiated and avoid apoptosis. Transfection of tumour suppressor genes into cancer cells has been effective at inducing apoptosis and/or arresting cell cycle (Seth 2005). Moreover, some tumour suppressor genes such as *p53* can also inhibit angiogenesis and tumour invasion, providing additional anti-tumour effects (Nishizaki et al., 1999; Seth 2000). This "bystander" effect is critical if tumour suppressor gene therapy is to be of any benefit as it is not possible with current or foreseeable vector technology to transduce every tumour cell within a tumour mass with the therapeutic gene. Vectors expressing tumour suppressor genes can be directly injected into the tumour masses; targeted to tumour cells or can be used *ex vivo* for killing tumour cells. In China, Adp53 has now been licenced as a cancer therapeutic for head and neck squamous carcinoma (Peng 2005).

In almost 12% of cancer gene therapy clinical trials, the gene of interest introduced into target cells is a tumour suppressor gene (Majhen et al., 2006). The *p53* tumour suppressor gene is one of the most frequently used genes in Ad gene therapy, because it plays a key role in cell cycle control, induces apoptosis and it is very often mutated in tumours (Ahn et al., 2002; Haviv et al., 2002; Wolf et al., 2004). Ad/*p53* gene therapy

inhibited *in vitro* growth of different cancer cells, such as lung cancer cells, colon cancer cells, head and neck tumour cells, ovarian cancer cells, and breast cancer cells (Spitz et al., 1996; Clayman et al., 1998; Swisher et al., 1999; Parker et al., 2000; Modesitt et al., 2001). In some clinical trials, *p53* gene therapy has shown promising results. 16 of 19 treated patients with prostate cancer were confirmed as having increased *p53* expression after Ad/*p53* treatment and subsequently showed augmented apoptosis in cancer cells (Pisters et al., 2004). It was also reported that the expression of functional *p53* protein was achieved after injection of Ad/*p53* in 15 patients with gliomas (Lang et al., 2003). In this study, 1 patient is still alive without any additional treatment; the other 14 experienced remission for 6 months and 2 of them survived longer than 1 year.

Combination of *p53* gene therapy with cytotoxic chemotherapy and radiation therapy has been investigated. Chemotherapy and radiation therapy induce G1 arrest and apoptosis in part by up-regulation of wt-*p53* following DNA damage. It has been reported that cells with mutated or null *p53* genes have increased resistance to chemotherapy or radiation therapy (Sanlioglu et al., 2004). Preclinical studies indicate that functional *p53* protein significantly sensitises tumour cells when Ad/*p53* is used in conjunction with administration of cytostatics such as cisplatin and doxorubicin (Pagliaro et al., 2003; Ganjavi et al., 2005). The synergistic inhibitory effects on tumour cell growth in human lung cancer have been exhibited by Ad-mediated wt-*p53* gene transfer in combination with docetaxel and radiation therapy *in vitro* and *in vivo* (Nishizaki et al., 2001). A phase II/III study is currently ongoing to evaluate the clinical efficacy of intralesional administration of Ad*p53* in combination with chemotherapy and radiotherapy in patients with locally advanced lung cancer (Swisher et al., 2002).

1.2.3.3 Suicide Gene Therapy

In the suicide gene delivery system, a gene encoding an enzyme that catalyses the conversion of a normally non-toxic agent to a toxic agent is delivered to the tumour cells. The toxic substance then eradicates the tumour cells (Moolten 1994). After insertion of the suicide gene constructs into the tumour cells, treatment with high doses of a relatively non-toxic pro-drug ensures that the transfected tumour cells are producing high concentrations of the cytotoxic drug (Yazawa et al., 2002). High concentration of the cytotoxic drug kills both the transfected cells and their neighbours (bystander effect). Thus, suicide gene therapy can cause tumour regression even when only a fraction of the tumour cells are transfected. The concept here is to achieve very high local toxin expression within the tumour mass while avoiding generalised systemic toxicity.

The most commonly used approach for suicide gene therapy is herpes simplex virus (HSV)-thymidine kinase (TK), followed by treatment with ganciclovir (GCV) (Moolten 1986). Viral TK phosphorylates GCV, an antiviral drug for herpes simplex virus. GCV is relatively non-toxic to humans. Monophosphate GCV is converted to the triphosphate form, a toxic substance, by TK. GCV triphosphate is incorporated into replicating DNA and stops chain elongation resulting in cell death. This approach has been used in the treatment of malignant mesothelioma. Results of phase I clinical trials of Ad-mediated HSV-TK transfer have demonstrated that intrathroracic injections of the Ad are safe. However, strong humoral and cellular immune responses against the adenoviral vector were seen (Molnar-Kimber et al., 1998; Sterman et al., 1998). Overall however, clinical efficacy with the TK approach has been disappointing. Nevertheless, a very

encouraging aspect seen in follow-up from one of the studies was durable responses in 2 out of 13 patients (> 6.5 years)(Sterman et al., 2005). Given the very limited gene expression seen in this study, the investigators concluded that the basis of the response was likely immune system activation rather than the HSV-TK. This has prompted the group to further pursue immune-based approaches (see below). Another strategy of suicide gene therapy is to transfect with *Escherichia coli* gene coding for cytosine deaminase (CD) followed by systemic use of 5-fluorocytosine (5-FC). CD transforms the non-toxic pro-drug 5-FC into the cytotoxic drug 5-fluorouracil (5-FU). A phase I study for the treatment of prostate carcinoma has demonstrated that adenovirus-mediated double suicide gene therapy (HSV-TK and CD) can be safely and successfully used in humans (Freytag et al., 2002). A number of other suicide gene therapies have been developed and tested in cell culture, laboratory animals, and some early clinical trials (Yazawa et al., 2002). However, none of these approaches have yet achieved the degree of efficacy needed for a role in routine clinical practice.

1.2.3.4 Immunogenetic Therapy

Immunogenetic therapy involves modulation of the immune system to stimulate anti-tumour immunity. The major benefit of an immunotherapy approach is the potential for eliminating widespread metastatic disease. However, all immunotherapy studies show that these approaches are much less effective when there is a large bulk of tumour present. De-bulking (either by surgery or other conventional treatment) enhances the effects. Cancer therapy approaches based on efforts to stimulate the immune system have received a great deal of attention for a number of years, and many have

incorporated gene delivery aspects (either through DNA vaccines or cytokines gene delivery for example) (Robinson et al., 1998; Rosenberg 2001).

Cancer gene vaccination is an approach based on activating components of the immune system in order to enhance their capacity for recognising and rejecting tumour antigens (Tuting et al., 1997). After presenting tumour antigens to the host immune system, effectors of the immune system are expected to be able to destroy tumour cells expressing the same antigens (Henderson et al., 2005). Initially tumour cells are harvested from the patients and then are grown *in vitro*. These cells are modified by transfection with one or more genes in order to be more recognisable to the immune system. Alternatively the modified cells are antigen-presenting cells such as dendritic cells or tumour infiltrating lymphocytes such as NK cells and cytotoxic T-lymphocytes specific for tumour antigens. It has been shown that administration of Ad encoding colorectal tumour specific antigen gp70 in the CT26 murine colorectal cancer cell model prolonged survival in 50% of animals and significantly reduced their tumour mass (Nakamura et al., 2002). Recent clinical trials with GVAX, a vaccine made from autologous tumour cells modified to express GM-CSF with an Ad vector showed some encouraging results for non-small cell lung cancer (Nemunaitis 2003). The initial phase I/II study demonstrated a clinical effect with 3 patients experiencing complete remission (not sustained) and 7 of 33 subjects achieving stable disease for 7 months. However, a second study using a stably transfected cell line to provide the GM-CSF to the tumour cells showed much less effect, even though levels of GM-CSF achieved were more consistent and higher than in the first study. A further phase II trial comparing GVAX alone to GVAX combined with cyclophosphamide resulted in 14 of 53 subjects

achieving stable disease and 1 patient experiencing stable disease for over 2 years. Median overall survival was between 5.4 months and 9.5 months (Cross et al., 2006).

Immunogenetic therapy is also being developed via the delivery of immunostimulatory genes, mainly cytokines, to the tumour *in vivo*. The type of genes can be IL-2, IL-4, IL-12, tumour necrosis factor, interferon-γ; GM-CSF; genes which have immunostimulatory activity or T-cell costimulatory molecules such as B7.1 and B7.2 (Seth 2005). For example AdCMVIL-12 is an Ad vector bearing the IL-12 gene under the control of the CMV promoter. Intratumoural injection of AdCMVIL-12 in animals with a single large tumour nodule implanted in the liver caused significant inhibition of tumour growth in a dose dependent manner. Animals treated with AdCMVIL-12 also showed protection against tumour rechallenge, and survival of animals was prolonged compared to controls (Barajas et al., 2001).

With regard to mesothelioma, a promising strategy that has reached the stage of human clinical trial is the use of gene delivery for IFN- β (Odaka et al., 2001). In pre-clinical animal studies it was shown by the Albelda group that the majority of mice bearing early mesothelioma lesions could be cured using AdIFN- β. This effect was dependent on the presence of functional CD8 T-cells, as the effect was lost with T-cell depletion or if immunocompromised mice were used. However, as with many immune-based approaches, successful therapy in the mice was exquisitely dependant on the size of the tumour at time of vector administration. Delay of even a couple of days resulted in substantially reduced efficacy. The strategy has recently been evaluated in a phase I clinical study and found to be safe, with some minor clinical responses seen in 4 out of 10 patients (Sterman et al., 2007). Use of higher doses is being considered.

1.2.3.5 Anti-angiogenic Gene Therapy

Angiogenesis is the formation of new blood vessels in which new vessels sprout and mature from pre-existing vasulature. It is a critical process required by solid tumours to support their growth. Angiogenesis is regulated by both angiogenesis-inducing factors and angiogenesis-inhibiting factors. For example, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), TNF-α, and IL-8 are endogenous stimulators of angiogenesis, and soluble VEGF receptor, matrix metalloproteinase (MMPs), endostatin, angiostain, IL-12 can inhibit angiogenesis (Persano et al., 2007). Regardless of the origin of tumour cells, tumours can be treated by blocking the development of new tumour blood vessels (Denekamp et al., 1998).

Gene therapy using anti-angiogenesis genes has shown promise in animal models. Gene transfer of angiostatin or endostatin decreases tumour angiogenesis and reduced tumour growth (Shi et al., 2002; Xu et al., 2003). However, some data indicated that angiostatin monotherapy could show efficacy only on certain tumour types, and endostatin may not be enough to starve rapidly growing tumours (Indraccolo et al., 2002; Joseph et al., 2003). A series of studies found a strong anti-proliferative effect of viruses-mediated angiostatin and endostatin gene transfer *in vitro* and significant anti-tumour effects in cancer models (Scappaticci et al., 2001; Schmitz et al., 2004; Isayeva et al., 2005; Raikwar et al., 2005). Although these studies suggest that long-term expression of both inhibitors may be effective as a preventative approach against recurrent and cancer metastases, regression of established tumours even by the combined treatment remains elusive. Combination of anti-angiogenic therapy with conventional therapies including radiotherapy, chemotherapy may offer a more promising therapeutic efficacy (Persano

et al., 2007). Initial reports combining a vasculotoxic drug with anti-angiogenic gene therapy has shown encouraging results (Kerbel 2006).

VEGF is now believed to play an important role in the angiogenesis and growth of solid tumours. VEGF released by tumour cells binds to receptors on endothelial cells (ECs), initiating a signalling cascade that results in new blood vessel formation. VEGF binds to two distinct tyrosine kinase receptors, Flt-1 and KDR, producing different signal transduction properties (Neufeld et al., 1999). A variety of studies have shown that overexpression of VEGF and its receptors Flt-1 and KDR occurs in various types of tumours, in contrast to low levels in normal tissues. It has also been shown in many tumours, including mesothelioma, that the tumour cells themselves express VEGF receptors thus establishing an autocrine growth loop. Numerous strategies, including monoclonal antibodies e.g. Bevacizumab, have been developed to target this pathway as potential treatment adjuncts to current chemotherapy protocols. A multicentre phase II trial of the combination of gemcitabine and cisplatin with bevacizumab has been conducted in patients with malignant mesothelioma at the University of Chicago (Kindler et al., 2005). Although the clinical studies show statistically significant benefits when it is added to conventional chemotherapy, the absolute benefits are modest. There appears to be a significant degree of redundancy in the signalling cascades needed for angiogenesis, and the inhibition of a single molecule (i.e. VEGF) is insufficient to have a major effect. Intra-tumoural expression of VEGF is closely associated with tumour growth and metastasis. The soluble fragment of VEGF receptor Flt-1 (s-Flt-1) has anti-angiogenic properties because of its antagonistic activity against VEGF. The delivery of targeted sFlt-1 to cultured ECs has been shown to inhibit the

proliferation of the cells confirming that expressed sFlt-1 bound to exogenous VEGF and blocked the binding to the full length Flt-1 receptor (Kim et al., 2005).

VEGF appears to be important for the progression and prognosis of malignant mesothelioma (Ohta et al., 1999). It is an autocrine growth factor for mesothelioma as well as stimulating vessel formation (Strizzi et al., 2001). Patients with mesothelioma have significantly higher serum levels of VEGF than patients with any other solid tumour (Linder et al., 1998). VEGF expression in mesothelioma correlates with microvessel density and Flt-1 expression (Konig et al., 1999; Ohta et al., 1999), and a high microvessel density is associated with poor survival in mesothelioma patients (Kumar-Singh et al., 1997; Ohta et al., 1999). These results provide evidence for the critical role of VEGF signalling in angiogenesis and solid tumour growth, and suggest that inhibitors of VEGF may have therapeutic benefit in mesothelioma patients. Recent studies have demonstrated promising results using the Flt-1 promoter in gene therapy. Moreover, the Flt-1 promoter exhibits a "liver off" phenotype when used in adenoviral vectors, an important attribute for reducing toxicity to this organ, which has a high propensity to take up Ad vectors that reach the systemic circulation (Nicklin et al., 2003). A study has shown the improvement of therapeutic efficacy of anti-VEGF receptor gene therapy in an experimental prostate cancer model. The novel strategy combines tumour cell-specific killing using a conditionally replicating oncolytic virus, termed Ad-hOC-E1, with tumour endothelium-specific targeting using an Ad vector secreting Flt-1, which is a soluble form of the VEGF receptor and behaves as a VEGF neutralizing agent (Jin et al., 2005). It is proposed that this combination of targeted anti-angiogenesis therapy may be an efficient tool for the treatment of cancer.

New gene therapy approaches are being designed around molecular features of tumour angiogenesis. The tumour vasculature is abnormal and when ECs form tumour blood vessels, ECs differ structurally and phenotypically from the normal vasculature. Compared to normal vessels, tumour vessels are characterized by greater permeability, the presence of fenestrate, transcellular holes and the loss of the basement membrane (Carmeliet et al., 1996; Mcdonald et al., 2000). New technologies including cDNA microarray and SAGE (serial analysis of gene expression) have been used to identify novel markers of angiogenesis (St Croix et al., 2000). It was reported that ECs undergoing angiogenesis had augmented expression of integrins and aminopeptidase N, which may be new receptors that bind specifically to tumour-associated ECs (Bhagwat et al., 2001; Kumar 2003). More knowledge about the molecular differences between tumour angiogenesis and normal angiogenesis (e.g. in wound healing) will help in the development of more specific therapies.

1.2.4 Immune Responses in Gene Therapy

1.2.4.1 Immune Responses

The success of gene therapy relies largely on gene delivery systems with less toxicity and immunity, high efficiency in gene transfer and the therapeutic gene expression in the targeted cells or tissues (Zhou et al., 2004). To date, one of the challenges of gene therapy is to understand the interaction of gene delivery vectors with host immune systems in order to improve the safety and effectiveness of these vectors.

The non-specific innate immune response develops promptly after a vector or transgene antigen penetrates the body (Janeway et al., 1997). The early phase of the host immune response is caused by neutrophils, macrophages and natural killer (NK) cells. The adaptive immune response including humoral response and cell-mediated response is fully activated later than the innate response. T lymphocytes mediated cellular immune responses and specific humoral immune responses are important parts of the challenges in gene therapy. The adaptive immune response can lead to elimination of the vector and infected cells from the body, and also recall a memory response upon re-infection of the same vector or transgene (Schagen et al., 2004). Viral vectors, which express immunogenic epitopes within the organism, are the most likely to induce an immune response. The immune response induced against viruses may be beneficial when the goal is vaccination or tumour lysis as is the case in the development of mesothelioma therapy. However, in most non-cancer applications the immune response is unwelcome and even in cancer therapy a balance must be struck between any potential helpful effect of the induced inflammation and the negative effects of host toxicity and possible premature loss of therapeutic genes and viral agents (Bessis et al., 2004).

1.2.4.2 Immune Responses Against Adenoviral Vectors

The immune responses against adenoviral vectors include both the innate and adaptive responses (Zhou et al., 2004). First-generation Ad vectors induce strong innate responses and adaptive responses (Bessis et al., 2004). It was reported that 90% of the viral genome was eliminated from the liver within the first 24h, after intravenous administration of a first-generation Ad vector to immunocompetent and immunodeficient mice due to viral uptake in the phagocytic Kupffer cells that line the

hepatic sinusoids (Schagen et al., 2004). This problem affects all current generation Ads because the capsid proteins in these vectors induce an obvious innate response rapidly after viral entry (Bessis et al., 2004). The adaptive response is far weaker with third-generation vectors however, which are deleted of all viral genes and thus do not suffer from the additional inflammatory stimulus of native viral gene expression.

The innate immune response to adenoviral vectors is dose-dependent and induced by the viral particles or capsid independent of viral replication or gene expression (Zhang et al., 2001; Liu et al., 2003; Brunetti-Pierri et al., 2004). Innate immune responses are triggered when pattern recognition receptors (PRR) recognise specific conserved patterns on pathogens, such as the adenovirus capsid. Toll-like receptors (TLRs), which are expressed primarily by macrophages and dendritic cells (DCs), are the most extensively studied PRR (Hensley et al., 2007). TLRs can recognise components of bacteria, viruses, and protozoa in addition to fungi, and regulate the synthesis of both antiviral type 1 interferons (IFN) and pro-inflammatory cytokines (Wang et al., 2007). These cytokine responses are important in controlling adenovirus replication and also provide an initiation signal for the adaptive immune response. Importantly however, activation of TLRs may have some benefits in cancer therapy. There are several studies now that show that activation of TLRs for example by poly I:C, CpG motifs etc can enhance an anti-tumour response (Lund et al., 2003; Krug et al., 2004). However, not all innate immune responses to Ad vectors are triggered by TLRs, and there are also other non-TLR pattern recognition receptors that are activated by Ad vectors (Hensley et al., 2005; Nociari et al., 2007; Zhu et al., 2007).

Specific immune responses leading to antibody production and T lymphocytes activation also occur within a few days after vector introduction. Adenoviruses induce infiltration by CD4+ and CD8+ T cells (Kafri et al., 1998; Molinier-Frenkel et al., 2000). In general, antigen-presenting cells (APCs), such as dendritic cells and macrophages, activate the cellular immune response towards Ad antigens (Schagen et al., 2004). These APCs directly present the viral antigens to CD4+ and CD8+ T cells, generating a cytotoxic response to the vectors. The humoral immune response is initiated by the binding of adenovirus particles to the surface immunoglobulin of B cells (Janeway et al., 1997). This results in CD4+ T-cell activation and induces differentiation of B cells to plasma cells. Antibodies produced by plasma cells are specific for viral capsid proteins. These antibodies can prevent infection by the vectors during subsequent gene therapy attempts. In humans, pre-existing immunity also develops in response to natural infections with wild type Ad. These antibodies can neutralize transduction by Ad vectors *in vivo* (Smith et al., 1996), activate the complement system, and induce an inflammatory response in the presence of Ad (Cichon et al., 2001). Clinical studies in which direct tumour injection of viral vectors have been used have interestingly not shown a close correlation between antibody levels and therapeutic effect, but this is more likely to be a problem if vector is planned to be administered systemically. Attempts to circumvent pre-existing antibodies include the use of serotypes of Ad as vectors that are less commonly encountered naturally, for example Ad serotype 35, or even the use of non-human Ads such as the ovine (sheep) adenovirus (Both 2004). As discussed in an earlier section, structural modification of Ad serotype 5 can also circumvent neutralising antibodies. Transient immunosuppression at the time of vector administration has also been proposed as a strategy to reduce early immune responses. With regard to mesothelioma, the Albelda

group administered systemic corticosteroids to a small group of subjects prior to the intrapleural administration of an Ad vector containing the HSV-TK gene. Decreased clinical signs of inflammation were seen compared to subjects not given steroids, but there was no increase in the efficiency of gene transfer (Sterman et al., 2000). Apart from Ad-specific Abs, neutralizing Abs might also be generated against the transgene product.

