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**TUMOUR GENE THERAPY USING ADENOVIRAL VECTORS EXPRESSING
TUMOUR NECROSIS FACTOR ALPHA**

By

ROBERT ANTHONY MARR, BSc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

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**TUMOUR GENE THERAPY USING ADENOVIRAL VECTORS EXPRESSING
TUMOUR NECROSIS FACTOR ALPHA**

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**Title: Tumour Gene Therapy Using Adenoviral Vectors Expressing Tumour Necrosis
Factor alpha.**

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Number of pages: 176

ACKNOWLEDGMENTS

I would like to thank my supervisor Dr. Frank Graham and my supervisory committee for their help and guidance over the years. I would also like to extend my thanks to Mary Hitt, Christina Addison, and Jonathan Bramson, for their technical assistance and constructive criticism which contributed to the generation of my manuscripts. Thanks are also extended to Stevie Baker for her support and assistance over the last year of my degree (sniffle). Finally I would like to thank my parents, Don and Carmela Marr (sniffle sniffle), for their moral and financial support over the years.

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CHAPTER I: ABSTRACT

The general focus of my project was the production of recombinant adenovirus vectors for use in immunotherapy of cancer. The basic strategy involved the infection of tumour cells with Ad-vectors expressing cytokines, inducing a local anti-tumour response. The cytokine of interest for my project was tumour necrosis factor alpha (TNF α), which I used for treatment of a murine transgenic model of breast cancer. TNF α is a pluripotent cytokine with a wide variety of physiological functions including antitumour activity. TNF α was originally discovered through the anticancer activity of sera of mice treated with endotoxin (Carswell et al., 1975). There are two known cell surface receptors for TNF α termed p55 and p75. Both receptors signal a variety of functions and some redundancy exists between them. However the p55 TNF receptor is the major activator of cytotoxicity and cytokine secretion, while the p75 TNF receptor is primarily responsible for proinflammatory and lymphoproliferative signals.

Perhaps the major limiting factor affecting the use of TNF α in tumour therapy is its systemic toxicity, through the induction of septic shock and cachexia (Tracey, 1995; Tracey et al., 1986). We have been investigating techniques which would reduce the systemic side effects of TNF α , while retaining its antitumour activity. We found that local expression of TNF α from within a tumour (transduced cells) alone is not enough to eliminate its lethal side effects, therefore two other approaches are being investigated in an attempt to deal with systemic toxicity induced by TNF α . The first was the

construction of an Ad vector expressing a membrane bound mutant of murine TNF α (see chapter III). The second approach involved specific targeting of the two cell surface receptors of TNF α . To accomplish this, we used Ad vectors expressing human TNF α , and a novel p75 TNF receptor specific mutant of murine TNF α for use in tumour immunotherapy (See chapters IV and V). It was found that restricting TNF α to the membrane produced a marked reduction in lethality while retaining near normal antitumour activity. Targeting the p55 TNF receptor proved to be ineffective, while targeting the p75 TNF receptor drastically reduced the lethality of the cytokine while retaining some antitumour activity. However the overall efficacy of this therapeutic technique was poor, as only a low percentage of mice were cured with our Ad-TNF vectors.

Literature Review

Adenoviruses

Adenoviruses were first identified through the observation of spontaneous degeneration of primary human cell cultures derived from adenoid tissue (Reviewed in (Shenk, 1996)). A large number of adenovirus serotypes have been isolated, characterized and identified as the etiologic agents causing a variety of diseases including acute respiratory infections, conjunctivitis, and gastrointestinal infections. Adenoviruses are classified as part of the *Adenoviridae* family belonging to the *Mastadenovirus* genus. There are at least 47 different human adenoviral serotypes based on the neutralizing

characteristics of antisera prepared against adenoviruses of other serotypes. These serotypes can be arranged into six subgroups (A to F) according to their ability to agglutinate red blood cells (among other properties). Adenovirus types 2 and 5 (subgroup C) are probably the best characterized of the serotypes, and complete genome sequences have been determined for both (Chroboczek et al., 1992; Roberts et al., 1984). The extensive research conducted on adenoviruses has provided unique insight into a variety of cellular mechanisms including cell cycle regulation, transcriptional regulation, and mRNA splicing. As well, adenoviruses have also proven very useful as vehicles for gene delivery into mammalian cells both for the purposes of research and gene therapy.

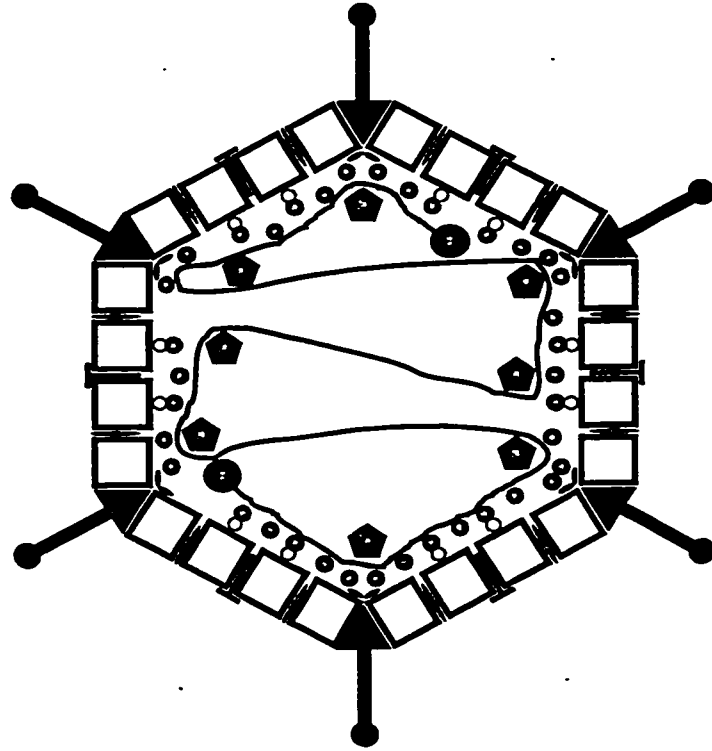
The Adenovirus Virion

The adenovirus virion consists of a linear double-stranded DNA molecule of approximately 36 kb containing short inverted terminal repeats (ITRs) at each end, packaged within the core of the virion. The protein shell or capsid of the virion is an icosahedron composed of 240 hexon and 12 penton units forming 20 triangular surfaces and 12 vertices. Each penton unit at the vertices is attached to a projecting fiber. The entire capsid is approximately 140nm in diameter (Shenk, 1996) (see fig 1-1).

The core of the adenovirus virion contains four proteins in association with the viral genome. Proteins V, VII, and mu are all found within the core associated with the viral DNA. Protein VII is thought to act similarly to nuclear histones providing a substrate for the viral DNA to wrap around. The fourth protein, termed the terminal

Figure 1-1: The adenovirus virion.

A cross sectional view of the adenovirus virion. The virion is composed of the outer capsid components (shown below on the left) and the inner core components (shown below on the right) including the viral DNA. This diagram was modified from Shenk, "Adenoviridae": The Viruses and Their Replication", In Fields Virology, 3rd Edition (1996).



- | | | | |
|---|--------------|---|-----------|
|  | Fiber (IV) |  | TP |
|  | Penton (III) |  | X |
|  | Hexon (II) |  | V |
|  | Protein IX |  | DNA + VII |
|  | IIIa | | |
|  | VIII | | |
|  | VI | | |

protein (TP), is covalently attached to the 5' end of the viral chromosome (Rekosh et al., 1977). The TP is attached via a phosphodiester bond between a key serine residue within TP and the 5' hydroxyl of each terminal cytosine residue of the Ad DNA. The function of this protein in Ad DNA replication will be discussed below. The adenovirus capsid is composed of seven proteins, the most abundant being polypeptide II which forms the trimeric hexon unit of the capsid (Horwitz et al., 1970). The penton base, found at the vertices of the capsid, is composed of a pentamer of polypeptide III. The fiber is composed of a trimer of polypeptide IV and projects outward from the penton base at each vertex functioning as the ligand for the primary Ad receptor (van Oostrum and Burnett, 1985), (Bergelson et al., 1997). The *hexon capsomere* is the primary unit forming the capsid while the penton fills the gaps produced at the vertices. Other proteins associated with the capsid (VI, VII, IX, IIIa) are thought to act by bridging various parts of the capsid and core as well as serving to stabilize the virion.

Adenovirus Infection

As mentioned above, the adenovirus first recognizes and binds to its receptor, the Coxsackie virus Adenovirus receptor (CAR), on the cell surface as the first step of infection. CAR is a 46kD transmembrane protein thought to possess two extracellular immunoglobulin-like domains (Bergelson et al., 1997). Internalization of the virus is mediated via an association between the α v integrins (α v β 3, α v β 5) on the target cell's surface and the penton base (Wickham et al., 1993). This association triggers

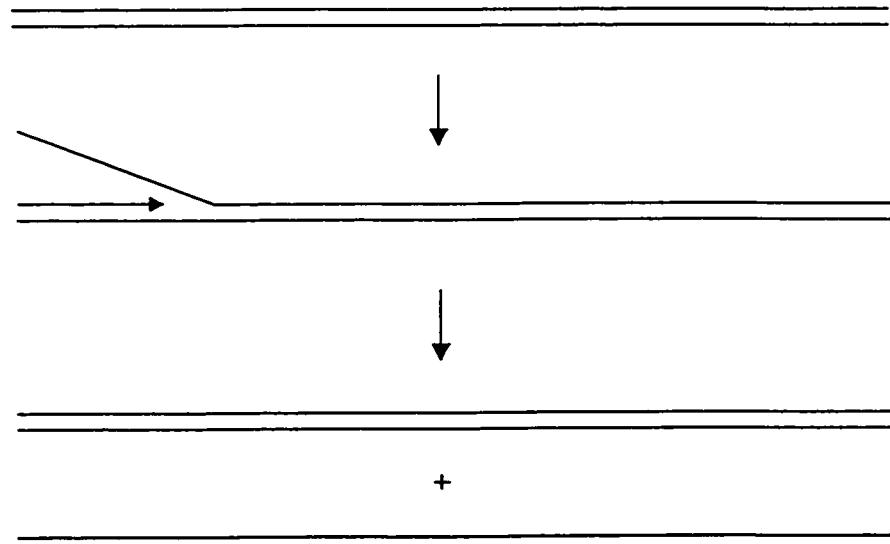
internalization of the virus via receptor-mediated-endocytosis. Viral escape from the endosome is mediated by the penton base and is triggered by the acidic environment inside (Pasten et al., 1986; Svensson, 1985). Once inside the cytosol, the virus is directed toward the nucleus, likely through association with microtubules mediated by hexon (Dales and Chardonnet, 1973). The viral DNA enters the nucleus by passing through the nuclear pores, leaving the capsid behind to be proteolytically degraded (Greber et al., 1993). The first gene products to be expressed from the viral genome are from the E1 region, which functions in transactivation of other early gene transcription (E2, E3, E4) as well as inducing cell cycle progression. Six to eight hours after early gene expression late gene synthesis is upregulated (L1-L5) in conjunction with the onset of viral DNA synthesis (reviewed in (Shenk, 1996)).

Adenoviral DNA replication is a two-step process involving the viral origin of replication contained within the ITR. The first round of replication produces one double stranded copy of the genome and a single stranded intermediate. Secondly, replication of the displaced single strand is primed by self-association mediated by the ITRs (see figure 1-2). Both cellular and viral encoded proteins contribute to replication of the genome. The viral encoded pre terminal protein (pTP) primes the initiation of DNA synthesis by providing a free hydroxyl group for the addition of dCTP by the viral encoded polymerase (Rekosh et al., 1977). Cellular nuclear factors I and III (NFI and NFIII) also contribute to the initiation of viral DNA replication, while NFII and the viral encoded (E2) 72kD DNA binding protein are required for chain elongation

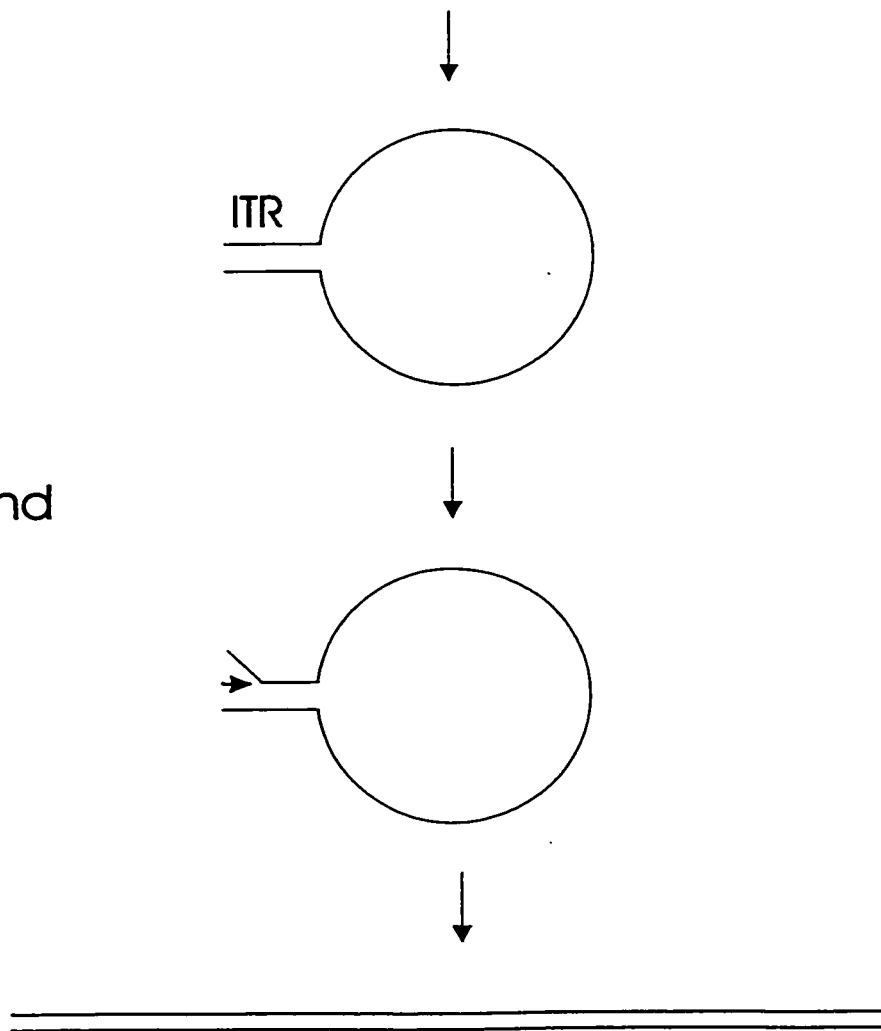
Figure 1-2: Adenovirus DNA replication.

Graphical representation of the two phases of adenoviral DNA replication. The first round involves replication of one strand primed by the TP at the origin of replication within the ITR. This produces a double stranded and a single stranded intermediate. The second round of replication is facilitated by complementary association of the ITR sequences to form a pan-handle structure which resembles the ends of the adenoviral genome. Replication primed from this structure produces the second complete double stranded copy of the adenoviral genome.

First Round



Second Round



(Chen et al., 1990; Lindenbaum et al., 1986; Nagata et al., 1983; Verrijzer et al., 1991). Incorporation of the replicated viral genome into the capsid requires only one cis acting element termed the packaging signal. This short sequence is situated at the left end of the genome near the ITR, and will only function when positioned near the ends of the chromosome (Grable and Hearing, 1992). Proteolytic degradation of the cytoskeleton and the induction of apoptosis facilitate escape of the new virus particles from the infected cells, resulting in release of approximately 10000 progeny viruses per cell (Green and Daesch, 1961).

Adenovirus Genome.

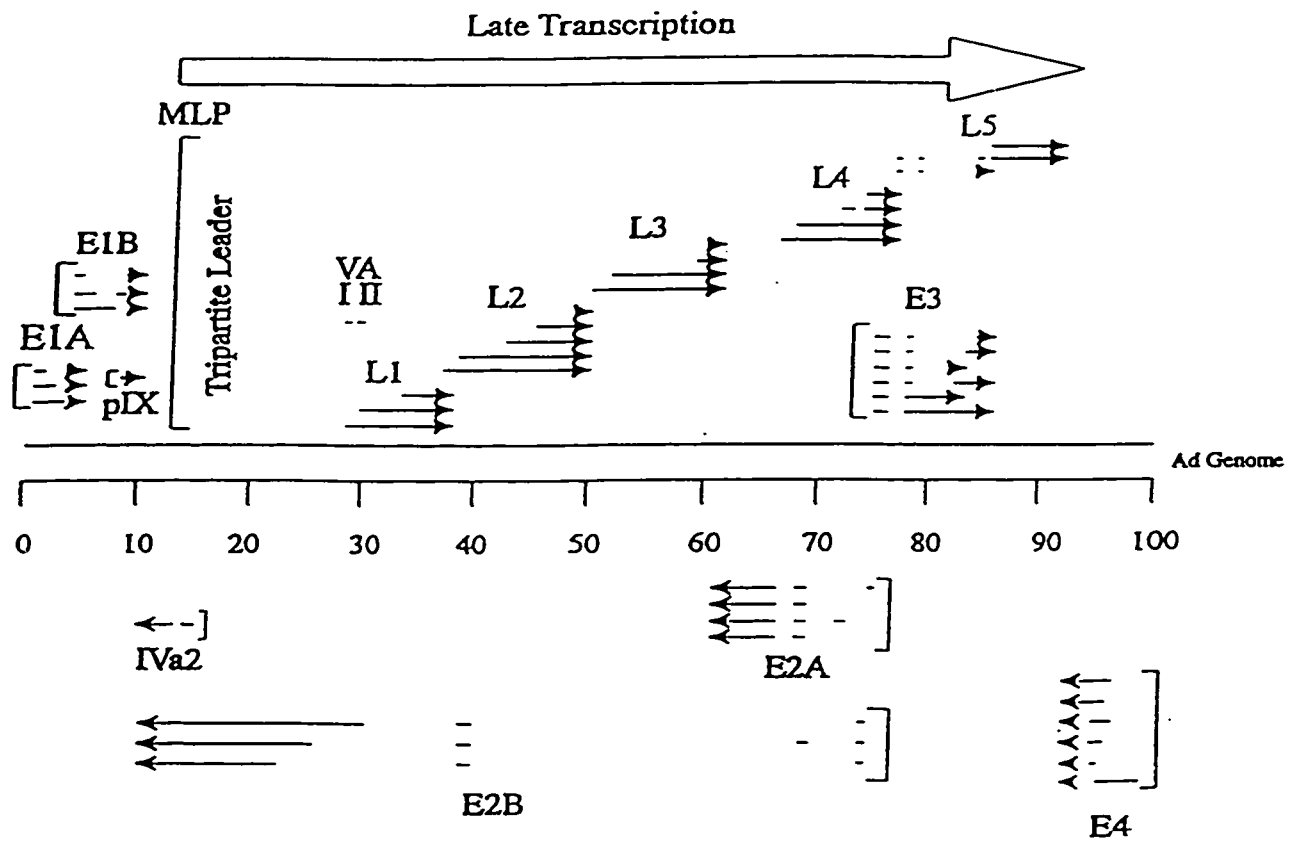
In terms of genome organization the Ad genome comprises five early regions (E1A, E1B, E2, E3, E4) and one late region which utilizes different polyadenylation sites to give five distinct mRNA families (L1 to L5) (see figure 1-3). Extensive splicing of the viral mRNAs results in synthesis of a variety of mRNA species encoding over 40 polypeptides (reviewed in (Shenk, 1996)) involved in all aspects of viral function. Each early region serves a variety of functions ranging from transactivation of viral and cellular genes, inhibition of apoptosis, host transcriptional and translational shut off, and immune evasion, while the late gene products are primarily virion structural components.

Early Region I

There are two groups of transcripts which are produced from E1, which utilize

Figure 1-3: Transcriptional map of adenovirus type 5.

All the major Ad transcript regions are indicated by brackets. All early transcripts are designated by an E, and the late transcripts by an L. Arrows represent the particular mRNA species. Those mRNAs derived from rightward transcription of the genome are positioned above the genome, and those mRNAs derived from leftward transcription are positioned below the genome. This diagram was modified from Shenk, "Adenoviridae": The Viruses and Their Replication", In Fields Virology, 3rd Edition (1996).



different promoters and polyadenylation signals (E1A and E1B). The E1A genes, (designated 12S and 13S) expressed first, are involved in activation of transcription and cell cycle progression. The two products differ in a 46 amino acid portion that is present in the 13S form and absent from the 12S (Downey et al., 1984). E1A expression is required for transactivation of all other viral promoters and deletion or mutation of E1A renders the virus replication deficient. The E1A proteins are thought to activate transcription and cell cycle progression through association with several cellular factors. One of the retinoblastoma (pRb) tumour suppressors, binds to the cellular E2F transcriptional activator and represses its function. Both E1A 12S and 13S can bind to pRb and prevent its repression of E2F (Bandara and La Thangue, 1991; Chellappan et al., 1991). The E1A proteins also modulate transcription through association with many other cellular proteins including the TATA binding protein (TBP), Dr1, ATF-2, and p300. The TBP binds to the TATA box which is a Thymine and Adenine rich region located in many promoter regions (Pugh and Tjian, 1992). TBP associates with the auxiliary transcription factor IID (TFIID), which mediates formation of the pre-initiation complex, facilitating transcription. Dr1 can bind to TBP and repress its activity (Inostroza et al., 1992). The association of E1A 12S with Dr1 is thought to relieve this repression and promote gene expression. ATF-2 is a member of the ATF family of transcription factors (Papavassiliou, 1994), and many cellular and Ad viral promoters contain ATF binding sites (Liu and Green, 1990). The p300 transcriptional coactivator is thought to promote cellular differentiation (Webster et al., 1988), and E1A blocks the ability of p300 to regulate gene

expression, thus facilitating entry into S phase (Arany et al., 1994).

The E1B gene products are involved in the prevention of apoptosis, as well as inducing host cell shut off. There are two major products produced from E1B: E1B-55kD and E1B-19kD (Green et al., 1982). E1B-55kD inhibits apoptosis through binding and repressing the activity of p53 (Kao et al., 1990) involved in cell cycle arrest and apoptosis. E1B-19K is a homolog of the Bcl-2 family of proteins that are involved in regulation of apoptosis. Bcl-2 act to inhibit apoptosis through dimerization with other pro-apoptotic family members (Bax, Bad) thus blocking apoptosis (reviewed in (Kroemer, 1997)). The relative abundance of the various pro and anti apoptotic factors determines the cells susceptibility to apoptotic stimuli. The E1B-19kD functions similarly to Bcl-2, thus protecting the cell from apoptosis. E1B-55kD is also thought to work in cooperation with E4 products to facilitate the block of host cell mRNA accumulation and to promote viral mRNA accumulation (Pilder et al., 1986; Sarnow et al., 1984). Both E1A and E1B regions are required for transformation of target cells as one is responsible for induction of cell cycle progression while the other blocks apoptosis induced by the former.

Early Region II

The products of E2 are all involved primarily in viral DNA replication. The DNA polymerase is a 140kD polypeptide, encoded by the viral E2b region, which also exhibits a 3'-5' exonuclease activity believed to be involved in proof reading (Field et al., 1984). The E2b encoded pTP is an 80kD polypeptide that complexes with the polymerase to initiate

DNA synthesis (Temperley and Hay, 1992). As discussed above pTP primes DNA synthesis by providing a free β -hydroxyl group from serine residue 562 for the formation of a phosphodiester bond with the 5' hydroxyl group of dCTP (the first base pair of the genome) (Smart and Stillman, 1982). The E2-72kD single stranded DNA binding protein is involved in binding to and protecting single stranded DNA replication intermediates produced during replication (van der Vliet and Levine, 1973). This protein is required for chain elongation and its presence greatly enhances the processivity of the viral DNA polymerase (Lindenbaum et al., 1986).

Early Region III

The early region 3 (E3) gene products are not required for viral replication and elimination of this region does not affect viral infection and virus production in cell culture. The products of E3 are involved in evasion of the host's immune system and protect the virus from multiple antiviral mechanisms. The E3-19kD protein is localized to the endoplasmic reticulum and functions through association with the antigen presenting domain of MHC class-I preventing antigen presentation on the cell surface (Burgert and Kvist, 1987). This is thought to protect Ad infected cells from recognition and lysis by specific cytotoxic T lymphocytes (CTLs). Another mechanism by which the host can control viral infection is through the production of TNF α from monocytes, macrophages, and lymphocytes. TNF α can induce apoptosis in virus infected cells (Koff and Fann, 1986) and adenoviruses lacking E3 have been found to be susceptible to TNF α

cytotoxicity (Gooding et al., 1988). The activation of phospholipase-A2 following activation of the TNFR-I (producing arachidonic acid) is an essential step in the cytotoxic response (Suffys et al., 1991). The E3-14.7kD and 10.4kD proteins are thought to interfere with arachidonic acid production (Zilli et al., 1992), thus protecting the cell from apoptosis. The transcription factor NF- κ B and its regulators can influence a cell's susceptibility to apoptotic stimuli. Activation of NF- κ B is believed to protect cells from apoptosis, through its transactivation of various genes including MYC, which in turn promote cell cycle progression through the upregulation of cyclins A and D3 (reviewed in (Foo and Nolan, 1999)). E3-14.7kD has been shown to interact with the cellular factor FIP-3, and this association is believed to interfere with FIP-3's ability to repress NF- κ B activity thus favoring cell survival (Li et al., 1999). In addition to the TNFR the Fas receptor can also trigger apoptosis, and can be used by cytotoxic lymphocytes (CTLs) to kill target cells (Stalder et al., 1994). Interestingly E3-14.7kD has been shown to block Fas induced apoptosis in Ad infected cells (Shisler et al., 1997). The E3-11.6kD protein is localized to the nuclear membrane and is thought to play a role in the induction of apoptosis near the end of the viral replicative cycle (Tollefson et al., 1996a; Tollefson et al., 1996b).

Early Region IV

Products of the adenovirus early region 4 (E4) have a seemingly less focused spectrum of activities compared to the other viral transcriptional units. Similar to E1A the

E4 encoded Orf6/7 polypeptide is able to transactivate transcription from the E2 promoter. This is also thought to occur through an association with E2F. It has been shown that Orf6/7 dimerizes to join two E2F factors and stabilize their binding to the E2 promoter, which contains two E2F binding sites (Obert et al., 1994). In addition to transactivation of viral transcription orf6/7 is thought to upregulate expression from cellular promoters which carry paired E2F binding motifs similar to the E2 promoter (Johnson et al., 1994). Both the Orf3 and Orf6 proteins are believed to facilitate accumulation of late viral mRNAs by enhancing mRNA transport and stability. Orf6 operates in conjunction with E1B-55kD to upregulate late viral mRNA export and inhibit host cellular mRNA export from the nucleus (Bridge and Ketner, 1990). Orf6 has also been reported to bind to p53 (independent of E1B-55kD) and repress its activity in a similar fashion to E1B-55kD (Dobner et al., 1996). The Orf4 polypeptide has been shown to downregulate both E1A and E4 promoter activity through its interaction with the cellular phosphatase (PP)2A (Bondesson et al., 1996; Kleinberger and Shenk, 1993). The E4Orf4-(PP)2A complex is thought to dephosphorylate / inactivate key cellular transcription factors (AP-1) involved in E1A mediated transcriptional upregulation. Recently Orf4 has also been implicated as a key factor in the final induction of host cell apoptosis during the viral life cycle in a pathway independent of p53 (Marcellus et al., 1998).

The Adenovirus Late Genes

Expression of Ad late genes coincides with the onset of viral DNA replication and

is regulated by the major late promoter (MLP). Several theories have been put forth to explain this delay in expression. One possibility is simply that the viral core needs a set amount of time to free itself from associated viral proteins before active transcription can begin from regions closer to the middle of the genome. This is supported by the observation that E1 and E4 gene products are the first to be expressed during infection. However, this can not fully account for the regulated / delayed expression of the Ad genes. Activation of the MLP is also controlled by viral encoded transcription factors. The viral “delayed early” IVa2 protein has been demonstrated to bind to and enhance MLP expression (Tribouley et al., 1994). Early gene products are believed to release the IVa2 promoter from its repressor(s) allowing it to transactivate the MLP. The late genes are expressed as one long transcript which utilizes alternative splicing and polyadenylation to produce the mRNAs encoding structural capsid components. All the late gene regions are divided into five families from L1 to L5 based on the usage of these different polyadenylation signals. The L1-52-/55kD polypeptide is not actually incorporated into the capsid but is thought to act as a scaffold protein facilitating capsid assembly (Hasson et al., 1989). L2 mRNAs encode penton and core proteins while L3 encodes hexon. The L3 region also encodes a protease which functions in facilitating capsid assembly by cleaving viral proteins VI, VII, VIII, and pTP into their mature forms (Tihanyi et al., 1993). L4 encodes polypeptide VIII (see figure 1-1), and L5 mRNA encodes the fiber (reviewed in (Shenk, 1996)).