Despite the above concerns, the immune response may not always work against the desired goal of achieving destruction of a particular cell type especially tumour cells. The stimulation of host-dependent antitumour immune responses is potentially beneficial for cancer therapy since it appears to induce immune reactions to both gene-modified tumour cells and their non-modified counterparts (Tang et al., 1993). This has the advantage of producing an effect in distant organs and may be advantageous in metastatic cancer.

The role of inflammatory and immune responses in mediating the antitumour effects associated with viral replication within tumours in human patients is not fully understood. Intratumoural viral replication might induce or augment the development of antitumoural immunity (Gomez-Navarro et al., 2000). In animal models, there is confirmation of cell-mediated immunity to tumour cells infected with a replicating herpes virus, but there is no published evidence obtained with adenoviral vectors. The investigation of this issue has been hampered by the fact that human serotype Ads do not replicate well in mouse cells, thus the use of replicating agents has focused on human tumour xenografts in immunodeficient mice. Further studies need to be done in immunocompetent animal models.

1.2.5 Conditionally Replicative Viruses

1.2.5.1 Conditionally Replicative Adenoviruses (CRAds)

Conditionally replicative adenoviruses (CRAds) are being developed as potential agents in the treatment of cancer. CRAds replicate only in tumour cells and destroy these cells through the natural process of adenoviral replication. In addition, the generated progeny viruses released from infected and lysed tumour cells may infect neighbouring tumour cells. The tumour will ultimately be destroyed via several rounds of replication and cell lysis (Figure 1-7)(Hedley et al., 2006). Two main approaches to engineer a conditionally replicative adenoviral vector have been evaluated (Alemany et al., 2000). The first strategy is to mutate or delete genes that are essential for replication of the virus in normal cells. In most cases, this involves deletions in the E1 region. The second is to control the expression of the viral genes to tumour cells through the use of tumour specific promoters. In this way, the viral replication is limited to the tumour cells, so as not to cause unnecessary toxicity to normal tissues. Both CRAds-based strategies have been rapidly translated into clinical trials.

The Ads with deletion of the *p53*-binding protein E1B-55kD or the retinoblastoma (pRb) binding site of the protein E1A are examples of limiting viral replication by deletions in viral genes (Bischoff et al., 1996; Fueyo et al., 2000). By far, the most extensively studied CRAd is the mutant ONYX-015 (also known as dl1520) that contains a deletion in the E1B-55kD gene. A key requirement for adenoviruses to replicate is the inactivation of *p53*. One of the functions of E1B-55kD is to bind and inactivate the tumour suppressor protein *p53*. In the absence of this gene, the actions of

Figure 1-7

CRAd infection of cancer cell

Re-infection of cancer cells

Tumor–specific replication and cancer cell lysis

CRAd dispersion
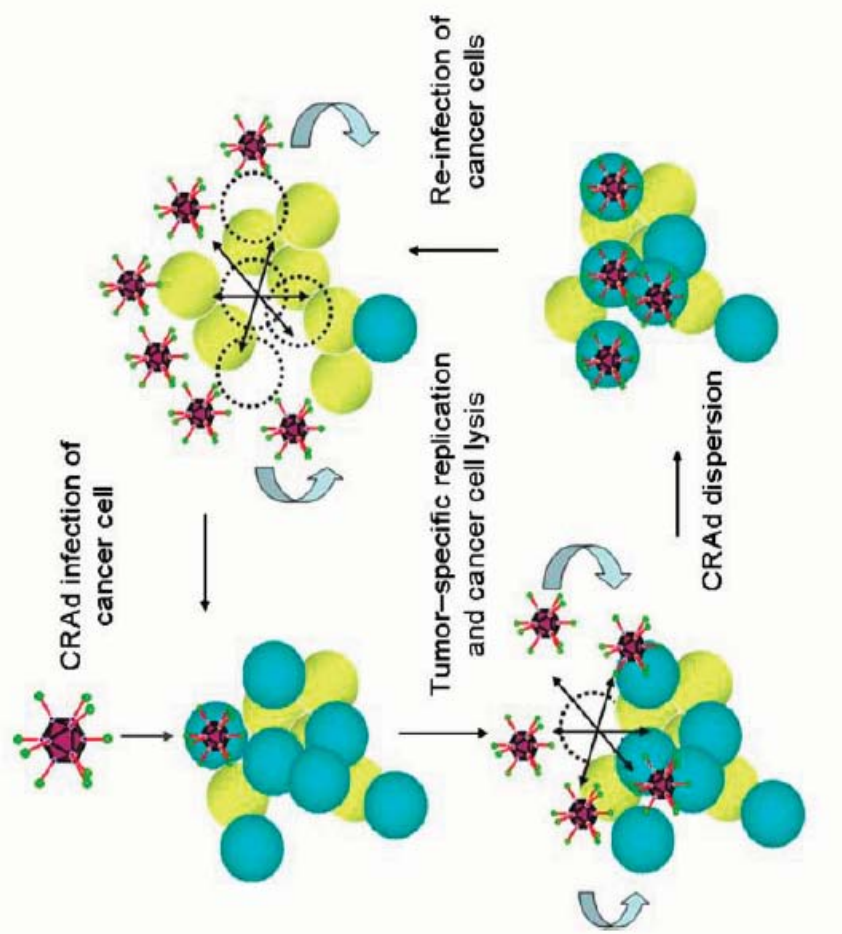
p53 would lead to apoptosis of the cell, and thus prevent efficient virus replication and subsequent spread. However, because *p53* is mutated in many common tumours; viral inactivation of *p53* is not required. Therefore, the mutant adenovirus ONYX-015 would replicate selectively in tumour cells (Bischoff et al., 1996). This study has reached phase I and II clinical trials for head and neck, pancreatic, ovarian, colorectal, lung, and oral carcinomas (Khuri et al., 2000; Mulvihill et al., 2001; Nemunaitis et al., 2001; Reid et al., 2001; Vasey et al., 2002; Rudin et al., 2003). Overall, these clinical trials have demonstrated that ONYX-015 is safe even when repeated high-level doses were injected, and provided evidence of virus replication and tumour necrosis *in vivo*. However, the principle of ONYX-015 replicating only in *p53*-defective cells has been questioned (Harada et al., 1999; Hay et al., 1999; Vollmer et al., 1999). The recent view on ONYX-015 specificity is that this virus replicates in cells with dysfunctional *p53*, regardless of the genetic defect causing this dysfunction. Moreover, oncolytic capacity of this virus is severely attenuated compared to wild type adenovirus probably due to the late virus mRNA transcription function of the missing E1B-55kD protein (Vollmer et al., 1999). Hence, the progression of second generation of E1B-55kD CRAds with improved tumour selectivity has continued (Davis et al., 2005). A second promising type of CRAd, known as AdΔ24 or its counterpart, dl922-947, carries a 24bp deletion in the adenoviral E1A gene at the binding site of the retinoblastoma gene (Rb) product. *Rb* normally binds to and inhibits E2F, a strong division-inducing transcription factor. Binding of *Rb* and release of E2F is needed for Ad replication in normal cells. In tumour cells, *Rb* is often already mutated, thus the *Rb* binding function is dispensable in these cells. AdΔ24 can only replicate in *Rb* mutant tumour cells, not in normal quiescent cells where E2F is bound to Rb. In *Rb* mutant tumours AdD24 can lyse both dividing and non-dividing tumour cells with great efficiency (Fueyo et al., 2000) and dl922-947

was shown to be effective in a range of tumour types *in vitro* and *in vivo* (Heise et al., 2000). However, although dl922-947 was attenuated effectively in quiescent normal cells, it did replicate in proliferating normal cells (Heise et al., 2000). In order to improve tumour specificity, Ad5-Δ24RGD, an infectivity-enhanced variant of AdΔ24 was constructed in an effort to improve the therapeutic window (Suzuki et al., 2001). Ad5-Δ24RGD contains a cyclic RGD peptide motif in the fibre HI-loop. This CRAd was found very effective in killing a variety of tumour models including glioma, ovarian and cervical cancers (Lamfers et al., 2002; Lam et al., 2003; Bauerschmitz et al., 2004). These studies demonstrate great promise for the development of CRAds that can achieve safe, selective, and effective tumour eradication. However, the limitation of the humoral response still needs to be studied (Hedley et al., 2006).

The second strategy to engineer a CRAd is the introduction of tissue or tumour specific promoters to control the expression of the viral genes essential for replication, such as E1A and E1B, to tumour cells. The first CRAd of this type is CV706 in which prostate specific antigen (PSA) promoter drives the expression of adenovirus E1A gene (Rodriguez et al., 1997). Other promoters have been employed to develop CRAds that specifically replicate in other types of cancer, such as α-fetoprotein promoter (AFP) in liver cancer (Hallenbeck et al., 1999), the MUC1 promoter in breast cancer (Kurihara et al., 2000), surfactant protein B promoter in lung cancer (Doronin et al., 2001). All these CRAds showed special replication in their target cells and demonstrated anti-tumour activity in preclinical models. However, tissue specific promoters for many different human cancers have not been characterized yet. Moreover, the promoters used are primarily tissue-specific rather than tumour-specific. CRAds relying on these promoters might cause toxicity to normal tissues. Recently, it has been reported that a novel

tumour-specific promoter, the survivin promoter, exhibits selective high activity in tumour cells and low activity in liver (Zhu et al., 2006). The viral infectivity of survivin-based CRAds is enhanced by incorporating a capsid modification (RGD in HI loop of Ad5 knob or use of the knob region of Ad serotype 3 F/3). The study indicates that the CRAd-S.F5/3 is an excellent candidate that can be translated into a clinical trial for treatment of human malignant mesothelioma.

Promoter – based CRAd strategies have also been developed based on more universal physical or other properties of tumours. For example, all solid tumours create an environment with low oxygen tension (hypoxia), so AdEHT2 was constructed, in which a promoter that contains hypoxia responsive elements (HREs) and estrogen responsive elements control E1A expression (Hernandez-Alcoceba et al., 2002). AdEHT2 can activate E1A expression by hypoxia in different cancer cell lines or by estrogens in estrogen receptor expressing cell lines (eg breast cancer), thus increasing the CRAds potential utility. Since tumours have high expression of telomerase reverse transcriptase (TERT), a TERT-specific CRAd could be efficacious against a wide variety of tumours and less cytotoxic to normal cells (Huang et al., 2003; Wirth et al., 2003). The growth of solid tumours requires new blood vessel formation. A number of genes, including *Flk-1* and *endoglin*, are over-expressed in angiogenic endothelial cells. Both AdFlk-1 with E1A under the control of the *Flk-1* promoter, and AdFlk-Endo with E1B under the control of the *endoglin* promoter replicate efficiently in human umbilical vein endothelial cells (HUVEC), not *Flk-1* and *endoglin* negative cells (Savontaus et al., 2002).

To date, clinical trials data with CRAds have demonstrated that these agents are safe but efficacy is poor (Young et al., 2006). It is now clear that convincing clinical responses have been observed only when virotherapy is combined with other approved therapeutic modalities (Khuri et al., 2000; Deweese et al., 2001). Several approaches have been employed to enhance anti-tumour efficacy. One approach is to incorporate enzyme pro-drug strategies or immunomodulatory genes into CRAds (Hermiston et al., 2002). For example, a cytosine deaminase/thymidine kinase (CD/TK) fusion gene was engineered into ONYX-015-based adenoviruses (Ad5-CD/TKrep). In two phase I clinical trials of prostate cancer, the prodrugs (5-fluorouracil and GCV) were administrated on day 3 after intraprostatic injection of Ad5-CD/TKrep (Freytag et al., 2002). In the second trial, conventional conformal radiotherapy was given during prodrug administration (Freytag et al., 2003). No dose-limiting toxicities were observed and prostate-specific-antigen (PSA) reductions were demonstrated in combination with prodrugs and/or radiotherapy in either trial. Incorporation of cytotoxic ligands (eg TNF, TRAIL) or immunostimulatory genes is another approach to enhance anti-tumour effect of CRAds (Hermiston et al., 2002; Ries et al., 2004; Bernt et al., 2005; Ren et al., 2006). This approach may provide more impressive results particularly when combined with conventional approaches.

1.2.5.2 Non-CRAd Replicative Agents

Since the first engineered replicative-selective virus to be used in humans, at least six different species have been translated into clinical trials. Newcastle disease virus (NDV) has anti-tumour effects in a range of cancers including glioma, colorectal cancer, and breast cancer (Pecora et al., 2002; Csatary et al., 2004). A phase I trial was conducted

with the NDV strain PV701 (Wellstat Biologics, Gaithersburg, MD) in patients with metastatic solid tumours. Dose-limiting toxicities were noted, and long-term intravenous NDV treatment is well tolerated. In a study, the NDV strain HUJ was administered intravenously to patients with glioblastoma with no dose-limiting toxicity, and infectious NDV was recovered from a tumour biopsy after 3 months maintenance therapy (Freeman et al., 2006). Reovirus is prevalent in the human population but not associated with any known human disease. Studies have shown that reovirus multiplies in tumour cells with activated gene of ras family or ras-signalling pathway while sparing normal cells (Strong et al., 1998; Figova et al., 2006). Data from phase I trials have shown no dose-limiting toxicities when intratumoural injection of reovirus in patients with glioma or prostate cancer. Attenuated strains of other viruses such as vaccinia virus, poliovirus, and measles virus have shown tumour selective oncolytic effects in pre-clinical models (Gromeier et al., 2000; Russell 2002; Zeh et al., 2002). Genetic modification of non-CRAds replicative agents to enhance anti-tumour efficacy and modulate antiviral immune responses may be employed for future clinical trials.

1.3 Matrix Metallo-proteinases (MMPs)

The clinical trials of CRAds to date have been limited more by inadequate efficacy than by toxicity. Spread of adenoviruses through tumour masses is limited by a number of issues including relative lack of viral receptors on cancer cells and by factors relating to the host's immune system. However even tumours composed of highly susceptible tumour cells in immunocompromised mice often fail to respond adequately to viral therapy. Thus, physical properties of the tumour, such as hypoxia, fibrosis and necrosis may also be an impediment. As discussed, newer approaches have sought to combine the action of CRAds with standard chemotherapy agents and radiotherapy (Lamont et al., 2000). A complementary strategy would be to devise a CRAd system that would not only have specificity for target tumour cells, but would enhance viral spread throughout a tumour mass. Improvements could possibly be achieved by expressing a gene that leads to the production of a secreted factor that breaks down fibrotic barriers. Ideal candidate molecules for this purpose are the MMP family that collectively have the capacity to break down all components of the extracellular matrix. Among the many MMPs that have been identified, MMP-2 and MMP-9 are thought to be key enzymes because they degrade type IV collagen, the main component of ECM (Stamenkovic 2003). Gene delivery for MMP-9 has already been achieved using Ad vectors (Baker et al., 1996). Therefore, the co-delivery of CRAds with MMP-9 might improve therapeutic efficacy by reducing tumour-associated fibrosis thereby enhancing viral spread through a tumour mass.

1.3.1 Extracellular Matrix (ECM)

The extracellular matrix (ECM) plays a key role in tissue architecture and homeostasis (Stamenkovic 2003). The ECM is a framework of proteins and proteoglycans secreted by and surrounding stromal fibroblasts. It is secreted locally and assembles into a network in the spaces surrounding cells. The ECM's main components are various glycoproteins, proteoglycans and hyaluronan. ECM also contains many other components: proteins such as fibrin, elastin, laminins, and various growth factors. In most organs, the most abundant glycoproteins in the ECM are collagens. Two major functions of proteoglycans are the support of cell adhesion and the binding of a host of latent growth factors (Iozzo 1998). Hyaluronan participates in the regulation of numerous cellular functions, prominent among which are adhesion, trafficking, and signalling (Laurent et al., 1992). ECM can serve many functions, such as providing support and anchorage for cells, providing a way of separating the tissues, and regulating intercellular communication. The ECM regulates a cell's dynamic behaviour. The ECM changes the structure and functions in response to a host of cellular stimuli. ECM remodelling is the result of multiple concurrent processes according to the initiating stimulus. Remodelling minimally requires two events: synthesis and deposition of ECM components on the one hand and their proteolytic break down on the other. Numerous proteases are involved in the proteolytic degradation of the ECM, most prominent among which are members of the matrix metalloproteinases (MMPs) family.

1.3.2 Matrix Metalloproteinases (MMPs)

The matrix metalloproteinases (MMPs) are a family of zinc-dependent neutral endopeptidase that regulate the movement of cells within the ECM and also have the ability to degrade all of its components (Hidalgo et al., 2001; Ohbayashi 2002). They are essential regulators of the cell's microenvironment through their control of extracellular proteolysis (Hojilla et al., 2003). The balance and regulated degradation of ECM proteins by MMPs is involved in many physiological processes including wound healing, tissue remodelling, angiogenesis, and embryo development (Sternlicht et al., 2001). However, recently *in vivo* studies of MMP function have shown that MMP also cleave numerous non-ECM protein substrates (Fu et al., 2007). At present, MMPs comprise a family of 25 related, yet distinct vertebrate gene products, of which 24 are found in mammals (Ra et al., 2007). MMPs have been identified and can be grouped into five subclasses according to their primary structure and substrate specificity: collagenases (MMP-1, MMP-8, MMP-13, and MMP-18), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, MMP-11), membrane-type MMPs (MT-MMPs) and others including metalloelastase (MMP-12) and Matrilysin (MMP-7) (Ohbayashi 2002). The general structure of the MMPs includes a signal peptide, a propeptide domain, a catalytic domain with a highly conserved zinc-biding site, and a haemopexin-like domain that is linked to the catalytic domain by a hinge region (Hidalgo et al., 2001)(Figure 1-8)(Purcell et al., 2002). In addition, the gelatinases (MMP-2 and MMP-9) contain a fibronectin type II within the catalytic domain, and MT-MMPs contain a transmembrane domain at the haemopexin-like domain. The haemopexin domain is absent in the smallest member of the MMP family, MMP-7 (Fu et al., 2008).

Signal Peptide | Propeptide | Fibronectin type II inserts | Zn^{++} | Hinge region | Haemopexin domain | Transmembrane domain
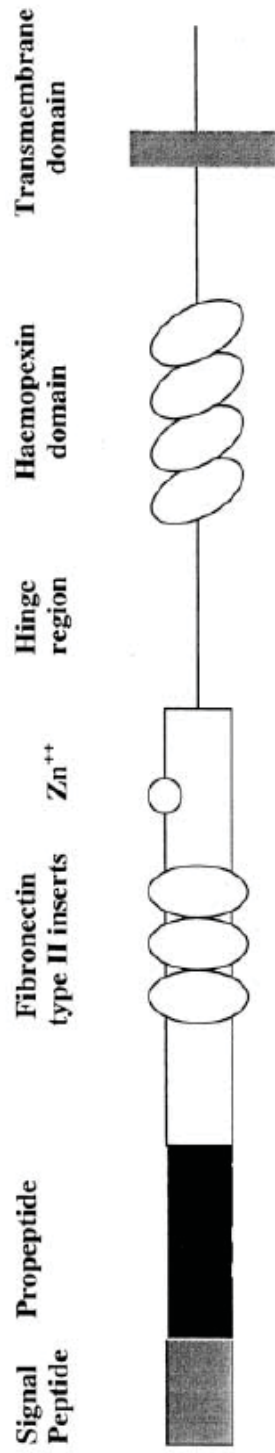
Fig.1-8 General structure of the matrix metalloproteinases (*Modified from* Kähäri and Saarialho-Kere, 1999; with permission.)

Adapted from Purell WT, et al. Hematol Oncol Clin N Am, 2002;16(5):1189-227

1.3.3 Regulation of MMPs

The biological functions of the MMPs are regulated highly at the level of gene expression and protein activation. Regulation of MMPs occurs on four points: alteration of gene expression, activation of latent zymogens, and inhibition by inhibitors of metalloproteinases (Stamenkovic 2003). In general, the MMP genes are expressed at low levels or not at all *in vivo* (Stamenkovic 2003). However, numerous cytokines (IL-4, IL-10) and growth factors (transforming growth factor TGF, epidermal growth factor EGF, VEGF) as well as physical cellular interactions provide stimuli that can rapidly induce MMP expression (Stamenkovic 2000). Regulation is also dependent on the activation of the zymogen/proenzyme-secreted form of MMPs. The pro-MMPs are secreted in their inactive form and required cleavage to a truncated form to become active (Ohbayashi 2002). The interaction, which is between a cysteine residue located in the propeptide portion of the molecule and the catalytic zinc atom, blocks access to the active site and cleavage of this site results in enzyme activation. Activated MMPs are capable of activating other MMPs (Murphy et al., 1999). The third level of control of MMPs occurs through inhibition of enzymatic activity by non-specific protease inhibitors including α2-macroglobulin and α1-antiprotese, and the specific tissue inhibitors of the metalloproteinases (TIMPs) (Kahari et al., 1999). The most thoroughly studied MMP inhibitors are TIMPs. The TIMPs are a family of four structurally related proteins (TIMP-1, -2, -3, -4), which exert a dual control on the MMPs by inhibiting both the active form of the MMPs and their activation process.

1.3.4 MMPs and Cancer

Increased levels of MMPs are seen in cancers with a high metastatic potential, which relates to their role in improving cell mobility. For this reason inhibitors of MMPs have been developed as potential anti-cancer agents. These inhibition strategies include pharmaceutical agents (eg Marimastat), antisense approaches, and use of TIMPs. With regard to the latter a number of preclinical studies have shown that gene delivery of TIMPs 2 and 3 in particular are capable of reducing tumour growth. To date however, clinical studies of pharmaceutical MMP inhibition have not shown much efficacy and they have not become established therapies. Based on the foregoing however, the notion of using MMP-9 expression as an agent to improve viral spread could have adverse consequences by actually enhancing tumour metastases. The impact of MMP-9 transient gene delivery on tumour metastases as described herein has not yet been assessed.

1.4 Summary

Mesothelioma is a deadly disease that is increasing in frequency. Currently available treatments are inadequate and median survival remains poor at around 12-14 months even with current "state-of-the–art" therapies. Clearly, new treatment options are needed. There has been a great deal of interest in gene and viral based therapies in recent years, with some promising developments but none of sufficient utility to enter standard clinical practice. Mesothelioma is an ideal target for replicating viral therapies in view of its propensity to remain localised to the pleural space until late in the disease. Most of the developmental work in gene and viral therapy has been based on the adenovirus, which has many attractive properties, although other agents are now emerging. However, replicating viruses alone have proven inadequate and further complementary strategies are needed.

In this thesis I have taken several steps in an effort to advance the development of viral therapy for mesothelioma. These steps include:

The development and evaluation of new CRAd agents based on promoters relating to the angiogenic pathway (VEGF and Flt-1) with optimised tropism.

Evaluation of CRAd efficacy in combination with an approach to break down extracellular matrix.

Identification and characterisation of an immunocompetent murine model of mesothelioma to provide a platform for the evaluation of CRAd and immunotherapy.

To evaluate the combination of replicating viral therapy in combination with CD40 activation in the above model.

To evaluate an alternate emerging replicating viral agent, attenuated measles vaccine as a candidate therapy for mesothelioma.

Chapter 2. Material and Methods

2.1 Materials

2.1.1 Cell Lines and Cell Culture

The human embryonic kidney cell line 293 was used in the amplification and titration of adenoviral vectors. This cell line is an E1 transcomplementing line developed by Frank Graham (Graham et al., 1977; Krougliak et al., 1995) to enable the production of E1 deleted adenoviruses. These cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM): F12 medium (50:50) (Gibco BRL Life Technologies, Grand Island, NY, USA), supplemented with 10% foetal bovine serum (FBS) (Trace Biosciences, Melbourne, Vic, Australia), with supplemental 2 mM L-glutamine, penicillin G (100u/ml) and gentamycin (50μg/ml)(Gibco BRL Life Technologies, Grand Island, NY, USA). A549 lung cancer cells were maintained in DMEM with bicarbonate, and supplements as described above.

Human mesothelioma cell lines included Ju77, ONE, H2596, H2052, H226, Lo, H28, H2373, MSTO, and murine mesothelioma cell lines included AE17, AE1, AE1750, AB1, and MM6. These 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 for human mesothelioma cell lines or 5% FBS for murine mesothelioma cell lines.

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured in M199/Earle's balanced salts medium (JRH Biosciences, Lenexa, KA, USA) with 20% FBS and supplemented as described (Gamble et al., 1993), and were used at passage 4 or less. Human bronchial epithelial cells BEAS-2B were cultured in Keratinocyte serum free medium (Gibco BRL Life Technologies, Grand Island, NY, USA), supplemented with bovine pituitary extract, recombinant epidermal growth factor, 2 mM L-glutamine, penicillin G (100u/ml) and gentamycin (50µg/ml)(Gibco BRL Life Technologies, Grand Island, NY, USA). All cells were maintained in a 5% CO_2 -atmosphere at 37°C.

2.1.2 Bacterial Culture

Escherichia coli (E.coli) strain DH5α were propagated in 1x LB (Lysogeny broth) medium or on plates containing 1x LB and 15g/L bacto-agar at 37°C for 12-18 h. Transformed DH5α were grown in 1x LB containing 100 µg/ml ampicillin (Boehringer Mannheim, Mannheim, Germany) or on 1x LB and 15g/L bacto-agar plates containing 100 µg/ml ampicillin. Bacterial cultures were stored in LB containing 15% glycerol at -70°C.

Competent DH5α cells were prepared by growing DH5α to log phase growth (OD_{600} ~ 0.6) in 50 ml LB broth. Cells were pelleted at 6000 rpm in JA-20 rotor (J2-21M/E centrifuge, BECKMAN) for 10 minutes (min) at 4°C and suspended in 25 ml ice-cold 0.1 M $MgCl_2$ followed by the same centrifugation condition as above. The pellets were resuspended in 12.5 ml ice-cold 0.1M $CaCl_2$ and incubated on ice for 20 min. Cells were then collected using the same centrifugation method and resuspended in 1.6 ml

ice-cold transformation buffer (10 ml 1M CaCl$_2$, 20 ml 80% glycerol, 70 ml H$_2$O). Cells were aliquoted in 200 μl volumes and stored at -70 °C.

BJ5183 are electrocompetent *Escherichia coli* cells used in the AdEasy^{TM} system for homologous recombination. BJ5183 cells are not *rec*A mutants but deficient in other enzymes that mediate recombination in bacteria (genotype: *end*A, *sbc*B$^-$, *rec*BC$^-$, *str*R) (Hanahan et al., 1984), and have been chosen because of their ability to efficiently generate stable homologous recombinants. To prepare electrocompetent BJ5183 bacteria, the cells were grown to an OD$_{550}$ ~ 0.8, then collected and washed twice with ice-cold 10% glycerol. Twenty-microliter aliquots of the electrocompetent BJ5183 cells were kept at -70 °C.

2.1.3 Plasmid Vectors

The pMV10-Flt-1 is a 8.5kb plasmid and constructed by Dr Stuart A. Nicklin (University of Glasgow, United Kingdom). Flt-1 promoter was cloned into the *Sph*I/*Xba*I site of pMV10 upstream of LacZ and the CMV polyadenylation signal.

The pshuttleCMV is an adenovirus transfer vector containing a multiple cloning site in which a cloned gene is under the control of the human CMV promoter for constitutive recombinant expression in a wide variety of cell lines. Upon homologous recombination in bacteria with a plasmid containing the ΔE1-ΔE3 adenovirus serotype 5 genome, a new plasmid is generated in which the expression cassette is inserted into the original E1 region of the Ad genome. This resulting plasmid is then transfected into 293 cells to

generate a recombinant adenovirus expressing the gene of interest under the CMV promoter.

The pAdEasy-1 (kind gift of Dr Koichi Takayama, Kyushu University Hospital, Japan) is a 33.4kb plasmid containing the adenovirus serotype 5 (Ad5) genome with deletions in the E1 (Ad5 nucleotides 1-3533) and E3 (Ad5 nucleotides 28,130-30,820) regions. Upon homologous recombination in bacteria with a transfer vector, in which a gene cassette of interest has been cloned, a new plasmid is generated with the expression cassette inserted into the E1 region of the adenovirus genome. The AdEasy-1 derived recombinant adenoviruses can be propagated in E1-expressing packaging cells, such as 293 or 911 cells. This plasmid contains a copy of the ampicillin resistance gene as well as the pBR322 origin of replication.

Both small-scale DNA and large-scale preparation of plasmid DNA from bacteria were performed using Qiagen Plasmid Extraction Kits (Qiagen Inc, Valencia, CA, USA).

Figure 2-1 Maps of Vectors
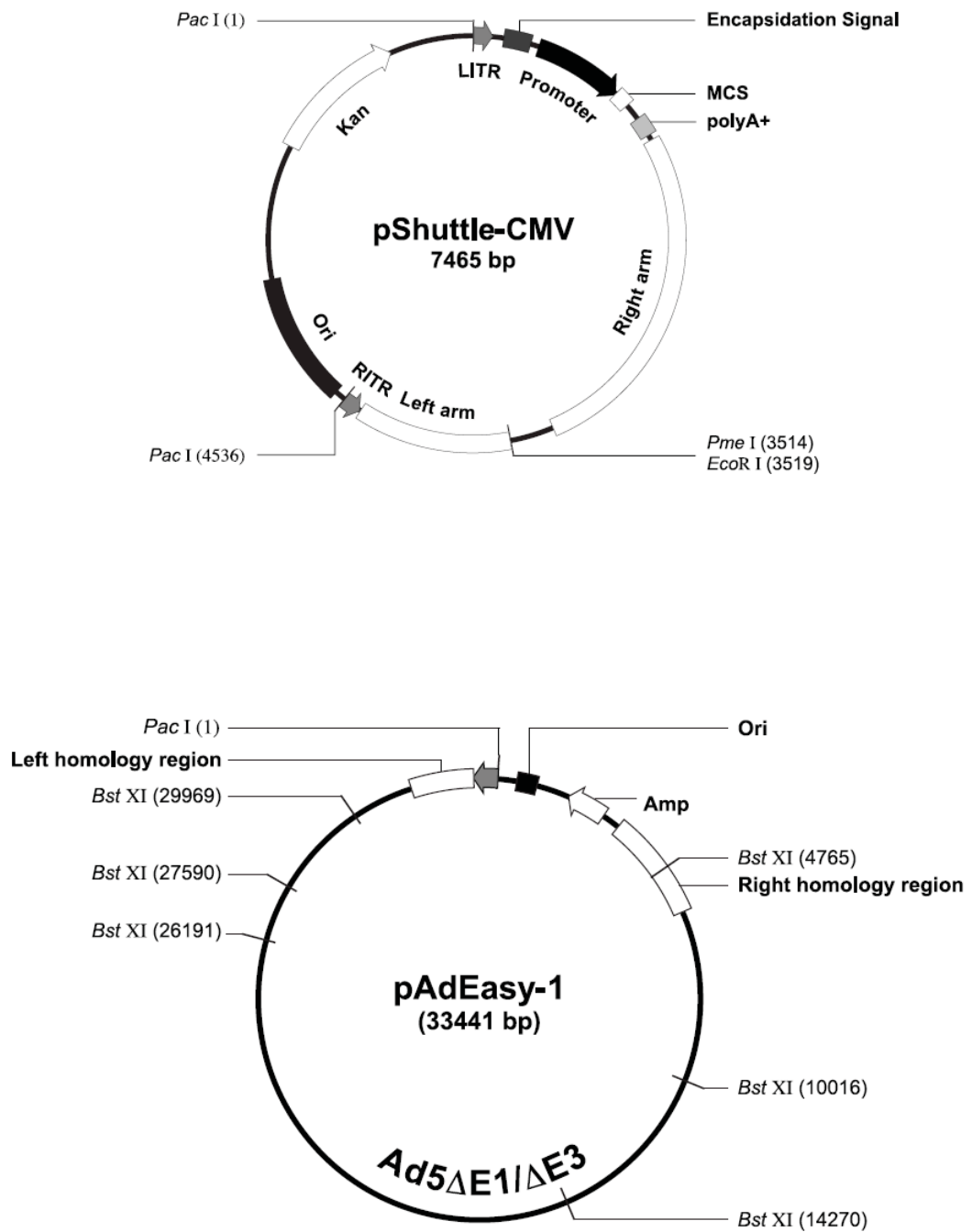
2.1.4 Oligonucleotide Sequences

All oligonucleotide sequences used in this project were synthesised by GeneWorks (GeneWorks Pty Ltd, Hindmarsh, SA, Australia). Oligonucleotide pellets were resuspended at the required concentration in sterile water. The sequences and coordinates of the oligonucleotide primers used in this study are listed in Table 2-1.

Table 2-1 Oligonucleotide sequences of primers used in this study

Name	Sequence	Region specific
Seq.Flt-1 Ad F	5'-agcagaagactgaggaaatgac -3'	Flt-1 in AdFlt-1E1
Seq.Flt-1 Ad R	5'-tccagccaggagacaaccac-3'	Flt-1 in AdFlt-1E1
Flt-1&E1 F	5'-aagtggttgtctcctggctgga -3'	Flt-1 &E1 in AdFlt-1E1
Flt-1&E1 R	5'-atcagccagtacctcttcgatc-3'	Flt-1 &E1 in AdFlt-1E1
E1F	5'-tatacccggtgagttc-3'	wt E1
E1R	5'-atcagccagtacctcttcgatc-3'	wt E1
Flt-1 mRNA F	5'-tgcttgaaaccgtagctgg-3'	Flt-1 mRNA
Flt-1 mRNA R	5'-tttcttcccacagtccaac -3'	Flt-1 mRNA
VEGF mRNA F	5'-gaagtggtgaagttcatggatgtc-3'	VEGF mRNA
VEGF mRNA R	5'-cgatcgttctgtatcagtctttcc-3'	VEGF mRNA
Cyclophilin F	5'-ggcaaatgctggacccaacacaaa-3'	House-keeping gene
Cyclophilin R	5'-ctaggcatgggagggaacaaggaa-3'	House-keeping gene

2.1.5 Antibodies

Table 2-2 Antibodies used in this study

Antibodies	Purpose	Company
Rabbit polyclonal IgG Flt-1 (C-17): sc-316	Western Blotting	Santa Cruz Biotechnology, Inc, California, USA
Rabbit polyclonal IgG VEGF (147): sc-507	Western Blotting	Santa Cruz Biotechnology, Inc, California, USA
Rabbit anti-Actin (I-19): sc-1616	Western Blotting	Santa Cruz Biotechnology, Inc, California, USA
Anti-human MMP9 monoclonal antibody	Western Blotting	R&D Systems, Minneapolis, USA
ImmunoPure ® Goat anti-mouse IgG, (H+L), Peroxidase Conjugated (31430)	Western Blotting	Pierce Biotechnology, Rockford, IL,USA
ImmunoPure ® Goat anti-rabbit IgG, (H+L), Peroxidase Conjugated (31460)	Western Blotting	Pierce Biotechnology, Rockford, IL,USA
Murine anti CD40 FGK45 antibody	Animal	Western Australian Institute for Medical Research, Perth, WA
Mouse anti-adenovirus monoclonal antibody (MAB8052)	Immunohistochemistry	Chemicon International Company, Single Oak Dr,USA
Rat anti-mouse CD8 (MCA1768)	Immunohistochemistry	AbD Serotec, Oxford, UK
Biotinylated anti-rat IgG (H+L) (BA-9400)	Immunohistochemistry	Vector Laboratories, Burlingame, CA, USA
Mouse anti-CAR (RmcB 05-644)	Flow Cytometry	Upstate Cell Signal Solutions, Charlottesville, VA, USA
Mouse anti-human CD46 (555948)	Flow Cytometry	BD Biosciences, San Jose, CA, USA
PE-conjugated Rat anti-mouse (340270)	Flow Cytometry	BD Biosciences, San Jose, CA, USA
PE-conjugated Rat anti-mouse CD8a (553033)	Flow Cytometry	BD Biosciences, San Jose, CA, USA
Alexa Fluor® 647-conjugated anti-mouse CD3 (51-0032-80)	Flow Cytometry	eBioscience, San Diego, CA, USA

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

2.1.6 Patient Pleural Fluid

Patients with suspected malignant mesothelioma were tested by chest X-ray and CT scan. Immunohistochemistry is important for determining that the tissue of origin is mesothelial and that it is malignant mesothelioma, especially epithelial membrane antigen (EMA), expression on the luminal aspects of the tumour. Immunohistochemical analysis of biopsy samples was used to confirm the patients with malignant mesothelioma. The analysis was performed in the Tissue Pathology of the Institute of Medical and Veterinary Science (IMVS, Adelaide, Australia). Pleural fluid from a confirmed patient was centrifuged at 1100 rpm for 5 min and cell pellets were resuspended in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, penicillin G (100u/ml) and gentamycin (50µg/ml). Following tumour cell adherence to the tissue culture flask, non-adherent blood cells were removed with several tissue culture media washes. Immunohistochemical analysis of the cultured cells revealed positive staining with E29 antibody against epithelial membrane antigen (EMA). Normally, after 7-14 days proliferation of adherent cells could be seen. These cells were then used for experimental analysis.

2.1.7 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 and C57 Black6 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.

2.1.8 Commonly Used Buffers and Solutions

<u>2XBES-buffered solution</u>

50 mM N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid

280 mM NaCl, 1.5 mM $Na_2HPO_4 \cdot 2H_2O$

<u>Ceasium Chloride (CsCl) solutions</u>

CsCl 1.33   227.1 gm CsCl/ml in 5mM Hepes pH 7.8, makes up 500 ml

CsCl 1.45   304.5 gm CsCl/ml in 5 mM Hepes pH 7.8, makes up 500 ml

CsCl solution filter 0.2 microns sterilise and store at 4^oC.

<u>Cell lysis buffer</u>

137mM NaCl, 10% Glycerol, 1% Triton X-100, 10 mM Tris-Cl (pH 7.4)

Store at 4^oC and just before using add 1 protease inhibitor cocktail tablet (Roche Diagnostics, Germany) to 10 ml of lysis buffer.

<u>Dialysis buffer</u>

10% Glycerol, l0mM Hepes, 1mM $MgCl_2$ pH7.4

<u>DOC lysis buffer</u>

20% of Ethanol, 100 mM Tris pH 9.0, 0.4% Sodium deoxycholate

<u>Ethidium bromide stock solution</u>

10 mg/ml Ethidium Bromide (Sigma) dissolved in ddH_2O to reach a concentration 4 µg/ml and store at 4^oC in a dark bottle.

2x HEPES Buffered Saline

140mM NaCl, 1.5mM $Na_2HPO_4.2H_2O$, 50mM HEPES

LB (1x)

10 g/L Bacto-tryptone, 5 g/L Bacto-yeast extract, 10 g/L NaCl

Running buffer (5x): pH 8.3

Tris 15 g/L, Glycine 72 g/L, SDS 5 g/L

SDS gel loading buffer (6x)

0.35 M Tris-HCl (pH 6.8), 10.28% (w/v) SDS, 36% Glycerol, 5% β-mercaptoethanol,

0.03% bromphenol blue

TAE (1x)

40 mM Tris-acetate, 1 mM EDTA

TBS (1x)

50 mM Tris pH 7.4, 135 mM NaCl

TBST (1x):

TBS+0.1% Tween-20

TE buffer pH 9.0

1 mM EDTA, 10mM Tris pH 9.0

Transfer Buffer (1x)

48 mM Tris, 39 mM Glycine, SDS (0.0375% w/v), 20% Methanol

Virus lysis buffer

0.1% SDS, 10mM Tris-HCl (pH7.4), 1mM EDTA

2.2 Methods

2.2.1 Construction of Vector for Homologous Recombination in Bacteria

The pshuttleCMVE1 is a gift from Dr Yasuo Adachi (OSAKA University, Japan), which is a CMV promoter driven adenovirus E1 region (37329-6337) shuttle vector. The pshuttleCMVE1 was digested with *EcoR*V/*Xho*I to remove the CMV promoter and then blunt-ended. The Flt-1 promoter (-748 to +284) was excised from the plasmid pMV10-Flt-1 using *Hind*III and *Xba*I, blunt-ended, then ligated with pshuttleCMVE1, forming pshuttleFlt-1E1 (orientation confirmed by DNA sequencing). Ad5(5/3)VEGFE1 were kindly provided by Dr Koichi Takayama (Kyushu University Hospital, Japan).