The adenoviral Virus Associated RNAs (VA RNAs) are expressed early after

adenoviral infection but are dramatically upregulated during the late phases of the infection. Both Ad 2 and Ad 5 express two VA RNAs each transcribed by the cellular RNA polymerase III (Mathews, 1990). These small RNAs are not translated and function to counter the translational block induced by α and β interferon, and the presence of large amounts of double-stranded RNA(dsRNA) present in infected cells. They are thought to act in maintaining efficient translation of viral transcripts during host shut off in the late phases of infection (Thimmappaya et al., 1982). It has been shown that the VA RNAs inhibit the activity of protein kinase R (activated by interferon and dsRNA) which can phosphorylate and inactivate eIF-2, the cellular translational initiation factor (Kitajewski et al., 1986). The VA RNAs are thought to be localized to areas of viral mRNA transcription thus selectively protecting viral translation while host translation is inhibited (Mathews, 1980).

Adenovectorology

Adenoviruses have become one of the most intensely studied and popular vehicles for the delivery of foreign genes to target cells. Of the adenoviral serotypes, Ad 2 and Ad 5 are the best characterized. These serotypes also belong to subgroup C whose members cause generally mild upper respiratory infections, and have not been shown to be oncogenic in rodents. Thus these serotypes have been developed and widely used for gene delivery and gene therapy. The adenovirus also exhibits a number of advantages which make it a popular gene vector system. Both Ad2 and Ad5 replicate to very high titers, and

infect a wide variety of mammalian cell types (both quiescent and replicating). Ad vectors also produce high levels of expression in transduced cells, and can be manipulated easily using recombinant DNA technology due to the fact that its genome is double stranded DNA, and purified viral DNA can generate infectious virus in transfected cells.

First Generation Ad vectors

The adenoviral type 5 capsid can package (or accommodate) DNA up to 105% of its normal genome size (36kb) (Bett et al., 1993). This allows for the addition of 1.8 kb of foreign DNA to the viral genome without preventing packaging of the recombinant virus, but leaves little room for the insertion of a heterologous promoter and cDNA. The E1 region has been shown to exhibit transforming properties, even though Ad2 and Ad5 have not shown oncogenic properties *in vivo*. Thus, the removal of E1 from the vector is preferable for several reasons. It reduces the risk associated with the Ad vector, increases the capacity for transgene insertion, and renders the vector replication incompetent. The removal of E1 sequences (keeping cis acting elements) results in the addition of 3.2 kb to the cloning capacity of the Ad5 vector (Bett et al., 1994). These E1⁻ vectors can be constructed and propagated in an E1 expressing 293 cell line (Graham et al., 1977).

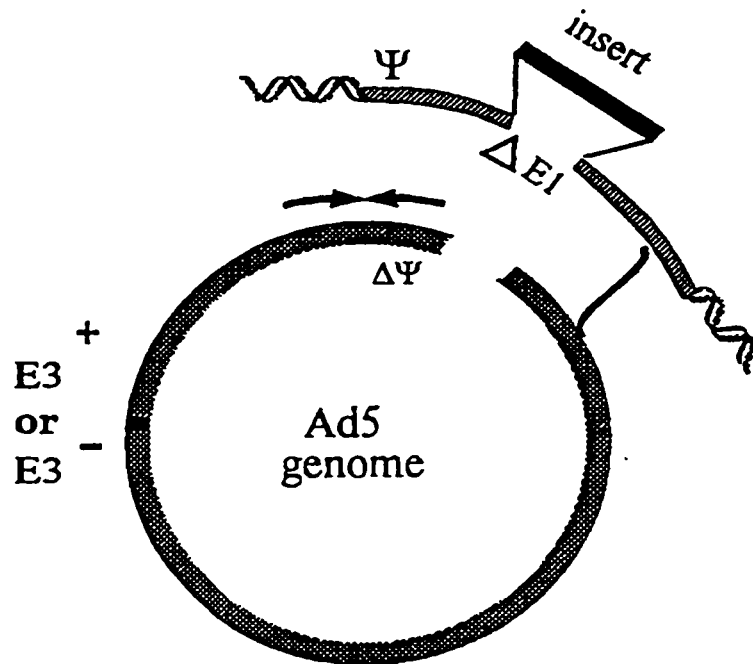
As described above, the E3 region is not required for adenoviral replication *in vitro* and is primarily involved in host immune evasion. Thus the E3 region has also been deleted from most modern Ad vectors allowing for further increased packaging capacity. Ad5 vectors with both E1 and E3 deletions can theoretically incorporate up to 8-8.5 kbp of

foreign DNA (Bett et al., 1994). A common method used to construct recombinant Ad vectors utilizes homologous recombination between adenoviral sequences. One of the most recent systems developed utilizes a “two plasmid system” in which the majority of Ad sequences are carried on a bacterial plasmid (with deletions in E1 and E3, and the packaging signal). This plasmid is cotransfected with a second “shuttle” plasmid carrying a packaging signal and a portion of the left end adenoviral sequences. Usually, the shuttle plasmid also contains a convenient multicloning site for the insertion of the desired cDNA and regulatory elements. This technique is viable because circular Ad genomes have the capacity to replicate within transfected permissive cells (Graham, 1984). Only a relatively small region of homology (1000bp) between the shuttle plasmid and the genome sized plasmid is required to facilitate efficient homologous recombination (McGrory et al., 1988). Once homologous recombination has occurred, a vector is produced which possesses the packaging signal, transgene, and all other elements required for viral propagation in 293 cells (see fig 1-4).

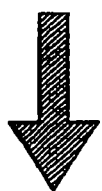
Transgenes can be inserted in E1 in either the leftward or rightward orientation, however, it has been reported that higher expression is attainable in the rightward orientation (Hitt et al., 1995). A potential problem with transgenes oriented rightwards is the possible interference with viral replication by extended transcription past the foreign cDNA. This is remedied by the use of a heterologous polyadenylation sequence immediately downstream of the transgene cassette, which also serves to increase transgene expression. A variety of promoters have been used to drive expression of transgenes in Ad

Figure 1-4: Construction of first generation Ad vectors via the two plasmid system.

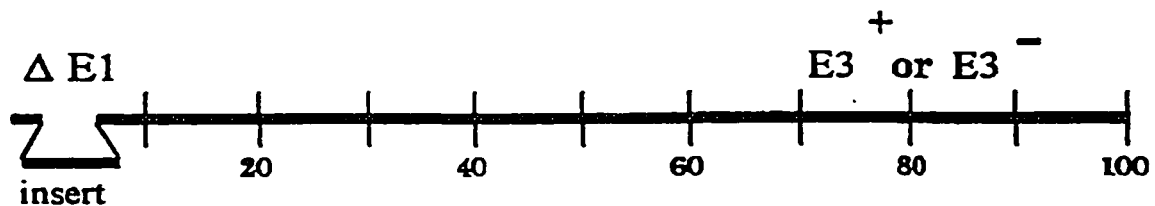
Both the shuttle plasmid with transgene insert and packaging signal (Ψ) (top right), and the Ad genome containing plasmid, without packaging signal (center left), were cotransfected into 293 cells. Homologous recombination between the two plasmids within a 2kb region common to both, generates an adenoviral genome containing the transgene insert, capable of replication in 293 cells. This figure was adapted from Christina Addison's PhD. thesis (Fig. 2-1), Construction and characterization of adenoviral vectors expressing cytokines for cancer immunotherapy (1997).



E3⁺
or
E3⁻



COTRANSFECTION
RECOMBINATION



vectors, and several have been directly compared including the β -actin promoter, human cytomegalovirus immediate early promoter (HCMV), the major late promoter (MLP), and the SV40 early and late promoters (Xu et al., 1995). Often it has been shown that the HCMV promoter is the strongest. Comparisons of the HCMV promoter with the murine cytomegalovirus immediate early promoter (MCMV) have suggested that the MCMV promoter is more efficient, particularly in murine cell lines (Addison et al., 1997b) (also see chapter IV). One may wish to express multiple transgenes in a single vector.

Although this can be accomplished by simply placing one expression cassette downstream of the other, transcription from the upstream cassette can reduce expression from the downstream cassette. Another option for the expression of two cassettes in a first generation Ad vector is to place one transgene in E1 and the other in E3. However, if one prefers to express multiple transgenes inserted in the same deletion area, an internal ribosomal entry site (IRES) can be used. This allows for efficient expression of multiple cDNAs using a single promoter and poly adenylation signal (Gurtu et al., 1996).

Disadvantages of Ad vectors

Perhaps the most common criticism of Ad vectors is that the adenoviral genome persists as an episome. Thus in replicating cells the vector is diluted out. The transient expression of Ad vectors is further exacerbated by the host immune response (reviewed in (Hitt et al., 1997)). Despite the deletion of the E1 sequences in first generation vectors, low levels of Ad gene expression do occur. Immune responses directed against existing

Ad vector sequences have been detected and result in reduced transgene expression and blockage of vector re-administration (Yang et al., 1994). Another problem with first generation Ad vectors is the potential production of replication competent adenoviruses (RCA). This can occur through homologous recombination between E1 sequences in the genome of 293 cells and residual E1 sequences in the vector backbone, producing an E1⁺ vector. This can complicate the use of these vectors for gene therapy, as a low level of replicating virus would enhance the antiviral immune response, and facilitate replication of the vector thus increasing expression of the transgene and the actual vector dose. In addition, replicating viruses would have a selective growth advantage in culture rapidly overtaking the gene vector titers. The production of RCA can be prevented through the use of other complementing cells which lack overlapping homologous sequences. These problems can also be addressed by further disrupting other viral sequences to construct 2nd and 3rd generation adenoviral vectors.

2nd and 3rd Generation Adenoviral Vectors

Second generation Ad vectors are those in which regions of the Ad genome, other than E1 and E3, have been disrupted and/or deleted. One of the most highly expressed genes in first generation Ad vectors is the E2a-72kD DNA binding protein (DBP). To address this background expression problem, vectors expressing temperature sensitive mutants of DBP have been developed which are not active at 37°C (Engelhardt et al., 1994). These vectors show an increased duration of expression *in vivo* and are also less

inflammatory. Ad vectors deficient in E1, E2a, and E3 were shown to produce substantially lower levels of residual late gene expression thus lowering the potential for immune activation (Amalfitano et al., 1998). Cell lines which would complement deletions in E1, E4, and pIX have been produced, which would allow for insertions of up to 11kb (Krougliak and Graham, 1995). Both E4 and pIX were placed under inducible controls to avoid cytotoxicity. Other sequences including E1b-55kD, pTP, and the 140kD-polymerase, have also been deleted from Ad vectors (reviewed in (Hitt et al., 1997)).

Third generation Ad vectors are those in which all or most of the Ad genome is deleted thus requiring the presence of a “helper virus” to provide the necessary trans acting factors. In theory, all that is required for replication and encapsidation of DNA is the ITRs and a packaging signal. Thus all other requirements could be provided in trans by the host cell and a helper virus. Several groups have developed strategies for achieving this goal. A capacity of up to 28kb has been achieved by (Kochanek et al., 1996), with the use of a helper virus with E1 and E3 deletions. This helper vector also contained a partial deletion in the packaging signal resulting in a 100 fold decrease in efficiency of encapsidation of the helper. Serial passage of this vector mixture resulted in as little as 1% helper contamination in vector preparations. More recently an improved helper dependent system has been developed in which *Cre* mediated recombination is used to remove a *floxed* packaging signal from an E1/ E3 deleted helper vector in *Cre* expressing 293 cells (Parks et al., 1996). This allows for up to 36 kb of foreign DNA to be inserted into a

recombinant helper-dependent vector. Serial passage of this vector mixture into 293^{Cre} cells and CsCl gradient purification, resulted in approximately 0.1% helper virus contamination. The use of a helper-dependent vector has been shown to greatly enhance the duration of expression of the transgene and reduce inflammation *in vivo* (Morral et al., 1998; Morsy et al., 1998; O'Malley et al., 1999).

Gene Therapy

Adenoviral vectors are being extensively investigated as gene therapy vectors for the treatment of a wide variety of diseases. One of the potential diseases which is actively being investigated as an target for Ad gene therapy is cystic fibrosis (CF). CF is a genetic disorder characterized by decreased chloride conductance and increased sodium uptake, resulting in respiratory failure. The treatment of CF with Ad vectors expressing the CFTR gene has shown some promise (Johnson et al., 1995). Some have reported transient correction of chloride transport with no vector associated complications (Zabner et al., 1993). However, reports have been variable as to the effectiveness and immunogenicity of this treatment (Hay et al., 1995; Knowles et al., 1995). Atherosclerosis is another disease to which Ad gene therapy has been applied. Ad vectors expressing the LDL receptor have been used in an attempt to increase the uptake and metabolism of low density lipids (LDL) with some success in animal models, showing transient reductions in LDL levels (Ishibashi et al., 1993; Kozarsky et al., 1994).

One of the most widely studied applications of Ad vectors in the treatment of

disease is for cancer gene therapy. Some of the disadvantages of Ad vectors may actually prove to be advantageous when applied to the gene therapy of cancer. The transient expression induced by adenoviral vectors is favorable for tumour therapy as one would not wish continued expression of a potentially inflammatory or toxic gene after the tumour has regressed. The host immune response against Ad vectors (1st generation in particular) could also act to enhance or promote an antitumour immune response. Direct intratumoural injection of Ad vectors might allow for the expression of the antitumour agent locally (from transduced tumour cells) thus enhancing the antitumour response and reducing any systemic side effects. Cancer gene therapy typically involves one of three approaches (or combinations of them). The first approach utilizes tumour suppressor genes. Tumour suppressor genes are commonly used to arrest or kill infected tumour cells. One of the most well-characterized tumour suppressor genes is p53. Approximately half of all tumours have some form of mutation in p53. P53 becomes stabilized in response to certain stimuli, including DNA damage, virus infection, and oncogenesis (de Stanchina et al., 1998; Kuerbitz et al., 1992; Zindy et al., 1998). It is a transcription factor which can activate genes involved in cell cycles arrest at the G1/S (p21^{waf1}) or the G2/S (CIP1, SDII) checkpoints. It can also induce apoptosis by influencing Bax expression and the expression of genes involved in regulating the cellular redox state (reviewed in (Kaelin, 1999)). Introducing wild type p53 via gene therapy, has proven to be somewhat useful for tumour therapy, through the induction of tumour growth arrest and regression (Liu et al., 1994), (Putzer et al., 1998)). Also cyclin-dependent kinase inhibitors such as

p21^{waf1} and p16^{ink4} have been used to treat tumours in animal models. Both induce cell cycle arrest through binding to and inactivating cyclin-dependent kinases, and Ad vectors expressing these cell cycle inhibitors have been shown to delay tumour growth in vivo (Eastham et al., 1995; Jin et al., 1995; Schreiber et al., 1999; Yang et al., 1995). The second approach involves the use of suicide genes. The herpes virus thymidine kinase (HSV-TK) gene and the cytosine deaminase (CD) gene confer sensitivity to the cytotoxic effects of ganciclovir and 5-fluorocytosine respectively. Both have shown promise for use in tumour gene therapy. An Ad vector expressing CD was shown to facilitate inhibition of tumour growth in nude mice (Hirschowitz et al., 1995). Similarly an Ad vector expressing HSV-TK facilitated complete tumour regressions in 20% of treated mice (Chen et al., 1994). Both the use of tumour suppressor genes and suicide genes in tumour therapy involve the induction of a bystander effect which kills tumour cells surrounding the transduced cell (reviewed in (Hitt et al., 1997)). Immunotherapy is the third commonly used method of cancer gene therapy. This strategy often involves the Ad-directed expression of immune activating cytokines which will break the tolerance to, or induce an immune response against, tumour associated antigens. This technique has the advantage of potentially being able to induce a systemic antitumour response which would seek out and destroy any tumour metastases. Tumour immunotherapy will be discussed in more detail below.

Tumour Biology

Oncogenesis

The process of oncogenesis generally starts with a breakdown in the proliferative controls of the cell cycle. Cellular factors whose expression or overexpression promote uncontrolled proliferation are called oncogenes. Many of these oncogenes fit into one of three categories: Protein kinases, G proteins, and transcriptional activators (reviewed in (Bishop, 1991)). Receptor tyrosine kinases (RTK) play a pivotal role in oncogenesis. These cell surface receptors typically exhibit an extracellular ligand binding domain, a transmembrane domain and a tyrosine kinase domain (reviewed in (Porter and Vaillancourt, 1998)). Receptor dimerization upon ligand binding facilitates autophosphorylation of key tyrosine residues and activation of signal transduction pathways leading to transformation (Biswas et al., 1985; Heldin et al., 1989). The serine/threonine kinases are part of a phosphorylation cascade known as the mitogen-activated protein kinase cascade (MAPK) leading to the activation of transcription factors involved in cellular transformation (Seger et al., 1995). A key player in this pathway is Raf which is activated via RTK phosphorylation through the signaling intermediate Ras (Wood et al., 1992). Ras is a member of the GTP binding protein family (G-proteins) and has been implicated in oncogenesis (Sukumar, 1989). Ras is activated by being recruited to the RTKs and activated through the association with adaptor proteins (e.g. Grb2) culminating in the exchange of bound GDP for GTP (Lowenstein et al., 1992). Phosphoinositide 3'-kinase (PI3-K), which phosphorylates lipids, is also an important

signal transduction protein (reviewed in (Porter and Vaillancourt, 1998)). PI3'-K is recruited to the membrane by activated RTKs and catalyzes the phosphorylation of membrane lipids which act as second messenger (Truitt et al., 1994). Transcription factors bind to DNA in the promoter/enhancer regions of genes and upregulate expression. Transcription factors which induce genes involved in cell cycle progression are the downstream targets of many proliferative signal transduction pathways involved in oncogenesis. The process of oncogenesis also involves inactivation of tumour suppressor genes. Tumour suppressors such as p53, pRb, and NF1 act to inhibit proliferation and counter the effects of oncogenes by inducing apoptosis (reviewed in (Bishop, 1991)).

Angiogenesis

Tumourigenesis is a multistep process beginning with the uncontrolled proliferation of the cell. A further step of tumourigenesis is the induction of new blood vessel formation to feed the expanding tumour mass. This development of new blood vessels from pre-existing capillary beds is called angiogenesis. Without the formation of new blood vessels the volume of a tumour is limited to a diameter of only a few millimeters (reviewed in (Bouck, 1990)). Tumours can induce angiogenesis by two approaches: i) the production / secretion of angiogenesis promoting factors, and ii) recruitment of cells which will secrete angiogenic factors (reviewed in (Folkman, 1992)). Many of the angiogenic factors secreted by tumour cells are growth factors which induce phenotypic changes in endothelial cells facilitating vessel formation. Some pro-

angiogenic polypeptides include basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), tumour necrosis factor alpha (TNF α), and vascular endothelial growth factor (VEGF). These proteins interact with endothelial cells to alter their proliferative capacity, motility, and interactions (degradation, attachment, detachment) with the extracellular matrix. Inflammatory cells attracted to the tumour site can also contribute to the angiogenic potential of a tumour. These cells include macrophages (Moore and Sholley, 1985) and mast cells (Dethlefsen et al., 1990). Many of these factors and the process of angiogenesis itself are also believed to enhance the metastatic potential of tumours.

Metastasis

The most critical aspect of cancer is the production of tumour metastases. For metastasis to take place the tumour cells must migrate and traverse the basement membrane of the capillaries and then re-attach and traverse at another location. This complex process requires changes in cell adhesion, motility, and proteolytic degradation of the extracellular matrix (ECM) (reviewed in (Aznavorian et al., 1993)). Integrins and adhesion molecules mediate cellular interactions with the ECM and other cells. Integrins are composed of a variety of $\alpha\beta$ subunit heterodimers which bind to specific types of ECM. The cadherin family of cellular adhesion molecules (CAM) mediate cell-cell interactions. The cadherins have been found to inhibit metastasis presumably by binding tumour cells together (Takeichi, 1991), while the integrins have generally been found to promote metastasis (Humphries et al., 1986; Saiki et al., 1989). This complex requirement

for attachment underscores the complicated interactions of the metastatic tumour cell with its surroundings.

For movement of a cell through the ECM repeated attachment to the ECM and release is required in a highly regulated and directional manner. Proteolysis of the ECM is required in this process. Matrix metalloproteinase expression has been linked to the metastatic potential of a tumour (Liotta and Stetler-Stevenson, 1991). These enzymes degrade various components of the ECM including collagens, gelatin, fibronectin, and proteoglycans. The degradation of these matrix components is required for the passage of cells through the ECM and basement membranes of capillaries.

Cellular motility has also been correlated with metastatic potential (Mohler et al., 1987). A wide variety of secreted factors including growth factors and ECM components have been shown to facilitate motility. Induction of cellular motility can be divided into two categories: chemotaxis and chemokinesis. Chemotaxis is the induction of directional movement of a cell towards the stimulus, while chemokinesis is the general induction of motility of a cell (in any direction). Thus the combined action of altered cellular adhesion, proteolytic degradation, and chemotaxis/chemokinesis facilitate the “escape” of tumour cells from the primary site to new locations in the body.

The Polyoma-middle-T Mammary Tumour Model

The use of transgenic murine systems is a powerful tool for investigating the role(s) of particular signal transduction pathways in oncogenesis. The polyoma virus

middle-T antigen is well known for its transforming ability, through association with a variety of cellular factors involved in signal transduction. Middle-T can act by triggering the tyrosine kinase activity of members of the c-src family of kinases (Cheng et al., 1988; Courtneidge and Smith, 1983; Kornbluth et al., 1986). It can also associate with and activate other cellular factors including the regulator subunit of PI3'-K (Courtneidge and Heber, 1987). A polyoma middle-T (PyMidT) breast cancer model has been made through the construction of a transgenic mouse which expresses the middle-T antigen under the control of the mouse mammary tumour virus promoter enhancer (MMTV) (Guy et al., 1992). These transgenic mice developed multifocal mammary adenocarcinomas rapidly, with frequent metastasis to the lung. It was later shown that explanted tumour cells from PyMidT mice could be cultured *in vitro* and injected subcutaneously into syngeneic mice, resulting in the establishment of a subcutaneous tumour (Addison et al., 1995a). These tumours were also shown to be treatable with Ad vectors expressing IL-2, resulting in the elimination of cancer in a percentage of treated mice. This tumour model is the primary tool used for the tumour gene therapy experiments presented in this thesis.

Tumour Immunology

The immunological state within the region of a tumour has been the subject of considerable research for quite some time. It has long been known that some tumours can be recognized by the immune system, and therapies based on provoking the immune system to attack tumours have been attempted since the first observations that acute

bacterial infections could mediate tumour regression (Bast et al., 1975a; Bast et al., 1975b). Tumours invariably employ a variety of strategies by which they avoid detection / elimination by the immune system, and even manage to exploit an inflammatory response to promote growth and metastasis. Researchers are currently exploring a variety of strategies with which to counter this immune evasion and facilitate eradication of the tumour.

The major immune cells involved in an effective antitumour response are T lymphocytes, natural killer (NK) cells, macrophages, and B lymphocytes (reviewed in (Verbik and Shantaram, 1995)). There are two major subgroups of T lymphocytes: the CD4⁺ (T helper) and the CD8⁺ (cytotoxic) populations. Helper T cells facilitate a productive immune response through the recognition of foreign antigens in the context of MHC-class II on professional antigen presenting cells, through the T cell receptor (TCR) and coreceptor (CD4). Once a particular antigen specific helper T cell has been stimulated it begins to proliferate and produces various factors (cytokines) involved in stimulating and guiding the immune response (Th1 vs. Th2). A T helper 1 (Th1) response is characterized by a primarily cellular mediated effector function and the production of cytokines such as IFN γ , and IL-12. A T helper 2 (Th2) type response is characterized by a primarily humoral or antibody mediated response with the production of cytokines such as IL-10. The cytotoxic T cell population (CTL) functions by specifically inducing the lysis of target cells presenting “foreign” antigen in the context of MHC-class I. The recognition of antigen is mediated by the TCR and CD8 coreceptor (Julius et al., 1993). In addition to

recognition by the TCR, T cells also require a costimulatory signal provided typically by B7 which binds and signals through its receptor (CD28) on the T cell (Freeman et al., 1989; Linsley et al., 1990). Failure to costimulate in the presence of specific antigen results in induction of anergy and T cell death (Harding et al., 1992).

T lymphocytes are the major effector cells which can mediate an effective antitumour response, however, other cell types can also play an important role. Natural killer cells are a subpopulation of lymphocytes that can kill tumour cells in a manner similar to that of CTLs. NK cells typically lack a TCR and CD4 / CD8 markers but are able to recognize and lyse target cells by several alternate mechanisms, including antibody dependent cell mediated cytotoxicity (ADCC) (Lanier et al., 1988). The other specific activating receptors on NK cells are poorly understood, however the role of inhibitory receptors which bind MHC-class-I is better understood. NK markers Ly49 and SW5E6 have been demonstrated to bind to MHC alleles H-2^d and H-2^b (respectively) and inhibit lysis of cells expressing those alleles (Karlhofer et al., 1992; Sentman et al., 1989). It is said that NK cells are able to recognize the absence of self (MHC deficient) as opposed to the presence of non-self (antigen with MHC). NK cells are also responsive to the cytokines produced by lymphocytes and other immune cells (IL-2, IL-12), which can induce them into a highly active form called a lymphokine activated killer (LAK) cell (Gately et al., 1992; Ritz et al., 1988). Macrophages function in the phagocytosis and destruction of foreign materials. Macrophages can have antitumour activity in a variety of ways. They can act as professional antigen presenting cells theoretically facilitating a

specific immune response against the tumour. Also, they can act directly against tumour cells through the production of cytotoxic factors such as tumour necrosis factor alpha (TNF α) or kill by direct contact with tumour cell resulting in apoptosis (Bucana et al., 1983). An antitumour role for B cells, mast cells, and eosinophils has also been suggested by various investigators (Sogn, 1998; Verbik and Shantaram, 1995).

Cytokines play a key role in any effective immune response. Perhaps the most fundamental cytokine required for immune activity is interleukin 2 (IL-2). IL-2 (also called T cell growth factor) is primarily produced by T cells, and is required for T cell proliferation. IL-2 also activates NK cells and enhances antibody production by B cells (Kohler and Sondel, 1989). IL-12 is a key mediator of the Th1 response and is produced primarily by monocytes/macrophages and B cells (D'Andrea et al., 1992). IL-12 has been found to promote LAK cell activity, activate CTL activity, promote the release of other cytokines such as IFN γ and TNF α , plus upregulate IL-2 and TNF α receptors on T cells (reviewed in (Verbik and Shantaram, 1995)). Tumour necrosis factor alpha (TNF α) is a key mediator of inflammation and has many antitumour properties. TNF α will be discussed in more detail below.

Immunotherapy of cancer

Tumours by their very nature are not significantly inhibited by the immune system. However, tumour cells and derived products have commonly been found to be antigenic but non-immunogenic. There are a variety of ways in which a tumour can escape

detection by the immune system (reviewed in (Sogn, 1998)). Tumour cells can modify the resident endothelium resulting in decreased infiltration of immune cells. The surrounding stroma can also be affected in a similar manner. Tumour cells themselves may be deficient in antigen presentation or the tumour may inhibit professional antigen presentation. Furthermore, T cell anergy can be induced through the absence of a costimulatory signal from the tumour cell, and T cell apoptosis through expression of the Fas ligand. Cytokines produced by tumour cell (IL-10, TGF- β) may also inhibit immune activation and effector function (Hisatsune et al., 1994; Strassmann et al., 1991).

Thus, the goal of immunotherapy is to stimulate the immune system and induce an effective antitumour immune response to eliminate the disease. A major attraction of this type of approach is that the induction of tumour specific immunity would potentially result in elimination of metastases, which are usually the main cause of fatality. It has been clearly demonstrated in murine models, that effective immune responses can be generated against even non immunogenic tumours (Liu et al., 1996). Investigators have used a variety of strategies to induce an immune response against tumours, including specific antibody administration, administration of stimulated T cells, and cytokine administration. Cytokine therapy is attractive as it is relatively simple to implement. It is based on the hope that the presence of high levels of cytokine will overcome any inhibitory effects mediated by the tumour. A major problem associated with this approach is the induction systemic of toxicity. Cytokines such as IL-2 and IL-12 have been shown to be toxic at doses required for tumour therapy (Atkins et al., 1997; Sondel et al., 1987). In this respect

adenovirus vectors may be well suited for tumour immunotherapy. Ad vectors, if injected into solid tumours, might allow for the possibility of locally expressed high levels of cytokine in the area of infection. First generation Ad vectors express transiently, which is desirable in tumour therapy as one does not typically want continued expression after tumour eradication. Also, the high level of immunogenicity of the first generation Ad vectors could advantageously act as an adjuvant.

Tumour Necrosis Factor α

TNF α is a multipotent cytokine with a variety of physiological activities. It was originally discovered through the anticancer activity of sera of mice treated with endotoxin (Carswell et al., 1975). It is associated with a wide variety of responses including the activation of immune cells, changes in the extracellular matrix, apoptosis, cachexia, and septic shock. TNF α is first produced as a 26 kD membrane bound precursor protein that is subsequently cleaved from the membrane by a metalloproteinase called TNF α converting enzyme (TACE) (Black et al., 1997; Tracey and Cerami, 1994). The secreted (mature) 17kD product acts as a homotrimer in the circulation. TNF α is secreted by a wide variety of cell types including macrophages, lymphocytes, and polymorphonuclear granulocytes (reviewed in (Fiers, 1991)). Its expression can be induced by bacterial endotoxin, virus infection, parasites, serum complement, antibody-antigen complexes, and cytokines.

TNF receptors

There are two cell surface receptors for TNF α referred to as p55 and p75. The extracellular domains of these receptors are homologous and contain four highly conserved cysteine rich regions. However, the intracellular domains are very different, and the receptors are believed to act through distinct signal transduction pathways (reviewed in (Fiers, 1991)). In many cases the full effect of TNF α requires activation of both receptors. It is thought the p75 TNFR can potentiate signaling through the p55 TNFR through a ligand passing mechanism, involving the association of TNF α with the lower affinity p75 TNF receptor and subsequent “passing” of TNF α to the higher affinity p55 TNF receptor (Dri et al., 1999; Tartaglia et al., 1993). Cooperation is also thought to occur through intracellular signaling, as both TNF receptor 2 associated factors 1 and 2 (TRAF1, TRAF2) binding domains have been shown to be required for potentiation of cytotoxicity (Declercq et al., 1998). The p55 TNFR has been shown to induce cytotoxicity, cytokine secretion, as well as mediating changes in the extracellular matrix, while the p75 TNFR is primarily involved in proinflammatory and proliferative signals (reviewed in (Sarraf, 1994)). The p75 TNFR is believed to be responsible for the TNF mediated activation of T cells (Tartaglia et al., 1991; Vandenabeele et al., 1992). It is the dominant receptor in human lymphoid tissue (Ryffel and Mihatsch, 1993), and human tumour infiltrating lymphocytes (TIL) were found to express the p75 TNFR exclusively (Trentin et al., 1995). Human TNF α has been found to be species specific, it does not bind to the murine p75 TNFR but does bind the murine p55 TNFR (Lewis et al., 1991; Ranges

et al., 1989).

As previously mentioned, TNF α displays many toxic side effects. The spectrum of toxic effects can be organized into acute and chronic. Large quantities in the serum can rapidly induce septic shock, vascular leak syndrome, and disseminated intravascular thrombosis (Tracey et al., 1986). Extended exposure to lower levels of TNF α can cause cachexia, marked by weight loss, and dehydration. It was once assumed that the p75 TNFR was primarily responsible for the induction of acute systemic toxicity. It has been demonstrated that human TNF α is much less toxic to mice compared to murine TNF α (Brouckaert et al., 1989). Since human TNF α specifically recognizes the murine p55 TNFR alone, it was thought the p75 receptor must mediate systemic toxicity. Subsequently, studies with gene knockout mice, demonstrated that both receptors are involved in the induction of septic shock, with the p55 TNFR being the primary mediator of septic shock and the p75 receptor providing a potentiating function (Bluethmann et al., 1994; Erickson et al., 1994). This has subsequently been supported by more recent data (Sheehan et al., 1995) (also see chapter V), including an experiment demonstrating the induction of toxicity in baboons by p55 TNFR agonist and not p75 TNFR agonists (Welborn et al., 1996).

Immunological activity of TNF α

TNF α is a key mediator of inflammation (reviewed in (Sarraf, 1994)). It has been shown to rapidly activate neutrophils, macrophages, NK cells, and T cells, and to induce

the production of other cytokines. TNF α is a potent inducer of IL-6 which in turn induces acute phase protein synthesis, fever, and lymphocyte proliferation. TNF α can also induce IL-1, GM-CSF, IL-8, and IFN γ production. Lymphocyte expression of the IL-2 receptor has also been shown to be induced by TNF α (Pimentel-Muinos et al., 1994). In a paper by Collins *et al.*, TNF α was shown to upregulate MHC class I expression on vascular endothelial cells and fibroblasts, demonstrating a potential effect on antigen presentation (Collins et al., 1986). In addition to cytokine production, TNF α mediates inflammation through its effects on the extracellular matrix. It can induce the expression of adhesion molecules (ELAM-I, I-CAM-1, VCAM-I) from vascular endothelial cells facilitating the infiltration of leukocytes into the affected area (Paleolog et al., 1994). TNF has also been demonstrated to act as a costimulatory molecule. The presence of membrane associated TNF α on the surface of CD4⁺ T cells has been shown to enhance antibody production, and I-CAM-I and B7-1 presentation from B cells, and this activity seems to be linked to the p75 TNF receptor (Aversa et al., 1993; Ranheim and Kipps, 1995).

Antitumour activity of TNF α

There are primarily three ways in which TNF α can kill tumour cells. The first is by direct cytotoxicity through the induction of apoptosis. This most likely plays a small role in tumour regression, as most tumour cells are resistant to the cytotoxic/cytostatic effects of TNF α in the absence of protein synthesis inhibitors (reviewed in (Balkwill et al., 1990)). For example meth-A-sarcoma cells are not sensitive to TNF α *in vitro*, but meth-A-sarcoma

derived tumours are highly sensitive *in vivo* (Nawroth et al., 1988). The second manner in which TNF α can kill tumours is through its effects on the tumour vasculature. TNF α induces a prothrombotic environment within the tumour vasculature, inducing hypoxia. TNF α inhibits thrombomodulin, and induces plasminogen activator inhibitor, and tissue factor procoagulant activity (Clauss et al., 1992; Prober, 1987). The tumour vasculature is uniquely sensitive to TNF α , as the quality of tumour vasculature is typically poor and disorganized containing long vessel loops high in catabolites and low in nutrients (Denekamp, 1993). Thus the disruption of tumour vessels has a greater chance of producing necrosis. Furthermore, factors secreted by tumour cells may cooperate with the pro-thrombotic effects of TNF α , as it has been demonstrated that factors isolated from meth-A-fibrosarcoma cells can sensitize endothelial cells to the procoagulant effects of TNF α (Clauss et al., 1990; Nawroth et al., 1988). Fibrin deposition occurs rapidly (30min) in meth-A-sarcoma tumours treated with TNF α , and treatment with anticoagulants reverses the necrotic effects (Shimomura et al., 1988). The third mechanism by which TNF α can induce tumour regression is through immune activation. TNF α has been shown to activate various immune cells (Palladino et al., 1987). In murine systems the antitumour activity of TNF α has been shown to be dependent on both CD4⁺ and CD8⁺ T cell populations, and to promote antitumour CTL activity (Asher et al., 1991; Blankenstein et al., 1991; Marincola et al., 1994). The multiple routes by which TNF α produces antitumour activity makes it an attractive candidate for tumour therapy.

CHAPTER II - MATERIALS AND METHODS

Recombinant DNA Techniques

Bacterial cell culture

Escherichia coli (*E. coli*) strain DH5 α (*supE44*, *hsaR17*, *recA1*, *gyrA96*, *thi-1*, *relA1*) was used to carry recombinant DNA plasmids. These bacteria were grown on solid phase Luria-Bertani broth agar (LBA) or in liquid culture in Luria-Bertani broth (LB) with the appropriate antibiotic (80 μ g/mL ampicillin). For the production of large scale quantities of plasmid, bacteria were grown in Super Broth (SB) supplemented again with the appropriate antibiotic.

Bacterial transformations

Recombinant plasmid DNA was introduced into bacterial cells by calcium chloride transformation. Calcium chloride competent cells were prepared by inoculation of LB with 1/100 volumes of an overnight culture of *E. coli*, and incubated at 37°C with shaking until an OD₅₉₀ of 0.375 was reached. The cells were then centrifuged for 10 min at 2000 xg at 4°C in pre-chilled polypropylene tubes. After decanting the supernatant, the cell pellets were resuspended in 10mL of ice-cold CaCl₂ solution (60mM CaCl₂, 10mM PIPES pH 7, 15% glycerol) and centrifuged again for 5 min at 1400 xg at 4°C. The cell pellets were again resuspended in 10mL of ice-cold CaCl₂ solution and let stand for 30 min on ice. The cells were recentrifuged and the pellet resuspended in 2mL of the ice-cold CaCl₂ solution.

The cell suspension was then aliquoted into microfuge tubes (200 μ L) and stored at -80°C until used. For transformation of cells with recombinant plasmids, 200 μ L of the competent *E coli*. were mixed with varying quantities of plasmid DNA (5 to 50 μ L), and incubated on ice for 10 min. After this, the mixture was heat shocked at 42°C for 2 min, and allowed to “recover” in 1 mL of LB for 30 to 60 min at 37°C. Following the transformation, cells were plated onto LBA containing the appropriate antibiotic, in various quantities. Potential bacterial colonies were then picked from the plates and screened for the proper plasmid (see below).

Small scale DNA preparations (mini preps)

Antibiotic resistant colonies were removed for agar plates with a sterile wooden pick or a metal loop, and inoculated into 2mL of LB plus antibiotic. After incubation overnight with shaking at 37°C, 1.5mL of each culture was aliquoted into microfuge tubes and centrifuged at 16000 xg for 3 min. The cell pellet was resuspended in 100 μ L of lysis buffer (50mM glucose, 25mM Tris-HCl pH 8, 10mM EDTA, Rnase 10 μ g/mL), and let sit for 5 min at room temperature (RT). Two hundred μ L of alkaline-SDS buffer (0.2N NaOH, 1% SDS) was added, and the sample was quickly mixed and incubated on ice for 5 min. One hundred and fifty μ L of 3M sodium acetate (pH 4.8) was then added and mixed, and incubated on ice for 20 min. The cell debris was then pelleted by centrifugation (16000 xg, 5 min) and the supernatant transferred to a fresh tube containing 1mL of isopropanol. After vortexing the mixture was centrifuged for 10 min at 16000 xg, and the supernatant

was aspirated off. The remaining pellet (may not be visible) was resuspended in 100 μ L of TE buffer (10mM Tris-HCl, pH 8, 1mM EDTA) and mixed with 200 μ L of 96% ethanol and centrifuged for 10 min at 16000 xg. The supernatant was aspirated off and resuspended with 150 μ L 70% ethanol and again centrifuged for 3 min at 16000 xg. The supernatant was then aspirated off and the pellet dried at 60°C for 5 min and resuspended in 50 μ L of TE buffer. This solution containing the plasmid DNA was then incubated at 60°C for 20 min before being stored at 4°C.

Enzymatic manipulation of DNA

Plasmid DNA was “cut” with restriction enzymes according to the manufacturer’s instructions. Restriction digested DNA was visualized by electrophoresis on a 1% agarose gel (Maniatis et al., 1989). DNA fragments were isolated from low melting temperature and regular agarose by excision of the desired fragment from the gel with a scalpel, and the DNA purified from the gel using a Wizard PCR DNA isolation kit (Promega), according to the manufacturer’s instructions. Sequence analysis of DNA was performed at the MOBIX central facility, McMaster University, via an automated sequencer. The ligation of DNA fragments for cloning was performed using T4 DNA ligase, according to the manufacturers instructions. Ligation reactions were incubated overnight at 14°C, or at room temperature for 2 hrs.

Preparation of large quantities of plasmid DNA (large scales)

For the production of large quantities of plasmid DNA, 1L of SB (plus antibiotic) was inoculated with 3mL of an 8 hrs bacterial culture, and incubated overnight with shaking at 37°C. Cells were collected by centrifugation at 6000 xg (10 min, 4°C). The cell pellet was then resuspended in 40mL lysozyme buffer (lysis buffer (see mini preps) plus 5mg/mL lysozyme), and incubated at room temperature for 20 min. Eighty mL of alkaline-SDS buffer was mixed in, and incubated for 5 min on ice. After the alkaline lysis 40mL of 5M potassium acetate solution (made by the addition of 147.2 g of KOAc to 300 mL of distilled (d) water, plus 57.5mL glacial acetic acid and adjusting final volume to 500mL with dH₂O), mixed and incubated on ice for 20 min. Ten mL of dH₂O were added and the mixture was centrifuged at 6000 xg (10 min, 4°C). The supernatant was then filtered through 2 to 3 layers of cheese cloth into 100 mL of isopropanol and let stand at room temperature (RT) for 30 min. The precipitated DNA was pelleted by centrifugation (6000 xg, 10 min, RT), and the resulting pellet resuspended in 7mL of TE. Cesium chloride was then added to the solution (1.1g per mL), mixed thoroughly to dissolve the CsCl, and left on ice for 30 min. After centrifugation (6000 xg, 20 min, 4°C) the supernatant was transferred to a Bechman 13mL quickseal ultracentrifuge tube, 200µL of 10mg/mL ethidium bromide was added, and the solution overlaid with light paraffin oil. After this, the tube was heat-sealed, mixed, and centrifuged to equilibrium in a Bechman Vt65.rotor (55000 rpm, 15°C, 18-20 hrs). Bands of plasmid DNA were visualized by the red colour produced by ethidium bromide. The plasmid DNA was removed from the tube with a

syringe (18 gauge needle), and the ethidium bromide was extracted 4X with 6 volumes of CsCl saturated isopropanol. Three volumes of TE were then added and the DNA was precipitated by the addition of 8 volumes of 96% ethanol. The DNA was pelleted by centrifugation (6000 xg, 4°C, 15 min), resuspended in 70% ethanol (pelleted again, 5 min), dried, and dissolved in 0.5-1mL of TE. Any possible contamination with nucleases was inactivated by incubation at 60°C for 20 min, before storage at -20°C. The final concentration of DNA was determined by fluorometric assay in a Hoeffer TKO fluorometer by comparison to a known DNA standard.

Polymerase Chain Reactions (PCR)

Amplification of small quantities of DNA for diagnostic purposes or cloning was done using the polymerase chain reaction (PCR). PCR reactions were conducted as described in Maniatis *et al.* (Maniatis et al., 1989) using a Perkin Elmer PCR thermocycler. PCR primers were synthesized at the MOBIX Central Facility, McMaster University.

Mammalian Cell Culture

All cell lines and tissue culture were grown in a 37°C, 5% CO₂ humidified incubator. All media reagents were obtained from Gibco, unless otherwise stated. All culture media was supplemented with 100U/mL penicillin, 100µg/mL streptomycin, 2mM L-glutamine, and 2.5µg/mL fungizone (Squibb Canada). Cells were routinely seeded into new dishes following two washes with phosphate buffered saline (137mM NaCl, 3mM

KCl, 4mM Na₂HPO₄, 1.5mM KH₂PO₄) (PBS) and incubated for 2-5 min with trypsin-EDTA (Gibco), with the exception of 293 cells which were passaged by washing twice with citric saline (130mM KCl, 15 mM sodium citrate). All cells were grown in 10% fetal bovine serum (FBS) unless otherwise stated (see appendix table A4-1).

Adenovirus Construction and Propagation

Rescue of recombinant adenovirus vectors

Recombinant adenovirus vectors expressing TNF α were generated by homologous recombination in 293 cells using the two plasmid system (see literature review) (Bett et al., 1994). The shuttle plasmids containing the gene cassette of interest (in E1) were cotransfected into 293 cells with the Ad-genomic plasmid pBHG10 using the calcium phosphate method (Graham and Van der Eb, 1973). Briefly, 10 μ g of salmon sperm DNA (sheared by vortexing), 5 or 10 μ g of shuttle plasmid DNA, 5 to 10 μ g of pBHG10, 25 μ L of 2.5M CaCl₂ (dropwise) was added to 0.5mL HEPES buffered saline (HeBS) (21mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), 137mM NaCl, 5mM KCl, 0.5mM Na₂HPO₄·H₂O, 5.6mM glucose, pH 7.1) (Hitt et al., 1995). This solution was mixed and let stand at RT for 15-30min, before it was added to 60mm dishes of 293 cells (70% to 80% confluent) containing 5mL of media. Dishes were then incubated overnight (37°C, 5% CO₂), after which the medium was removed and cells were overlaid with 10mL of agarose medium (0.5% agarose, 293 medium plus 0.2% yeast extract). After solidification of medium the dishes were stored in an 37°C incubator (5% CO₂) and

monitored for viral plaque formation (7 to 15 days). See appendix for diagrams of all the shuttle plasmids used to rescue recombinant Ad vectors.

Plaque purification of recombinant Ad vectors

To isolate newly rescued Ad vectors, an agar plug containing the viral plaque was taken from the dish using a Pasteur pipette, and stored in 1mL of PBS supplemented with 0.68mM CaCl₂ and 0.5mM MgCl₂ (BPS⁺⁺)(plus 10% glycerol), at -80°C. Serial dilutions of the agar plug were made in PBS⁺⁺, and 0.2mL of each dilution were used to infect confluent 60mm dishes of 293 cells. This was done by removing the medium from the cells, adding the virus mixture to the dish, and incubating the cells for 30-45 min at 37°C (5% CO₂). Following this the dishes were overlaid with agarose-medium as described above, and incubated until visible plaques were seen. At that time several well isolated plaques were picked (as before), and 0.2mL of the agar plug mixture was used to infect a 60mm dish of 293 cells (as above). After the infection, 5mL of 293 cell medium was added to the dishes, and left to incubate at 37°C (5% CO₂). Once complete cytopathic effect (CPE) was observed on the infected dishes (ie. 95%+ cells rounded up and or lifted off of the surface of the dish), the cells were allowed to settle for 15 min at RT, and the supernatant was removed, glycerol added to 10%, and stored at -80°C. The residual cells left on the dish were digested in 0.5mL of pronase-SDS (500 µg/mL pronase, 10mM Tris pH 7.4, 10mM EDTA, 0.5% SDS) at 37°C overnight. The cell lysate was then transferred to a microfuge tube, the DNA extracted with 1X volume of buffer-saturated phenol, and

precipitated with 1/10 volume 3M NaOAc and 2X volumes of 96% ethanol. The DNA was pelleted by centrifugation (16000 xg, 15 min). After washing the DNA pellet first in 70% ethanol and second in 96% ethanol, the pellet was dried and resuspended in 50 μ L TE. The structure of the recombinant Ad vector DNA was analyzed by restriction enzyme digest (HindIII), and purified isolates, with the correct digest band pattern on an agarose gel, were further amplified and used in subsequent experiments.

Production of high titer viral stocks

Medium was removed from dishes of 293 cells and virus inoculum from high titer stock or plaque purification (20 μ L or 150 μ L respectively, in 1mL of PBS⁺⁺, per 160mm dish) was added. After incubation at 37°C (5%CO₂) for 30-45 min medium was added and the cells incubated until complete CPE was observed. At this point the cells were scraped into the medium, collected in a 50mL corning tube, and centrifuged 6000 xg for 15 min. For the storage of high titer crude lysate, the cell pellet was resuspended in PBS⁺⁺ + 10% glycerol (1mL per 160mm dish) and stored at -80°C until needed. If the viral stock solution was to be further purified (concentrated) by CsCl banding the cell pellet was resuspended in 0.1M Tris-HCl (pH 8) (15mL Tris-HCl per 30 160mm dishes). For the infection of 293N3S cells (in suspension) three liters of 293N3S “spinner” cell culture (5x10⁵ cells/mL in Joklik’s medium supplemented with 5% fetal bovine serum) was centrifuged at 2300 xg for 30 min. Half of the conditioned medium was saved and the remaining cell pellet was resuspended in 300mL of fresh Joklik’s medium. High titer crude

lysate was added to the mixture (4mL per 3L spinner culture or a multiplicity of infection of 1 to 5). Cells were incubated at 37°C with stirring for 1-1.5 hrs, after which the infected cells were transferred to the original spinner flask containing 3L of culture medium (50% fresh, 50% conditioned). The infected spinner cells were then incubated at 37°C with stirring until 85-95% of cells were observed with inclusion bodies. After this, the cells were harvested by centrifugation at 2300 xg for 30 min, and resuspended in the residual conditioned medium. The cells were then centrifuged in 50mL polypropylene tubes at 600 xg, and finally resuspended in 30mL 0.1M Tris-HCL (pH 8) and stored at -80°C until needed.

Inclusion body staining

To determine if the spinner cell infection is near completion, inclusion bodies produced by the virus are determined. At various time-points post infection, a 1.5mL sample of cells is taken and centrifuged at 600 xg for 5 min. The cell pellet was resuspended in 0.5mL of 1% sodium citrate, and let stand at RT for 10 min. One half mL of Carnoy's fixative (3:1 methanol : glacial acetic acid) was added, and the cells were incubated further for 10 min. An additional 2mL of Carnoy's was added, and the cells were centrifuged for 5 min at 600 xg. The cell pellet was resuspended in 5-10 drops of Carnoy's, and a drop of this mixture was placed onto a microscope slide and allowed to dry (1 hr). The inclusion bodies were made visible by staining with 1-2 drops of orcin stain (2% orcin in 50% glacial acetic acid). Inclusion bodies appeared as refractile densely-

staining (red) nuclear localized particles.

Cesium chloride gradient banding of virus

High titer stocks of virus were treated with 1/10 volumes of 5% sodium deoxycholate (30 min, RT) and mixed regularly. Afterwards 1/100 volumes of 2M $MgCl_2$ and 1/200 volumes of a DNase I solution (100mg/mL DNase I in 10mL of 20mM Tris-HCl pH 7.4, 50mM NaCl, 1mM dithiothreitol, 0.1mg/mL bovine serum albumin, 50% glycerol). The solution was mixed by inversion and incubated at 37°C for 1 hr (with occasional mixing). The solution was then centrifuged 6000 xg for 15 min at 5°C. A step gradient was prepared with 0.5 mL of 1.5d CsCl solution gently overlayed with 3 mL of 1.35d CsCl solution, and again with 3 mL of 1.25d CsCl solution, in a SW41 ultracentrifuge tube. Five mL of the cell lysate supernatant were gently overlayed onto this gradient. The sample was centrifuged at 35,000 rpm in a SW41 rotor at 10°C for 1 hr. Adenoviral bands appeared as a grey/blue zones located at the interface between the 1.25d and 1.35d solutions. These virus bands were collected and pooled into SW50.1 ultracentrifuge tubes and the tubes were topped up with the 1.35d CsCl solution, mixed well, and centrifuged in a SW50.1 rotor at 35000 rpm, 4°C, for 18-22 hrs. Virus bands were collected in 0.5-1mL volumes, and dialyzed at 4°C in 500mL 0.01M Tris-HCl (pH 8) for 4 hrs, and again in fresh Tris-HCl overnight. Dialysis was performed in a Slide-A-Lyzer dialysis cassette. Afterward, glycerol was added to the virus preparation (to 10%), and stored at -80°C until needed.

Plaque titration of viral stocks

To determine the concentration of adenovirus vectors, plaque titrations were performed. Virus stocks were serially diluted in PBS⁺⁺, and 0.2mL was used to infect 60mm dishes of confluent 293 cells (as above). Cells were overlaid with agarose medium (as above) and incubated until visible viral plaques appeared (7 to 15 days). The average number of plaque forming units (p.f.u.) were determined from plaque counts.

Infection of Mammalian Cells in Culture

The number of cells per dish was determined by trypsinization and counting of cells on a hemocytometer. After removal of the medium, virus was added to the monolayer (1mL for 150mm dish and 0.2mL for a 60mm dish, in PBS⁺⁺), as to produce a multiplicity of infection (moi) of 50 pfu per cell (typically). The moi may differ between experiments. After incubation for 30-45 min (37°C, 5%CO₂), the cell culture medium was replaced (25mL for 150mm dishes, and 5mL for 60mm dishes) and cells were placed in an incubator (37°C, 5%CO₂). Cell culture supernatants were sampled at various time points post infection and analyzed for expression of the viral transgene by various methods.

Molecular Biological Techniques

Immuno-blots (Western Blots)

Cell culture supernatants from cells transduced with Ad vectors, were added to 1X volume of 2X polyacrylamide gel electrophoresis (PAGE) loading buffer (125mM Tris-

HCl pH 6.8, 4% SDS, 20% glycerol, 10% β -mercaptoethanol, 0.2% bromophenol blue) and boiled in a water bath for 2-3 min. The samples were then separated on a 12.5% acrylamide gel (Maniatis et al., 1989) by electrophoresis at 5-10 mAmps overnight. The acrylamide gel was then electrotransferred onto a nitrocellulose membrane (Immobilon-P). This was accomplished by placing the gel between a Scotch brite pad, and 2 pieces of 3M Whatmann paper (on either side) with the nylon membrane against the gel facing the anode. Prior to this the nylon membrane was soaked in methanol, then water, and finally transfer buffer. The entire gel "sandwich" was immersed in transfer buffer (20mM Tris-HCl, 150mM glycine pH 8, 20% v/v methanol), and electrotransferred at 1 amp for 3 hrs (at 4°C). The membrane was then placed in blocking solution (5% powdered milk in PBS) at RT for 2 hrs. Once blocking was complete, the membrane was incubated in 20-50mL of primary antibody solution (1:100 to 1:1000 dilution of antibody in blocking solution), with gentle shaking for 1 to 4 hrs at RT. The membrane was washed very well (4X) in 100-200mL of PBS. The membrane was then incubated in 15-40mL of the secondary antibody solution (1:1000 to 1:5000 dilution of HRP-conjugated secondary antibody in blocking solution), with shaking for 1 to 4hrs at RT. After washing vigorously again, the membrane was subjected to chemiluminescent detection using the ECL system (Amersham) according to the manufacturer's protocol. After treatment with ECL the membrane was wrapped in plastic wrap and exposed to Kodak XAR-5 film for 30 sec to 30 min until a satisfactory signal was observed. For western blot analysis of human TNF α the primary antibody was polyclonal rabbit anti human TNF α (Chiron) at a dilution of 1:1000.

The secondary antibody was HRP conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology) at a 1:2000 dilution. Murine TNF α was detected using a polyclonal goat anti-mouse TNF α antibody (R&D Systems) at a dilution of 1:500, and a secondary HRP conjugated donkey anti-goat antibody (Santa Cruz Biotechnology) at a dilution of 1:1000.

Co-immunoprecipitations

One hundred μ L of pre-swollen (PBS) protein-G sepharose (Pharmacia) was added to 0.5 mL of cell culture supernatant. Two μ g of anti-murine p55 or p75 TNFR antibody (55R-286, and TR75-89 respectively), and 2 μ g of soluble murine p55 or p75 TNFR (R&D Systems) was added respectively. The mixture was rotated for 4 hrs at 4°C, then centrifuged for 2 min (16000 xg). The pellet was resuspended in fresh PBS and centrifuged again. This was repeated for a total of six washes. The pellet was then resuspended in 50 μ L of protein loading buffer (1X), and boiled for 3 min. The sepharose beads were then centrifuged down (as before) and 15-25 μ L of the supernatant was loaded onto a polyacrylamide gel (12.5%) for SDS-PAGE and immuno-blot analysis.

Flow cytometry

MT1A2 cells were infected with Ad vectors at a MOI of 50 pfu per cell. One day post infection the cells were trypsinized (1X trypsin-EDTA), diluted with 1:10 volumes of FBS, centrifuged (2300 xg), washed once in MEM (20mM HEPES), and finally resuspended in MEM (20mM HEPES) at a concentration of 1×10^6 cells per mL. Flow cytometry was

performed on these cells by Dr. Denis Snider's laboratory (Immunology Facility, McMaster University). Cells were stained with primary rat anti-murine TNF α antibody (Immunocorp Science), and secondary fluorescein(FITC)-conjugated goat anti-rat IgG antibody (Southern Biotechnology Associates). Viable cells were selected for analysis by excluding propidium iodide (PI) positive (dead) cells.

Detection of TNF α Expression / Activity

TNF α ELISA

Biotrak Kits, designed for the detection of human and murine TNF α , were purchased from Amersham, and used according to the manufacturer's protocol. The concentration of TNF α was calculated by extrapolation from a standard curve produced by standard dilutions of recombinant TNF α . The kits used horseradish peroxidase (HRP) conjugated streptavidin to detect biotinylated anti-TNF α antibodies.

TNF α bioassay

The cytotoxicity bioassay measured direct killing of TNF α sensitive cell lines (A673/6 or L929). Cells were dispensed into 96 well plates (2.5 or 5×10^4 cells respectively, in $50 \mu\text{L}$), and incubated overnight at 37°C (≈ 20 hrs) in growth medium. The next day the medium was aspirated and replaced with $40 \mu\text{L}$ of medium plus soyabean trypsin inhibitor (SBTI) ($100 \mu\text{g}/\text{mL}$) and cycloheximide (Sigma) ($20 \mu\text{g}/\text{mL}$). Ten μL of samples (cell culture supernatants from infected cells) or standards were added to the microtiter wells and

incubated at 37°C overnight (\approx 18 hrs). Afterwards 10 μ L of 5mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was added to each well and incubated for 4 hrs, after which 50 μ L of 50% dimethylformamide (20%SDS pH 4.7) was added. Following an additional overnight incubation, the OD₅₇₀ was measured. For the quantitation of TNF α , the measured OD₅₇₀ from diluted samples was compared to the OD₅₇₀ obtained from serial dilutions of a standard TNF α recombinant stock solution. Cycloheximide, SBTI, MTT, and recombinant murine TNF α used as the standard were purchased from Sigma, St. Louis, MO, USA. Recombinant human TNF α was purchased from R&D Systems, Minneapolis.