2.2.2 Generation of Recombinant Adenoviral Plasmids by Homologous Recombination in *E.coli*

Efficient recombination is highly dependent on the competency of the bacteria cells used. Typically, 0.5-1.0 µg of pshuttleFlt-1E1 was linearized with *PmeI*, purified by phenol/chloroform extraction and ethanol precipitation, and mixed with 1.0 µg of supercoiled pAdEasy-1 (Ad5 or Ad3 knob) in a total volume of 6.0 µl. Twenty microliters of electrocompetent *E.coli* BJ5183 cells were added, and electroporation was performed in 1.0mm cuvettes at 2KV, 200Ω, and 25 µF in a Bio-Rad Gene Pulser electroporator. The cells were immediately placed in 500µl L-Broth and grown at 37°C

for 20 min. One hundred twenty-five microliters of the cell suspension then was inoculated onto each of four 10-cm Petri dishes containing L-agar plus 50μg/ml of kanamycin. After 16-20 h growth at 37°C, 10-25 colonies per dish generally were obtained. The smaller colonies, which usually represented the recombinants, were picked and grown in 2 ml of LB containing 50μg/ml of kanamycin. Clones were first screened by analysing their supercoiled sizes on agarose gels, comparing them to pAdEasy-1 controls. Those clones that had inserts were further tested by *PacI* restriction endonuclease digestion. Once confirmed, supercoiled plasmid DNA pAdFlt-1E1 was transformed into DH5α cells for large-scale amplification by electroporation. In such cases, 1.0 μl of plasmid DNA (~100 ng) in 15μl of water was mixed with 5.0 μl of electrocompetent DH5α cells in a total volume of 20.0 μl, and electroporation was performed as described above.

2.2.3 Production of Adenoviruses in Mammalian Cells

Approximately 2×10^5 293 cells per well were plated in 6-well culture plates 24 h before transfection, by which time they reached 50-70% confluency. Cells were washed once with no FBS DMEM:F12 medium, then 5% FBS medium was added to each well and the plates returned to the CO_2 incubator for 1 h before transfection. Eighteen micrograms of recombinant adenoviral vector pAdFlt-1E1, digested with *PacI* and ethanol-precipitated were used for transfection of 6 wells in 6-well plate. A transfection mix was prepared by adding 18μg of linearized plasmid DNA with 675μl filter dd H_2O, 75μl of 2.5M CaCl and 750μl of 2xBES. After incubation at room temperature for 10~20 min, the transfection mix was added to the cells. After 16~24 h at 37°C, the mediumcontaining the transfection max was removed, and 5% FBS growth medium

was added. Transfected cells were monitored for cytopathic effect (CPE) and collected 7-10 days after transfection by scraping cells off wells and pelleting them along with any floating cells in the culture. After 3 cycles of freezing in an ethanol/dry ice bath and rapid thawing at 37°C, 1 ml of viral lysate was used to infect 293 cells in a 75 cm^2 flask when it was 70% confluent. After 3-5 days, viruses were harvested as described above. Half of viral lysate was used to infect 2x 150 mm^2 tissue culture plates. When 50% lysis was observed, one plate of infected cells was harvested for adenovirus DNA miniprep, and the infected cells in another plate were for viral amplification.

2.2.4 Validation of Viral Construction

2.2.4.1 Viral DNA Extraction

Adenovirus DNA miniprep was used to separate the packaged adenovirus DNA from the host nucleic acid. The infected cells from one 150mm^2 tissue culture plate were pelleted from the medium by centrifugation and resuspended in 400µl TE buffer pH 9.0. To this, 400µl of DOC lysis buffer was added and mixed with pipetting 15 times. The solution was transferred to a 1.5 ml eppendorf tube and 8µl of 500mM spermine-HCl added then incubated on ice for 15 min. Then spun at 14,000 rpm for 4 min at 4ºC. The supernatant was transferred to a clean eppendorf tube and 4µl of 10mg/ml RNase A added, then incubated at 37ºC for 10 min. To this 60µl of 10% SDS, 20µl of 0.5M EDTA and 40µl of 10mg/ml proteinase K were added and incubated at 37ºC for 1 hour (h). Phenol: chloroform extraction was performed, taking no more than 900µl. Then, 30µl of 5M NaCl was added and the tube filled with isopropanol (>600µl). This was mixed then spun for 10 min and 4ºC at 14,000 rpm. The solution was removed and the

pellet washed with 70% ethanol and air-dried. The pellet was resuspended in 25µl of TE buffer. The viral DNA was then used for PCR and sequencing.

2.2.4.2 PCR and DNA Sequencing

The recombinant viruses could be simply screened by PCR. The Seq.Flt-1 Ad F and Seq.Flt-1 Ad R primers were used for checking Flt-1 promoter. The PCR program used for the amplification of the Flt-1 promoter consisted of 95°C for 5 min, to ensure complete denaturing of the DNA, followed by 35 cycles of 95°C for 1 min, the specific annealing temperature 58°C for 1 min then extension at 72°C for 1 min, and final step of 72°C for 10 min. The expected size of the PCR products was 900 bp. The viral Flt-1—E1 junction region was confirmed by PCR with the primers Flt-1&E1 F and Flt-1&E1 R. The specific annealing temperature was 52°C. The expected sizes of the PCR products should be 312 bp.

For each reaction, 0.5 µl (5 units/µl) of polymerase Taq Gold (Roche Diagnostics Australia Pty Ltd, Castle Hill, NSW, Australia) was added to a final volume of 50 µl containing a pair of primers (10 pmol each), 50 ng template DNA, 4 µl 25 mM MgCl$_2$, 1 µl 10 mM deoxynucleotide triphosphates (dNTPs), and 5 µl 10 x PCR buffer. PCR was performed for 30-35 cycles using the Perkin Elmer DNA Thermal Cycler 4800 (PerkinElmer Life and Analytical Sciences, Shelton, CT, USA). PCR amplified DNA was examined by 1% agarose gel electrophoresis (100 volts) and visualised under UV Transilluminator (Ultra-Lum Inc, Carson, CA, USA).

Further verification was performed by sequence analysis (Applied Biosystems BigDye®

Terminator v3.1 Cycle Sequencing Kit; Foster City, CA, USA). Flt-1 promoter and the

viral Flt-1–E1 junction region were confirmed by sequencing with the primers seq.Flt-1

Ad F and E1R. Sequencing conditions involved 25 cycles of: 30 sec denaturation at

96ºC, 15 sec annealing at 50ºC and 4 min extension at 60ºC. Sequencing DNA was

cleaned by using isopropanol method. The samples were forwarded to the Molecular

Pathology Sequencing Centre, IMVS, Adelaide for sequence analysis.

2.2.5 Viral Amplification and Purification

Viral stocks for each vector were produced in 293 cells using a standard protocol.

Briefly, 10 ml of cell lysates from 150 mm^2 tissue culture plate (see 2.2.3) or 10µl of

viral stock were added to 400 ml of 5% DMEM: F12 medium, 20 ml of which was

added to each of 20 x 150 mm^2 tissue culture plates which were at least 90% confluent

with 293 cells. This was equivalent to 5-10 particle forming units (pfu) per cell. When

CPE were seen, typically 36-48 h s after infection, the cells and medium were

harvested. The cells were pelleted from the mediumby centrifugation at 800 rpm for 5

min. The individual cell pellets were combined and then stored at -70°C until required

for purification.

In order to lyse the cells, three freeze/thaw cycles were performed. The cell lysate was

centrifuged at 3,500 rpm for 20 min at 4°C to pellet the cell debris. The supernatant

containing the virus was collected and then purified by double caesium chloride

gradient. The discontinuous CsCl gradient removes the majority of cellular

contaminants and defective viral particles. The gradient was established by pipetting 4

ml of CsCl 1.33 into a centrifuge tube (Beckman Coulter, CA USA) and then carefully backfilling with CsCl 1.45. The viral stock was very gently loaded on top of gradient. PBS was used to ensure the centrifuge tube was full to maximum. Balance tubes were prepared in the same manner, using PBS to complete the volume requirements of the centrifuge tube. The centrifuge tubes were carefully balanced using an electronic balance (Model HF300G, A&D Co., Ltd., Tokyo, Japan) and then placed in a pre-cooled Beckman Ultracentrifuge (SW41 rotor, Beckman Coulter, Fullerton, CA, USA) for 2.5 h at 18,000 rpm at 4°C. There were usually 2 viral bands seen in the centrifuge tube after this first spin: a lower band containing the viral particles of interest and an upper band containing incomplete viral particles. There was also a layer of cellular residue on top of the bands. Only the lower viral band obtained from this centrifugation was collected. The continuous CsCl gradient was then performed to completely separate infectious from defective viral particles. The band, which was collected from the first spin, was loaded onto a second caesium chloride gradient prepared in the manner described previously, then centrifuged in the ultracentrifuge at 25,000 rpm for 22-24 h at 4°C. There was only one band present following the second centrifugation step. This band was collected, and CsCl was removed by dialysis. The viral band was dialysed at 4°C using a Slide-A-Lyzer® dialysis cassette (Pierce Biotechnology Inc, Rockford, IL, USA). The dialysis buffer was used at 200x the volume of the collected band and at least 3 changes of buffer were used. The dialysed virus was collected, aliquot into 1.5 ml polypropylene tubes, labelled and stored at -70°C until required.

2.2.6 Viral Particle Titration

The titre of the viral product was determined using the OD_{260} absorbance method (viral particle method, VP) and the tissue culture infectious dose ($TCID_{50}$) method. These methods measured viral particles per ml and plaque forming units (pfu) per ml respectively providing a basis for comparison to ensure that when using the viruses for animal experiments similar amounts of viral particles and infecting particles could be delivered.

2.2.6.1 Viral Particle Method

This method is simply based on evaluation of particles in solution correlating to DNA content. The extinction coefficient is $1.1x10^{12}$ viruses per OD_{260} unit. This method does not discriminate between potentially infective complete viral particles and defective viral particles. A 20µl aliquot of virus was thawed to 4°C. The virus was diluted with virus lysis buffer in a 1:5 ratio. A blank sample containing dialysis buffer and virus lysis buffer was also prepared under the same conditions. The samples were incubated at 56°C with shaking for 10 min, and then placed in the cuvette for analysis in the Beckman DU®640 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). To determine the number of viral particles, the stock absorbance was multiplied by the dilution factor and the extinction coefficient $1.1x10^{12}$ as follows:

(OD_{260}) x (viral dilution) x $1.1x10^{12}$ = _____VP/ml

2.2.6.2 TCID$_{50}$ Method

The TCID$_{50}$ method is based on the development of CPE in 293 cells using end-point dilutions to estimate the titre. 293 cells in 2% DMEM: F12 were plated in 96-well plates to give a final concentration of 1×10^4 cells per well. Serial dilutions of the viral stock were prepared in 2% medium and incubated with 293 cells. The presence or absence of CPE, after 10 days, in each well was determined. The test is valid if the negative controls have no CPE, and the lowest viral dilution shows 100% infection and the highest viral dilution shows 0% infection.

The titre is determined using the KÄRBER statistical method (Fields et al., 1996):

For 100µl of dilution the titre is $T = 10^{1+d \, (S-0.5)}$

Where d= Log 10m of the dilution (=1 for ten-fold dilution)

And S= the sum of the ratios (always starting from the first 10^{-1} dilution)

These measures were performed on each virus grown and harvested. Comparisons were made with the previous titres of prior generations obtained to ensure that the quality of the viral stocks was maintained.

Figure 2-2: Example of calculating TCID$_{50}$

Typical Results of TCID$_{50}$
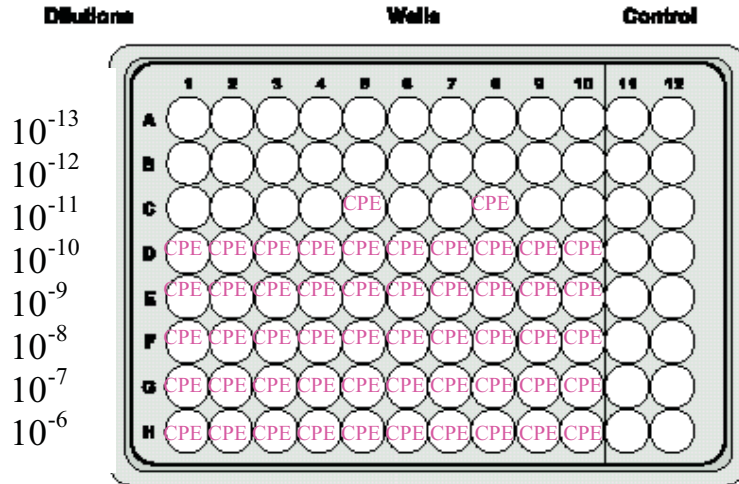
Results: Determine the ratio of positive wells per row

Dilution	Ratio
10^{-6}	10/10=1
10^{-7}	10/10=1
10^{-8}	10/10=1
10^{-9}	10/10=1
10^{-10}	10/10=1
10^{-11}	2/10=0.2

S=sum of the ratios (starting form 10^{-1})= 1+1+1+1+1+1+1+1+1+1+0.2=10.2

Titre: $T = 10^{1+d\,(S-0.5)} =10^{1+1(10.2-0.5)}$ for 100µl aliquot virus

$T = 10^{10.7}$ TCID$_{50}$ for 100µl aliquot virus

$T = 10^{11.7}$ TCID$_{50}$ per ml aliquot virus

TCID$_{50}$= 5x 10^{11}

2.2.7 Wild Type Exclusion

The recombinant viruses can be screened for wild type exclusion by PCR. The E1F and E1R primes were used for checking wt E1 transcomplementing region. DNA from Adwt was used as a positive control. The PCR program consisted of 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 1 min, final step of 72°C for 10 min. The expected sizes of the PCR products are 177 bp.

2.2.8 RNA Extraction and RT-PCR

Total RNA extracts were made from different cell types using the RNA-Bee RNA isolation reagent (Iso-Tex Diagnostics, Inc., Friendswood, Texas, USA). Suspension cultures of each cell type (5×10^6 cells) were pelleted, 1 ml RNA-Bee was added and the cells were homogenised by passing through a pipette several times to ensure lysis. The samples were transferred to eppendorf tubes and 0.2 ml chloroform was added followed by vigorous shaking for 15-30 seconds (sec). After 5 min incubation on ice, the sample was spun at 12,000 g for 15 min at 4°C. The aqueous upper phase containing the RNA was then transferred to fresh reaction tubes and precipitated by adding 500µl isopropanol. Samples were incubated at RT for 10 min and then centrifuged at a 12,000g for 10 min at 4°C. The precipitated RNA was washed in 1 ml of 75% ethanol, vortexed briefly and re-spun at 7500g for 5 min at 4°C. The RNA samples were air dried for 5-10 min. RNA was dissolved in RNase-free water by diethyl-pyrocarbonate (DEPC) treatment.

Reverse transcription-polymerase chain reaction (RT-PCR) was used for the detection of VEGF and Flt-1 mRNA expression levels in different mesothelioma cell lines. For a Reverse Transcription (RT, RNA is first reverse transcribed into cDNA) reaction, 1-2 micrograms of RNA is used. RNA is first incubated at 65°C for 5 min to denature RNA secondary structure and then place immediately on ice. Other components of RT are added to the reaction including 0.5mM dNTPs, 10 units of RNase inhibitor, 1μM of Oligo-dT primer, 4 units of Omniscript Reverse Transcriptase and 10x buffer RT in a total 20μl volume. RT reaction is extended at 37°C for 1 h. Subsequent PCR amplification using VEGF mRNA F & R primers and Flt-1 mRNA F & R specific for VEGF and Flt-1 mRNA. The PCR program used for the amplification of the VEGF mRNA consisted of 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, the specific annealing temperature 50°C for 1 min and 72°C for 1 min, final step of 72°C for 10 min. The expected sizes of VEGF mRNA are 408 bp and 541 bp. The specific annealing temperature of Flt-1 was 55°C and the size was 613 bp. The housekeeping Cyclophilin gene was 355 bp and the PCR conditions were as follows: 22 cycles of denaturation (95°C, 30s), annealing (64°C, 30s), and extension (72°C, 30s).

2.2.9 Western Blotting

Western Blotting is a method to determine, with a specific primary antibody, the relative amounts of the protein present in different samples. In general, the process of Western blotting includes preparation of cell lysates, polyacrylamide gel running, membrane transfer, and antibodies incubation and detection.

The cells were collected and lysed with lysis buffer. The protein concentration of the cell lysates were determined by Bio-Rad protein assay reagent (Bio-Rad Life Sciences Group, Hercules, CA, USA) and the samples were standardised for protein content and 30-40 μg protein samples were reduced in 5% β-mercaptoethanol in SDS gel loading buffer. Polyacrylamide gels were set-up using the Bio-Rad gel preparation and electrophoresis apparatus (Mini-PROTEAN® 3 Cell Bio-Rad Life Sciences Group, Hercules, CA, USA). Gels consisted of a 12% resolving layer and a 5% stacking layer. The resolving gel was formulated to contain 1.67 ml dH$_2$O, 2 ml 30% acrylamide solution, 1.25 ml 1.5 M Tris pH 8.8 and 50μl 10% SDS. The gel was polymerised by addition of 50μl of 10% (w/v) Ammonium Persulfate (APS) and 5μl N, N, N', N', - tetramethylethylenediamine (TEMED) (Bio-Rad Life Sciences Group, Hercules, CA, USA). The stacking gel was formulated to contain 1.735 ml dH$_2$O, 425μl 30% acrylamide solution, 300μl of 1M Tris pH 6.8, and 25μl of 10% SDS, and was polymerised by addition of 25 μl of 10% APS and 2.5 μl of TEMED. After boiling for 5 min, the samples were loaded onto the gel and the gel was run at 100 volts for 1-2 h approximately. Proteins were transferred from the gel to a polyvinylidene difluoride membrane (Amersham Biosciences, Munich, Germany) occurred under cold conditions at 100 volts for 1 h. The membranes containing transferred proteins were blocked in 10% non-fat milk in TBS-T for 30~60 min before incubating with primary monoclonal antibodies or polyclonal antibodies. Under certain circumstances, blocking and incubation with primary antibodies were extended to overnight at 4°C followed by 3 washes (15 min each) with TBS-T. The secondary immunoreaction was performed using horseradish peroxidase-linked goat anti-mouse or goat anti-rabbit IgG for 1 h at room temperature. After 3 washes (15 min each) with TBS-T, the blots were detected using ECL reaction (Amersham Biosciences, Munich, Germany). X-ray film exposed to

blots (Amersham Biosciences, Munich, Germany) was fixed and developed using an Ilfospeed 2240 X-ray film developer (Ilford, Marly 1, Switzerland).

2.2.10 Adenoviral Infection

Mesothelioma cells were cultured in tissue culture flasks and then plated in the appropriate plate (6, 24, 96 well) in triplicate depending on the application intended. Normally, $2x10^5$ cells per well were seeded in 6-well plates, $5x10^4$ cells per well in 24-well plates, $1x10^4$ cells per well in 96-well plates. For example 24-well plates were seeded at $5x10^4$ cells per well. Each treatment well was infected with viruses at the multiplicity of infection (MOI) of 1,10,100 pfu per cell in 500µl 2% FCS medium for 2 h. Cells were washed once with serum-free medium and then replaced with 2 ml fresh complete medium and left for the required length of time prior to analysis. In uninfected wells 2% medium was used as a control-infecting agent and was also replaced with complete medium.