TNF α proliferation assay

CT6 cells (donated by Mike Rothe, Tularik, Inc.) were deprived of IL-2 for 24 hrs before being aliquoted into 96 well microtiter plates (5x10⁴ cells per well in 40 μ L growth medium (without IL-2)) with 10 μ L of samples (transfected cell culture supernatant) or standards. Three days later 10 μ L of 5mg/mL MTT was added to each well and incubated for 4hrs, after which 50 μ L of 50% dimethylformamide (20%SDS pH 4.7) was added. Following an additional overnight incubation, the OD₅₇₀ was measured. For receptor blocking, soluble p55 mTNFR (R&D Systems, Minneapolis, MN, USA) was added to the appropriate wells to a concentration of 33 μ g/mL.

Tumour Studies

Preparation of polyoma-middle-T tumour cells

Tumours were surgically removed from mammary glands of MT⁺ female mice, and placed in a Petri dish, containing approximately 10mL of collagenase-dispase (Boehringer) solution (0.25mg/mL collagenase-A, 2.5mg/mL dispase-II in PBS). Forceps and razor blades were used to slice tumours into smaller chunks. After this, the tumours were further divided with forceps and scissors. The “processed” tumour tissue was transferred to a 100mL bottle, and filled to a total volume of 25mL with collagenase-dispase. Next the mixture was stirred vigorously for 1.5-2 hrs at 37°C. The bottle was left to stand for 2 min, then the upper layers of the mixture were carefully transferred into a 50mL Corning tube (carefully not to suck up too many of the larger floaty things). This was centrifuged (2300 xg), washed in MEM, and resuspended in approximately 22mL of medium. The cells were then plated onto 11 150 mm dishes (2mL of cell mixture plus 23mL of fresh culture medium and incubated overnight, 37°C, 5%CO₂). The next day the old media was removed and replaced with fresh media. Cells grew to confluence within 2-4 days.

Generating polyoma-middle-T tumour bearing mice

Polyoma-middle-T (PyMidT) cell monolayers were trypsinized vigorously using 2X trypsin-EDTA for 15 min, and harvested into a 50 mL Corning tube with about 5mL of fetal bovine serum. The cells were centrifuged (2300 xg, 5 min) and washed several times in sterile PBS. The final concentration of PyMidT tumour cells was adjusted to 2.5x10⁶

cells/mL. Syngeneic FVB mice were injected with these cells subcutaneously on the right hind flank using a 1 mL syringe and 25g needle (200 μ L per mouse to deliver 5×10^5 cells). Sizable tumours (approximately 60mm³) developed within 18-21 days post injection.

Tumour immunotherapy

Established PyMidT tumours were directly injected with various concentrations of Ad vectors expressing cytokines in 50 μ L of PBS, with an insulin syringe (Becton Dickinson). Ad vector preparations used for tumour immunotherapy were purified and concentrated using the CsCl banding procedure (above). Tumours were measured prior to injection with calipers, and every 2-7 days post-injection. Tumour volumes were calculated assuming a prolate spheroid using the equation: $\pi/24$ (length \cdot (width + depth)²). Mice which underwent complete tumour regression were challenged 2-4 months later with PyMidT cells (as before) on the left hind flank.

Production of hematoxylin and eosin stained tumour sections

Tumours were removed from treated mice, cut in two with a razor and fixed in 10% Neutral Buffered Formalin (NBF) for 18-24 hrs. After fixation the tissue was immersed in 50% ethanol, then 60% ethanol, for 30 minute periods until finally being stored in 70% ethanol. Tumours were then paraffin embedded, sectioned and stained with hematoxylin and eosin (H&E) at the pathology central facility, McMaster University.

Preparation of tumour homogenate for TNF α ELISA

Tumours were removed from mice and snap frozen in liquid nitrogen and stored at -80°C. Thawed tumour tissue was then homogenized in 0.5mL of PBS (plus 100 μ M phenylmethylsulfonylfloride and 10 μ g/mL aprotinin, using a Tissueimizer (Janke & Kunkel, Ultra-Turrax). Afterwards the sample was sonicated (Branson Sonifer 450) twice for 10 seconds. The homogenate was cleared by centrifugation (16000 xg) and stored at -80°C until assayed by ELISA.

Preparation of serum for TNF α ELISA

Blood from sacrificed mice was collected by extraction from the thoracic cavity (after cervical dislocation) with a plastic disposable pipet. The collected blood was left at RT for one hour and then centrifuged gently (2000 xg). The serum was collected from the clotted blood and stored at -80°C until assayed by ELISA.

Cytotoxic Lymphocyte Assay

For the detection of cytotoxic T lymphocytes (CTL), spleens from immunized mice were removed and minced into single cell suspensions by crushing with a fine metal mesh in PBS plus 1% FBS. Cells were suspended in 0.83% NH₄Cl for 5 min on ice (to remove erythrocytes), after which they were washed and resuspended in PBS (1% FBS). Polyoma-mid-T expressing MT3 cells were exposed to 5000 rads of γ -radiation then cocultured with splenocytes (1.2x10⁷ splenocytes:1.2x10⁵ stimulators) in RPMI-1640 medium (plus

20mM HEPES, and 100 μ M β -mercaptoethanol), for 5-6 days. Stimulated spleen cells were incubated for 6 hrs with ^{51}Cr -labeled MT3 target cells or labeled nonspecific targets (PT₀₅₁₆ cells), at ratios of 90:1, 30:1, 10:1, and 3.3:1 (effector to target) in 300 μ L total volume. Target cells were prepared by incubation for 1 hr in 100 μ L (1 μ Ci/ μ L) of fresh ^{51}Cr (sodium chromate, Dupont), after which they were washed 5 times by repeated centrifugation (2300 xg) and resuspension in CTL medium. The lysis step was performed in V-bottomed microtiter plates at 37°C (5%CO₂). As an additional negative control, 10 μ L of anti-CD3 antibody (hamster anti-mouse CD3 (145-2C11) ascites at a dilution of 1:8) was added to control wells. The radioactivity released by the lysis of target cells was determined by adding 80 μ L of cell medium to a glass tube and measuring γ -radiation using a γ -radiation counter. The percentage of specific ^{51}Cr release was then determined by the equation:

$$\frac{(\text{Experimental release} - \text{spontaneous release}) \times 100}{(\text{Maximum release} - \text{spontaneous release})}$$

Spontaneous and maximum release were determined by incubating target cells with medium alone (no effector splenocytes) or with 1N HCl respectively.

CHAPTER III: Tumour Immunotherapy using an adenoviral vector expressing a membrane-bound mutant of murine TNF α

Introduction

Tumour necrosis factor alpha is a multipotent cytokine with a plethora of physiological functions. It can attack tumours in three ways: i. direct cytotoxicity; ii. disruption of tumour vasculature; and iii. immune activation. Unfortunately TNF α is also involved in a variety of disease processes including septic shock (Tracey, 1995; Tracey et al., 1986). The systemic administration of TNF α for tumour therapy has been associated with acute systemic toxicity. Physicians have attempted to deal with this problem through the use of isolated limb perfusion (ILP) techniques. This approach has met with some success in reducing the systemic toxicity associated with TNF α while retaining the antitumour activity of the cytokine (reviewed in (Lejeune et al., 1998)). However, ILP is limited to the treatment of affected limbs and requires surgery. A more effective and less complicated means of delivering TNF α to the tumour site would be highly desirable. Gene therapy is ideally suited for this task, as the transduction of tumour cells with a TNF α expression cassette would allow for the local and continuous production of the cytokine from the tumour cells themselves. First generation adenovirus vectors are well suited for this task, as they can infect a wide range of mammalian cells, and can express transient but high levels of the transgene. Therefore we constructed an Ad vector expressing wild type murine TNF α for testing in tumour gene therapy of a murine transgenic polyoma-middle-T

breast cancer model developed by Dr. William Muller's laboratory (Addison et al., 1995a; Guy et al., 1992). However, it was anticipated that sufficient quantities of murine TNF α would diffuse from the tumour site, to cause lethality at doses required for efficacy. For this reason a second Ad vector was constructed which expressed a membrane bound mutant of murine TNF α . The mutant, used in our studies, was previously shown to be active and uncleavable from the membrane (Decoster et al., 1995). This was done in the hope that the presentation of TNF α on the cell surface of transduced cells would activate target cells via cell to cell contact, but restrict cytokine exposure to the tumour site. Thus, by preventing escape of TNF α into the circulation, the systemic toxicity would be reduced while retaining local antitumour activity. Both these vectors were characterized and used in tumour gene therapy experiments in syngeneic mice bearing transgenic polyoma-middle-T (PyMidT) tumours, through direct injection of the vectors intratumourally.

The second author of this paper Christina Addison contributed by assisting the first author with the cytotoxic T lymphocyte (CTL) assay by preparing all the cell lines involved and assisting with all other steps in the assay (see fig. 3-4). Christina Addison also originally generated the MT1A2, PT₀₅₁₆, and MT3 cell lines (see appendix table A4-1). Dr. Denis Snider provided the flow cytometric analysis of the expression of membrane bound TNF α on the surface of transduced MT1A2 cells (see figure 3-2). Dr. William Muller provided our laboratory with the PyMidT mouse mammary tumour model. Dr. Jack Gauldie assisted through providing access to the pathology lab run by Brian Hewlett. Dr. Frank Graham supervised and provided direction (as well as lab space, reagents, and a pay

check) for all of the work done for this manuscript. The first author Robert Marr performed all the remaining work which lead to the completion of this manuscript including the design and performance of the experiments. Dr. Graham also contributed by proof reading the manuscript before its submission.

Tumour immunotherapy using an adenoviral vector expressing a membrane-bound mutant of murine TNF α

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The most limiting factor regarding the use of TNF α in tumour therapy is systemic toxicity. The expression of membrane-bound (nonsecreted) TNF α within a tumour may serve to reduce systemic toxicity while retaining anti-tumour activity. Two adenovirus (Ad) vectors were constructed: (1) Ad-mTNF-wt expressing wild-type murine TNF α ; and (2) Ad-mTNF-MEM expressing a mutant non-secreted (membrane-bound) form. Only the Ad-mTNF-wt vector induced high levels of TNF α secretion in transduced cells (approximately 400 ng/10⁶ cells), however, both vectors induced efficient cell surface expression as detected by FACS. These vectors were used in tumour immunotherapy trials in a murine transgenic breast cancer model. High

serum concentrations of mTNF α (approximately 1 ng/ml) were detected only in Ad-mTNF-wt-treated mice, while both vectors induced substantial disruption of tumour pathology. The wt TNF vector was highly toxic, killing 12 of 16 mice at a dose of 5 × 10⁸ p.f.u., whereas the Ad-mTNF-MEM vector showed low toxicity killing three of 27 at the same dose. Both vectors induced partial, and in some cases, permanent tumour regressions, with cured mice displaying protective immunity and specific CTL activity against the tumour. These results indicate that the use of a nonsecreted form of TNF α can result in a relatively large reduction in systemic toxicity with little or no reduction in antitumour activity.

Keywords: tumour therapy; adenovirus vector; tumour necrosis factor α ; gene therapy; membrane-bound TNF; immunotherapy

Introduction

Tumour necrosis factor alpha (TNF α) was originally discovered through the anticancer activity of sera of mice treated with endotoxin.¹ It is associated with a wide variety of responses involving the activation of immune cells, alterations in the extracellular matrix, antiviral activity, cachexia, septic shock and more.^{2,3} TNF α is first produced as a cell surface 26 kDa protein, most of which is then cleaved from the membrane by metalloproteases, to produce the mature secreted 17 kDa form.² TNF is secreted by many cell types including macrophages, lymphocytes, polymorphonuclear cells, astrocytes and Kupffer cells.^{2,4} Its expression can be induced by bacterial endotoxins, viruses, parasites, complement, antibody/antigen complexes and cytokines.⁴

TNF α can act against tumours in three basic ways. The first is through direct cytotoxicity against tumour cells. Induction of apoptosis is the primary mechanism of direct cytotoxicity by TNF α , mediated through engagement of the p55 TNF receptor present on most cell types.^{2,5} However, most normal and tumour cells are resistant to direct killing by TNF α .⁶ The second mechanism is through effects on the tumour vasculature. It has been shown that TNF α can promote intravascular throm-

basis within tumours, inducing a hypoxic/necrotic environment.^{2,7-9} The third antitumour mechanism is through immune activation. TNF α is a key mediator of inflammation, and can rapidly activate neutrophils, macrophages and NK cells, as well as induce the production of other cytokines (IL-6, IL-1, GM-CSF and IL-8) and adhesion molecules.^{4,10} The antitumour activity of TNF α has been shown to be due, in part, to T cell (CD4/CD8) activation and to induction of tumour-specific immunity.¹¹⁻¹³

Perhaps the major limiting factor preventing the clinical use of TNF α in tumour therapy is its induction of systemic toxicity due to septic shock and cachexia.^{4,14,15} The use of adenoviruses for delivery of TNF α might serve to circumvent this problem. There are many advantages to using adenovirus vectors to express potentially toxic cytokines for tumour therapy. The vector can produce high levels of continuous/local expression when directly administered to the tumour site, which could serve to reduce the systemic side-effects associated with the cytokine, while enhancing the local antitumour activity.¹⁶ Furthermore, the so-called first generation Ad vectors only transiently express the transgene, thus avoiding long-term stimulation of the immune system.^{17,18}

Two mTNF α expressing Ad vectors were used in immunotherapy of a murine transgenic breast cancer model.¹⁹ The first vector expressed the complete wild-type mTNF α cDNA. Because secreted cytokines can

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Received 15 May 1997, accepted 7 July 1997

diffuse into the circulatory system from the site of production we constructed a second vector which expressed a genetically engineered murine TNF α (Δ 1-9K11E), first described by Decoster *et al*,²⁰ that had been altered to a nonsecreted (membrane-bound) form, to prevent its dissemination. Our results show that both vectors had significant antitumour activity, but that the mutant vector was far less toxic.

Results

Expression of TNF α from Ad vector-transduced cells

Two vectors have been constructed which express murine TNF α (Figure 1b), the first expressing the complete wild-type mTNF α cDNA (Ad-mTNF-wt) and the second expressing a mutant form of mTNF α which prevents its enzymatic cleavage from the membrane (Ad-mTNF-MEM) while retaining bioactivity.²⁰ The mutant cDNA generated by PCR mutagenesis of the wild-type cDNA, has amino acids one to nine deleted and lysine 11 mutated to glutamic acid.

To measure TNF expression, cells were infected at a multiplicity of infection (MOI) of 50 p.f.u. per cell and culture supernatants were harvested at various times, frozen (-70°C), and later assayed for mTNF α activity using a TNF α -sensitive cell line (A673/6), as described in Materials and methods. Ad-mTNF-wt directed high levels of expression (up to 400 ng/10⁶ cells) in human MRC5 and murine MT1A2 cells (Figure 2a). However, negligible levels of secreted TNF α were detected from Ad-mTNF-MEM-transduced cells. Expression of secreted TNF α by Ad-mTNF-wt-infected cells was also confirmed by ELISA (data not shown).

To detect membrane-associated TNF, FACS analysis was done on MT1A2 cells transduced with either Ad-mTNF-wt or Ad-mTNF-MEM (Figure 2b). Similar levels of expression were detected on the surface of cells transduced with both mTNF α vectors (79 and 77% positive, respectively), while only background levels of staining were observed on mock-infected and control virus (Ad-d170-3)-infected cells.

In vivo expression of TNF α from Ad vectors

The tumour model used for our *in vivo* studies was generated through subcutaneous injection of normal syngeneic mice with mammary tumour cells derived from transgenic mice carrying polyoma middle-T antigen (PyMidT) under the control of the MMTV promoter.^{19,21,22} These subcutaneous tumours were injected with 5 \times 10⁶ p.f.u. of Ad-mTNF-wt, Ad-mTNF-MEM or control vector (Ad-d170-3). Blood was taken from mice killed on days 1 and 3 and sera were assayed for mTNF α by ELISA. Some Ad-mTNF-wt-treated mice became sick (appeared wasted with ruffled fur and rapid shallow breathing) on day 2 and were killed. In addition to blood, tumours were also removed from the animals and snap-frozen or fixed in 10% neutral-buffered formalin (NBF) for later histological analysis. High levels of mTNF α were detected in the serum of mice injected with the Ad-mTNF-wt vector (Figure 3a), particularly on day 2 when visible signs of toxicity were most apparent. In contrast, only background levels of mTNF α were detected in the serum of mice injected with Ad-mTNF-MEM or Ad-d170-3, whereas significant levels of mTNF α were detected by

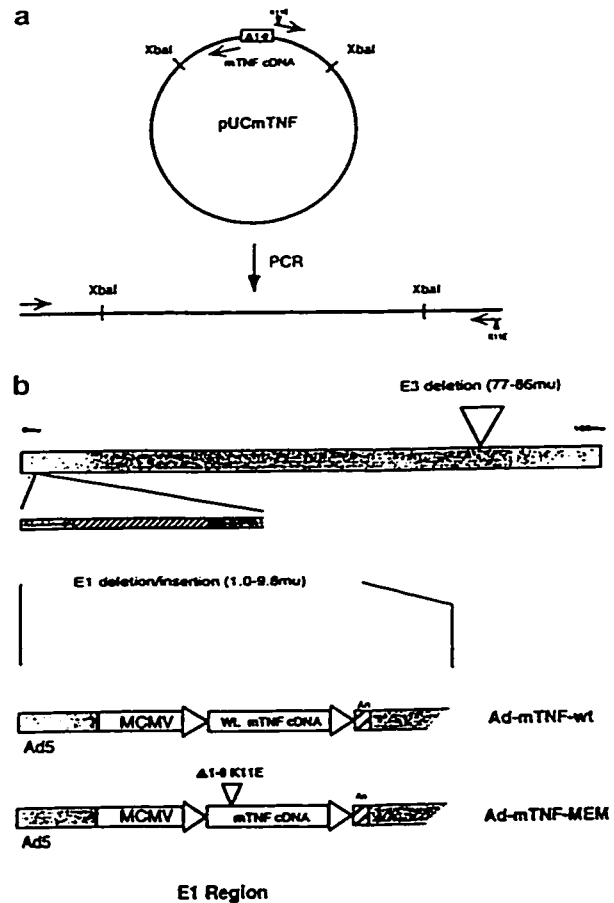


Figure 1 (a) The deletion/point mutation Δ 1-9 K11E was generated by PCR mutagenesis. Primers AB7329 (containing point mutation) and AB7330 (shown as small arrows) were used to drive replication leftward and rightward, respectively, from the outside of the deleted area within the mTNF α cDNA. The linear product was self ligated to produce the plasmid p Δ 1-9K11E containing the mutated cDNA. Vent polymerase was used for the PCR reaction since it produces primarily blunt-ended amplification products. (b) Ad vectors containing TNF α expression cassettes. Both vectors carry the mTNF α cDNA in an E1/E3-deleted Ad-5-based vector. Ad-mTNF-wt expresses the complete mTNF cDNA, and Ad-mTNF-MEM expresses a membrane-bound mutant of mTNF α , where amino acids one to nine have been deleted and lysine 11 is mutated to glutamic acid.²⁰ Both vectors utilize the MCMV promoter to drive expression.

ELISA in homogenized tumour tissue from mice treated with both mTNF α vectors (Figure 3b). Tumour pathology from treated mice was examined to determine if bioactive TNF α was present in Ad-mTNF-MEM-treated tumours. Haematoxylin and eosin stained tumour sections showed considerable disruption of tumour morphology marked by vast areas of cells undergoing pycnosis within tumours injected with either mTNF α vectors, while normal pathology was observed in Ad-d170-3 injected tumours (Figure 4). These data demonstrate that the membrane-bound mutant is not readily released into the circulatory system, but is present and bioactive in vector-infected tissues.

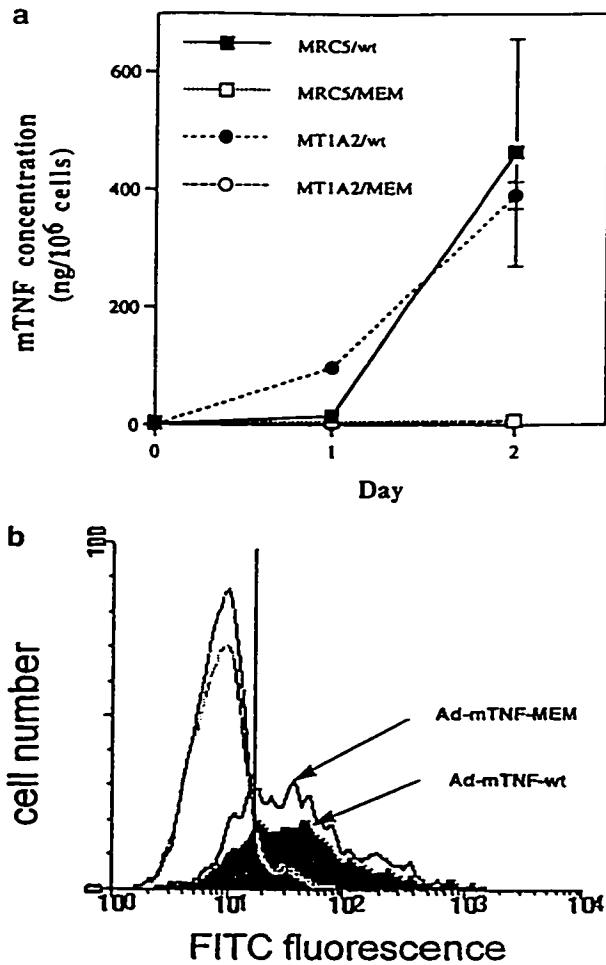


Figure 2 *In vitro* expression in cells transduced with murine TNF α vectors. (a) Human MRC5 (squares) and murine MT1A2 (derived from primary transgenic tumour cells, circles) cells were transfected with Ad-mTNF-wt (closed symbols) or Ad-mTNF-MEM (open symbols) at an MOI of 50 p.f.u. per cell (error bars represent standard error). (b) FACS analysis of membrane-bound mTNF α expressed from MT1A2 cells. MT1A2 cells were cultured with no virus (light dotted line), or transduced with control virus (grey line), Ad-mTNF-wt (black solid), or with Ad-mTNF-MEM (black line) at an MOI of 50 p.f.u. per cell. Isolated cells were stained with rat anti-TNF α followed by FITC-anti-rat IgG. Viable cells were gated for analysis by exclusion of PI-positive (dead) cells. Ninety-eight per cent of cells incubated with rat IgG control and FITC-anti-rat IgG had fluorescence to the left of the vertical line. The percentage of positive cells is shown with arrows.

Antitumour activity of murine TNF α vectors

Mice bearing subcutaneous PyMidT tumours were used in immunotherapy experiments to determine the toxicity associated with a single injection of Ad vectors as well as the effects on tumour progression. Mice were injected intratumorally with various doses of Ad-mTNF-wt, Ad-mTNF-MEM or Ad-d170-3, after which the tumour volume was monitored weekly. Table 1 summarizes the toxicity and antitumour response induced by the various Ad vectors.

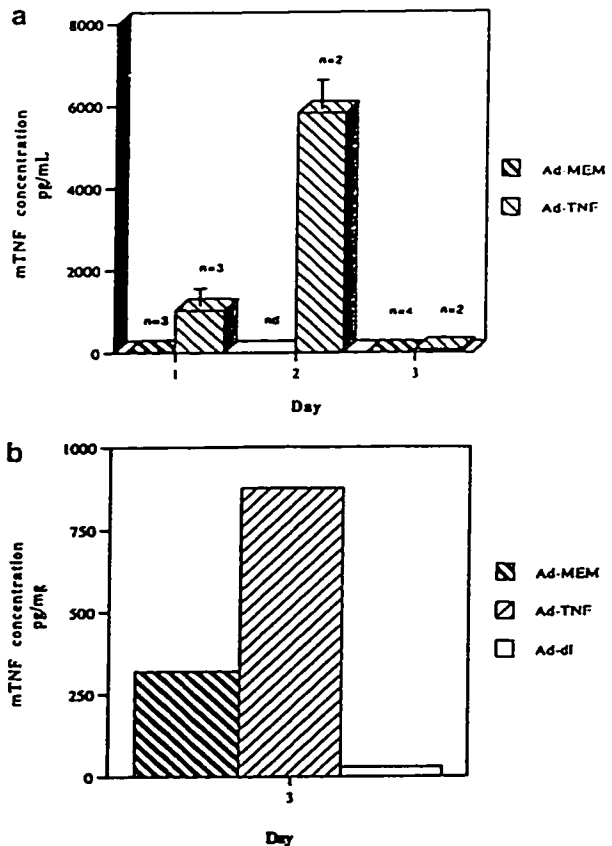


Figure 3 (a) *In vivo* expression of murine TNF α . Mice were injected intratumorally with 5×10^6 p.f.u. of Ad-mTNF-MEM (Ad-MEM) or Ad-mTNF-wt (Ad-TNF), then killed at 1, 2 or 3 days after injection. Serum was collected from dead mice and assayed for mTNF α by ELISA as described in Materials and methods. No murine TNF α was detected from mice injected with control (Ad-d170-3) virus (not shown). Note: error bars represent standard error except where $n = 2$, in which case the spread between data points is given. (b) Supernatants from homogenized tumours taken 3 days after injection with 5×10^6 p.f.u. of Ad-mTNF-MEM (Ad-MEM), Ad-mTNF-wt (Ad-TNF) or control Ad-d170-3 (Ad-d1) ($n = 1$), were assayed for mTNF α by ELISA, as described in Materials and methods.

The Ad-mTNF-wt vector proved to be quite toxic showing a dose-dependent rate of mortality. Approximately 50, 75 and 100% of the mice died or had to be killed within 2-3 days after injection at doses of 1×10^6 , 5×10^6 and 1×10^7 p.f.u. respectively. However, mice surviving doses of 1 and 5×10^6 showed partial or complete tumour regressions, with tumours shrinking to at least half of their original volume before relapsing (12 and 25% at 1 and 5×10^6 p.f.u., respectively), or completely disappearing (25 and 50% at doses of 1 and 5×10^6 p.f.u., respectively). Conversely the Ad-mTNF-MEM vector showed a substantial reduction in systemic toxicity: only approximately 11-15% lethality was observed at doses of 5×10^6 and 1×10^7 p.f.u., respectively. Most importantly, the tumours of mice treated with these doses of Ad-mTNF-MEM underwent partial or complete regressions at frequencies similar to those observed in mice surviving

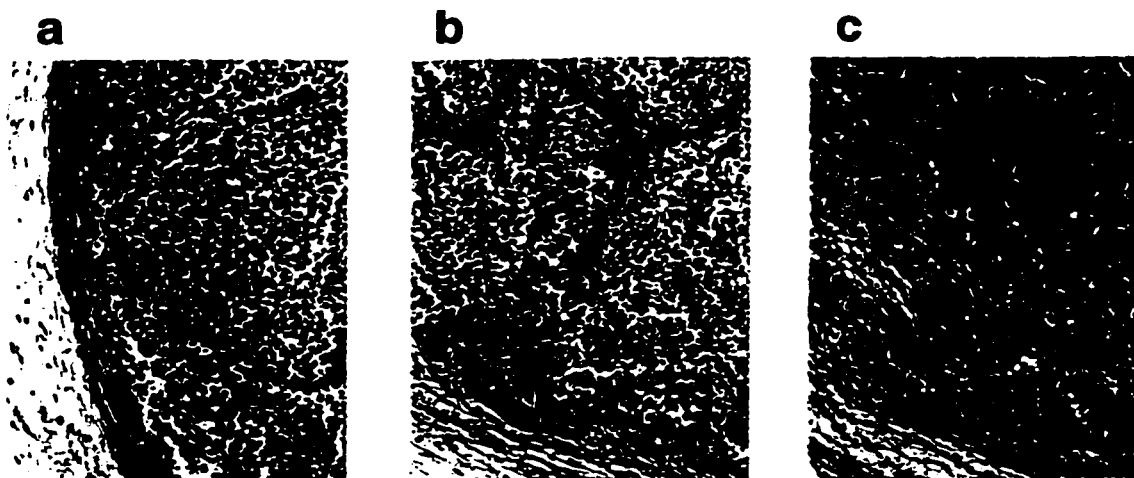
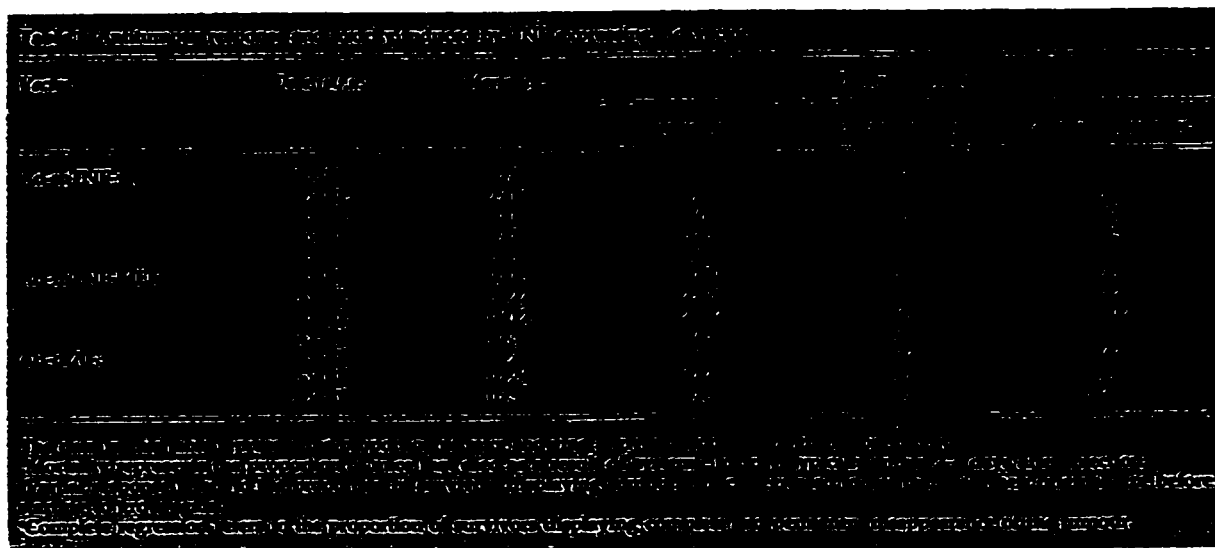


Figure 4 Tumour morphology of mice intratumorally injected with 1×10^6 p.f.u. of Ad-mTNF-wt (a), 5×10^6 p.f.u. of Ad-mTNF-MEM (b) or 5×10^6 p.f.u. of control Ad-d170-3 (c). Tumours were removed 48 h after injection, fixed in 10% NBF, paraffin-embedded, thin-sectioned, and stained with haematoxylin and eosin. Massive disruption of the tumour pathology was visible 2 days after injection from tumours treated with both TNF α vectors, while normal tumour pathology is observed in control virus-injected tumours.



treatment with Ad-mTNF-wt. Typically in cured mice tumours disappeared within 3–5 weeks after injection with either vector. Ad-d170-3-injected mice showed no tumour response as well as no systemic toxicity. These results show that both wild-type and membrane-bound mutant mTNF α vectors can induce a potent antitumour response, with the mutant form being far less toxic.