2.2.11 Luciferase Assay

Ad vectors containing the gene for firefly luciferase (Luc) were used to assess the transduction capability of specific vectors in the cell lines of interest. Cells may be more or less resistant to infection depending on a variety of factors including their CAR expression and specific modifications to the viral structure, which may make infection more efficacious. Cells were plated in 24-well plates as previously described. The cells were infected at the similar 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).

2.2.12 Crystal Violet Staining

All mesothelioma cell lines were assessed for their response to infection with the adenoviral vectors being examined. These cell viability studies were carried out with uninfected cells and cells infected with control virus in parallel. A standard method of infection was employed for all experiments. At various time points the cells were washed with PBS and then fixed with 10% buffered-formalin for 10 min. Following this the cells were stained with 100µl of 0.1% crystal violet in 2% ethanol for 10 min, and then immersed in cold water to remove excess dye. The viable cells were stained with the dye. The plates were air dried overnight. The following day the fixed cells were lysed with 200µl of 10% acetic acid and transferred to a 96-well plate, which was analysed on plate reader (MR 5000/7000 Microplate Reader, Dynatech Laboratories Inc., Chantilly, VA.) at a wavelength of 570nm. The standards curves for each cell line were established concurrently for each time point and then used to calculate the actual cell counts from the OD readings using a linear regression model.

2.2.13 Flow Cytometry

Flow cytometry was employed to detect the presence of coxsackie adenoviral receptor (CAR) and CD46 on mesothelioma cell lines. This method was adapted from the indirect immunofluorescent method described by Miller (Miller et al., 1998). Briefly, 2×10^5 cells were detached using standard trypsin methods and resuspended in 1 ml 2x Hepes Buffered Saline. The samples were then centrifuged at 4000 rpm for 1 min and the supernatant discarded. The cells were agitated, then washed with Isoton & 0.1% BSA (wash buffer) and centrifuged again at 4000 rpm for 1 min and the supernatant discarded. The cells were stained with 10µl of mouse monoclonal anti-CAR antibody 5µg/ml stock (or no primary antibody as negative control), and incubated at room temperature for 15 min in the dark, then washed with 2 ml wash buffer and centrifuged as previously. Five microliters of secondary antibody PE-conjugated RAMγ (Rat anti-mouse) was added to each sample. The samples were incubated at room temperature for 15 min in the dark, then washed with 2 ml wash buffer, centrifuged as previously and the supernatant discarded. They were then resuspended in 50µl wash buffer and analysed with a FACSCalibur flow cytometer and CellQuest software (BD Biosciences, San Jose, CA, USA).

Flow cytometry was also used to measure CD8 T-cells expression in tumours by staining with appropriate fluorescent conjugated monoclonal antibodies. AE17 tumours were harvested from C57 Black6 mice. Tumour samples were disaggregated using a 'Medimachine' (BD Biosciences, San Jose, CA, USA) for 2 min to prepare a single cell suspension. Effectiveness of the procedure was checked by staining a cytospin preparation of the disaggregated tissue with May Grunwald Giemsa. The staining was

performed by the Haematology Laboratory at the Women and Children Hospital (Adelaide, SA). The single cell suspension was then centrifuged at 4000 rpm for 1 min and the supernatant discarded. The cells were agitated, then washed with wash buffer and centrifuged again at 4000 rpm for 1 min and the supernatant discarded. The cells were stained with 2.5 µl of PE-conjugated anti-CD8a (BD Biosciences, San Jose, CA, USA) and 2.5 µl Alexa Fluor 647-conjugated anti-CD3 (eBioscience, San Diego, CA, USA), and incubated at room temperature for 15 min in the dark, then washed with 2 ml wash buffer and centrifuged as previously. They were then resuspended in 50µl wash buffer and analysed with a FACSCalibur flow cytometer and CellQuest software (BD Biosciences, San Jose, CA, USA).

2.2.14 Tumour Xenograft Model Establishment

Human mesothelioma cells ($5x\ 10^6$ H226) or murine mesothelioma cells ($5x\ 10^5$ AE17) were suspended in 100µl PBS, and then inoculated subcutaneously into the right flanks of Balb/C nude mice or C57B/6 mice using 27G insulin syringes (Terumo, Elkton, MD, USA). The tumour volume was measured twice weekly by the same operator using standard procedure and same calliper (Expert PVC 140F, Draper Tools Limited, Hampshire, Australia). Tumour volume was calculated using the equation: Volume = ½ x length x width^2. The tumours were allowed to achieve a volume of 50 mm^3, and then mice were randomly allocated to treatment groups prior to treatment.

2.2.15 Virus Injection in Tumour Xenografts

Mice were injected intratumorally with a single dose of $1x10^9$ pfu of adenovirus in 100µl PBS. Control mice received PBS vehicle alone. To investigate the effect of co-delivery of CRAds with MMP-9, mice received a mixture of these two different viruses ($1x10^9$ pfu each virus in a final volume of 100µl PBS). Control mice received PBS alone. Tumour diameters were measured daily until the tumour size reached ~10x10 mm, then the mice were sacrificed. Survival was also monitored and plotted against time after virus injection. To evaluate the combination of viral therapy and immunotherapy, viral injection commenced when a small lump was apparent range from 3-4 mm in diameter. Some heterogeneity in tumour size was unavoidable, so as far as possible tumours of various sizes were equally distributed between the groups. Tumours were injected $1x10^9$ pfu of adenoviral vectors in 100µl PBS or PBS alone. Two day later, mice receiving activating anti-CD40 antibody FGK45 received 100µg in 100µl of PBS intraperitoneally three times in 6 days. The tumour volume was measured every day. Mice were culled till the tumour size reached ~10x10 mm. Survival was also monitored and plotted against time after treatment commenced.

2.2.16 Immunohistochemical Staining

2.2.16.1 Immunohistochemistry on Paraffin-embedded Tissue Sections

The mice were euthanised by using CO_2 in an ethical fashion. The tissues from each mouse were removed, and fixed in 10% buffered-formalin (ACE Chemical Co., Camden Park, SA, Australia). The tissues were processed into paraffin blocks after

overnight fixation and sections cut at 5µm. Paraffin embedded specimens and H&E staining were performed by the Tissue Pathology Laboratory at the IMVS.

For immunohistochemistry, sections of tissues were prepared on poly-L-lysine-coated slides (Polysine™, Menzel-Gläser, Germany) and dewaxed at 60°C for 1 h. The slides were re-hydrated through xylene, three washes of 5 min duration, then washed in 95%, 90%, 70% ethanol for 5 min at each concentration. The slides were then washed in de-ionised water twice for 5 min each wash. Endogenous peroxide in the section was blocked with 0.3% (v/v) hydrogen peroxidase in PBS for 30 min, and then washed with PBS-Tween twice for 5 min each wash. Adenovirus antigen detection was performed using methods and supplied by DAKO ("DAKO ARK", Dako, Carpinteria, CA, USA). The primary mouse anti-adenovirus antibody was first complexed with a biotinylated anti-mouse secondary antibody (DAKO). The antibody complex was then applied to the sections and incubated for 15 min, rinsed in PBS, and incubated in streptavidin-peroxidase for 15 min. Diaminobenzidine/hydrogen peroxidase was used as the chromogen substrate for 5 min. All slides were counterstained with hematoxylin (Vector Laboratories Inc, Burlingame, CA, USA) after washed with water, and then blued in running water for 2-3 min. Slides were dehydrated in 80%, 100% ethanol and xylene, and then mounted using Depex (VWR International Ltd, Poole, England) and cover slips.

2.2.16.2 Immunohistochemical Staining of Frozen Tissue Sections

Tissues were snap frozen in compound-embedding medium (OCT, SAKURA, Tokyo, Japan), and 10-µm sections were collected on poly-L-lysine-coated slides using a

cryostat. Sections were allowed to air dry for at least 1 h, and then fixed in ice-cold 5% acetone in methanol for 10 min. Slides were washed with 0.2% Triton-X in PBS twice for 5 min each wash. Endogenous peroxide in the section was blocked with 0.3% (v/v) hydrogen peroxidase in methanol for 30 min, then washed with 0.2% Triton-X in PBS twice for 5 min each wash. Avidin/Biotin block were performed for 15 min each (Vector Laboratories, Inc. Burlingame, CA, USA). Sections were incubated with mouse CD8 primary antibody (AbD Serotec, Oxford, UK) diluted in buffer containing 2% blocking serum for 1 h, followed by washing 0.2% Triton-X in PBS 3 times for 5 min each wash. A biotinylated secondary antibody anti-rat IgG (Vector Laboratories Inc, Burlingame, CA, USA) was applied to the slides for 10 min, and then followed by washing as above. Immunostaining was visualised by incubating streptavidin horseradish peroxidase for 15 min ("DAKO ARK", Dako, Carpinteria, CA, USA) and diaminobenidine-H_2O_2 for 5-10 min until the desired stain intensity develops and rinsed in tap water. All slides were counterstained, cleared and mounted as described in 2.2.16.1.

2.2.17 Statistical Analysis

Statistical significance was calculated using GraphPad (San Diego, CA) PRISM. Student's test was used to determine differences between two populations. One-way ANOVA was used to determine differences between more than two populations. All data were expressed as the mean ± SD except tumour value data which were presented as mean ± standard error of the mean (SEM). Differences were considered significant when the P was <0.05.

Survival curves and tumour size change were analysed by statisticians in the Faculty of Health Sciences, University of Adelaide. A P-value of less than 0.05 was required for statistical significance. P-values were adjusted for multiple comparisons using the stepdown Sidak method. All analyses were performed using SAS version 9.1 (Cary, NC, USA).

Chapter 3. VEGF and Flt-1 Promoter-based CRAds

Kill Human Mesothelioma Cells

3.1 Introduction

Conditionally replicative adenoviral (CRAd) therapy is a promising approach for cancer treatment. Adenoviral vectors are effective tools for gene delivery because of their superior *in vivo* gene transfer efficiency in a wide spectrum of both dividing and non-dividing cell types. The most important obstacle for adenoviral gene therapy is low selectivity of the existing vectors and low efficiency of gene transfer. The relatively disappointing gene transfer efficacy seen in cancer gene therapy studies and the increase in knowledge about Ad biology prompted the development of replicating virus as a therapeutic strategy. As discussed in the Introduction, some form of control of replication is needed to maximise toxicity for the target tumour cells to minimise toxicity to normal tissues. Some degree of "targeting" is achieved by the direct injection of tumour with virus, but some release of virus into the systemic circulation can be expected, especially if a replicating virus is used. The liver is at particular risk of toxicity in this setting because of its high propensity to take up Ad vectors.

Employment of tumour/tissue specific promoters can reduce toxicity, increase safety, and improve the therapeutic index (St George 2003). Gene therapy using anti-angiogenesis genes has shown promise in preclinical models on mice (Scappaticci et al., 2001). VEGF is believed to play a very important role in tumour-associated angiogenesis in lung cancer as well as other solid tumours. VEGF activity is very low in

normal liver. Recent studies have demonstrated promising results using the Flt-1 promoter in gene therapy directed at the vasculature. Importantly, the Flt-1 promoter exhibits a "liver off" phenotype when used in adenoviral vectors (Nicklin et al., 2003).

In mesothelioma, both VEGF and Flt-1 are known to be upregulated relative to normal tissues. Therefore, I chose to evaluate CRAds based on the use of the VEGF or Flt-1 promoter to control replication for their ability to kill mesothelioma cells and for their selectivity.

To test our hypothesis, the native CRAd E1A promoter was placed with the human Flt-1 promoter and oncolysis was evaluated in various human mesothelioma cell lines, tumour cells from mesothelioma patients and human xenografts in immune deficient mice. The VEGF promoter driven CRAds were kindly provided by Dr Koichi Takayama (Takayama et al., 2007). This study highlights the potential and drawbacks in using the Flt-1 and VEGF promoters when delivering local and systemic human gene therapy for mesothelioma.

3.2   Adenovirus Receptors Expression in Human Mesothelioma Cells

Optimal therapeutic effect of CRAds depends on high efficiency infection of target cells. The native receptor for the dominant serotype Ad used in gene therapy studies (Ad5) is the coxsackie and adenoviral receptor, CAR. Several studies have now indicated that many human primary tumours have low CAR levels, whereas CD46 tends to be expressed at high levels on most tumours, Ad serotype 3 infects via CD46. The

expression of CAR and CD46 had been determined in 13 human mesothelioma lines by flow cytometry. We found variable CAR expression in these cell lines (Fig.3-1). The lowest CAR level was shown in H226, H2052 and H2596 cell lines. However, all mesothelioma cell lines tested showed strong CD46 expression. Based on these findings I chose to evaluate CRAds with Ad3 tropism as well as the original Ad5 tropism.

3.3 Construction of Ad5 (5/3) Flt-1E1

The overall strategy developed here is diagrammed in Fig.3-2 and involved three steps. The techniques are described in more detail in the Methods chapter, but outlined briefly here. First, the pshuttleCMVE1 was digested with restriction endonucleases to remove the CMV promoter and then blunt-ended. The Flt-1 promoter was excised from the plasmid pMV10-Flt-1, blunt-ended, then ligated with pshuttleCMVE1, forming pshuttleFlt-1E1. Second, the pshuttleFlt-1E1 was cleaved with *PmeI* to linearize it and transformed together with a supercoiled adenoviral vector pAd5(5/3)Easy-1 into *E.coli* strain BJ5183. Recombinants pAd5(5/3)Flt-1E1 were selected with kanamycin and screened by restriction endonuclease digestion (Fig.3-3A). Third, the recombinant adenoviral constructs were cleaved with *PacI* to expose its inverted terminal repeats and transfected into a packaging cell line 293. Recombinant adenoviruses were generated within 7-10 days. After viruses were harvested, adenoviruses DNA miniprep was performed. Validation of the recombinant viruses can be simply screened by PCR (Fig.3-3B) and DNA sequencing. Then viruses were amplified by infecting 293 cells and purified by double caesium chloride density centrifugation. The titres of the viral products were determined using the OD_{260} absorbance method and $TCID_{50}$ method. The recombinant viruses can also be screened for wild type exclusion by PCR (Fig.3-3C).

Fig. 3-1 Expression of CAR and CD46 in human mesothelioma cell lines by flow cytometry. Cell pellets from13 human mesothelioma cell lines and Huh7 cell line were collected and stained with CAR or CD46 antibody. The percentage of CAR and CD46 was enumerated by flow cytometry. The level of CAR expression in mesothelioma lines is variable, but strong CD46 expression could be detected in all mesothelioma lines tested. 3 of replicate experiments was performed. Data were presented as the mean ± SD (Figure provided by N. Milic).

Fig. 3-2 Generation of a recombinant adenovirus using AdEasy TM

Step 1: cDNA cloning in transfer plasmid
For replicative constructs, the E1 region (normally deleted from AdEasy plasmids to render them replication defective) has been reconstituted using a fragment of the wild type Ad genome.

Step 2: *In vivo* homologous recombination in bacteria

Step 3: Virus production in 293 cells or A549 cells
Viruses containing the Flt-1 or VEGF promoters driving E1 gene (for controlled replication) were constructed using standard techniques of homologous recombination and transfection into 293 cells (which contain E1 to allow replication defective Ads to grow) or A549 cells (which do not have E1) respectively.

Taken from He TC, et al. Proc Natl Acad Sci USA,1998; 95 (5): 2509-14
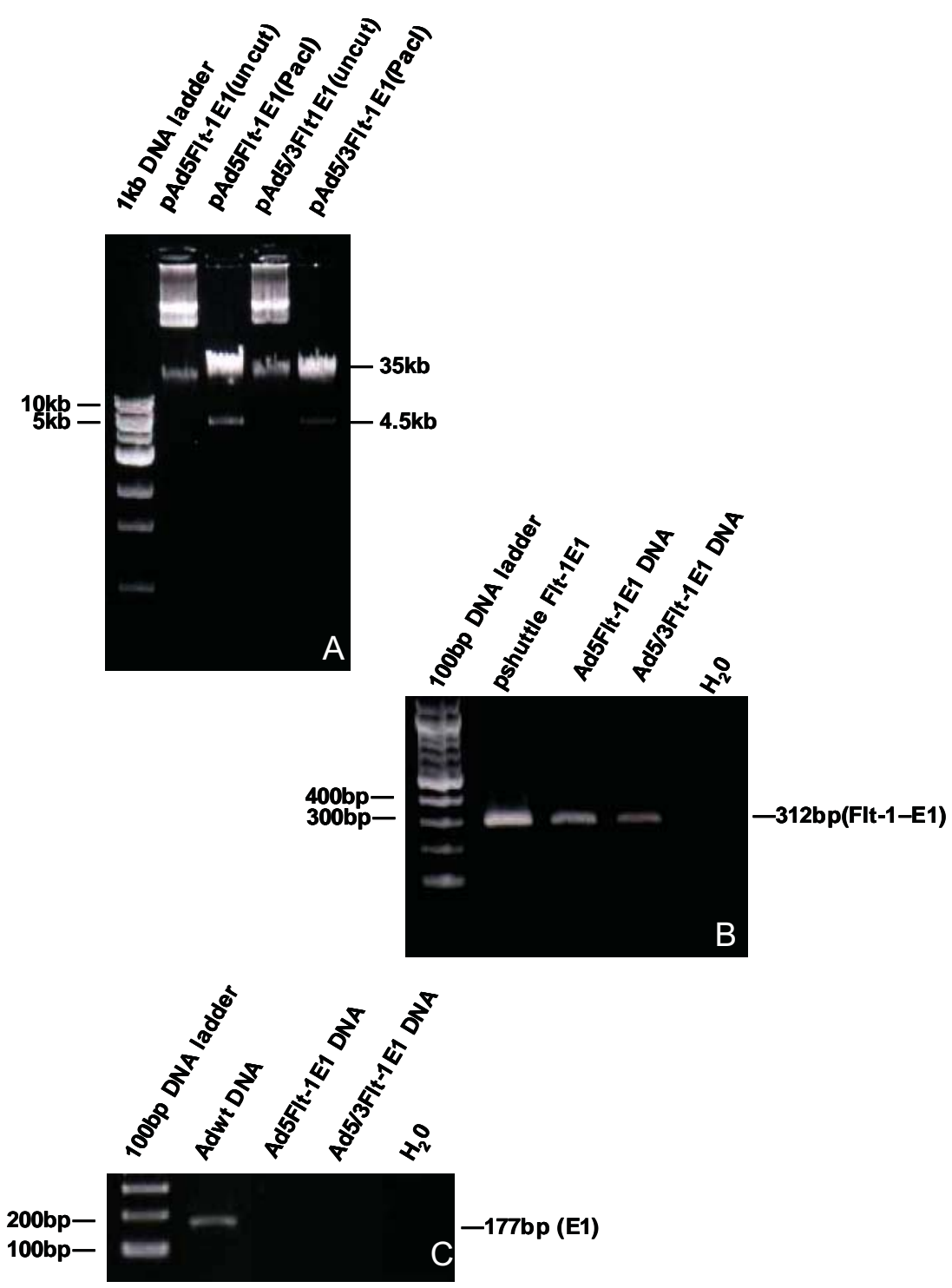
Fig. 3-3 Validation of viruses containing Flt-1 promoter driving E1 expression. A.Screening recombinants by plasmid size and restriction enzyme analysis. The restriction digest with PacI yields a large fragment of approximately 30kb, and a smaller fragment of either 3kb or 4.5kb. B.Screening of Flt-1—E1 junction area of recombinant viruses by PCR. PshuttleFlt-1E1 as a positive control. C.Screening of recombinant viruses for wild type exclusion by PCR. Ad5Flt-1E1 and Ad5/3Flt-1E1 are ensured not contaminated with wild type Ad.