Antitumour immunity

Immune memory against the transgenic tumour cells could provide a mechanism for protection against potential metastasis. In similar studies using Ad vectors expressing IL-2, IL-4 and IL-12 in this tumour model, we have achieved long-term protection and specific cytotoxic

T lymphocyte (CTL) activity.^{21–23} To investigate whether Ad mTNF vector treatment had the same effect, cured mice were tested for their ability to reject a secondary challenge with the transgenic tumour cells. Approximately 2 months after the tumour was cleared, two Ad-mTNF-MEM-treated and three Ad-mTNF-wt-treated mice were challenged with the transgenic middle-T tumour cells (see Materials and methods). All challenged mice were immune to the second injection, while naive mice developed tumours within a normal time frame (approximately 18 days).

The detection of antitumour immune memory suggested that tumour-specific precursor CTL were present in cured mice. Five months after the second challenge,

spleens were removed from Ad-mTNF-MEM-treated mice and tested for PyMidT-specific CTL activity. In both mice, strong specific CTL activity was detected against MT3 cells which express PyMidT (Figure 5). Negative controls included the use of PT₀₅₁₆ target cells (PyMidT negative) and anti-CD3-treated splenocytes, both of which generated background levels of ⁵¹Cr release. Also, splenocytes from naive mice showed background levels of activity (not shown).

Discussion

Using adenovirus vectors to express TNF α within a tumour produces a dramatic antitumour response. Unfortunately, direct administration of the vector to the tumour does not adequately prevent the toxicity observed by others (reviewed in Refs 2 and 3). Levels of the cytokine sufficient to cause a high incidence of mortality are still released into the circulation. To prevent high levels of circulating TNF we constructed an Ad vector which directs the expression of a nonsecreted membrane-bound form of murine TNF α , and have demonstrated the efficacy of using this vector to deliver and express this mutant within the tumour tissue, resulting in reduced systemic toxicity while retaining antitumour activity. The wild-type TNF α vector did seem to have somewhat more antitumour activity than the mutant membrane-bound form at equal doses. This could be due to a reduced interaction with immune and endothelial cells when using the membrane-restricted form. However, we believe that in this case the benefits of restricted exposure to TNF α outweigh the disadvantages. As well, an increase in the dose of the Ad-mTNF-MEM vector seems to compensate to a large extent for this slightly reduced antitumour activity. More importantly, it is clear that large doses of TNF α did not enter the circulatory system following Ad-mTNF-MEM administration, while a strong response was still initiated within the tumour. This reduction in circulating TNF correlated with a reduction in mortality associated with the cytokine. The

fact that high doses of the Ad-mTNF-MEM vector were still toxic to a relatively small proportion of the mice indicates that this system is not perfect. There was no dose of the Ad-mTNF-MEM vector that was able to cure mice while not causing systemic toxicity; thus the problem of systemic toxicity has not been eliminated. The low levels of toxicity seen in Ad-mTNF-MEM-treated mice could be a result of leakage of intracellular mTNF α into the system from dead or dying cells. Alternatively, if some of the vector disseminated from the tumour, toxicity could have been induced by the expression of membrane-bound mTNF α , in vital organs such as the liver. It has been previously shown in this laboratory that the injection of subcutaneous tumours with Ad vector can lead to substantial expression of the transgene in major organs such as the liver.²⁴ Attempts have been made to assay for TNF α in the livers of treated mice by ELISA, but they were unsuccessful due to a high background signal. It is also possible that the induction of other potentially toxic cytokines, such as IFN γ , could be responsible at least in part for the low levels of toxicity induced by Ad-mTNF-MEM.

We have not yet fully elucidated the nature of the antitumour response induced by the mTNF expressing Ad vectors. PyMidT tumour bearing mice successfully treated with Ad-mTNF-MEM or Ad-mTNF-wt demonstrated long-term immunity by rejecting a challenge with PyMidT tumour cells. This long-term immunity was likely to be mediated by memory T cells, and indeed, a high level of PyMidT-specific CTL activity was detected in mice tested. Our results are in agreement with previous findings in this field. In murine systems the administration of recombinant TNF α has been shown to promote antitumour CTL immunity.¹² In addition, Marincola *et al*¹³ showed that implanted methylcholanthrene induced murine sarcoma cells, engineered to secrete hTNF α , showed significant growth inhibition when injected into animals which possessed pulmonary metastases of the parental (wild-type) cell type. This inhibition was shown to be CD4 and CD8 cell-dependent by immunodepletion, indicating the involvement of T cells in the antitumour activity of TNF α .

Contrary to our results, Karp *et al*²⁵ 1992 found that only secreted and not membrane-bound human TNF α -transduced tumour cells prevented tumour growth, albeit there are many differences between our two experimental systems. They used a model involving the subcutaneous injection of retrovirally transduced tumour cell lines (205F and 203 E4) into mice, in contrast to our model of directly injecting pre-established PyMidT tumours with Ad vectors. However, the most notable difference between the two systems is their use of human rather than murine TNF α . Most TNF α studies in the mouse have been done using human TNF α . Much of this was done before the species specificity of the cytokine was fully understood. Human TNF α does not bind the murine p75 TNF receptor, but does bind the p55 receptor.²⁶⁻²⁸ One advantage to the use of human TNF α in the murine system is the lack of systemic toxicity. It has been estimated that in mice hTNF α is 50 times less toxic than mTNF α . This implies that the p75 TNF receptor is primarily responsible for the induction of systemic toxicity. However, it has recently been suggested that the reduced toxicity of hTNF α in the murine system is a result of the lack of cooperation elicited when both receptors are bound. In fact it appears that the p55 rather than the p75

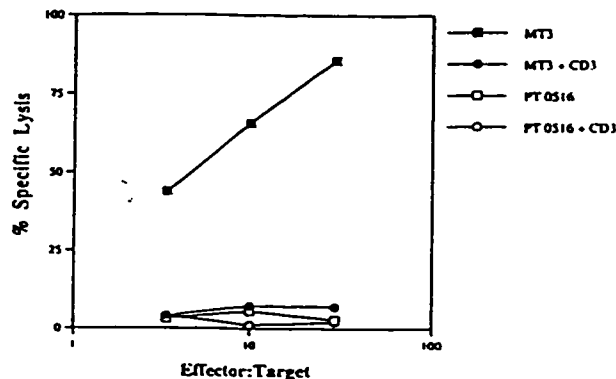


Figure 5 Specific CTL activity of splenocytes from an Ad-mTNF-MEM-cured mouse. Spleen cells were stimulated with irradiated MT3 cells expressing PyMidT, for 6 days, after which specific chromium release was assayed from labelled MT3 targets (solid symbols) and control (PyMidT negative, PT₀₅₁₆ cells) targets (open symbols). Anti-CD3 antibody treatment was used as an internal control (circles). Splenocytes from a naive mouse produced background levels of chromium release (not shown). Similar results were obtained in a repeat experiment.

TNFR is more strongly involved in the induction of systemic toxicity.^{29,31} The p75 TNF receptor (TNFR) is involved in proliferative and proinflammatory signals, as well as TNF-mediated activation of T cells.^{32,33} As one would predict, hTNF α does not activate murine T cell lines *in vitro*.²⁸ It has been demonstrated that the p75 receptor is the dominant TNFR in human lymphoid tissue, and in particular, human tumour infiltrating lymphocytes (TIL) were found to express exclusively the p75 TNFR.^{33,34} Therefore, we predict that the use of murine TNF α in a murine system would be a better model for the behaviour of human TNF α in the human system, since the full range of physiological effects are induced.²

The immunological activity of transmembrane TNF α may also be of particular interest. It has been suggested that the membrane-bound form of TNF α is the major activator of the p75 TNF receptor on T cells, and even provides a costimulatory signal to B cell when expressed on T helper cells.^{35,36} A membrane-bound mutant of human TNF α has been constructed by Perez *et al*.³⁷ This human mutant varies from the murine mutant, used in this study, in that amino acids one to 12 were deleted and there are no point mutations. It was found to be non-secreted and retained the ability to signal cell death through cell to cell contact with sensitive cells. It is conceivable that such a mutant could be useful in the treatment of human cancers. However, as with other potentially toxic cytokine therapies, combinations with other cytokines (IL-2, IL-12, IFN γ etc) may improve the effectiveness of the treatment. Work in this area is presently ongoing in our laboratory as well as in others.

Materials and methods

Cell culture

All cell culture media and reagents were purchased from GIBCO (Mississauga, Ontario, Canada) except for Fungizone which was purchased from Squibb (Montreal, Quebec, Canada). MRC5 cells (ATCC CCL-171; Rockville, MD, USA) were cultured in minimal essential medium (α -MEM) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2.5 μ g/ml Fungizone, and 10% fetal bovine serum (FBS). 293 Cells and MT1A2 cells (derived from primary transgenic PyMidT expressing tumour cells) were cultured in supplemented MEM F11 (as above).^{19,22,38} PT₀₅₁₆ cells (a non-transformed murine kidney cell line, PyMidT negative) and MT3 cells (a murine kidney cell line, derived by transduction of PT₀₅₁₆ cells with the PyMidT cDNA) were cultured in Dulbecco's MEM supplemented as above. In addition, MT3 cells were cultured in G418 (400 μ g/ml) until the time of use. The TNF α -sensitive A673/6 human cell line (obtained from Dr Jean Marshall, McMaster University, Hamilton, Ontario, Canada) was cultured in α -MEM supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin and 5% FBS.

Vector construction

The murine TNF α cDNA (pUCmTNF α) was kindly donated by Dr Graeme Dougherty (Terry Fox Laboratory Vancouver, British Columbia, Canada). The cDNA was inserted into pMH4 placing TNF transcription under the control of the MCMV promoter and the SV40 poly A signal (pmTNF-wt).³⁹ A deletion/point mutation of the

mTNF α cDNA(Δ 1-9/K11E) was constructed using PCR mutagenesis (Figure 1a). The gene was amplified from the circular plasmid pUCmTNF α using the primers AB7330 (5'TGTGAGGGTCTGGGCCATAGA) and AB7329 (5'GACGAGCCTGTAGCCCACGTCCTAGCA) which primed PCR replication leftward and rightward outside the deletion respectively (AB7329 contained the point mutation K11E), and Vent polymerase (New England Biolabs, Mississauga, Ontario, Canada). The mutated linear product was self ligated to generate the plasmid p Δ 1-9K11E, then cut with *Xba*I (Boehringer Mannheim, Laval, Quebec, Canada) to isolate the altered cDNA, which was inserted into pMH4 (as above) to yield pmTNF-MEM. Sequence analysis confirmed the presence of the deletion/mutation and the absence of any undesired PCR related mutations. These plasmids were later cotransfected with pBHG10 using the calcium phosphate method to obtain the desired Ad viral vectors, Ad-mTNF-wt and Ad-mTNF-MEM, containing the wild-type and mutant mTNF α cDNAs, respectively.⁴⁰ Adenoviral vectors were isolated and propagated in 293 cells as previously described.⁴⁰ Ad-d170-3 which has a deletion in E1 and a deletion/substitution in E3, was used as a negative control vector.⁴¹ All viruses used were purified by banding in CsCl gradients.⁴⁰

TNF α bioassay

The bioassay used to quantify secreted TNF activity measured direct killing of a sensitive cell line (A673/6). Cells were dispensed into 96-well plates (2.5×10^4 cells in 50 μ l medium per well) and incubated overnight at 37°C (approximately 20 h). The next day the medium was replaced with 40 μ l of cell culture medium plus soybean trypsin inhibitor (SBTI) (100 μ g/ml) and cycloheximide (20 μ g/ml). Ten microlitres of the standards or samples were added to the microtitre wells and incubated at 37°C overnight (approximately 18 h). Afterwards, 10 μ l of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added to each well, and the plates were incubated for 4 h, after which 50 μ l of 50% dimethyl formamide (20% SDS, pH 4.7) was added. Following an additional overnight incubation, the OD₅₇₀ was measured, and the TNF α concentration calculated with reference to the OD₅₇₀ obtained from serial dilutions of a standard TNF α recombinant stock solution. Cycloheximide, SBTI, MTT and the recombinant murine TNF α used as the standard were purchased from Sigma (St Louis, MO, USA).

FACS analysis

MT1A2 cells were transduced at an MOI of 50 p.f.u. per cell with Ad-mTNF-wt, Ad-mTNF-MEM, Ad-d170-3 (E1/E3-deleted control virus) or uninfected (mock). Twenty-four hours after infection the cells were harvested and resuspended in MEM supplemented with 20 mM Hepes for FACS analysis. Cells were stained with rat anti-TNF α monoclonal antibody purchased from Immunocorp Science (Montreal, Quebec, Canada), followed by FITC-anti-rat-IgG (Southern Biotechnology Associates, Birmingham, AL, USA). Viable cells were gated for analysis by exclusion of propidium iodide (PI)-positive (dead) cells.

Tumour studies

Transgenic mice carrying the PyMidT oncogene under the mouse mammary tumour virus promoter/enhancer

(MMTV) were generated by Guy *et al.*¹⁹ Breast tumours from transgenic females were explanted, and mechanically homogenized in collagenase (0.025% w/v)/dispase (0.25% w/v) (Boehringer Mannheim, Laval, Quebec, Canada). Tumour cells were then cultured in MEM F11 plus 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine, and 2.5 μ g/ml Fungizone for 24–48 h at 37°C (until confluent). The cultures were trypsinized (0.1% trypsin, 1.06 mM EDTA; Sigma), washed and resuspended in phosphate-buffered saline (PBS), then injected subcutaneously (1×10^6 cells in 200 μ l PBS per animal) into the right hind flanks of syngeneic FVB mice (Charles River, Canada). Reasonably large tumours (approximately 96 mm²) developed by 18–21 days after injection. These tumours were then directly injected with various doses of Ad vectors in 50 μ l PBS and tumour progression was then monitored through routine measurements of tumour size with callipers. Tumour volume was calculated from the longest diameter and average width assuming a prolate spheroid. For secondary challenges transgenic tumour cells were prepared and injected (as described above) in the left hind flanks of mice 2 months after primary tumour regression.

mTNF α ELISA

Blood from killed mice was collected on days 1 and 3 after Ad vector injection (some Ad-mTNF-wt mice were killed on day 2 due to sickness), and the serum was snap-frozen in liquid nitrogen, then stored at -70°C until analysed by ELISA. Tumours were removed from mice that had been killed, snap-frozen in liquid nitrogen, homogenized in 0.5 ml PBS (100 μ M phenylmethylsulfonyl fluoride and 10 μ g/ml aprotinin), and sonicated twice for 10 s. The homogenate was cleared by centrifugation (5 min in microfuge at room temperature) and stored at -70°C for later assay by ELISA. An ELISA kit specific for murine TNF α (Biotrak; Amersham, Bucks, UK) was used to quantify TNF α levels. The assay was carried out according to the manufacturer's directions using streptavidin-conjugated HRP to detect specifically mTNF α coupled with biotinylated anti-TNF α antibodies.

Tumour morphology

Tumours were removed from treated mice, cut in two with a razor and fixed in 10% NBF for 18–24 h. After fixation, the tissue was immersed once in 50% ethanol, then 60% ethanol for 30 min periods each, then stored in 70% ethanol. NBF fixed tumours were paraffin-embedded, sectioned and stained with haematoxylin and eosin.

PyMidT-specific CTL assays

Spleens were removed from immunized mice and minced into single cell suspensions by passage through a metal mesh in PBS plus 1% FBS. Splenocytes were resuspended in 0.83% NH₄Cl for 5 min on ice, after which they were washed and again resuspended in PBS plus serum. PyMidT expressing MCT3 cells were exposed to 50 Gy γ -radiation then cocultured with splenocytes (1.2×10^7 splenocytes: 1.2×10^5 stimulators) in RPMI-1640 (10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine, 20 mM Hepes, and 100 μ M β -mercaptoethanol) for 5–6 days. Stimulated spleen cells were incubated for 6 h with ⁵¹Cr-labelled MCT3 target cells or nonspecific control IT₁₆₃₉ target cells (100 μ Ci/ 10^6 cells) at ratios of 90:1, 30:1, 10:1 and 3.3:1 (effector:target)

in V-bottomed microtiter plates at 37°C. As an additional negative control, anti-CD3 antibody-treated wells (hamster anti-mouse CD3 (145-2C11) ascites at a 1:8 dilution, 10 μ l per well) were also used. The percentage specific chromium release was then determined:

$$\frac{(\text{Experimental release} - \text{spontaneous release}) \times 100}{(\text{Maximum release} - \text{spontaneous release})}$$

Spontaneous and maximum release were determined by incubating target cells with medium alone or with 1 N HCl, respectively.

Acknowledgements

We would like to thank Dr Graeme Dougherty from the Terry Fox Laboratory, Vancouver, British Columbia for his donation of the murine TNF α cDNA. We would also like to thank Jonathan Bramson and Mary Hitt for their help and suggestions. This work was supported by grants from the National Cancer Institute of Canada (NCIC), the Medical Research Council of Canada (MRC), the Canadian Breast Cancer Initiative, and London Life Insurance. FLG is a Terry Fox Research Scientist of the NCIC and WJM is an MRC Scientist. All PCR primer synthesis and DNA sequencing was done at the Mobix Central Facility, McMaster University, Hamilton, Ontario.

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Summary

Expression / activity of membrane bound murine TNF α mutant

Expression of murine TNF α was detected in the cell culture supernatant of cells transduced with Ad-mTNF-wt (Ad vector constructed to express wild type murine TNF α). Levels up to 0.4 μ g/mL were detected after two days post infection. No secreted TNF α activity was detected from cells transduced with Ad-mTNF-MEM (Ad vector expressing the membrane bound mutant of murine TNF α). However the expression of murine TNF α was detected on the surface of cells transduced with both Ad vectors by FACS analysis. Murine TNF α was detected in the serum of mice intratumourally injected with Ad-mTNF-wt only, while murine TNF α was detected in tumours injected with either vectors.

Reduced toxicity of membrane bound mutant expressed from Ad vector

The Ad-mTNF-wt vector proved to be highly toxic to treated mice, killing 8 of 8 mice at a dose of 1×10^9 pfu, and 12 of 16 mice at a dose of 5×10^8 pfu. Even at a dose as low as 5×10^7 pfu this vector killed 2 of 7 treated animals. However, the Ad-mTNF-MEM vector killed only 2 of 13 mice at a dose of 1×10^9 pfu, and 3 of 27 mice at 5×10^8 pfu. No toxicity was observed at 1×10^8 pfu. This demonstrated a marked reduction in lethality by the use of a membrane bound mutant.

Antitumour activity of membrane bound TNF α

Within 24 hours post vector injection a high degree of intratumoural necrosis was

observed from tumours treated with both murine TNF α vectors. The Ad-mTNF-wt vector cured 2 of 4 mice at the dose of 5×10^8 pfu, and 2 of 8 mice at the dose of 1×10^8 pfu, while the Ad-mTNF-MEM vector cured 2 of 11 mice at a dose of 1×10^9 pfu and 1 of 24 mice at 5×10^8 pfu.

Immune memory and CTL activity

All mice cured were resistant to challenge with a second injection of PyMidT tumour cells. One mouse cured by the Ad-mTNF-MEM vector was tested and shown to have PyMidT specific CTL activity.

CHAPTER IV: Tumour Immunotherapy in Mice using Adenovirus Vectors

Expressing Human TNF α

Introduction

Human tumour necrosis factor alpha is species specific. In the murine system human TNF α does not recognize the murine p75 TNF receptor, while it binds normally to the p55 TNF receptor (Lewis et al., 1991; Ranges et al., 1989). It has also been shown that human TNF α is much less toxic to mice compared to the murine form (Brouckaert et al., 1989). This led us to the hypothesis that targeting of the TNF receptors could result in reduced systemic toxicity while retaining antitumour activity. Since human TNF α was p55 specific and showed reduced toxicity, two Ad vectors were constructed expressing human TNF α . Both vectors expressed the mature form of human TNF α secreted under the interferon gamma signal peptide. The Ad-HCMV-TNF vector utilized the human cytomegalovirus virus immediate early promoter (HCMV) to drive expression of the transgene, and the Ad-MCMV-TNF vector utilized the murine cytomegalovirus immediate early promoter (MCMV) to drive expression. Both these vectors were tested in tumour immunotherapy experiments (as in Chapter III), to determine the lethality and antitumour properties. Also, direct comparisons of the efficiencies of the two promoters were made.

The second author of this manuscript, Dr. Mary Hitt, generated the Ad-HCMV-TNF vector (see fig. A2-1), and also provided useful insight into the work which generated this manuscript. Dr. William Muller provided the PyMidT mouse mammary tumour model.

Dr. Jack Gauldie provided access to the pathology laboratory run by Brian Hewlett. Dr. Frank Graham supervised and provided direction (as well as lab space, reagents, and a pay check) for all of the work done for this manuscript. The first author Robert Marr performed all the remaining work which lead to the completion of this manuscript including the design and performance of the experiments. Both Dr. Graham and Dr. Hitt also contributed by proof reading the manuscript before its submission.

Tumour therapy in mice using adenovirus vectors expressing human TNF α

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Received December 17, 1997; Accepted January 9, 1998

Abstract. Induction of systemic toxicity is a major factor limiting the use of TNF α in tumour therapy. The local expression of TNF α from Ad vector infected tumour cells, might result in reduced systemic toxicity and induce an enhanced local antitumour response. Two adenoviral vectors expressing human TNF α were constructed for use in tumour immunotherapy, one utilizing the HCMV promoter (Ad-HCMV-TNF) and the second utilizing the MCMV promoter (Ad-MCMV-TNF). Both vectors induced the secretion of hTNF α from transduced cells *in vitro*, however the MCMV promoter directed stronger expression in murine cells. Expression from the two vectors was also kinetically different with the MCMV promoter inducing earlier expression, from murine tumour cells *in vitro* and *in vivo*. Both vectors induced intratumoural necrosis, however only the Ad-MCMV-TNF vector induced systemic toxicity and significant antitumour activity when directly injected into tumours, killing 8 of 20 mice and inducing partial (2 of 12) and permanent (1 of 12) tumour regressions at a dose of 5×10^8 pfu/mouse. These data indicate that hTNF α expressed from an Ad vector is considerably toxic to mice while inducing a moderate antitumour response.

Introduction

Tumour necrosis factor α (TNF α) is a multipotent cytokine with a variety of physiological effects including antitumour activity (1,2). Mature (secreted) TNF α is a 17 kD polypeptide which functions as a homotrimer that binds to two cell surface receptors (p55 and p75). Both these receptors have homologous extracellular domains, but share little homology intracellularly, and signal through distinct signal transduction pathways (3-5). The p55 TNF receptor is primarily responsible for signalling a variety of responses including cytotoxicity (1,6), and cytokine

secretion (7,8), while the p75 TNF receptor is primarily responsible for lymphoproliferative signals (6).

Perhaps the greatest problem limiting the use of TNF α in tumour therapy is its induction of systemic toxicity: TNF α is a key mediator of septic shock and cachexia (8-10). Human TNF α binds the murine p55 TNF receptor but does not bind the murine p75 TNF receptor (4,11,12). It has also been demonstrated that hTNF α is 50 times less toxic to mice when compared to the murine form which binds both receptors (1). Thus use of the less toxic hTNF α cytokine to treat murine tumours could be a useful model in which to evaluate the possibility of using p55 specific agonists to reduce toxicity in the human system. Furthermore, the expression of cytokines from adenoviral vectors could allow further reduced systemic toxicity and enhanced antitumour activity, by inducing continuous TNF α expression from infected tumour cells, resulting in high TNF α levels locally within the tumour. Here we describe the construction of two Ad vectors expressing human TNF α using the human cytomegalovirus (HCMV) or the murine cytomegalovirus (MCMV) promoters to drive expression and assess the efficacy of using these vectors for tumour therapy in a polyoma middle-T transgenic breast cancer model.

Materials and methods

Cell culture. Cell culture media and reagents were purchased from Gibco (Mississauga, Ontario, Canada) with the exception of Fungizone[®] purchased from Squibb (Montreal, Quebec, Canada). Human fibroblast MRC5 (ATCC CCL-171) cells were cultured in minimal essential medium (α -MEM), murine melanoma B16BL6 cells (13) were cultured in F-15 medium. E1 complementing human embryonic kidney 293 cells (14), MT1A2 (15) cells (cell line derived from polyoma middle-T expressing transgenic mammary tumour cells), and primary tumour cells from the same line of transgenic polyoma middle-T (PyMidT) mice (16) were cultured in MEM-F11. The TNF α sensitive human cell line A673/6 was cultured in α -MEM supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin and 5% fetal bovine serum (FBS). All other cell culture media were supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2.5 μ g/ml Fungizone[®], and 10% FBS.

Vector construction. Ad vectors were constructed by cotransfection of 293 cells using the calcium phosphate cotransfection

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Key words: adenovirus, gene therapy, immunotherapy, tumour therapy, human tumour necrosis factor α , HCMV promoter, MCMV promoter

technique with the Ad-genomic plasmid pBHG10 (17) plus the appropriate E1 shuttle plasmid containing expression cassettes (illustrated in Fig. 1A). Ad-HCMV-TNF (Fig. 1B) was constructed by cotransfection with the shuttle plasmid pCMV-TNF containing the mature human TNF α cDNA fused to a sequence encoding the INF γ signal peptide under the control of the human cytomegalovirus immediate early (HCMV) promoter (15). Ad-MCMV-TNF was constructed by cotransfection with pBM4-TNF (generated by insertion of the hTNF expression cassette from pCMV-TNF into pMH4) containing the murine cytomegalovirus immediate early (MCMV) promoter (18). The hTNF α cDNA (fused with INF γ signal peptide) was obtained from Dr Patrick Hwu (NCI, Bethesda, MD, USA). Adenoviral vectors were isolated, propagated in 293 cells, and purified using CsCl gradients as previously described (19).

Tumour studies. Transgenic mice carrying the polyoma middle-T oncogene expressed from the mouse mammary tumour virus promoter/enhancer were generated by Guy *et al.* (16). Breast tumours from transgenic females were explanted, and homogenized in collagenase (0.025% w/v) and dispase (0.25% w/v) (Boehringer Mannheim, Laval, Quebec, Canada). Tumour cells were then cultured in MEM-F11 plus 10% fetal bovine serum, 1% pen/strep, 1% L-glutamine, and 1% Fungizone[®] for 24–48 h at 37°C (until confluent). The cells were then trypsinized (0.1% trypsin in 1.06 mM EDTA, Sigma, St. Louis, MO, USA), washed and resuspended in phosphate buffered saline (PBS), after which they were injected into syngeneic FVB mice subcutaneously (1×10^6 cells in 200 μ l of PBS) on the right hind flank. Sizable tumours (96 mm³) developed in 18–21 days post injection. These tumours were then directly injected with various doses of Ad vectors in 50 μ l of PBS. Tumour progression was monitored through routine measurements with callipers.