3.4 VEGF and Flt-1 Expression in the Human Mesothelioma Cells

3.4.1 VEGF and Flt-1 mRNA Expression

I wished to develop a strategy for the therapy of mesothelioma based on the use of CRAds in which the VEGF and Flt-1 promoters control the expression of E1. Although VEGF and Flt-1 expression has been previously reported, I wished to test the expression of these genes in the mesothelioma cell lines I had available, as well as a number of control lines. *In vitro*, I first investigated the panel of human mesothelioma cell lines, along with human umbilical vein endothelial cells (HUVEC), 293, BEAS-2B airway epithelial cells and A549 adenocarcinoma cells as controls for Flt-1 and VEGF expression using RT-PCR. Cyclophilin was used as a housekeeping gene. There are four structural variants of VEGF (VEGF 121, VEGF 165, VEGF 189, and VEGF 206) resulting from alternative mRNA splicing in the regions encoding the cytoplasmic domains. Fig.3-4 showed amplification of a 408 bp fragment (representing VEGF 121 cDNA) and a 541 bp fragment (representing VEGF 165 cDNA) in all cell lines tested. The results showed that the density of each band (VEGF 121 and VEGF 165) was similar in 9 human mesothelioma cell lines and other cell lines. The PCR bands corresponding to VEGF 189(615 bp) and VEGF 206 (666 bp) could not be detected. It indicated that VEGF 121 and VEGF 165 were dominant isoforms in these cell lines. Amplification with Flt-1-specific primers generated a 613 bp product in positive control cell lines, HUVEC and 293 cell lines. However, no Flt-1 mRNA was detected in most of the human mesothelioma cell lines and other cell lines although very faint specific
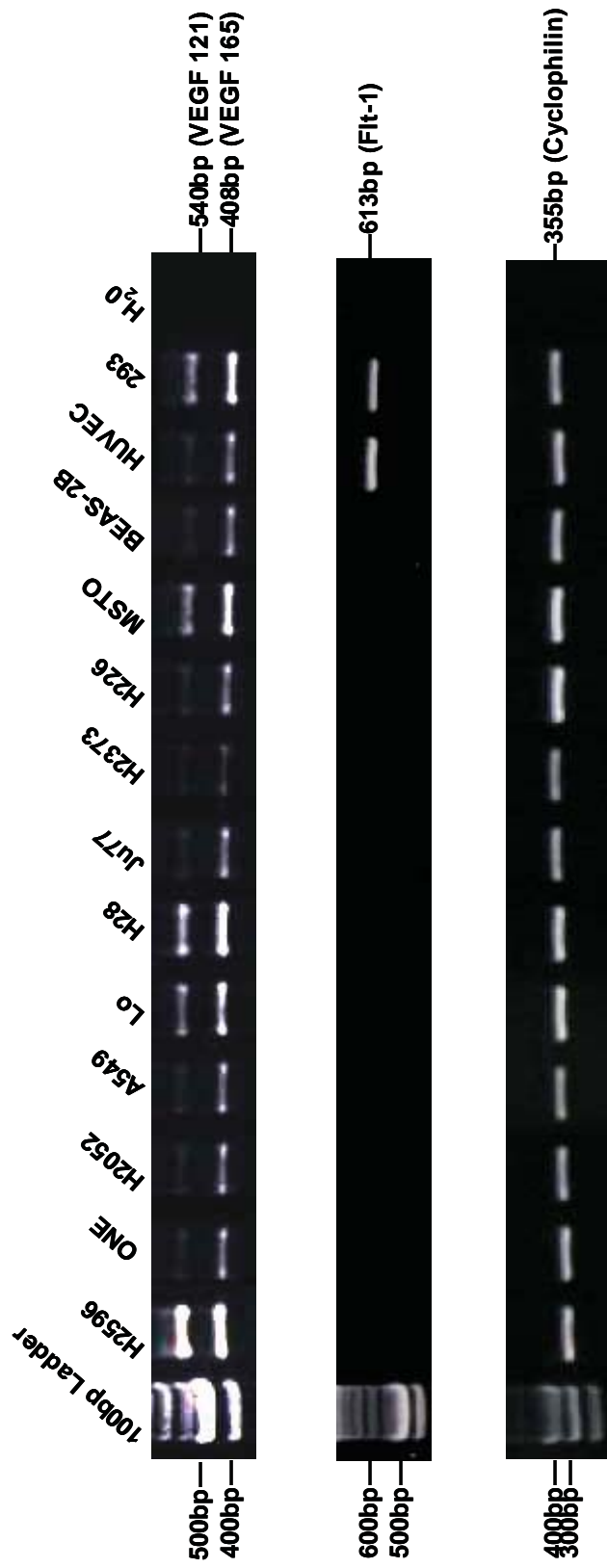
Fig. 3-4 Expression of VEGF and Flt-1 mRNA in human mesothelioma cell lines. mRNA was extracted from 13 cell lines and was reverse-transcribed into cDNA, followed by PCR amplification using primers specific for Cyclophilin (355bp), VEGF(540bp and 408bp) or Flt-1 (613bp). Shown were ethidium bromide-stained agarose gel containing RT-PCR amplification products from each cell line: A549 and BEAS-2B were used as control cell lines for VEGF mRNA. HUVEC and 293 were used as positive controls and A549 cell line as a negative control for Flt-1 mRNA, and Cyclophilin as a house-keeping gene.

Flt-1 bands could be detected in ONE, Lo, MSTO cells under UV transilluminator which does not show on the printed image.

3.4.2 VEGF and Flt-1 Protein Expression

Western blot analysis was used to investigate the correlation between mRNA expression and protein expression for VEGF and Flt-1 in the human mesothelioma cell lines. As shown in the Fig.3-5A, the VEGF protein expression (42 kDa) was demonstrated in all human mesothelioma cell lines tested and the positive control cell line, A549, and VEGF protein expression was detected in the low VEGF expressing control cell line, BEAS-2B. Figure 3-5B showed the Flt-1 protein expression in the human mesothelioma cell lines. According to datasheet of Flt-1 (C-17) antibody from Santa Cruz Biotechnology, Inc, this antibody can detect up to 3 bands of Flt-1 with molecular weight around 80 kDa. The Flt-1 protein levels were similar in 9 human mesothelioma cell lines, but varied in control cell lines. Positive control cell line 293 showed much higher concentration of Flt-1 protein than another positive cell line HUVEC. In the low VEGF expressing cell line BEAS-2B, very weak expression of Flt-1 could be detected. The detection of Flt-1 protein in the mesothelioma lines when the RT-PCR results had been negative was surprising, but the positive PCR results in the control HUVEC line suggested there was no technical problem. The results may reflect very low turnover of the Flt-1 receptors in the mesothelioma cells or a very short half-life of RNA under the conditions tested.
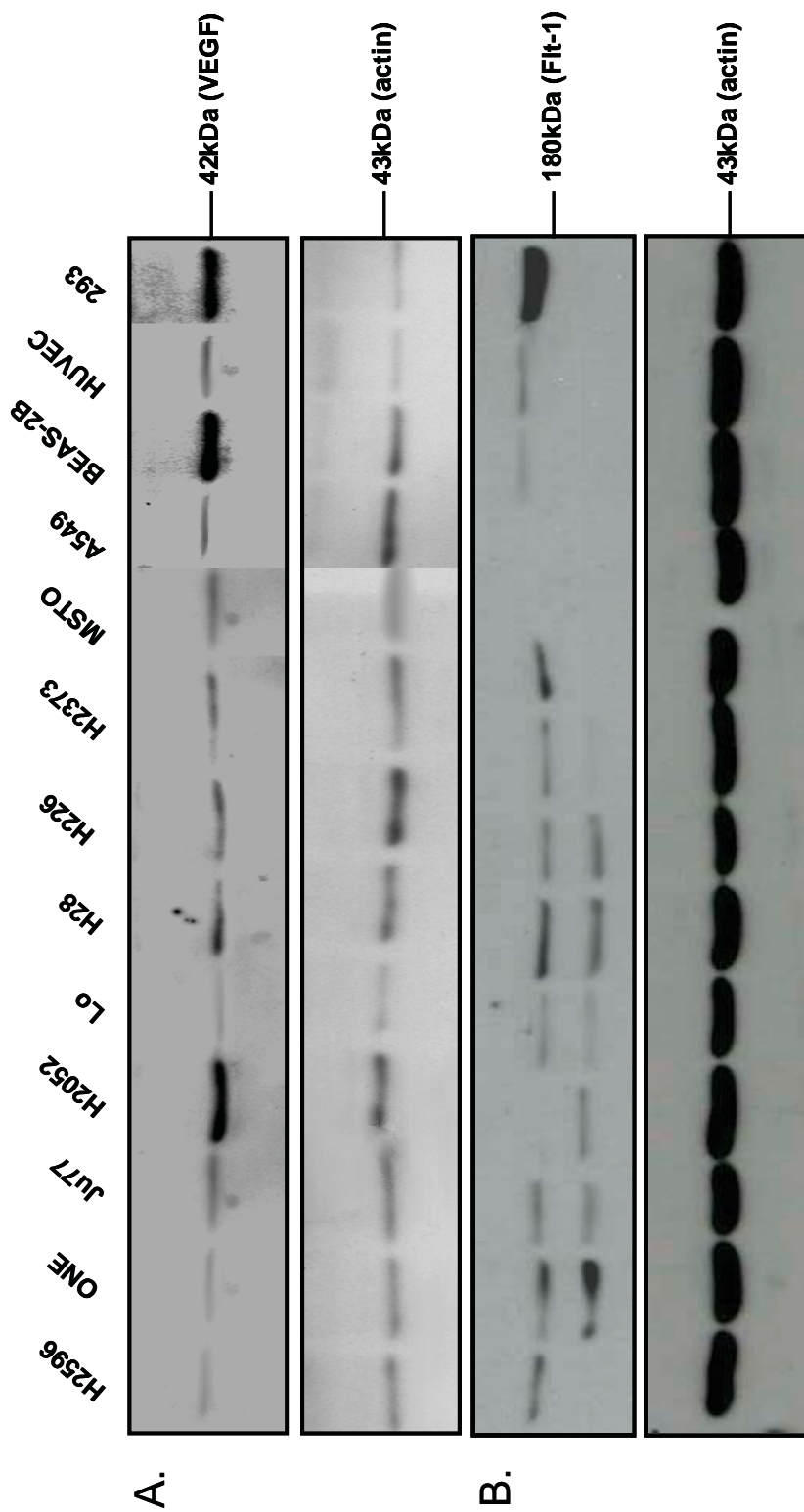
Fig. 3-5 Expression of VEGF (A) and Flt-1 protein (B) in human mesothelioma cell lines. The cells were collected and lysed with lysis buffer. The protein concentration of the cell lysates were determined by Bio-Rad protein assay reagent and the samples were standardised for protein content and 30-40 μg protein samples were reduced in 5% β-mercaptoethanol in SDS gel loading buffer. A549 cell line was used as a positive control and BEAS-2B as a negative control for VEGF protein. HUVEC and 293 cell lines were used as positive controls and A549 cell line as a negative control for Flt-1 protein, actin was a house-keeping protein.

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3.4.3 Evaluation of Primary Cells from Mesothelioma Patients

To take the evaluation of Flt-1 and VEGF expression further I then used primary material obtained from patients with mesothelioma. The patients with malignant mesothelioma had been evaluated by thoracic medicine physicians using standard approaches including chest X-ray and CT scan. Samples used for my analysis were obtained during procedures that were being undertaken for clinically indicated reasons (either diagnostic tap or biopsy, or during palliative drainage of fluid in subjects with already confirmed mesothelioma). Informed consent was obtained from all subjects involved. Histopathological diagnosis of mesothelioma was made by pathologists at the Institute of Medical and Veterinary Science in the course of normal clinical practice. On several occasions I obtained pleural fluid at the time of palliative drainage in which case I confirmed as far as possible that the fluid contained mesothelioma cells. Pleural fluid from confirmed patients was cultured, and the cultured cells were stained with E29 antibody against epithelial membrane antigen (EMA). Representative cytopathological features of malignant mesothelioma are shown in Fig.3-6, which shows the tumour cells from one patient were stained with the anti-EMA antibody. Immunohistochemical analysis was used to confirm all patients with malignant mesothelioma tested positive to EMA in this study. Most of the patient material obtained was used for cell killing assays (described later), as the cells tended to grow slowly and availability was limited.
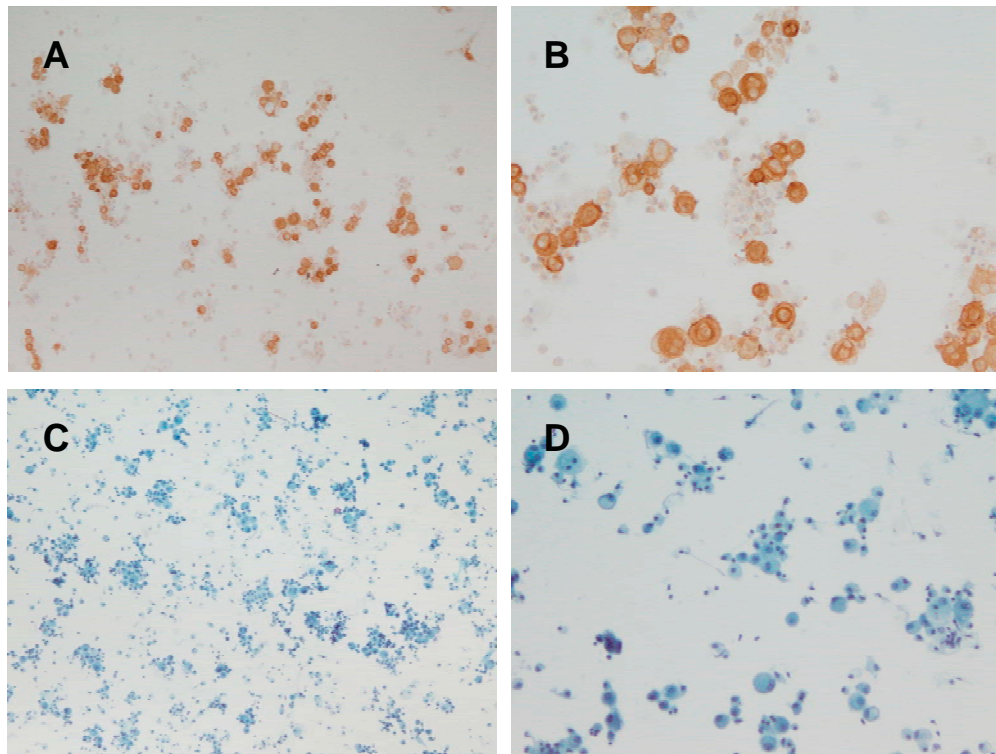
Fig. 3-6 Cytopathological features of malignant mesothelioma. Patient pleural fluid was centrifuged and these cells were cytospun following collection. Cell pellets were stained with E29 antibody against epithelial membrane antigen (EMA). Immunostaining of mesothelioma cells showed positive staining for epithelial membrane antigen (EMA) in the pleural fluid. A: positive malignant mesothelioma cells, x40 magnification, B: positive malignant mesothelioma cells, x100 magnification; C: EMA negative non-malignant cells, x40 magnification; D: EMA negative non-malignant cells, x100 magnification.

3.4.4 VEGF and Flt-1 mRNA and Protein Expression in the Mesothelioma Patient Cells

The VEGF and Flt-1 RNA status of mesothelioma cells from pleural fluid were analysed by reverse transcription and RT-PCR. From the data shown in Fig.3-7A, VEGF and Flt-1 mRNA expression could be detected in cells from a human mesothelioma patient. A549, HUVEC and 293 cell lines were used as controls for VEGF and Flt-1 mRNA. The result showed amplification of a 408 bp fragment (representing VEGF 121 cDNA) and a 541 bp fragment (representing VEGF 165 cDNA) in all cell lines tested. The PCR bands corresponding to VEGF 189 (615 bp) and VEGF 206 (666 bp) could not be detected. In the four types of cells, 293 and mesothelioma cells from patient showed relatively high expression of mRNA. Amplification with Flt-1-specific primers generated a 613 bp product in patient cells and positive control cell lines, HUVEC and 293. No Flt-1 mRNA expression was detected in the A549 cell line. Mesothelioma cells from patient show high VEGF and Flt-1 mRNA levels.

Furthermore, I investigated the correlation between mRNA expression and protein expression for VEGF and Flt-1. A549, HUVEC and 293 cell lines were used as controls for VEGF and Flt-1 protein as well. As shown in Fig.3-7B, the VEGF and Flt-1 protein expression levels varied in all tested cell lines. Flt-1 protein expression was detected in the patient cells, but not in the A549 cell line, which correlated with Flt-1 mRNA expression. Comparison between Fig.3-7A and 7B revealed VEGF and Flt-1 mRNA expression level correlated with VEGF and Flt-1 protein expression level positively.

Based on these results, further evaluation of VEGF and Flt-1 promoter CRAds was felt to be rational.

A.
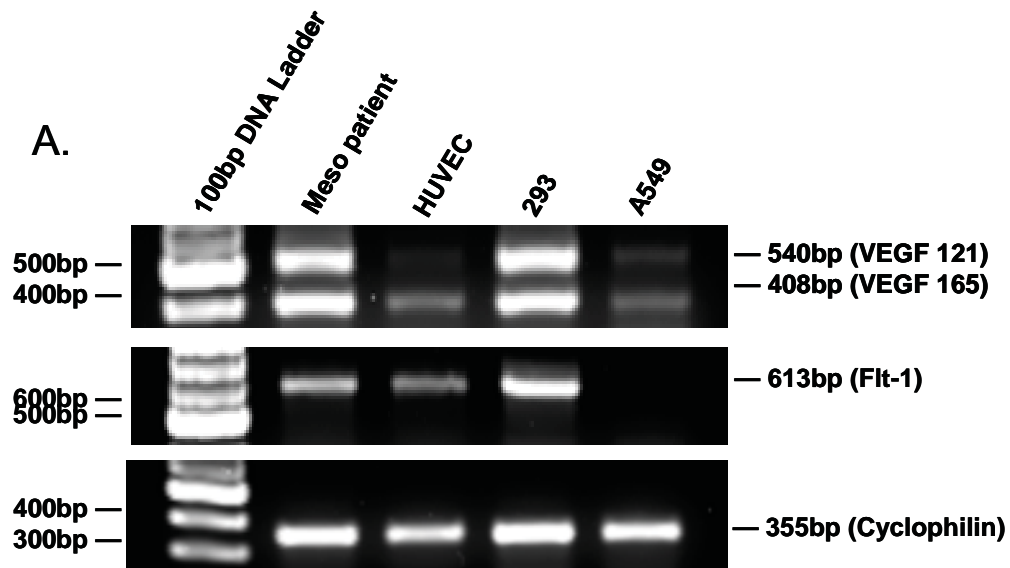
Fig. 3-7A VEGF and Flt-1 mRNA expression in a human mesothelioma patient. Total RNA extracts were made from mesothelioma patient cells, A549, HUVEC and 293 cell lines. RT-PCR was used for the detection of VEGF and Flt-1 mRNA expression levels. A549, HUVEC and 293 cell lines were used as controls for VEGF and Flt-1 mRNA using RT-PCR method. Cyclophilin was used as a house-keeping gene. Mesothelioma cells from patient show high VEGF and Flt-1 mRNA levels.

B.
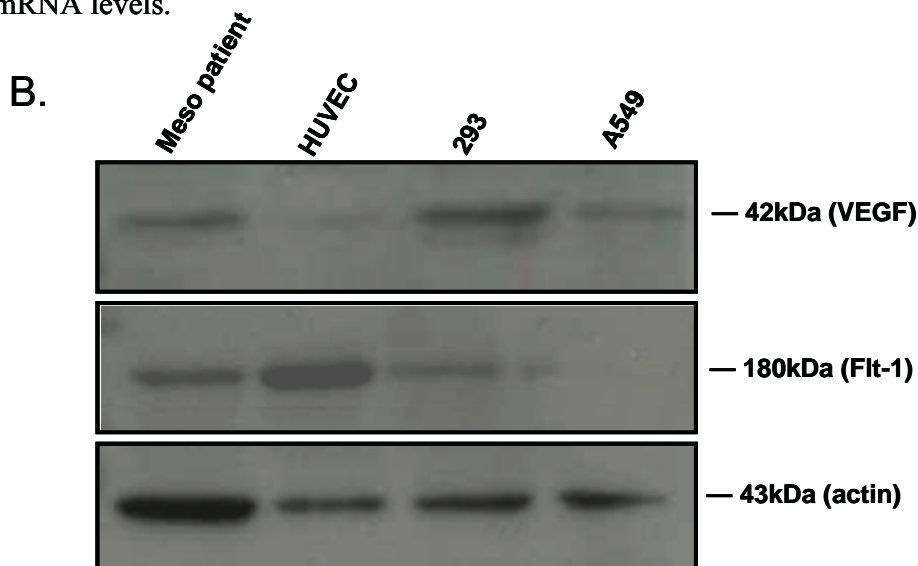
Fig. 3-7B VEGF and Flt-1 protein expression in a human mesothelioma patient. The cells (human mesothelioma patient cells, HUVEC, 293 and A549) were collected and lysed with lysis buffer. Western blotting was performed on cell lysates. A549, HUVEC and 293 cell lines were used as controls for VEGF and Flt-1 protein. Actin was used as a house-keeping protein. VEGF and Flt-1 protein expression could be detected in mesothelioma cells from patient.