Preparation of samples for expression assays. *In vitro* expression of TNF α was analysed following infection of 60 mm dishes of MRC5, B16BL6, MT1A2, or primary PyMidT cells at a multiplicity of infection (moi) of 50-plaque forming units (pfu) per cell, sampling cell supernatants at various time points post infection, and storing samples at -70°C until the time of assay. *In vivo* expression was determined following direct injection of PyMidT tumours with 5×10^8 pfu of Ad vector, after which blood from sacrificed mice was collected on days 1, 3 and 7 post injection, and the serum was snap frozen in liquid nitrogen then stored at -70°C until analyzed by ELISA.

TNF α bioassay. The bioassay used to quantitate secreted TNF activity measured direct killing of a TNF sensitive cell line (A673/6). Cells were dispensed into 96-well plates (2.5×10^4 cells in 50 μ l per well), and incubated overnight at 37°C (=20 h). The next day the media was aspirated and replaced with 40 μ l of media plus soyabean trypsin inhibitor (SBTI) (100 μ g/ml) and cycloheximide (Sigma) (20 μ g/ml). Ten μ l of the standards or samples were added to the microtiter wells and incubated at 37°C overnight (=18 h), after which 10 μ l of 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were added to each well and

incubated for 4 h. Finally 50 μ l of 50% dimethylformamide 20% SDS, pH 4.7, were added and following an additional overnight incubation, the OD₅₇₀ was measured, and the TNF α concentration calculated with reference to the OD₅₇₀ obtained from serial dilutions of a standard TNF α recombinant stock solution. Cycloheximide, SBTI, and MTT were purchased from Sigma, St. Louis, MO, USA. Recombinant human TNF α used as the standard was purchased from R&D Systems, Minneapolis, MN, USA.

hTNF α ELISA. An ELISA kit specific for human TNF α (Biotrak; Amersham, Bucks, UK) was used to quantify TNF α levels. The assay was carried out according to the manufacturer's directions using streptavidin-conjugated HRP to specifically detect hTNF α coupled with biotinylated anti-TNF α antibodies.

Tumour morphology. Tumours were removed from treated mice, cut in two with a razor and fixed in 10% neutral buffered formalin for 18–24 h. After fixation the tissue was immersed in 50% ethanol, then 60% ethanol, for 30 min periods until finally being stored in 70% ethanol. Tumours were then paraffin embedded, sectioned and stained with haematoxylin and eosin.

Results

***In vitro* and *in vivo* expression from Ad-TNF vectors.** Two adenovirus type 5 vectors were constructed with hTNF α expression cassettes transcribing rightwards within the E1 region of an E1/E3 deleted vector (Fig. 1). Both vectors expressed the mature 17 kD form of human TNF α fused with the INF γ signal peptide. Ad-HCMV-TNF utilized the HCMV promoter to drive expression of the transgene. Recent data from our laboratory has shown that the MCMV promoter directs higher levels of expression than the HCMV promoter particularly in murine cells (15), therefore the second vector (Ad-MCMV-TNF) was constructed utilizing the MCMV promoter.

Both vectors directed high levels of expression in transduced human MRC5 cells and relatively low levels of expression from murine B16BL6 cells as detected by ELISA and bioassay (Fig. 2). The Ad-HCMV-TNF vector induced expression levels up to about 3 μ g per 10^6 cells, while the Ad-MCMV-TNF vector directed expression levels up to about 4 μ g per 10^6 cells (assayed by ELISA) in transduced MRC5 cells (Fig. 2A). However, the difference in expression was much greater in transduced murine B16BL6 cells, with the HCMV vector producing levels of TNF α up to only 4 ng per 10^6 , while the MCMV vector directed levels of expression up to 25 ng per 10^6 (assayed by ELISA) (Fig. 2B). Similar trends of expression were also detected by bioassay based on cytotoxicity on a TNF α sensitive cell line (A673/6) (Fig. 2C and D). Expression levels in injected primary PyMidT transgenic mammary tumour cells and the MT1A2 mammary tumour cell line, derived from the same transgenic mouse model, were also assayed by bioassay (Fig. 2E). The HCMV vector induced expression levels of up to 0.5 and 1.25 μ g per 10^6 cells from transduced MT1A2 and primary PyMidT cells respectively, whereas the MCMV vector directed expression

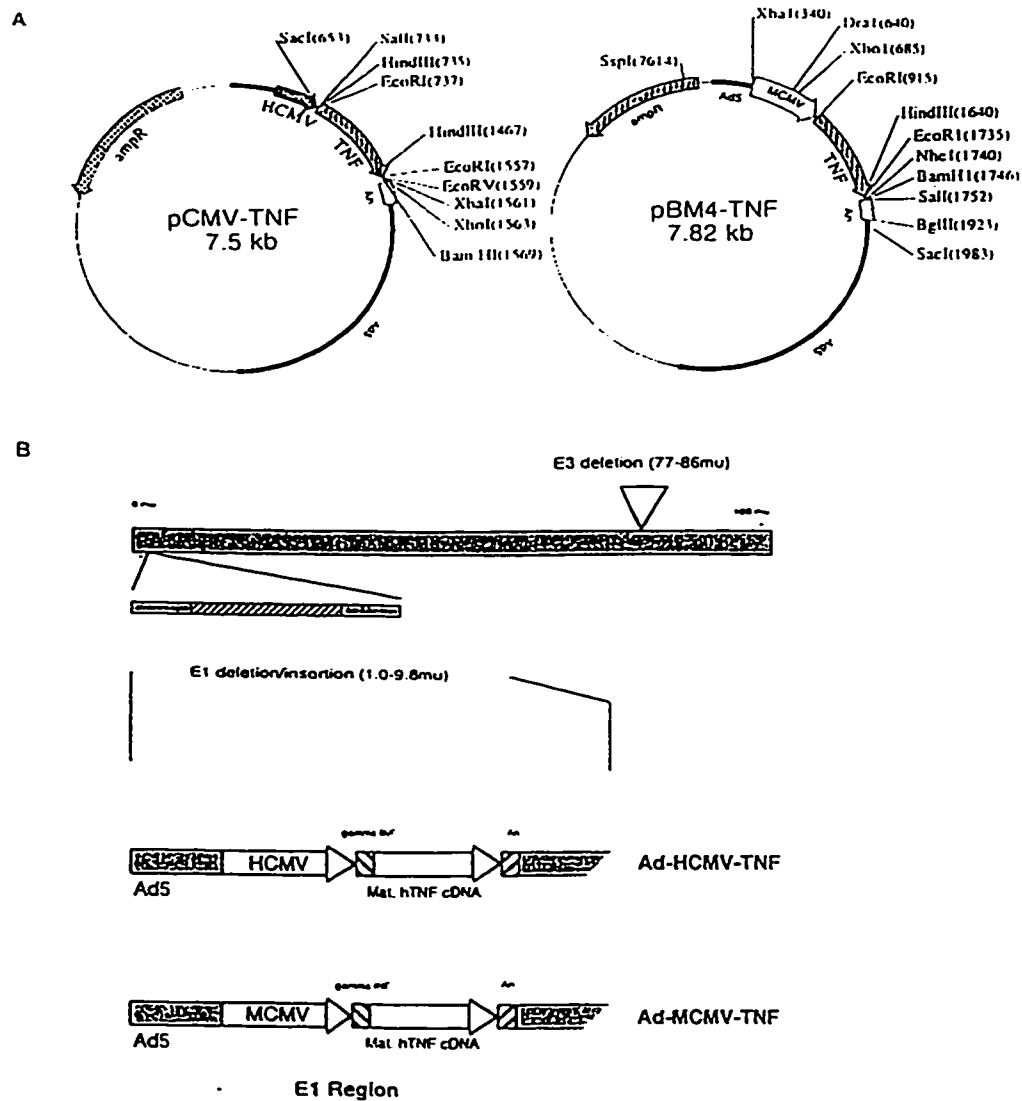


Figure 1. Generation of Ad vectors encoding hTNF α . A, Maps of shuttle plasmids. The shuttle plasmids contain Ad sequences +22 to 341 and +3524 to 5790 (indicated by solid bar). Cotransfection of 293 cells with pBHG10 and either pCMV-TNF or pBM4-TNF generated the vectors Ad-HCMV-TNF and Ad-MCMV-TNF respectively. B, Ad vectors expressing human TNF α . Both vectors express the mature (secreted) TNF α cDNA fused with the TNF α signal peptide and flanked by the SV40 polyadenylation signal. Expression of the transgene is driven by the human cytomegalovirus IE promoter (HCMV) in the Ad-HCMV-TNF vector while the murine cytomegalovirus IE promoter (MCMV) is used in the Ad-MCMV-TNF vector.

levels up to about 1.2 and 1.5 μg per 10^6 cells respectively. Furthermore, the kinetics of expression in murine tumour cells differed between the two vectors, with expression progressively increasing to maximum levels over three days in Ad-HCMV-TNF transduced cells, and peak levels being expressed as soon as 24 h in Ad-MCMV-TNF transduced cells (Fig. 2E). No TNF α was detected from mock infected cells (not shown).

Serum levels of human TNF α were assayed from mice intratumourally injected with 5×10^8 plaque forming units (pfu) of Ad-TNF vectors. Similar serum levels were detected in mice directly injected with either vector (Fig. 3) (three days post injection) as detected by a hTNF α specific ELISA.

However, the Ad-MCMV-TNF vector directed maximum expression levels 24 h post injection in contrast to the Ad-HCMV-TNF vector which showed minimal expression levels at that time. In addition, only background levels of expression were detected at day 7 (not shown). No hTNF α was detected from mice treated with a control 'empty' vector (Ad-d170-3) (not shown). These data are consistent with the *in vitro* expression kinetics (above).

Tumour morphology. To assess the effect of the Ad-hTNF vectors on tumour morphology, mice bearing PyMidT tumours were injected intratumourally with 5×10^8 pfu of either Ad-d170-3 (control), Ad-HCMV-TNF, or Ad-MCMV-TNF

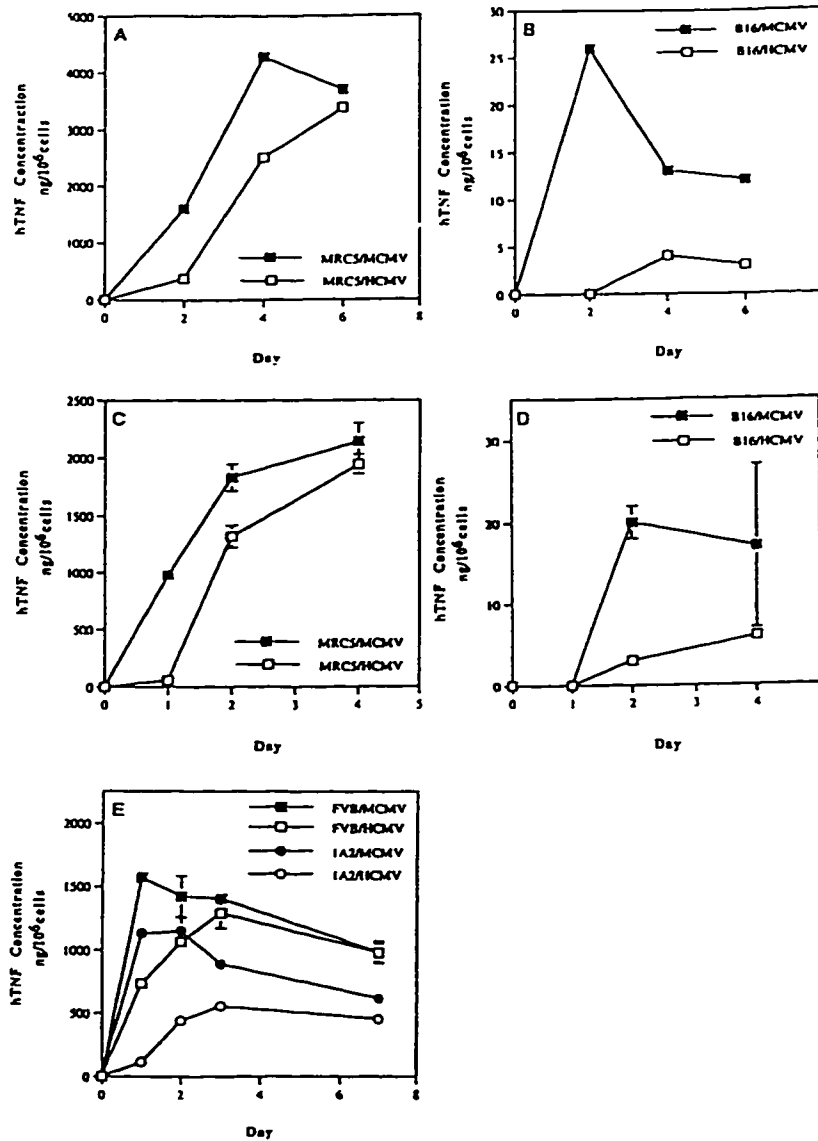


Figure 2. *In vitro* expression of human TNF α . Human MRC5 cells (A, C), murine B16BL6 cells (B16) (B, D), and murine MT1A2 (IA2) and primary murine PyMidT (FVB) cells (E), were transduced at a moi of 50 pfu per cell, with either Ad-HCMV-TNF (open symbols) or Ad-MCMV-TNF (closed symbols) and sampled at various time points. Sampled cell culture supernatants were assayed by hTNF α specific ELISA (A, B) or by a bioassay utilizing a TNF α sensitive cell line (A673/6) (C-E). Error bars represent standard deviation (ELISA data not done in duplicate).

Afterwards tumours were removed from sacrificed mice on days 1, 3, and 7 post injection, and fixed in 10% NBF for histological analysis (Fig. 4). No disruption of tumour morphology was observed in control virus treated tumours (Fig. 4A and B) compared to PBS treated tumours (not shown). Ad-HCMV-TNF treated tumours did not display any significant disruption of tumour pathology on day one (Fig. 4C), but necrosis was visible three days post injection, marked by large disrupted areas of the tumour containing cells undergoing pycnosis (Fig. 4D). In contrast Ad-MCMV-TNF treated tumours showed high amounts of disruption on both days (Fig. 4E and F). No significant disruption of tumour pathology was

visible in tumours by 7 days after treatment with any vector (not shown). This trend in the induction of necrosis was also consistent with the expression data described above, and indicated a marked difference in the kinetics of expression between the HCMV and the MCMV promoters both in cell culture and *in vivo*. In addition it is demonstrated that hTNF α expressed from Ad vectors can induce substantial intratumoural necrosis, even at doses as low as 2×10^7 pfu per mouse (not shown).

Effect of Ad-TNF vectors on tumour progression. Mice bearing PyMidT tumours on the right hind flank were directly injected

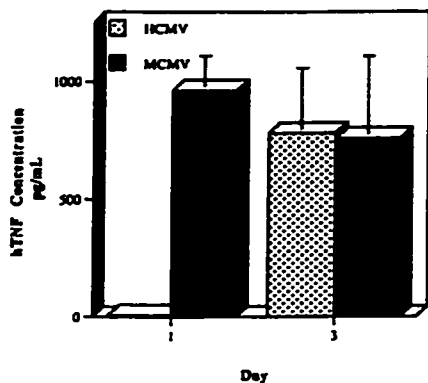


Figure 3. *In vivo* expression of human TNF α . Mice (n=3) were intratumorally injected with 5×10^8 pfu of Ad-HCMV-TNF (HCMV), or Ad-MCMV-TNF (MCMV), then sacrificed at 1 and 3 days post injection. Sera was collected from sacrificed mice and assayed for hTNF α by ELISA (see Materials and methods). Error bars represent standard error.

with various doses of Ad-HCMV-TNF, Ad-MCMV-TNF, or Ad-dl70-3 (control) (Table I). The HCMV vector did not show any toxicity at a dose of 5×10^8 pfu, but also did not induce any significant antitumour response manifested as either partial (tumour regresses to half its original size or less) or complete tumour regressions. In contrast the MCMV vector proved to be quite toxic killing 8 of 20 mice within 1-3 days post injection, at a dose of 5×10^8 pfu. Doses of 1

Table I. Antitumour response and toxicity induced by hTNF expressing Ad vectors.

Vector	Dose (pfu)	Mortality ^a	Tumour response		
			No resp.	Partial resp. ^b	Complete regression ^c
Ad-HCMV-TNF	$5E+08$	0/13	13/13	0/13	0/13
	$2E+08$	0/5	5/5	0/5	0/5
Ad-MCMV-TNF	$2E+09$	4/4	-	-	-
	$1E+09$	4/4	-	-	-
	$5E+08$	8/20	9/12	2/12	1/12
	$1E+08$	0/8	4/8	4/8	0/8
	$2E+07$	0/3	3/3	0/3	0/3
Ad-dl70-3	$5E+08$	0/24	24/24	0/24	0/24

^aMortality represents the proportion of mice which died as a result of systemic toxicity, typically within 1-3 days post injection; ^bPartial response refers to the proportion of survivors displaying tumours which regressed to less than half their original size before starting to grow again; ^cComplete regression refers to the proportion of survivors displaying complete and permanent disappearance of the tumour.

and 5×10^8 pfu of Ad-MCMV-TNF produced small numbers of partial regressions (4/8 and 2/12 respectively) or complete tumour regressions (1/12 at 5×10^8 pfu).

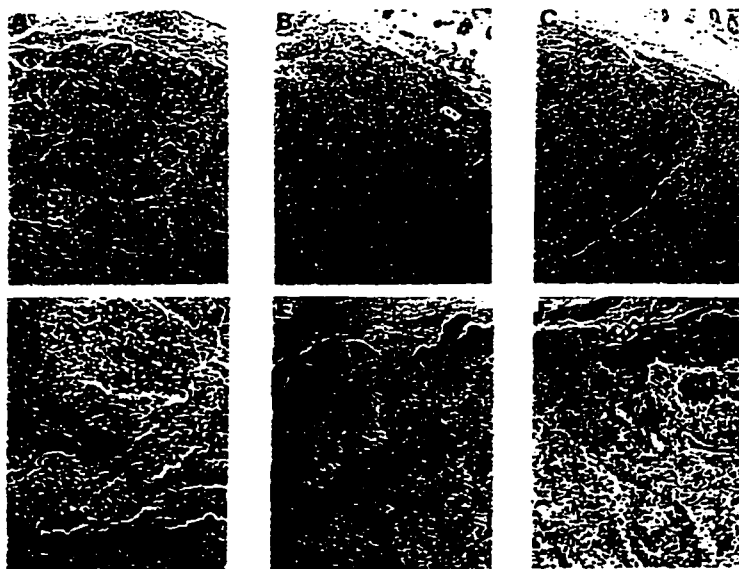


Figure 4. Tumour morphology of mice intratumorally injected with 5×10^8 pfu of Ad-dl70-3 (A, B), Ad-HCMV-TNF (C, D) and Ad-MCMV-TNF (E, F). Tumours were removed from sacrificed mice on days one (A, C, E) and three (B, D, F) post injection, fixed in 10% NBF, paraffin embedded, thin sectioned, and stained with haematoxylin and eosin.

Discussion

Previously the HCMV promoter was shown to induce highly efficient expression from Ad vector transduced cells (20). Our data supports this conclusion, however our results obtained from comparison of vectors containing HCMV and MCMV promoters were also consistent with those of Addison *et al.* (15), demonstrating that the MCMV promoter is more potent both *in vitro* and *in vivo* than the HCMV vector, particularly in murine systems. *In vivo* both Ad-TNF vectors described show the ability to induce intratumoural necrosis, however the vector possessing the MCMV (Ad-MCMV-TNF) promoter proved to be much more toxic than the HCMV vector (Ad-HCMV-TNF). In addition only the MCMV vector showed significant antitumour activity when directly injected into PyMidT tumours. This increased activity could be a result of the rapid onset of expression observed by the MCMV vector compared to the HCMV vector. Continuous administration of low doses of hTNF α has been shown to induce tolerance to the effects of the cytokine (1), also it has been established *in vivo* that TNF α can induce the secretion of soluble TNF binding proteins (soluble TNF receptor shedding) into the circulation (2,21). In addition, the pre-treatment of cells with TNF α can induce the expression of protective proteins. Perhaps the slower kinetics elicited by the Ad-HCMV-TNF vector allowed for time for such a down regulatory response to occur thus reducing the systemic toxicity and antitumour response induced by that vector.

The antitumour activity of TNF α can manifest itself in three different ways. The first is through direct cytotoxicity to tumour cells, by inducing apoptosis through engagement of the p55 TNF receptor (1,6). However most normal and transformed cells are resistant to the direct cytotoxic effects of TNF α (2,22). The second mechanism by which TNF induces tumour regression is through immune activation. TNF α is a key mediator of inflammation, and can activate neutrophils, macrophages and NK cells, as well as induce the production of other immune factors such as IL-6, IL-1, GM-CSF, IL-8 and adhesion molecules (8,23). The antitumour activity of TNF α has been reported to be due in part to T cell activation and has the ability to induce tumour specific immunity (24-26). Considering the rapid nature of the induction of necrosis (24 h) in our experiments, it is unlikely that the activation of T cells is involved, although the activation of other immune cells (neutrophils, M ϕ , NK cells) may be involved. The third mechanism by which TNF mediates tumour regression is through its effects on tumour vasculature. It has been shown that TNF α can promote intravascular thrombosis within tumours, inducing ischemic necrosis (1,27,28). Tumour vasculature is uniquely susceptible to TNF α induced coagulation, as the quality of tumour vessels is typically very poor, being low in nutrients and high in catabolites (2,6), due to the disorganized/abnormal development of tumour vessels. This is the most likely mechanism by which rapid intratumoural necrosis is induced.

Mutants of hTNF α have been generated with specificity for only the p55 TNF receptor (29,30). A p55 receptor specific form of human TNF α was found to induce fibrinolysis, coagulation, and neutrophil degranulation in treated baboons at levels comparable to the wild-type protein (31). It is

conceivable that such a mutant could be used for tumour therapy in humans. As a model for tumour therapy, however, expression of hTNF α from Ad vectors shows little promise due to the high levels of toxicity and low frequency of tumour regression in response to this cytokine. However, Ad-hTNF vectors may provide a useful murine model system for the further investigation of the use of p55 TNF receptor specific agonists for treatment of human cancers.

Acknowledgements

We would like to thank Brian Hewlett and Donna Crosshwaite for their help with the tumour morphology. This work was supported by grants from the National Cancer Institute of Canada (NCIC), the Medical Research Council of Canada (MRC), the Canadian Breast Cancer Initiative, and London Life Insurance. F.L.G. is a Terry Fox Research Scientist of the NCIC and W.J.M. is an MRC Scientist.

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Summary

MCMV promoter is more efficient than HCMV promoter

In vitro both vectors produced similar levels of expression in human cells, while the MCMV vector seemed to be more effective in murine cells. Expression from transduced primary PyMidT cells, and MT1A2 cells showed that the MCMV promoter directed high levels of expression sooner than the HCMV promoter (24 hrs and 72 hrs for maximal expression respectively). This trend was also reflected in the serum levels of human TNF α detected from treated mice, and in the induction of intratumoural necrosis by these vectors. The Ad-MCMV-TNF vector cured 1 of 12 mice and produced partial regressions in 2 of 12 mice at a dose of 5×10^8 pfu, while the Ad-HCMV-TNF vector produced no partial or permanent tumour regressions.

Human TNF α is highly toxic to mice

The Ad-MCMV-TNF vector was also found to be highly toxic to treated mice, killing 4 of 4 mice at a dose of 1×10^9 pfu and 8 of 20 mice at 5×10^8 pfu. The Ad-HCMV-TNF vector did not show any lethality to treated mice. This demonstrates that the MCMV promoter may be better suited to gene therapy in mice. Furthermore, it is clear that the targeting of the p55 TNF receptor results in high levels of systemic toxicity and little antitumour efficacy.

CHAPTER V: A p75 TNF Receptor Specific Mutant of Murine TNF α Expressed from an Adenovirus Vector Induces an Antitumour Response with Reduced Toxicity

Introduction

The TNF receptors are highly homologous in their extracellular domains, but differ considerably intracellularly. The receptors are believed to signal via distinct signal transduction pathways and have separate functions. The p55 TNF receptor is a major mediator of cytotoxicity, and cytokine secretion. The p75 TNF receptor is primarily responsible for proliferative and proinflammatory signals (reviewed in (Sarraf, 1994)). As mentioned above, human TNF α is much less toxic to mice compared to murine TNF α . Considering the species specificity of human TNF α , many had thought the p75 TNF receptor was primarily responsible for inducing systemic toxicity. However, it has now become clear that it is the p55 TNF receptor that is responsible for induction of systemic toxicity, while the p75 TNF receptor plays a potentiating role (Bluethmann et al., 1994; Erickson et al., 1994). Thus, targeting the p75 TNF receptor could result in further reduced toxicity while retaining the ability to activate an antitumour immune response. Many mutants of human TNF α have been characterized with specificity for either the p55 or p75 TNF receptors. However, none had been identified for the murine form. Human and murine TNF α are highly homologous, particularly in the region which recognizes the p55 TNF receptor. Therefore, we constructed an Ad vector expressing a double point mutant of murine TNF α (D142N-A144R) which was homologous to the most p75 TNF receptor

specific form of human TNF α (D143N-A145R) (Loetscher et al., 1993). The product of this vector (Ad-mTNF-75) was tested for its specificity for the p75 TNF receptor, and its performance in tumour gene therapy experiments was also tested.

The second author of this manuscript, Dr. Mary Hitt, originally generated the NDL cell line (see appendix table A4-1), and also provided useful insight into the work which generated this manuscript. Dr. William Muller provided the PyMidT mouse mammary tumour model. Dr. Jack Gaudie provided access to the pathology laboratory run by Brian Hewlett. Dr. Frank Graham supervised and provided direction (as well as lab space, reagents, and a pay cheque) for all of the work done for this manuscript. The first author Robert Marr performed all the remaining work which lead to the completion of this manuscript including the design and performance of the experiments. Both Dr. Graham and Dr. Hitt also contributed by proof reading the manuscript before its submission.

Revised

A p75 TNF Receptor Specific Mutant of Murine TNF α Expressed from an Adenovirus Vector Induces an Antitumour Response with Reduced Toxicity

Running Title:

"Tumour therapy using a mutant p75TNFR specific mTNF α "

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Key Words: Adenovirus, Gene Therapy, Immunotherapy, Tumour Therapy, TNF α , TNF receptor.

Abstract

The toxic effects of TNF α have greatly limited its use in tumour therapy. Recently, clear evidence has been obtained linking the p55 TNF receptor to the induction of systemic toxicity. We have generated a p75 murine TNF receptor (mTNFR) specific mutant of murine TNF α (D142N-A144R), cloned this gene into a recombinant adenovirus vector (Ad-75), and studied its efficacy for tumour immunotherapy of a murine transgenic breast cancer model.

Cell culture supernatants from Ad-75 transduced cells showed no cytotoxic activity on L929 cells, but retained the ability to induce proliferation of a murine T cell line (CT6), and this activity was not blocked by soluble p55 mTNFR. Furthermore it was shown that the mutant form of mTNF α was able to co-immunoprecipitate only with the p75 mTNFR, and not with the p55 mTNFR. Tumours injected with Ad-75 became necrotic and mice injected with up to 1×10^9 pfu showed no mortality, while both wild type murine and human TNF vectors induced lethality at doses of 1 and 5×10^8 pfu. All Ad-TNF vectors induced partial or permanent tumour regressions, with cured mice showing immune memory against the tumour. These results demonstrate that a p75 mTNFR receptor agonist expressed from a recombinant adenovirus vector does not induce mortality at doses which cause tumour regression.

Introduction

Tumour necrosis factor alpha (TNF α) was first identified through the detection of

antitumour activity in the sera of mice treated with endotoxin (1). Mature TNF α is a 17kD polypeptide which recognizes two cell surface receptors (p55 and p75 TNF receptors) (2). These receptors are highly homologous in their extracellular domains but show little homology between their intracellular domains, and are believed to act via distinct signal transduction pathways (3,4). The p55 TNF receptor (TNFR) is primarily responsible for signalling a variety of responses including cytotoxicity (2, 5), and cytokine secretion (6,7), while the p75 TNF receptor is primarily responsible for lymphoproliferative signals (5), and activation of T cells. TNF receptors are ubiquitously expressed on nearly all cell types, however the p75 TNFR is preferentially expressed by lymphoid cells.

Human TNF α does not recognize the murine p75 TNFR, but binds to the p55 TNFR (8,9). Also it is estimated that hTNF α is 50 times less toxic in mice compared to the murine form (2). This observation suggested that the p75 TNFR is primarily responsible for the induction of systemic toxicity, however it has become clear in recent years that the reduced toxicity exhibited by hTNF α in mice is a result of the lack of cooperation between signalling through both receptors and not to lack of activation of the p75 TNFR. Furthermore, it is now believed that the p55 TNFR is primarily responsible for the induction of systemic toxicity (10,11,12).