3.5 Luciferase Gene Expression in Human Mesothelioma Cells

3.5.1 Luciferase Gene Expression in Human Mesothelioma Cell Lines

The activities of the VEGF and Flt-1 promoters in the adenovirus context were analysed by infection of cells with luciferase expression vectors in 9 human mesothelioma cell lines and control cell lines A549, BEAS-2B, HUVEC and 293 (Fig 3-8). The cells were infected with Ad5CMVLuc, Ad5Flt-1Luc, or Ad5VEGFLuc, all of which have Ad5 knob tropism, along with Ad5/3CMVLuc and Ad5/3VEGFLuc that have Ad3 tropism. An Ad5/3Flt-1Luc vector would have been ideal for completeness but this was not constructed and was not considered critical, as the main issue being assessed was whether Ad5 or Ad3 tropism would be best and I felt the available vectors could address that question. As far as possible, viral batches were checked for comparable particle to pfu ratios using the standard assay on 293 cells. As can be seen, the luciferase expression in this line was fairly comparable with the VEGF constructs although the Ad5/3CMVluc construct had slightly lower expression in these cells than the Ad5CMVLuc counterpart.

For the vectors containing the CMV promoter no real benefit was seen for Ad3 tropism over Ad5, in fact for the most part expression was slightly lower with the Ad3 vector as was noted for the 293 cells. There was however one important exception, which was the H2596 line, which had the very lowest level of CAR expression (from Fig 3-1). In this line, an advantage of Ad3 tropism could be seen. One the other hand for the weaker (but
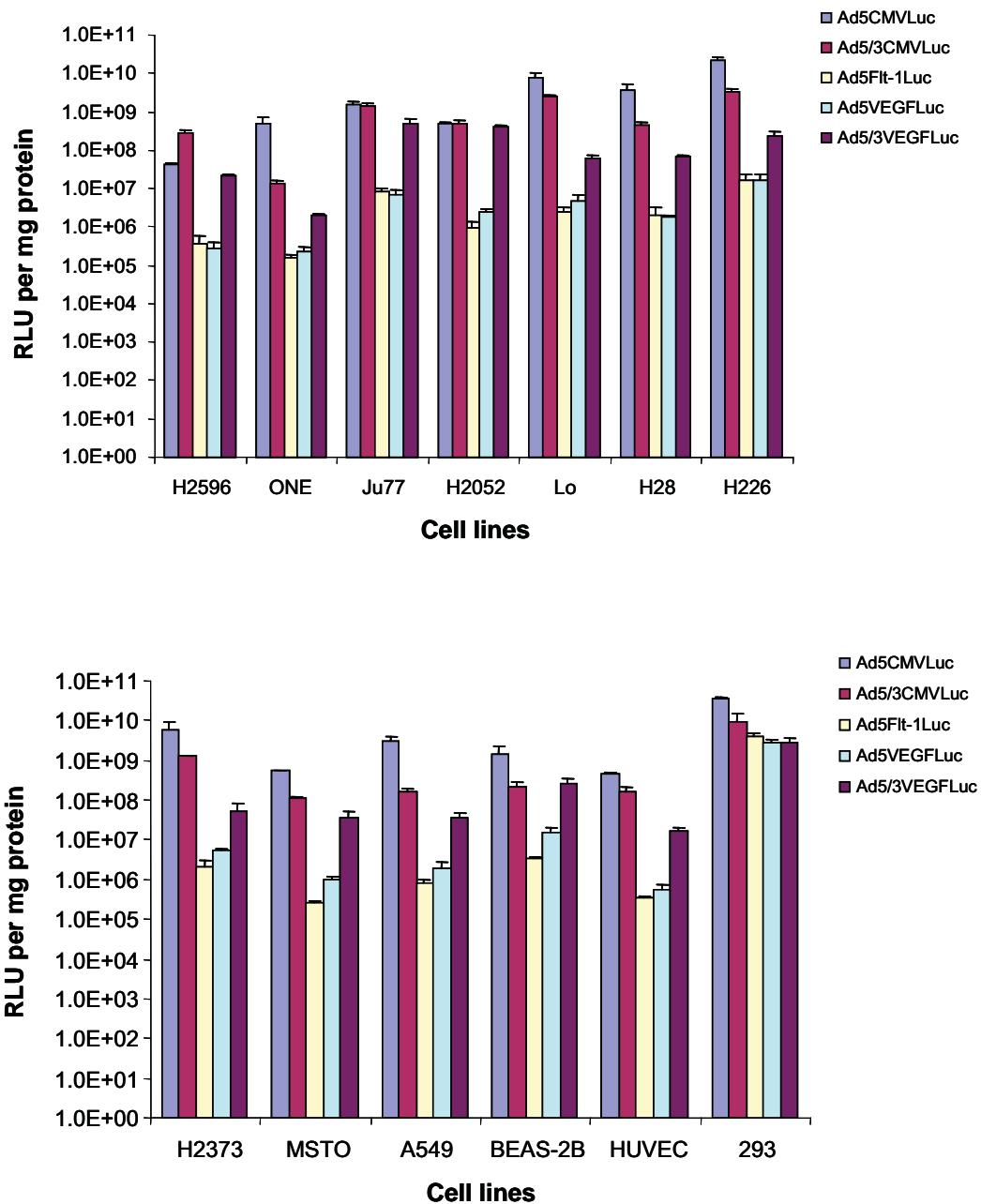
Fig. 3-8 VEGF and Flt-1 promoter-driven luciferase expression from Ad vectors. 9 human mesothelioma cell lines, A549, BEAS-2B, HUVEC and 293 cell lines were infected with at an MOI of 100 and luciferase activity was measured 48 h later. Ad5 tropism VEGF-luciferase vector demonstrated higher promoter activity than Flt-1-luciferase vector in all cell lines compared to 293 cell line. Evaluation of gene transfer using Ad5/3 versus Ad5 tropism VEGF-luciferase vectors indicates a general advantage for Ad5/3. Data were presented as the mean ± SD of triplicate wells.

putatively more specific) VEGF promoter constructs, Ad3 tropism had an advantage for all lines tested.

With regard to selectivity, I could not draw a conclusion in regard to VEGF, as all tested lines had been positive for VEGF expression. With regard to Flt-1, expression in the Flt-1 positive 293 cells was 1000-fold or higher than for the various mesothelioma lines that had not demonstrated Flt-1 expression by PCR. However, expression in the Flt-1 positive HUVEC line was surprisingly lower than expected. The reason for this is uncertain but promoter activity is known to diminish with cell passage number. I certainly found that Flt-1 protein expression in the HUVEC was quite low, despite the earlier positive PCR results. On the other hand the Flt-1 protein expression in the 293 cells was very strong.

3.5.2 Luciferase Gene Expression in Mesothelioma Patient Cells

I assessed the VEGF and Flt-1 promoter activities in the Ad vectors containing the luciferase gene as a reporter in mesothelioma cells from patients. The cells were infected at an MOI of 100 and luciferase activity was measured 48 h later. Figure 3-9 shows luciferase gene expression in one patient's cells. Ad5/3 tropism VEGF-luciferase vector demonstrated higher promoter activity than Ad5 tropism VEGF-luciferase vector. No difference was seen using the different CMV tropism vectors. Expression with the VEGF promoter was stronger than with the Flt-1 promoter. I also detected CMV, VEGF and Flt-1 promoter activities in the Ad vectors in different patients, and got similar result as above.
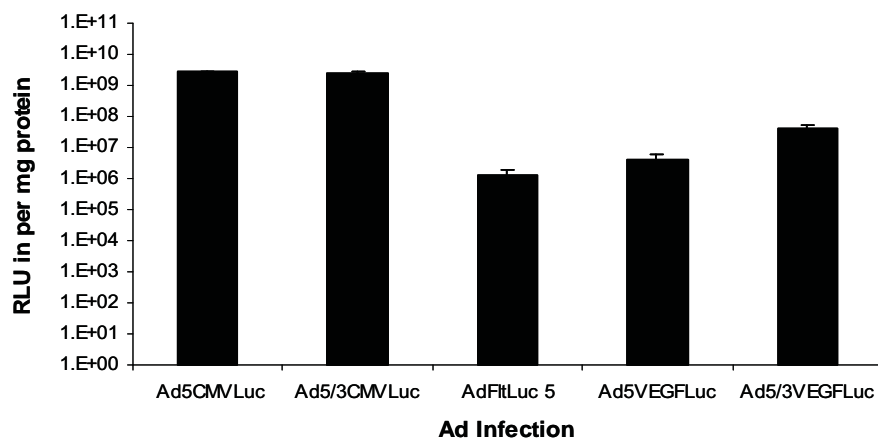
Fig. 3-9 VEGF and Flt-1 promoter-driven luciferase expression from Ad vectors in mesothelioma cells from one patient. Cells were infected with at an MOI of 100 and luciferase activity was measured 48 h later. Mesothelioma patient cells showed the slightly stronger VEGF promoter activity than Flt-1 promoter activity. Data was presented as the mean ± SD of triplicate wells.

3.6 Cell-killing Efficacy of VEGF and Flt-1 CRAds

3.6.1 Flt-1 and VEGF Promoter-based CRAds Kill Human Mesothelioma Cells

To demonstrate the cell-killing capacity of Ad5(5/3)VEGFE1 and Ad5(5/3)Flt-1E1 in human mesothelioma cell lines, I infected 9 cell lines, one positive cell line A549 (known to be highly permissive for wild type Ad5 replication) and one "normal" airway epithelial cell line BEAS-2B with small amounts of each virus (10 pfu/cell) to allow multiple cycles of viral replication over the ensuing 4-6 days. I then stained the attached cells with crystal violet and counted viable cells by measuring OD_{570} (Fig. 3-10) at various time points as shown. All analyses are done in triplicate and an image of the plates at the final assessed time point is shown. Susceptibility of mesothelioma lines tested here varies quite widely, even with wild type Ad. Lo and MSTO cell lines were quite sensitive to killing with wild type Ad, the Flt-1 and VEGF viruses, comparable to the known permissive A549 lung cancer line. H226 cell line was sensitive to killing with Flt-1 CRAds and Ad5/3VEGFE1, but was resistant to wild type Ad and Ad5VEGFE1. The cell-killing efficacy of all replicative viruses tested here showed no difference in H28 cell line. Some cell lines, H2052, H2373 and H2596, were resistant to wild type Ad and Ad5Flt-1E1, but they were sensitive to killing with VEGF CRAds and Ad5/3Flt-1E1. In ONE and Ju77 cell lines, VEGF CRAds and Ad5/3Flt-1E1 induced more cell death than wild type Ad and Ad5Flt-1E1. These replicative viruses still showed some cell killing effect in control cell line BEAS-2B though there are 40% of cells were alive 120 h after infection. All analyses presented here were conducted with
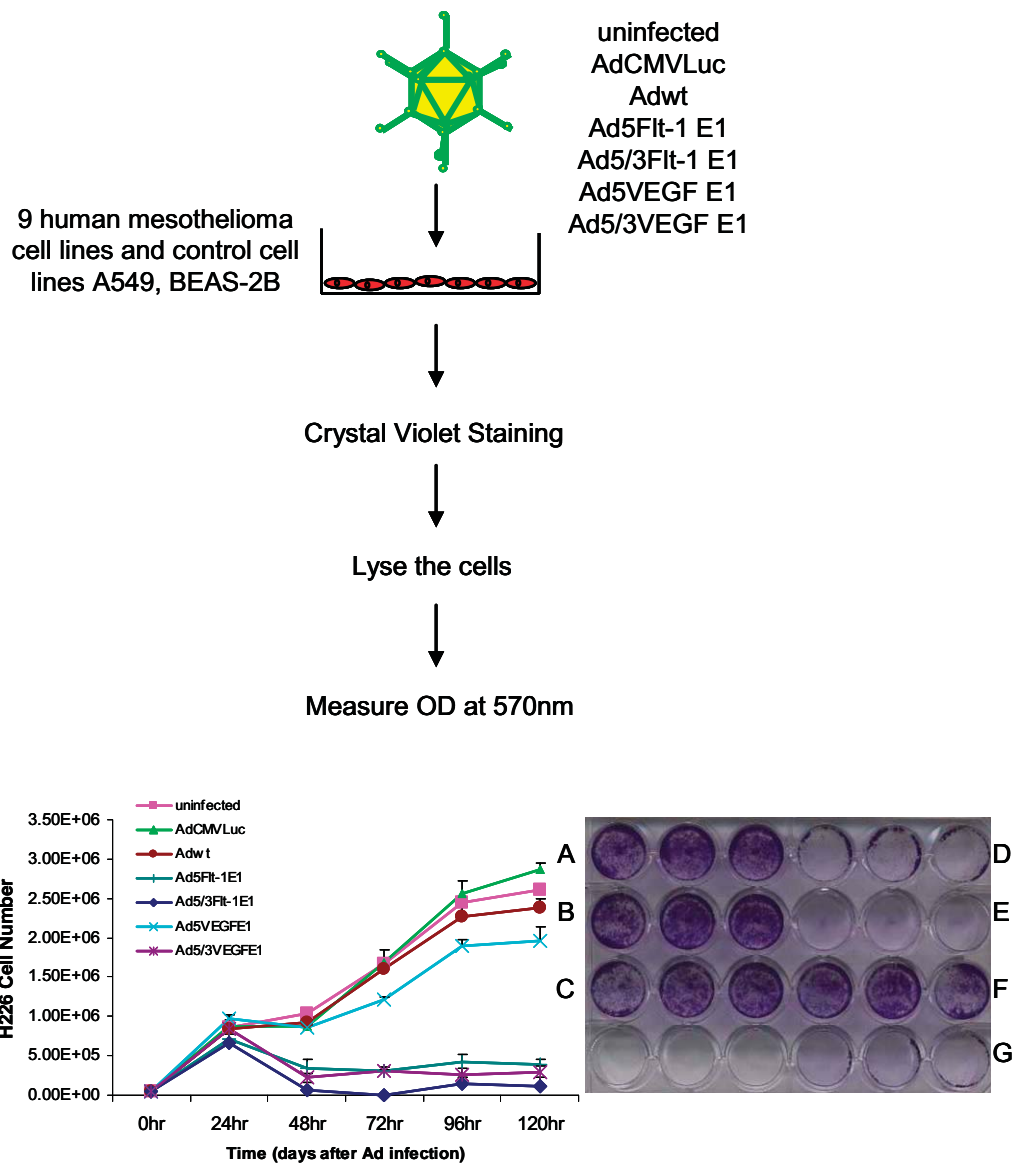
Fig. 3-10 Oncolytic potency of the CRAds in human mesothelioma cell lines.
Cells were infected with different viruses: A.uninfected B.AdCMVLuc C. Adwt
D. Ad5Flt-1E1 E. Ad5/3Flt-1E1 F. Ad5VEGFE1 G. Ad5/3VEGFE1. The attached
cells were stained with crystal violet 4-6 days post-infection and cell viability was
determined by measuring OD_{570nm}. Data were presented as the mean± SD of
triplicate wells. 3 of replicate experiments was performed.
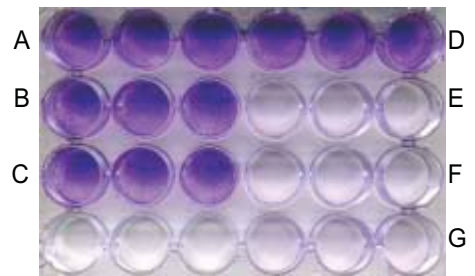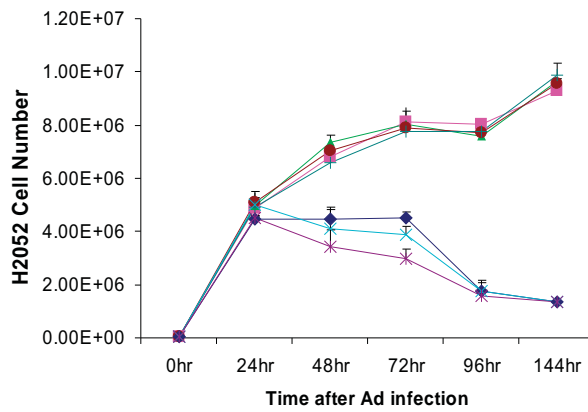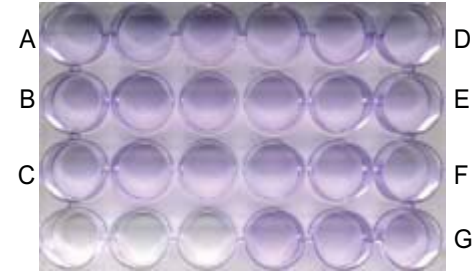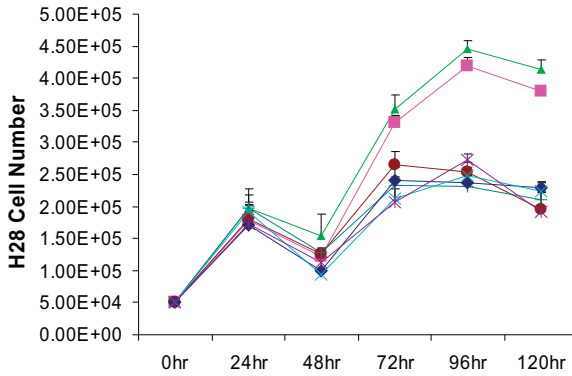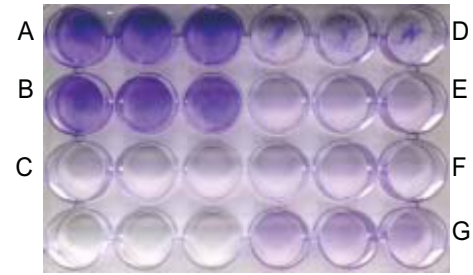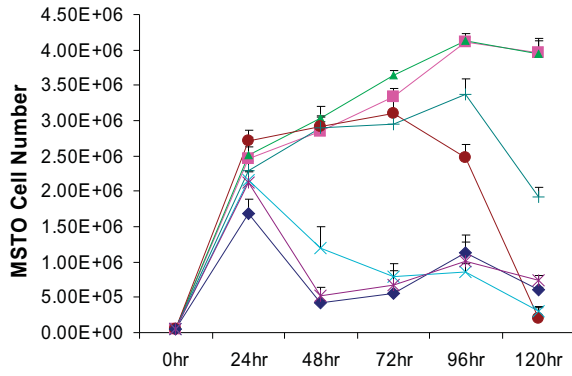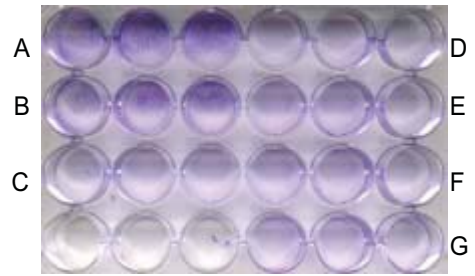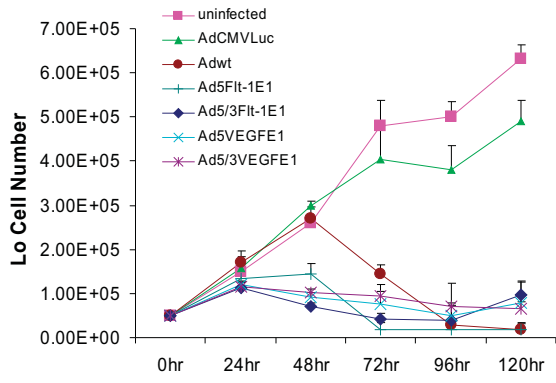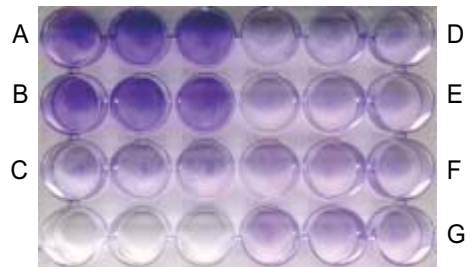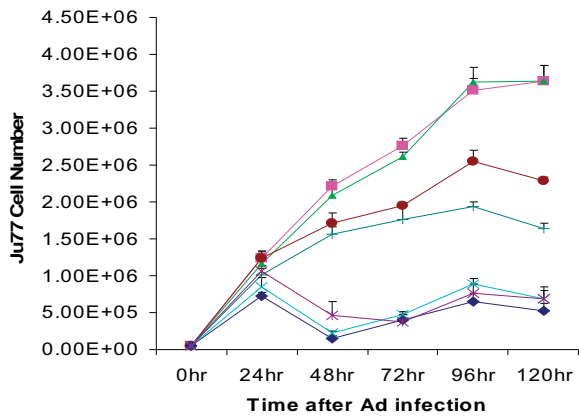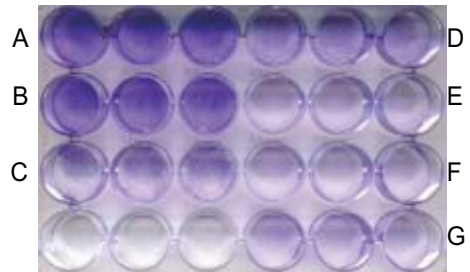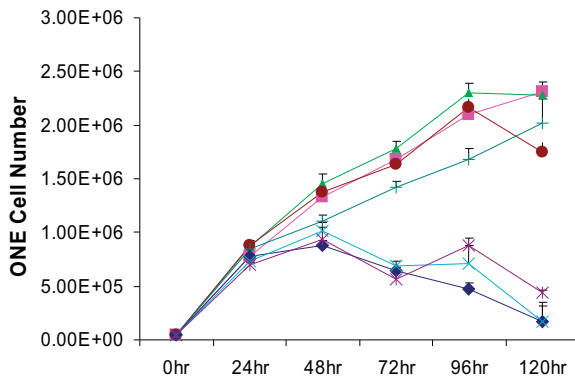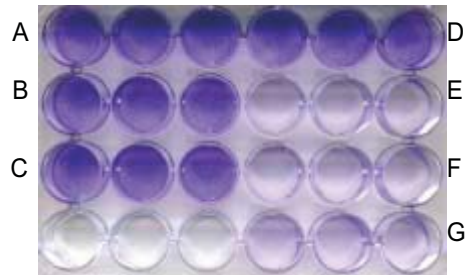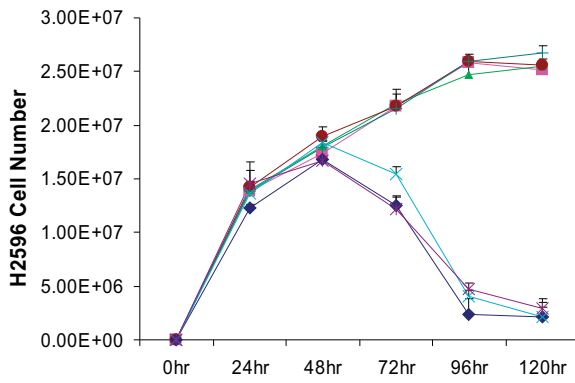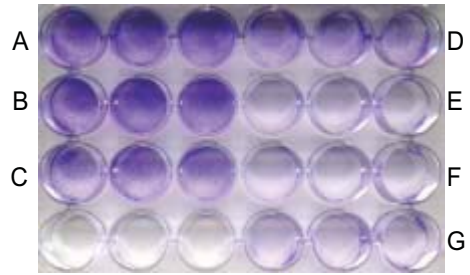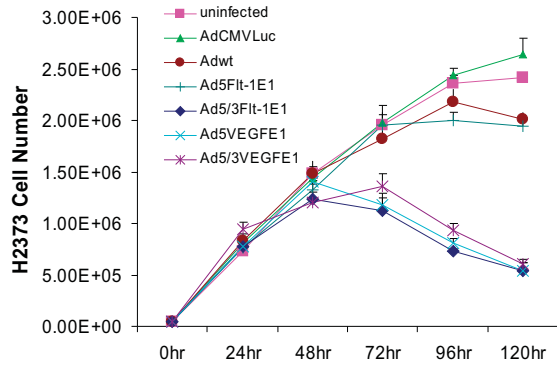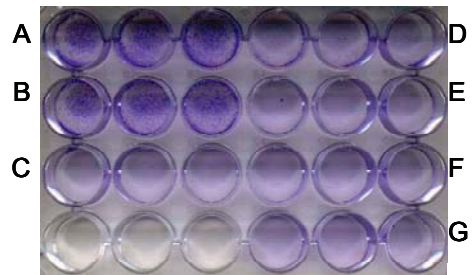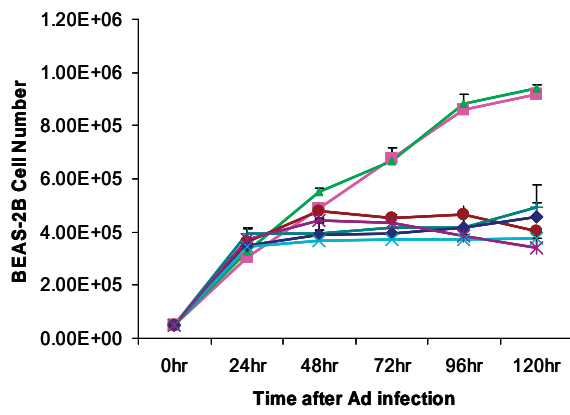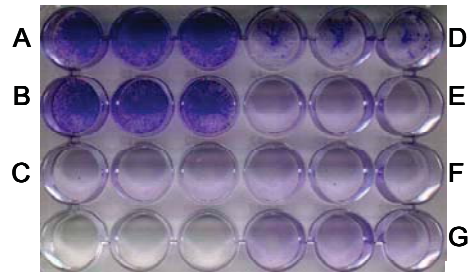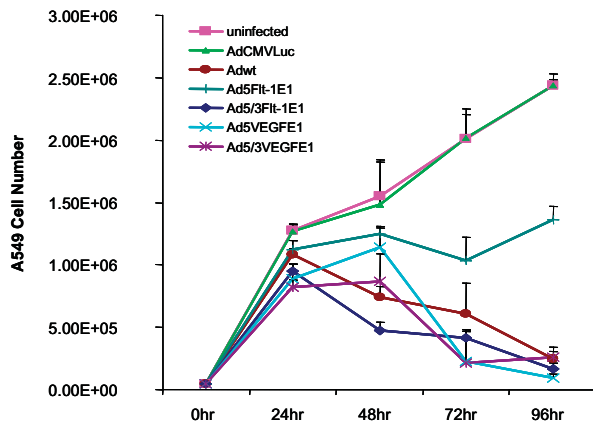
the same batches of virus and great care was taken to ensure consistency with the technical aspects of the experiments. The heterogeneity of results suggest a complex interplay between cellular factors such as the activity of the various promoters and cell receptors as well as other undefined properties of the cells.

3.6.2 Flt-1 and VEGF Promoter-based CRAds Kill Human Mesothelioma Cells from Patients

To demonstrate the cell-killing capacity of Ad5 (5/3) VEGFE1 and Ad5 (5/3) Flt-1E1 in mesothelioma cells from patients, I infected the cells with small amounts of each virus (10 pfu/cell) to allow multiple cycles of viral replication over the ensuing 4 days. I then stained the attached cells with crystal violet and counted viable cells by measuring OD_{570}. The cell killing effect of Flt-1 and VEGF promoter-based CRAds in three different patients with mesothelioma was shown in Figure 3-11. The result from one patient (Fig. 3-11A) showed that the cells had only modest susceptibility to wild type Ad and VEGF promoter-based CRAds, but they were quite sensitive to killing with Flt-1 CRAds. The cell-killing efficacy of wild type Ad and Flt-1 and VEGF promoter-based CRAds was assessed in cells from other two patients (Fig. 3-11B,C), and the cells were more sensitive to killing with Flt-1 CRAds than VEGF CRAds. No consistent difference was seen between Ad5 and Ad3 tropism. In conclusion, Flt-1 promoter-based CRAds had a significant cytotoxic effect to the human mesothelioma cells from patients.
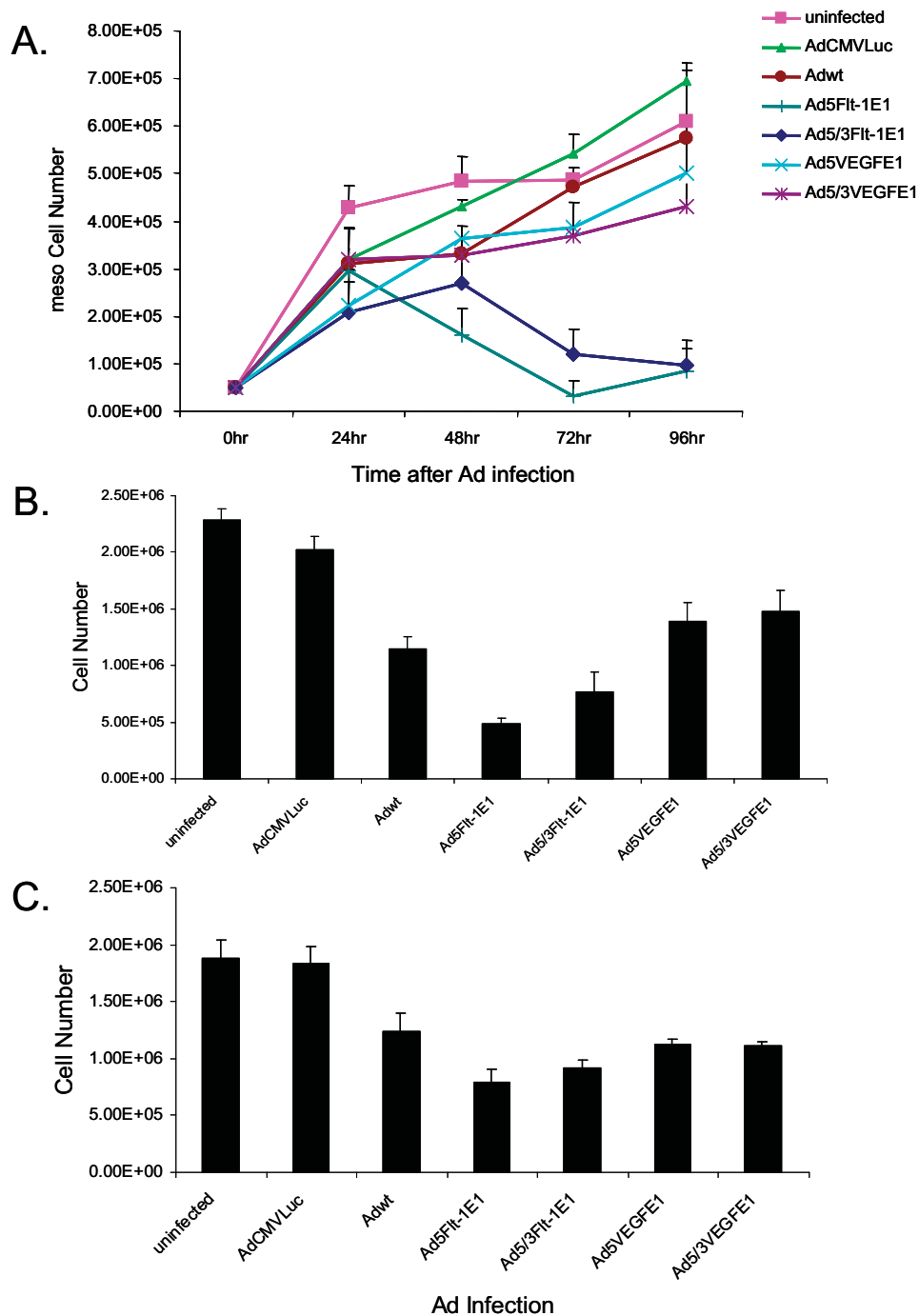
Fig. 3-11 Flt-1 and VEGF promoter-based CRAds killed human mesothelioma cells from patients. Cells were infected with different viruses. The attached cells were stained with crystal violet and counted viable cells by measuring OD_{570}. A, B, C represented 3 different patients with mesothelioma. A: CRAds killed mesothelioma cells from one patient at different time points. B,C: CRAds killed mesothelioma cells from other patients 96 h post infection. Data were presented as the mean± SD of triplicate wells. Each experiment was performed once.

3.7 Discussion

Transcriptional activity of cell-specific promoters typically correlates with the level of expression of the corresponding endogenous gene. Other factors beyond gene expression affect the relationship between mRNA levels and protein expression, including for example the rate of degradation and any post-translational processing of the protein being assessed. As shown in Fig.3-4 and 3-5, I found a reasonable concordance between VEGF mRNA expression level and protein expression level. The results with Flt-1 analysis showed for the most part undetectable mRNA levels in the mesothelioma cells (apart from very faint bands in 3 lines) despite positive signals in the HUVEC and 293 cells. Protein levels were variably low, but the 293 results were strong and concordant with the PCR results. Despite the positive PCR results in HUVEC it appears that expression may be variable depending on culture conditions. I suspect that by the time enough cells had grown up for the infection assays that Flt-1 expression was waning. In the protein analysis the Flt-1 expression in the HUVEC was no stronger than the mesothelioma cells and much less than the 293 cells. Importantly, I was able to extend my analyses to primary mesothelioma material. The major difference between this material and the cell lines was the much more readily detectable Flt-1 mRNA results in the patients, suggesting higher gene expression *in vivo* than in the lines. This would fit with previous publications in which Flt-1 expression has been detected by immunohistochemistry in mesothelioma biopsies.

I next assessed the transgene expression driven by VEGF and Flt-1 promoters in the Ad context *in vitro*. In all of the lines tested, luciferase expression was achieved using VEGF, Flt-1 or CMV promoters. An advantage of Ad3 over Ad5 tropism was seen for

the VEGF vectors but not for the CMV vectors except for the H2596 line that had the very lowest levels of CAR expression. This suggested that the high efficiency of the CMV promoter was overcoming any advantage of Ad3 tropism in most of the cells. I did perform additional studies using lower doses of AdCMV vectors but even here was unable to convincingly demonstrate an advantage for Ad3. A previous report by Zhu et al had found 2-5x greater expression with AdCMV vectors having Ad3 tropism compared to Ad5, but different cell lines were used (Zhu et al., 2006). In my studies using the VEGF promoter here, the advantage of Ad3 tropism ranged up to 100-fold in the tumour lines, despite equivalence in 293 cells. On balance, it seems reasonable to propose Ad3 tropism as the preferred option, and it seems that the advantage may be greater for those promoters that are relatively weaker. In general, tissue-specific promoters that are used in gene delivery or replicating vectors are weaker than CMV.

Viruses containing the Flt-1 promoters driving E1 gene expression were constructed using standard techniques of homologous recombination in *E.coli* and production in mammalian cells. The recombinant adenoviral constructs can be transfected into 293 cells (which contain E1 to allow replication defective Ads to grow) or A549 cells (which do not have E1). The advantage of using the A549 cells is the avoidance of any possible homologous recombination with cellular E1 sequences and generation of wild type recombinants. I tried to produce Flt-1 promoter-based CRAds in A549 cells, but the yields were very poor. This may relate to the low activity of the Flt-1 promoter in these cells. In 293 cells high yields of Flt-1 promoter-based CRAds could be obtained relative easily and these were screened by PCR to detect any emergence of wild type virus. The VEGF CRAds were obtained through a previously established collaboration.

I screened a panel of human mesothelioma cell lines to evaluate the cell-killing capacity of VEGF and Flt-1 promoter-based CRAds. The aims were primarily to assess cell killing efficacy and as far as possible to get some information about selectivity. In regard to the latter, this is often problematic, as cells in culture do not necessarily represent normal tissues. Even primary lines have different replication kinetics and other factors such as interactions with extracellular matrix are missing and could impact upon viral replication. Most publications in the area present negative control cells with low activity of the promoter under evaluation. Selectivity is never absolute however, and even CRAds that have entered human clinical trial show some replication in normal cells – for example the CRAD AdΔ24 discussed in the introduction will replicate in normal proliferating cells. Despite these concerns, the limitations in human clinical trials are not due to excess and uncontrolled replication leading to toxicity, but rather poor cell killing efficacy for the tumours.

The cell killing assays were interesting in a number of respects. The variability between the lines was quite marked and difficult to encompass within a single unifying hypothesis. It appears that complex interactions between tropism and promoter strength are affecting replication and cell killing, as well as other undefined cellular factors. By way of example, the H2596 cells were found to have very low levels of CAR and high levels of expression of CD46. Thus, greater cell killing would be predicted with Ad3 tropism vectors and that was indeed the case with the AdFlt-1 CRAds (i.e. Ad5/3Flt-1E1 >> Ad5Flt-1E1). These cells also appeared completely resistant to wild type Ad5 virus. The Ad3 versus Ad5 differential was also seen to some extent with the VEGF CRAds at the early time points but was much less impressive than the differential seen with the Flt-1 CRAds. This difference in response might be related to the relative

activity of the VEGF promoter versus the Flt-1 promoter in this line. As was seen with the luciferase gene transfer studies, greater promoter activity appears to blunt to some extent the differences due to viral tropism. Looking across the responses in the various lines, the general trend is that the Ad3 tropism vectors are at least as good as, if not better than the Ad5 versions. In no line did the Ad5 version appear better than Ad3. Thus, despite the heterogeneous responses, the summary of the cell line studies suggests development of Ad3 tropism CRAds appears rational as a design.

I was able to extend the studies of cell killing to the analysis of primary mesothelioma cells in short-term culture obtained from malignant pleural effusions. In these analyses the Flt-1 CRAds in particular showed good killing effect. In each of the cases studied, the Flt-1 CRAds were more efficacious than wild type Ad.

The above studies have shown that new viruses were created that have greater cell killing capacity for many mesothelioma cells than does wild type adenovirus. However, demonstrating improved selectivity of cell killing for tumour versus normal cells is more problematic. Compared to the cancer cell lines the "normal" BEAS-2B cells had a relatively blunted susceptibility to all of the viruses, including wild type. Certainly the new CRAds did not appear worse than wild type in this regard, so one could argue for an improved "therapeutic window". However, more extensive testing in systems designed to represent normal tissues would be desirable. To this end Zhu et al used freshly obtained human liver slices and were able to demonstrate reduced transgene expression using Ad vectors containing putative tumour-specific promoters (versus CMV) driving luciferase expression, but replication was not assessed. Ideally such a system would be useful to adapt for assessment of replication toxicity but this is not

available to me at this time. The "liver-off" phenotype of the Flt-1 promoter has already been assessed using a various Ad vectors carrying reporter genes in rodent models (Reynolds et al., 2001). I had hoped that the A549 cell line would be a useful "Flt-1 negative" control, but clearly some replication of Flt-1 CRAds was seen in this line, although replication was limited in that I was unable to use the line to produce vector stocks. The fact that this line is frequently used to produce viral stocks is a testament to its generally high permissivity for Ad replication. Thus even a small amount of promoter leakage could cause toxicity in these cells. Strategies to improve the selectivity of the Flt-1 CRAd could include re-engineering of the vector with blocking sequences (such as a poly-A sequence) inserted between the left ITR and the Flt-1 sequence if unacceptable toxicity was found on testing with more relevant "normal" human substrates, such as the liver slice technique (Rots et al., 2006). Even this advanced technique would not completely address liver toxicity issues however because it is now known that liver tropism *in vivo* is dependant on interaction of virus with blood coagulation factors (Waddington et al., 2008).

Despite the above comments concerning selectivity, as noted previously the major limitation in CRAd development clinically has been efficacy. As a next step in my evaluation, in the subsequent chapter I have extended evaluation to an *in vivo* murine model in which human tumour xenografts are established in nude mice. This model uses the H226 line, which as seen here has susceptibility to both Flt-1 and VEGF CRAds.