The treatment of cancer with a p75 TNFR specific agonist should result in greatly reduced systemic toxicity compared to that induced by wt TNF α , as well as being able to activate an antitumour immune response. Point mutants of human TNF α have been generated which show specificity for either the p55 hTNFR or the p75 hTNFR (13,14).

Since human and murine TNF α are highly homologous, particularly in the domains which recognize the p55 TNFR, we constructed a point mutant of murine TNF α (D142N-A144R) analogous to the human TNF mutant (D143N-A145R). Following rescue of this mutant gene into an Ad vector we were able to demonstrate that this murine mutant is p75 mTNFR specific. In addition we have investigated the efficacy of an adenovirus expressing this mutant for tumour immunotherapy.

Materials and Methods

Cell Culture

Cell culture media and reagents were purchased from GIBCO (Missauga, Ontario, Canada) with the exception of Fungizone[®] purchased from Squibb (Montreal, Quebec, Canada). MRC5 (human lung fibroblasts) cells (ATCC CCL-171) were cultured in minimal essential medium (α -MEM). Ad-5 E1-complementing human embryonic kidney cells (293), MT1A2 cells (a line derived from polyoma middle-T transgenic primary mammary tumour cells), and transgenic polyoma middle-T (PyMidT) primary tumour cells were cultured in MEM-F11 (15,16,17,18). The NDL cell line (generated from mutant transgenic neu mammary tumour cells) was cultured in Dulbecco's medium supplemented with 30 ng/mL epidermal growth factor (EGF) (Boehringer Mannheim, Laval, Quebec) (19). The TNF α sensitive murine cell line L929 was cultured in RPMI-1640 supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2 mM L-glutamine, 20 mM HEPES,

and 10% fetal bovine serum (FBS). CT6 cells (murine T cell) were also cultured in RPMI-1640 supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2 mM L-glutamine, 10 μ M B-mercaptoethanol, 20 U/mL IL-2 (Gibco BRL), and 10% FBS. All other cell culture medium was supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2.5 μ g/mL Fungizone[®], and 10% FBS.

Vector Construction

A cDNA encoding the mature (ie secreted form) murine TNF α cDNA fused with the IFN γ signal peptide cDNA was mutated using the four primer PCR technique with the primers 5'GGCTATTGAAACTAATGCAA^{3'} (Outside Rightward), 5'ACAATGCTTCCATCAAACGA^{3'} (Outside Leftwards), 5'AAGTACTTAAACTTTCGGGAGTCCGGG^{3'} (Inside Rightward), and 5'CCCGGACTCCCGAAAGTTTAAGTACTT^{3'} (Inside Leftwards), Vent[®] polymerase (New England Biolabs, Mississauga, ONT), and the plasmid pV1L-TNF (containing the construct) as the template (20). The double point mutant (D142N-A144R) was cloned into pMH4 creating the plasmid pmTNF-75 (21).

The Ad-mTNF-75 (Ad-75) (Fig. 1) vector was generated by in vivo recombination between the Ad-genomic plasmid pBHG10 and the shuttle plasmid pmTNF-75, which express the mutant secreted mTNF α cDNA under the control of the murine cytomegalovirus (MCMV) promoter and the SV40 poly adenylation signal (22). Adenoviral vectors were isolated, propagated in 293 cells, and purified (in CsCl gradients)

as previously described (23).

Expression assay preparation

In vitro expression assays were done by infecting cells at a multiplicity of infection (moi) of 1, 10, and 50 plaque forming units (pfu) per cell, sampling cell culture supernatants at various time points post infection, and storing samples at -70°C until the time of assay.

TNF α bioassay / Cytotoxicity Assay

The cytotoxicity bioassay measured direct killing of a TNF sensitive cell line (L929). Cells were dispensed into 96 well plates (5×10^4 cells in 50 μ L), and incubated overnight at 37°C (\approx 20hrs) in growth medium. The next day the medium was aspirated and replaced with 40 μ L of medium plus soyabean trypsin inhibitor (SBTI) (100 μ g/mL) and cycloheximide (Sigma) (20 μ g/mL). Ten μ L of samples or standards were added to the microtiter wells and incubated at 37°C overnight (\approx 18 hrs). Afterwards 10 μ L of 5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was added to each well and incubated for 4 hrs, after which 50 μ L of 50% dimethylformamide (20%SDS pH 4.7) was added. Following an additional overnight incubation, the OD₅₇₀ was measured. For the quantitation of TNF α , the measured OD₅₇₀ from diluted samples was compared to the OD₅₇₀ obtained from serial dilutions of a standard TNF α recombinant stock solution. Cycloheximide, SBTI, MTT, and recombinant murine TNF α used as the standard were

purchased from Sigma, St. Louis, MO, USA. Recombinant human TNF α was purchased from R&D Systems, Minneapolis, MN, USA.

CT6 proliferation assay

CT6 cells (donated by Mike Rothe, Tularik, Inc.) were deprived of IL-2 for 24 hrs before being aliquoted into 96 well microtiter plates (5×10^4 cells per well in 40 μ L growth medium (without IL-2)) with 10 μ L of samples (transfected cell culture supernatant) or standards. Three days later 10 μ L of 5mg/mL MTT was added to each well and incubated for 4hrs, after which 50 μ L of 50% dimethylformamide (20%SDS pH 4.7) was added. Following an additional overnight incubation, the OD₅₇₀ was measured. For receptor blocking, soluble p55 mTNFR (R&D Systems, Minneapolis, MN, USA) was added to the appropriate wells at a concentration of 33 μ g/mL.

Receptor Binding by TNF *In vitro*

For co-immunoprecipitations 500 μ L of ND1 cell culture supernatants from Ad vector transduced cells (moi 50 pfu per cell) were co-immunoprecipitated with 2 μ g of hamster anti-p55 mTNFR antibody (55R-286) or hamster anti-p75 mTNFR antibody (TR75-89) (both acquired from Dr. Robert D. Schreiber's laboratory (Washington University, St. Louis, MO)) (12), 2 μ g of soluble p55 mTNFR or p75 mTNFR respectively (R&D Systems, Minneapolis, MN) and protein -G Sepharose[®] (Pharmacia Biotech, Quebec, Ontario, Canada) (100 μ L pre-swollen slurry) in PBS for four hours. Neither

mTNFR antibody blocked TNF α binding. Sepharose beads were washed 6 times in PBS.

Samples were analyzed by SDS PAGE on a 12.5% polyacrylamide gel, and transferred to a nitrocellulose membrane (Immobilon-PTM, Millipore, Bedford, MA, USA). Western blotting was done by blocking the membrane with 5% powdered milk in PBS, then incubating with primary polyclonal goat anti mouse TNF α antibody (R&D Systems) for 4hrs at room temperature or overnight at 4°C. After extensive washing the membrane was incubated with secondary horseradish peroxidase (HRP) conjugated donkey anti goat antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 4hrs at room temperature. For detection of human TNF α , polyclonal rabbit anti mouse hTNF α antibody was used as the primary antibody (Chiron) (2hrs at room temp), and HRP conjugated goat anti-rabbit antibody was used as the secondary antibody (Santa Cruz Biotechnology, Inc.) (1hr at room temp.), this was done after blotting for the murine form.

Tumour studies

Transgenic mice carrying the polyoma middle-T oncogene under the Mouse Mammary Tumour Virus promoter/enhancer (MMTV) were generated by Guy *et al.*, 1992 (18). Breast tumours from transgenic females were explanted, and homogenized in collagenase (0.025% w/v) and dispase (0.25% w/v) (Boehringer, Mannheim, Laval, Quebec Canada). Tumour cells were then cultured for 24 to 48 hrs at 37°C (until confluent), then trypsinized (0.1% trypsin, 1.06mM EDTA in PBS, Sigma, St. Louis, Missouri, USA), washed, and resuspended in phosphate buffered saline (PBS), after which they were

injected into syngeneic FVB mice subcutaneously (5×10^5 cells in $200 \mu\text{L}$ of PBS) on the right hind flank. Visible palpable tumours (96mm^3) developed in 18-21 days post injection. These tumours were then directly injected with various doses of Ad-vectors in $50 \mu\text{L}$ of PBS. Tumour progression was monitored through routine measurements with callipers. For re-challenge, cured mice were injected with 5×10^5 tumour cells subcutaneously on the left hind flank.

Tumour morphology

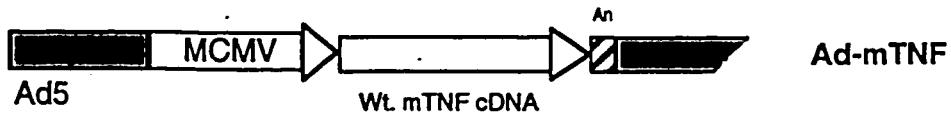
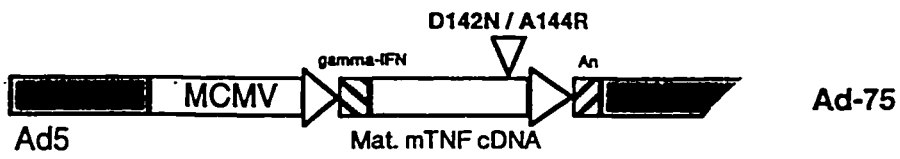
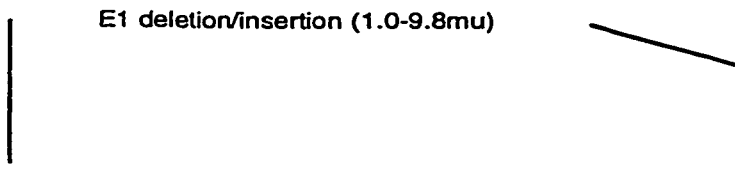
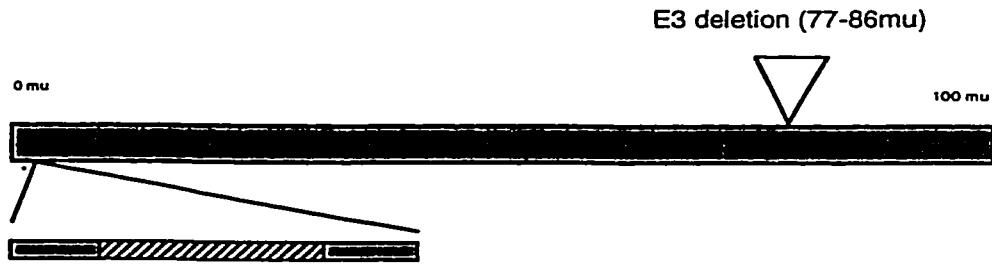
Tumours were removed from treated mice, cut in two with a razor and fixed in 10% Neutral Buffered Formalin for 18-24 hrs. After fixation the tissue was immersed in 50% Ethanol, then 60% Ethanol, for 30 minute periods until finally being stored in 70% Ethanol. Tumours were then paraffin embedded, sectioned and stained with haematoxylin and eosin.

RESULTS

In Vitro Expression of Ad-mTNF-75

A double point mutant of murine TNF α (D142N-A144R) was constructed similar to that previously reported for human TNF α , for optimal p75 TNFR specificity (13) (see materials and methods). This mutant murine cDNA fused with IFN γ signal peptide coding sequences, expressed under the control of the murine cytomegalovirus immediate early promoter (MCMV) and a downstream SV40 polyadenylation signal, was rescued into an E1/E3 deleted adenovirus type 5 vector (Ad-mTNF-75) (Fig 1). Other vectors used are also

Fig. 1. Ad vectors containing TNF α expression cassettes: Both Ad-75 (Ad-mTNF-75) and Ad-hTNF (Ad-MCMV-TNF) (23) vectors express the mature murine and human TNF α cDNAs respectively, fused with the IFN γ signal peptide. Ad-mTNF (Ad-mTNF-wt) (22) contains the entire murine TNF α cDNA. All TNF α expressing vectors utilize the MCMV promoter and a SV40 poly adenylation signal. All vectors are E1/E3 deleted Ad-5 based vectors.



illustrated, which express the wild type murine and human forms of TNF α also under the control of the MCMV promoter (24, 25).

To determine if Ad-mTNF-75 induced expression of the mutant TNF α cDNA, human fibroblast MRC5 and mouse adenocarcinoma derived NDL and MT1A2 cells were transduced with Ad-mTNF-75 at multiplicities of infection of 1, 10 and 50 pfu per cell. For comparison, cells were also transduced with Ad vectors expressing wild type murine TNF α (Ad-mTNF-wt or Ad-mTNF) (24), wild type human TNF α (Ad-MCMV-TNF or Ad-hTNF) (25) (Fig. 1) or an E1/E3 deleted control vector (Ad-BHG Δ E1,3). Expression of the mutant protein was detected by western blot analysis with a polyclonal anti mouse TNF α antibody (Fig 2). Comparable expression levels of mutant and wild type mTNF α were detected from transduced MT1A2 cells (Lanes 4 & 7). Furthermore, expression of mutant mTNF α was also detected in transduced NDL and MRC5 cells (Lanes 5 & 6). Interestingly, it appears that the electrophoretic mobility of the mutant mTNF α band is slightly greater than that of wild type TNF α . Expression levels of wild type human and murine TNF α were quantitated by a bioactivity assay using a TNF α sensitive cell line (L929). Levels of TNF α up to approximately 500 ng/ml were detected in culture supernatant from transduced murine cells and about 5 to 10 fold lower levels from infected human MRC5 cells (Table I).

Cytotoxicity and Proliferation Assays

The induction of cytotoxicity by TNF α is primarily signalled through the p55 TNFR (2,5). Therefore we tested the cytotoxic activity of cell culture supernatants from

Fig. 2. TNF expression in Ad-vector transduced cell supernatants: Detection of mutant (D142N-A144R) mTNF α expression by Western Blot with a polyclonal goat anti mouse TNF α antibody. Expression was detected from supernatants of Ad-mTNF or Ad-75 transduced MRC5, NDL, and MT1A2 (1A2) cell lines as described in the materials and methods. Samples (12.5 μ l of supernatant per well) were mixed 50:50 with loading buffer. Negative control: Ad-BHG Δ E1,3 transduced MT1A2 cells.

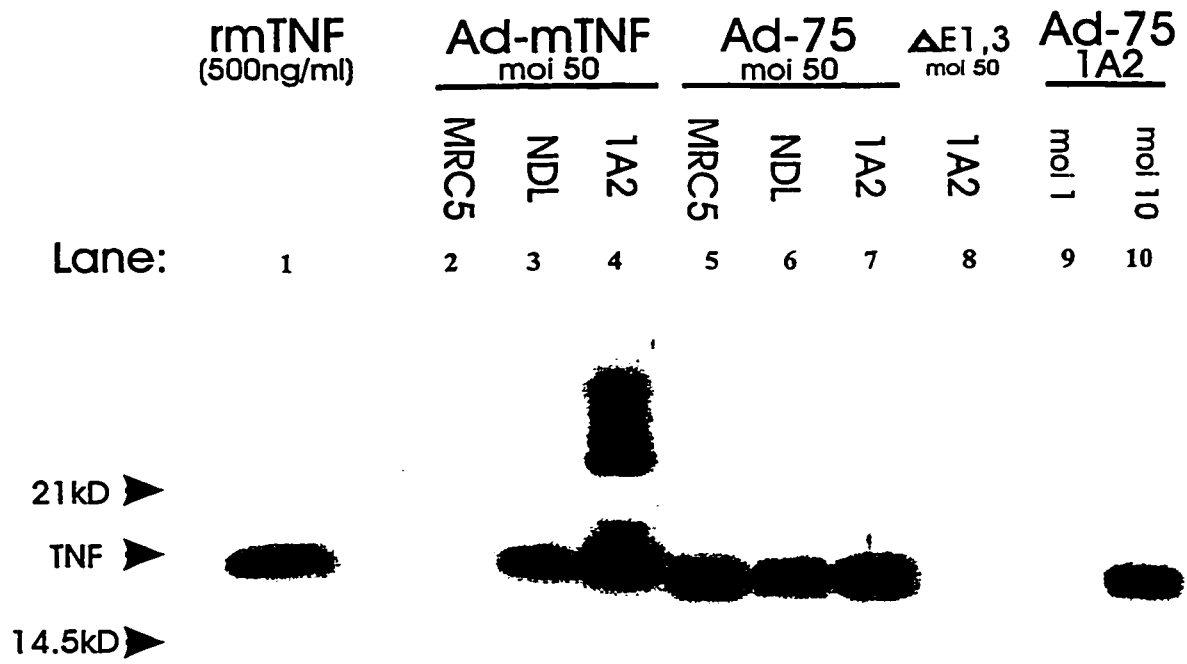


Table 1: Concentration of TNF expressed from transduced cells as detected by bioassay on L929 cells.

<i>Cell Line</i>	<i>Vector</i>	<i>[TNF]ng/ml</i> ¹	<i>Std. Error</i> ²
MRC5	Ad-mTNF	50	3
NDL	Ad-mTNF	437	5
MT1A2	Ad-mTNF	519	6
MRC5	Ad-hTNF	91	1
NDL	Ad-hTNF	262	7
MT1A2	Ad-hTNF	531	11

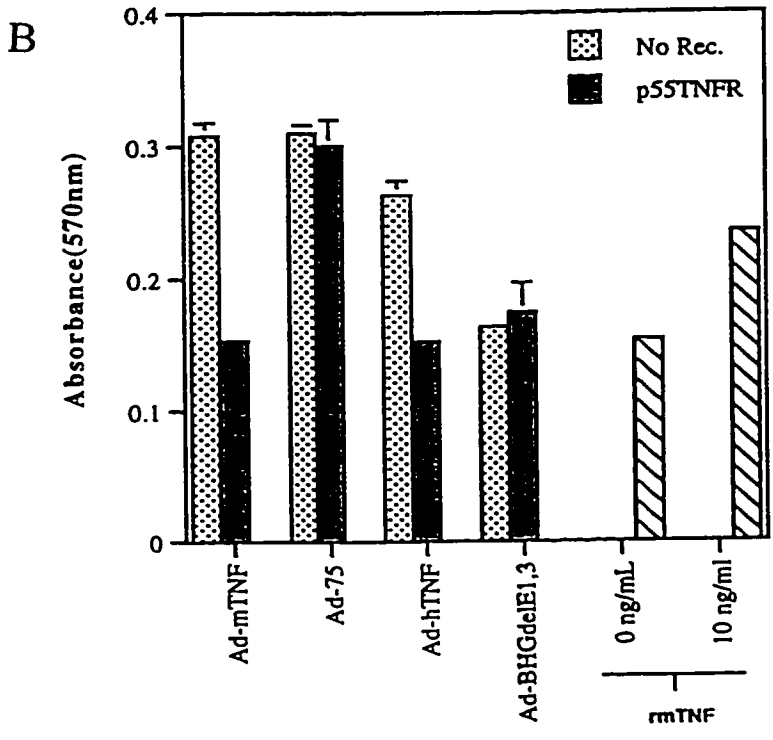
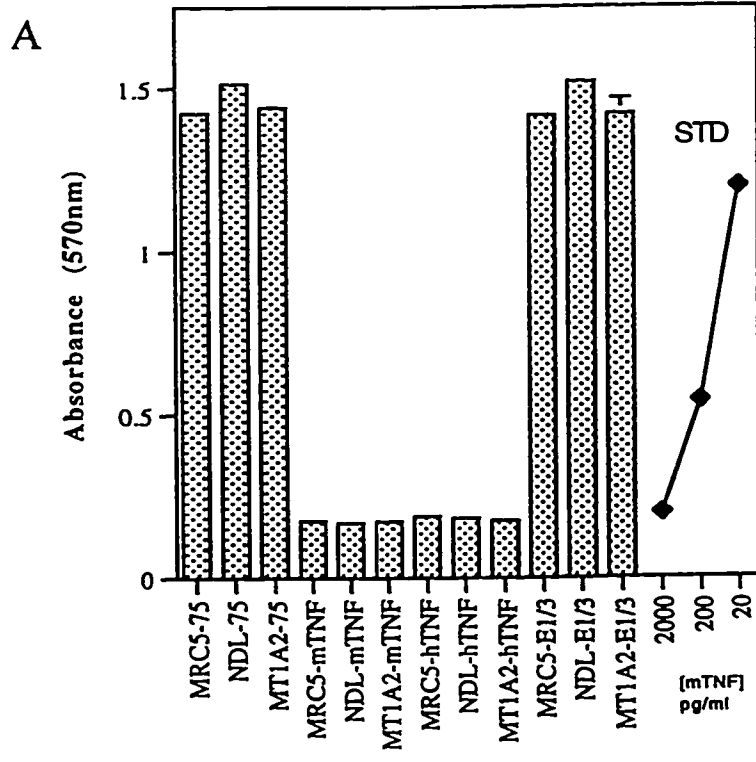
¹ Concentration of TNF alpha in culture supernatants from cells transduced with various Ad-vectors at a moi of 50pfu/cell (48 hrs post infection).

² Standard error (n = 4)

cells transduced with the mutant and wild type TNF α vectors, on the well characterized L929 cell line. Both wild type murine (p55 and p75 TNFR specific) and human TNF α (p55 TNFR specific) expressed from MRC5, NDL, and MT1A2 cells were cytotoxic to L929 cells, as was demonstrated by greatly reduced absorbance (570nm) levels (Fig. 3A), indicating the lack of chromogenic processing of MTT by viable cells (see materials and methods). However no cytotoxic activity was detected from cells transduced with the Ad-75 vector when compared to a control Ad-BHG Δ E1,3 vector. Cytotoxicity generated by standard concentrations of recombinant murine TNF α showed that near background absorbance (ie. maximal cytotoxicity) was induced by a concentration of only 2000pg/mL. The approximate concentration of mutant TNF α in cell culture supernatant was similar to that of Ad-mTNF transduced cells (\approx 400ng/mL) as estimated by western blot analysis (Fig. 2). Thus, even at a mutant mTNF α concentration roughly 100 to 200 times higher than that needed to induce maximal cell death by recombinant wild type protein, no cytotoxicity was observed. These data strongly suggest that the mutant form of mTNF α no longer binds to the p55 mTNFR.

Proliferative signals are commonly induced through activation of the p75 TNFR (5,12). Therefore we tested the ability of cell culture supernatants from cells transduced with the mutant and wild type vectors to induce the proliferation of a murine T cell line (CT6) (proliferation was measured by MTT as well) (see Materials and Methods). All three forms of TNF α induced the proliferation of CT6 cells (Fig. 3B), with the wild type and mutant murine TNF α vectors inducing the highest levels. Furthermore, the addition of

Fig. 3. *In vitro* bioactivity of mutant mTNF α : A: Cytotoxicity (MTT) assay on TNF α sensitive L929 cells with 10 μ L supernatants from Ad-75 (75), Ad-mTNF (mTNF), Ad-hTNF (hTNF), and Ad-BHG Δ E1,3 (E1,3) transduced MRC5, NDL, and MT1A2 cells (moi = 50pfu/cell). Cytotoxicity of standard dilutions of recombinant murine TNF α is indicated by the line graph on the right. B: Proliferation assay on the TNF α responsive CT6 cell line (detected by MTT), treated with 10 μ l supernatants from Ad vector transduce MT1A2 cells (moi = 50pfu/cell). Solid bars represent wells treated with soluble murine p55 TNF receptor (33 μ g/mL). Proliferation of CT6 cells in response to recombinant murine TNF α is indicated by cross hatched bars on the right.



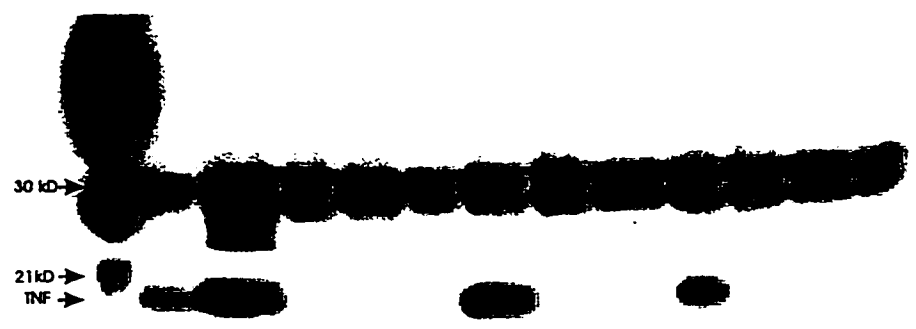
soluble murine p55 TNFR blocked activity by both murine and human TNF α , but had no effect on the mutant form. The ability of the mutant protein to induce proliferation of CT6 cells in both the presence and absence of soluble p55 mTNFR suggests that the mutant specifically activates only the p75 mTNFR.

***In vitro* binding of mutant and wild type TNF α to TNF receptors**

The physical associations of the various forms of TNF α with the murine p55 and p75 TNF receptors were demonstrated by co-immunoprecipitating ligands secreted by vector infected cells, with soluble forms of the receptors. Hamster anti-mouse p55 and p75 mTNFR antibodies were used to immunoprecipitate the murine receptors. Both these antibodies were chosen for their ability to bind the respective receptor without blocking TNF binding (12). Co-immunoprecipitates were analyzed by western blot analysis with primary anti murine and primary anti human TNF α antibodies. Wild type murine TNF α was co-precipitated with both p55 and p75 mTNFR (Fig. 4; Lanes 2 & 3). However only the p75 and p55 mTNFR co-precipitated the mutant murine and wild type human forms of TNF α respectively (Lanes 7 & 10). Unknown cross reacting bands appeared at approximately 30kD in all sample and control lanes. In all cases TNF α was not precipitated in the absence of either mTNFR. These results clearly demonstrate that the double point mutant (D142N-A144R) specifically binds to the murine p75 TNFR.

Fig. 4. *In vitro* binding of mutant and wt TNF α to TNFR: Co-immunoprecipitation / Western Blot of wild type human and murine TNF α , and mutant (D142N-A144R) murine TNF α in 500 μ L culture supernatant from NDJ cells transduced with the respective Ad vectors (moi = 50pfu/cell) using soluble murine p55 and p75 TNF receptors (2 μ g per precipitation), as described in the materials and methods. Receptors were immunoprecipitated by specific antibodies which did not interfere with ligand binding (2 μ g per precipitation). Co-immunoprecipitates were analyzed by Western Blot, first for murine TNF α , and second for human TNF α (see Materials and Methods). sTNFR refers to soluble TNF receptor.

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13
Ligand		mTNF				75				hTNF			
sTNFR		p55	p75			p55	p75			p55	p75		
TNFR Ab	Marker	p55	p75	p55	p75	p55	p75	p55	p75	p55	p75	p55	p75



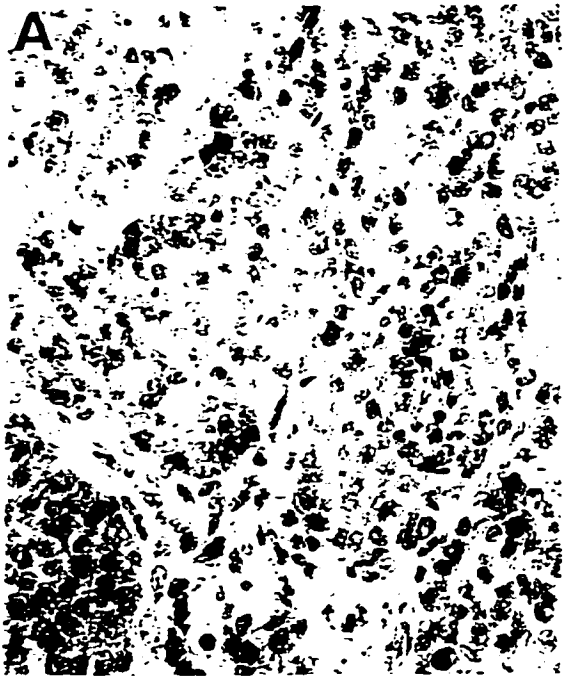
Effect of the mutant form of mTNF α on tumour pathology

Mice bearing transplanted polyoma middle-T tumours (PyMidT) were injected intratumourally with 5×10^8 pfu of Ad vectors. Two days post injection the tumours were removed from sacrificed mice, and processed for paraffin sectioning. Haematoxylin and eosin stains of tumour sections showed that, by comparison to normal tumour morphology as exhibited by tumours injected with control vector (Fig 5A), the mutant form of mTNF α was able to induce intratumoural necrosis (Fig 5B), as did the wild type vectors (Fig. 5C,&D), however the extent of the necrosis induced by Ad-75 was significantly lower. It should also be noted that the Ad-75 vector did not induce significant levels of necrosis at a lower dose of 1×10^8 pfu (not shown), while it has been previously determined that both wild type vectors readily induced intratumoural necrosis at that dose (24, 25).

Systemic and antitumour effects of the mutant form of mTNF α

Previous studies have suggested that the triggering of the p55 and not the p75 TNFR is primarily responsible for the induction of systemic toxicity (10,11,12), and that triggering of the p75 TNFR is responsible for signalling the activation/proliferation of lymphoid cells. Therefore, we decided to test the systemic/antitumour effects of the Ad-75 vector directly injected into PyMidT tumours. Both the wild type TNF α expressing vectors (Ad-mTNF & Ad-hTNF) have previously been tested in this tumour model (24, 25). These vectors proved to be quite toxic to treated mice killing 12 of 16 and 8 of 20 injected animals in two experiments, while curing 2 of 4 and 1 of 12 respectively at a dose of 5×10^8

Fig. 5. Tumour morphology: Haematoxylin and eosin staining of paraffin sections of tumours from mice treated with control (Ad-BHG Δ E1,3) vector (A), Ad-75 (B) (* necrotic region), Ad-mTNF (C) and Ad-hTNF (D), two days post intratumoural injection with 5×10^8 pfu per mouse (200X).



pfu per mouse. We chose to use these vectors along with the mutant vector for a direct comparison *in vivo*.

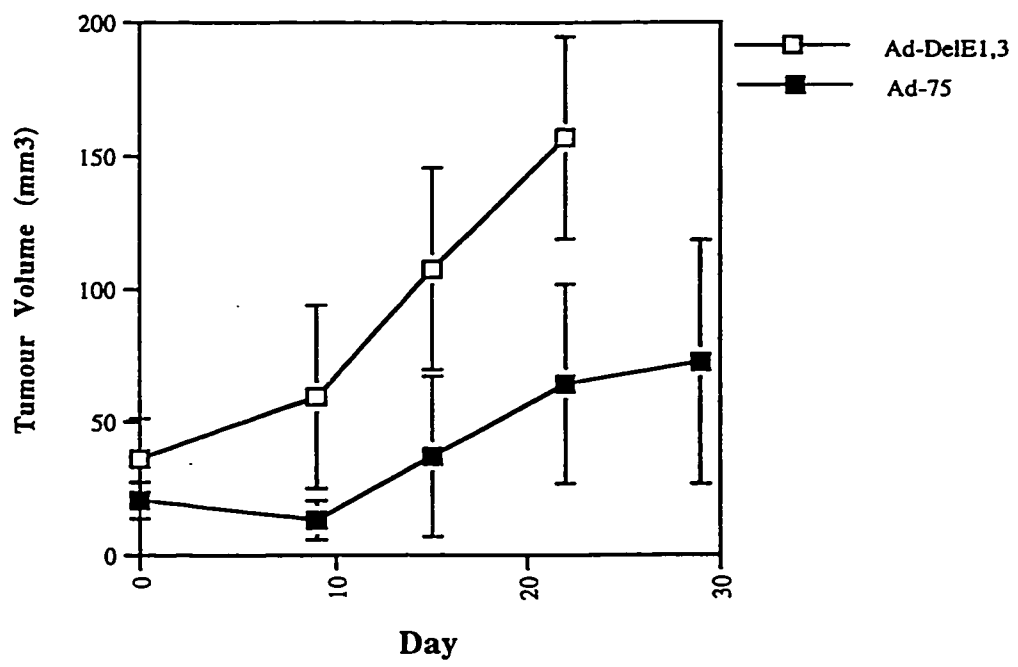
Table II shows the results of the immunotherapy treatments with the various Ad vectors. The Ad-75 vector showed no mortality even at a dose of 1×10^9 pfu, which in previous experiments with both wild type vectors resulted in 100% mortality within 1-3 days (24, 25). Both wild type vectors showed lethality at doses of 5 and 1×10^8 pfu, killing 2 of 4 and 3 of 8 (Ad-mTNF) and 3 of 5 and 1 of 5 (Ad-hTNF) mice respectively. No gross toxicity was observed in control vector injected mice. Even though the Ad-75 vector showed no mortality, it did induce an antitumour response in treated mice, causing partial regressions in 5 of 15 mice and permanent elimination of the tumour in 2 of 15 mice at a dose of 5×10^8 pfu. Mice surviving treatment with the wild type vectors also showed an antitumour response, with one in two being cured at a dose of 5×10^8 pfu (Ad-mTNF) and one in four being cured at a dose of 1×10^8 pfu (Ad-hTNF), as well as inducing partial regressions (see Table II). Figure 6 displays the growth kinetics typically exhibited by tumours treated with Ad-75 (5×10^8 pfu). All mice cured in these experiments were resistant to subsequent challenge (two months later) with PyMidT tumour cells, which implied the induction of immune memory against the tumour. These data clearly show that a TNF α mutant that functions through activation of the p75 mTNFR alone does not induce gross mortality while retaining the ability to induce an antitumour response.

Table 2: Antitumour response and toxicity induced by intratumoural injection with TNF expressing Ad vectors.

<i>Vector</i>	<i>Dose(Pfu)</i>	<i>Mortality</i>	<i>Tumour Response</i>		
			<i>No Response</i>	<i>Partial Response</i>	<i>Complete Regression</i>
Ad-75	1E+09	0/9	8/9	1/9	0/9
	5E+08	0/15	8/15	5/15	2/15
	1E+08	0/14	11/14	3/14	0/14
	2E+07	0/4	4/4	0/4	0/4
Ad-mTNF	5E+08	2/4	1/2	0/2	1/2
	1E+08	3/8	2/5	3/5	0/5
Ad-hTNF	5E+08	3/5	2/2	0/2	0/2
	1E+08	1/5	2/4	1/4	1/4
Ad-BHGdelE1,3	1E+09	0/4	4/4	0/4	0/4
	5E+08	0/11	10/11	1/11	0/11
	1E+08	0/12	12/12	0/12	0/12

- 1 Mortality represents the proportion of mice which died as a result of vector administration, typically within 1-4 days post injection.
- 2 Partial response refers to the proportion of survivors displaying tumours which regressed to less than half their original size before starting to grow again.
- 3 Complete regression refers to the proportion of survivors displaying complete and permanent disappearance of the tumour (within 2-4 weeks post vector injection).

Fig. 6. Tumour Growth Curve: Tumour growth kinetics after intratumoural injection of Ad-75 and Ad-BHGΔE1,3 (5×10^8 pfu) (n = 4). Tumour volume was determined through routine measurements with calipers. Error bars represent standard errors.



Discussion

The p55 TNF receptor has been implicated as the primary receptor for the induction of systemic toxicity by TNF α (10,11,12). Our *in vivo* experiment data support this conclusion by showing that, compared to the wild type forms of murine and human TNF α , a p75 TNFR specific form showed no lethality, however less obvious side effects may have been present. Furthermore, the fact that p75 TNFR activation does have immune stimulatory properties (3,5,7), allows one to specifically bypass TNF α related toxicity while retaining the ability to activate an antitumour response. Here we have shown such an application of a p75 TNFR specific ligand and compared it with ligands which bind both receptors (mTNF α) or only the p55 receptor (hTNF α).

We have not yet fully characterized the nature of the antitumour response induced by this receptor specific mutant, however the detection of immune memory against the tumour suggests that an immune response is involved. As well, it has been demonstrated that mice cured by cytokine expressing Ad vectors, including Ad-mTNF and Ad-75 (24, 26), showed PyMidT specific CTL activity (data not shown). The one mouse cured by the human TNF α vector has yet to be tested for PyMidT specific CTL activity. The role of the immune system in the antitumour activity of TNF α is well established (7,27,28,29,30). By comparing the effects of human and murine TNF α on murine T cell lines transfected with the human p75 TNFR cDNA, Vandenabeele *et al.* (7) showed that activation of the p75 TNFR was able to specifically induce cytokine secretion by T cells. Recently the effects of administration of a p75 TNFR specific mutant of human TNF α were tested in baboons (31),

and the results of this study indicated that this form of TNF α did not induce hypotension, IL-6 or IL-8 production, or skin necrosis as did wild type and p55 TNFR specific ligands. However a modest infiltration of lymphocytes and macrophages was detected in the dermis.

A comparison of the effectiveness of the Ad-75 vector at doses of 5×10^8 and 1×10^9 pfu indicates a potential inhibitory effect at higher doses, however greater numbers of mice are needed to confirm this (see Table II). TNF α has been implicated as part of the immune regulatory mechanism modulating T cell responses through elimination of reactive T cells (32,33). TNF α was shown to be an important mediator of T cell anergy in HIV infection and TNF α alone was shown to inhibit CD4+ T cell function (34). Other examples of the suppressive properties of TNF α have been described (35,36). Perhaps higher levels of mutant mTNF α produced at the dose of 1×10^9 pfu induced an immunosuppressive response.

The data presented in Table II show that exclusive activation of the p55 TNFR by human TNF α can produce an antitumour response in up to half of treated animals. The p55 TNFR is a powerful inducer of a variety of immunologic/antitumour activities, including the induction of cytokine secretion, neutrophil activation, endothelial cell activation (6,27,37,38), and cytotoxicity (2,5). The induction of intratumoural thrombosis is a major mechanism by which TNF α can induce rapid tumour necrosis (2,39,40,41). As well, Van der Poll et al., 1996 (38) demonstrated that only wild type and p55 specific agonists induced coagulation and fibrinolysis in baboons. Considering the dominant role of the p55 TNFR in endothelial cell activation and cytotoxicity one would not predict that a p75

TNFR specific agonist would induce intratumoural necrosis in contrast to our observations (Fig. 5). We did note however, that the degree to which the necrosis was induced was somewhat less in Ad-75 treated mice. It is possible that the overproduction of TNF α generated by the Ad vector could account for this observation. It is also possible that production of the mutant protein induced the secretion of endogenous mTNF α or other inflammatory proteins, at levels sufficient to cause tissue necrosis but not systemic toxicity. The observation that hTNF α induced proliferation of the CT6 cell line was also unexpected, considering that lymphoproliferative signals are primarily induced via the p75 TNFR. It is however possible that the longer incubation time with TNF α used in our assay (three days compared to one) (3), is at least partly responsible for the discrepancy between our data and results observed by others.

The results of our studies in our murine tumour model suggest that a p75 TNFR specific agonist may be very useful for the treatment of human cancers if the non toxic and antitumour properties of Ad-75 could be extrapolated to human patients. The expression of such a mutant from an Ad vector would be advantageous, considering adenoviral vectors allow for the possibility of local and transient high level expression of the transgene over a period of several days. However work must still be done to improve the effectiveness of this approach, possibly through the combination of TNF with other cytokines (IL-2, IL-12) and costimulatory molecules (B7). Work on this and other approaches is presently ongoing.

Acknowledgements

We would like to thank Dr. David Goeddel and Dr. Mike Rothe (Tularik, Inc., San Francisco, CA) for providing us with the CT6 cell line, and Dr. Robert D. Schreiber and Dr. Cora Arthur (Washington University School of Medicine, St. Louis, MO) for providing us with the murine TNF receptor specific antibodies (55R-286 and TR75-89). All PCR primer synthesis and sequencing was done at the Mobix Central Facility, McMaster University, Hamilton, Ontario, Canada. This work was supported by grants from the National Cancer Institute of Canada (NCIC), the Medical Research Council of Canada (MRC), the Canadian Breast Cancer Initiative, and London Life Insurance. F.L.G. is a Terry Fox Research Scientist of the NCIC and W.J.M. is a MRC Scientist.

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Summary

Mutant D142N-A144R is p75 receptor specific

The homologous mutant of human TNF α (D143N-A145R), was previously shown to be specific for the human p75 TNF receptor (#36 Loetscher *et al.* 1993). Here it is shown that TNF α with a mutation D142N-A144R is specific for the murine p75 TNF receptor. This was demonstrated by showing a lack of cytotoxic activity on L929 cells (p55 specific function), while retaining the ability to induce proliferation of CT6 cells. Furthermore the proliferation was not blocked by the addition of soluble p55 TNF receptor to the assay. Also, a physical association of the mutant TNF with the p75 TNF receptor alone was demonstrated by co-immunoprecipitating the mutant ligand with either the p55 or p75 TNF receptors.

Mutant murine TNF α showed reduced toxicity

Direct injection of Ad-mTNF-75 (expressing the double point mutant of mTNF α) failed to induce lethality at doses as high as 1×10^9 pfu. While both the Ad-mTNF-wt (wild type murine) and Ad-hTNF-wt (wild type human, also called Ad-MCMV-TNF) both produced lethality at lower doses.

Antitumour activity of mutant murine TNF α

Mice treated with the Ad-mTNF-75 vector showed some partial (5 of 15) and

permanent (2 of 15) tumour regressions at a dose of 5×10^8 pfu. Cured mice were resistant to subsequent challenge with PyMidT tumour cells on the left hind flank. Thus, a p75 TNF receptor agonist showed drastically reduced systemic toxicity while retaining some ability to regress tumours.

CHAPTER VI: DISCUSSION

Tumour necrosis factor alpha has been the subject of a great deal of research ever since its discovery as an anticancer agent in the sera of mice treated with endotoxin (Carswell et al., 1975). Its apparent anticancer properties led to its testing in patients as a possible cancer treatment. The direct injection of recombinant TNF α into the circulation or intratumourally has been shown to induce toxicity at doses required for therapeutic effects (Kimura et al., 1987; Sherman et al., 1988). Patients treated systemically showed a relatively low minimum tolerated dose and responded with organ failure and hypotension, with little antitumour effect. More recently clinicians have used isolated limb perfusion (ILP) or isolated organ perfusion (IOP) to deal with the toxicity induced by TNF α . ILP entails surgical isolation of the vascular root and connection to a heart and lung machine, which enable the constant perfusion of therapeutic agents. This procedure allows for the addition of approximately 10 times the normal minimal tolerated dose, and in combination with other therapeutic agents (melphalan), resulted in the complete regression of 70% of patients with *in transit* melanoma metastasis (reviewed in (Lejeune et al., 1998)). Even though this procedure has met with some success, it is still limited to regional therapy of advanced cancer, with ultimately little effect on patient survival. Also, the requirement for surgery is labour intensive and unpleasant. Thus improved methods for the delivery of TNF α to the tumour site are needed.

In recent years the use of viral vectors to deliver therapeutic genes for the treatment of cancer has become the subject of intense investigation. The potential for local

expression of anticancer agents within the region of the tumour is an obvious advantage of gene therapy, and Ad vectors are ideally suited for this task (discussed above). However, our observations have indicated that the local expression of TNF α from the tumour site is insufficient for the prevention of systemic toxicity. We have shown that an Ad vector expressing wild type murine TNF α induced high levels of lethality at doses required for antitumour activity (see chapter III). However, the restriction of TNF α to the cell surface resulted in a drastic reduction in lethality. It is also apparent that this form of TNF α retains the ability to cause partial and permanent tumour regressions. A membrane bound mutant of human TNF α has been characterized (Perez et al., 1990), allowing for the possibility of testing this type of therapy in human cancer patients.

Targeting either of the TNF receptors has also met with some success in our tumour model. Using human TNF α to specifically activate the p55 TNF receptor was shown to provide little protection from the lethal side effects of TNF α . Human TNF α failed to produce a powerful antitumour response as well. Targeting the p75 TNF receptor with the double point mutant showed a drastic reduction in lethality, however this mutant also failed to produce a powerful antitumour response. It should be noted that in both cases (membrane bound mutant, and p75 specific mutant) the antitumour activity of the treatment was reduced in comparison to wild type TNF α . The reduction in activity of the membrane bound mutant could be a result of reduced availability of TNF α for target cells. The antitumour activity of Ad-mTNF-MEM appeared to follow a positive dose response, and a higher dose of the vector partially compensated for the reduced activity. The reduction in

efficacy was more pronounced for the p75 receptor specific mutant. As stated above, activation of both TNF receptors has commonly been found to produce the greatest induction of TNF activity (see literature review). Also, the lack of p55 activation would result in the loss of any antitumour contribution provided by that receptor. The elimination of lethality, should have enabled us to increase the dosage to levels in which a higher degree of antitumour activity might be observed. This, however, was not the case. The higher dose used did not show any indication of increased antitumour activity. In addition to the ability of TNF to activate the immune system, TNF has also been implicated as a down-regulator of the immune system. It was shown to be involved in the induction of CD4⁺ T cell anergy by HIV gp120 (Kaneko et al., 1997). Similar to Fas, TNF α was also shown to induce apoptosis in T cells (CD8⁺) through activation the p75 TNF receptor (Zheng et al., 1995). This could explain the lack of increased efficacy at greater doses, as the higher levels of TNF α produced may have lead to immunosuppression rather than immune stimulation. In all cases, mice cured by the Ad TNF vectors were resistant to subsequent challenge with PyMidT tumour cells, demonstrating the induction of protective immune memory, critically important to the elimination of metastasis. The specific involvement of the immune system in regression of the primary tumour was not directly determined, however, considering the lympho-specific effects of the p75 TNF receptor, it is likely that immune activation was involved in regressions induced by the Ad-mTNF-75 vector. It is also likely that an immune response was also involved in regressions caused by the other Ad-TNF vectors, as it has been shown that the antitumour properties of TNF α

involve an immune response (see Literature Review) (Asher et al., 1991; Blankenstein et al., 1991; Marincola et al., 1994).

Although the techniques used were effective for reducing the lethality associated with TNF α , the antitumour activity is low relative to similar treatments with other cytokines such as IL-2 and IL-12 (30–40% complete regressions) (Addison et al., 1995a; Addison et al., 1995b; Bramson et al., 1996a). The combination of TNF treatment with other cytokine and or cytotoxic treatments could prove fruitful. However, the various mutants used in the above tumour therapy experiments have also been used in combination with Ad vectors expressing IL-2 and IL-12 (see Appendix). No combination of Ad-TNF vector with Ad-IL-2 or Ad-IL-12 vector produced any significant improvement in antitumour activity, with the exception of the combination of Ad-mTNF-MEM with an IL-12 expressing Ad vector (Ad-mIL-12) (Bramson et al., 1996b), in which case a dramatic increase in lethality was also observed.

The constructed Ad-TNF vectors could prove to be useful tools for the investigation of the role(s) of TNF α in other physiological processes. Lei *et al.* found that ovalbumin (OVA) induced airway eosinophilia in CD40L knockout mice, was dependent on the combination of IL-4 and TNF α (Lei et al., 1998). This was done through the co-administration of Ad vectors expressing IL-4 and murine TNF α (Ad-mTNF-wt). Ad-mTNF-wt was also used in a model of pulmonary inflammation, in which it was suggested that TNF α induced fibrosis through the induction of TGF- β (Sime et al., 1998). Mutant forms of human TNF α specific for the p75 TNF receptor (recombinant protein) have been

introduced into baboons, showing greatly reduced systemic toxicity similar to our findings (Welborn et al., 1996). The receptor specific TNF α expressing Ad vectors could prove to be useful for the investigation of the functions of either TNF receptor within the murine system. The Ad-mTNF-MEM vector could prove useful for the investigation of the specific role of the membrane bound form of TNF α , or locally expressed TNF α , in a variety of physiological processes. Furthermore, the partial success of the Ad-mTNF-MEM vector in tumour therapy, demonstrated the principal that a normally secreted cytokine can be restricted of to the cell surface and remain effective. This strategy could be applied to other toxic cytokines such as IFN γ , IL-2, and IL-12 using membrane anchored fusion proteins. This type of therapy would require a gene delivery technique, and adenoviral vectors would be ideal for such a task. With respects to tumour therapy, the techniques described here show that the lethality associated with TNF α can be reduced while retaining the ability to regress tumours, however the overall efficacy is poor. Fewer than 9% of the mice were cured with the Ad-mTNF-MEM vector (at doses of 1×10^9 and 5×10^8 pfu, with none cured at doses of 1×10^8 and 2×10^7 pfu) and approximately 5% were cured with the Ad-mTNF-75 vector (at doses of 1×10^9 , 5×10^8 and 1×10^8 pfu, with none cured at a dose of 2×10^7 pfu). Thus we were not able to increase the overall therapeutic index of our TNF α gene therapy techniques through mutation of TNF α .

CHAPTER VII: REFERENCES

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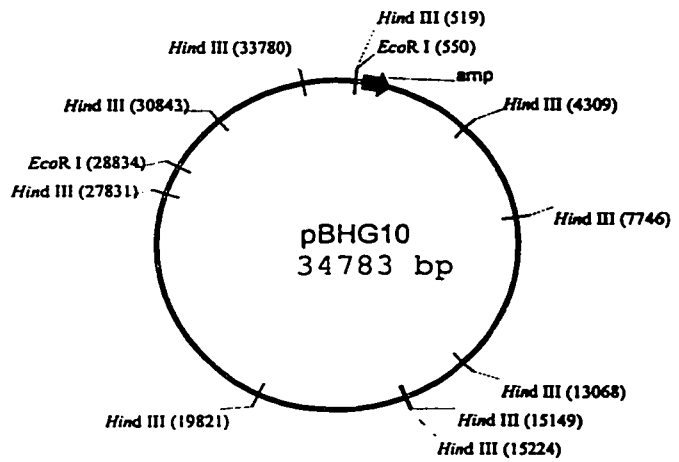
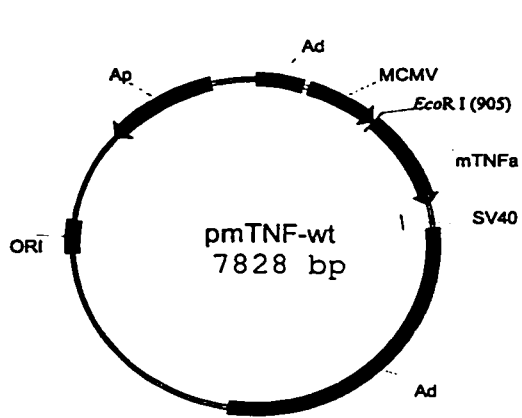
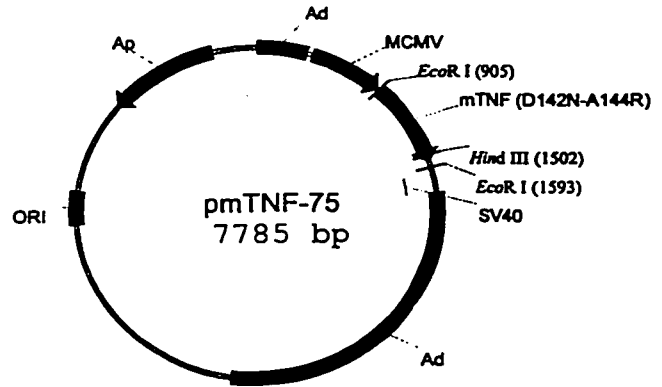
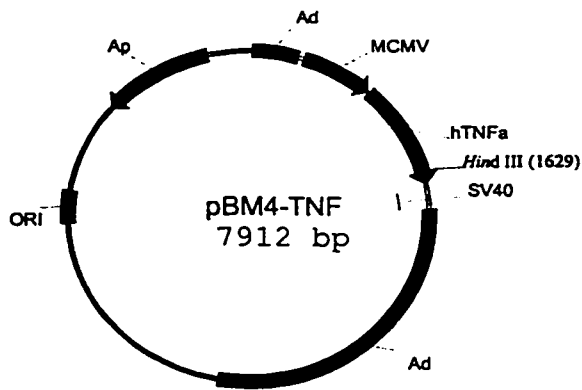
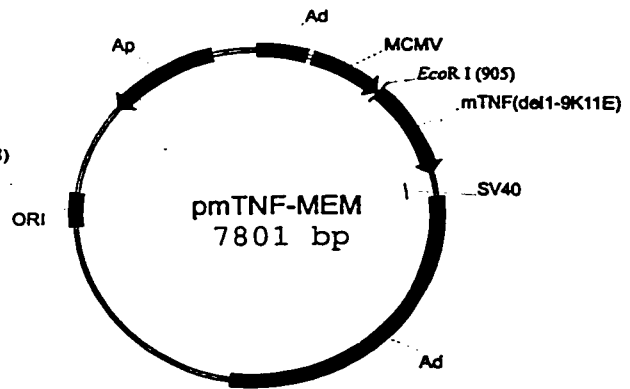
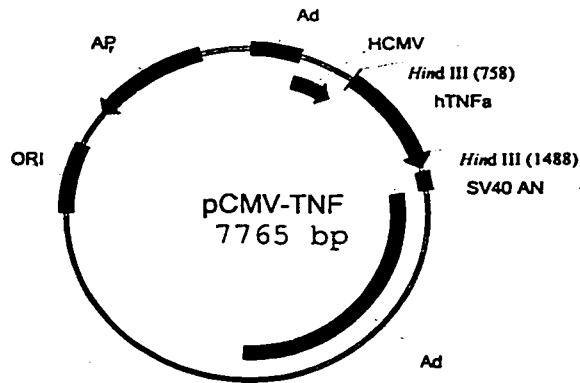
Zilli, D., Voelkel-Johnson, C., Skinner, T. and Laster, S.M. (1992) The adenovirus E3 region 14.7 kDa protein, heat and sodium arsenite inhibit the TNF-induced release of arachidonic acid. *Biochem Biophys Res Commun* 188(1), 177-83.

Zindy, F., Eischen, C.M., Randle, D.H., Kamijo, T., Cleveland, J.L., Sherr, C.J. and Roussel, M.F. (1998) Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes Dev* 12(15), 2424-33.

APPENDIX-1

Figure A1-1: Plasmids used to construct recombinant adenoviral vectors expressing various forms of TNF α .

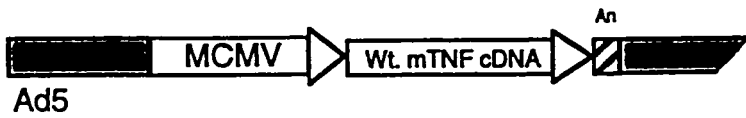
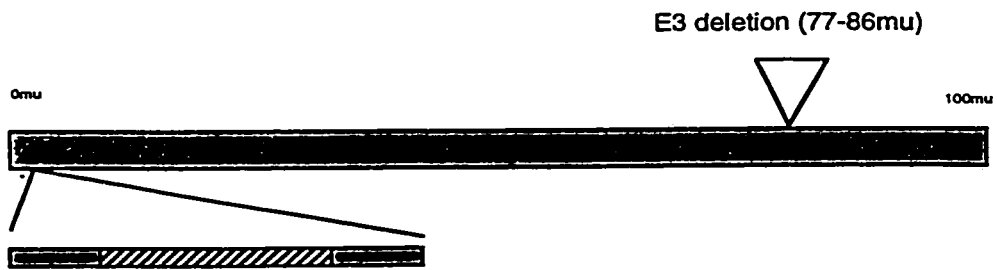
The “shuttle” plasmids pCMV-TNF, pBM4-TNF, pmTNF-wt, pmTNF-MEM, and pmTNF-75 were cotransfected into 293 cells with the Ad-genomic plasmid pBHG10. The shuttle plasmids generated the Ad vectors Ad-HCMV-TNF, Ad-MCMV-TNF, Ad-mTNF-wt, Ad-mTNF-MEM, and Ad-mTNF-75 respectively.



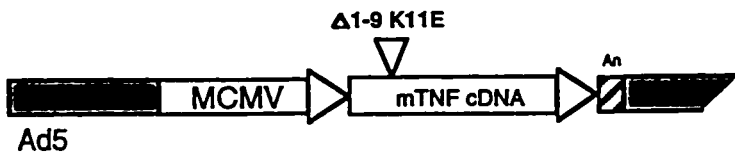
APPENDIX-2

Figure A2-1: Left end diagrams of the five Ad vectors used in this thesis.

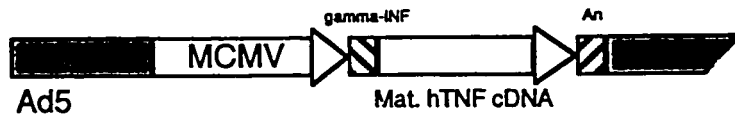
All vectors utilized the SV40 polyadenylation signal (An). The promoter (MCMV or HCMV) is indicated by rightward arrows as all vectors expressed rightward from within the E1 region of the adenovirus genome. The interferon- γ secretory peptide is indicated by the cross-hatched sections to the left of the TNF transgene (gamma-IFN). Mature (secreted) TNF α cDNA sequences are indicated as well (Mat.). Human and murine TNF α cDNAs are designated by hTNF and mTNF respectively.



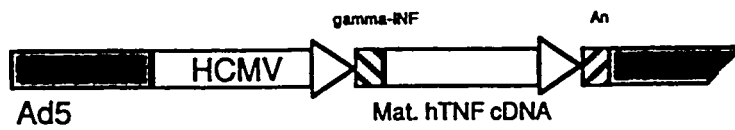
Ad-mTNF-wt



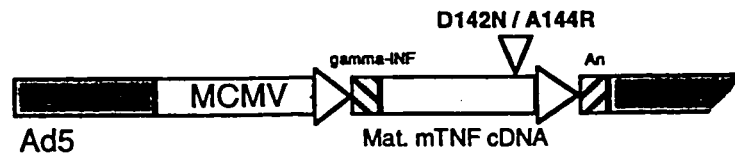
Ad-mTNF-MEM



Ad-MCMV-TNF



Ad-HCMV-TNF



Ad-mTNF-75

APPENDIX III: Immunotherapy by co-injection of two Ad vectors expressing cytokines.

All the combinational immunotherapy was conducted by co-injection of Ad vectors directly into the tumours (middle-T) implanted in syngeneic FVB mice (see Chapter II, Materials and Methods).

Table A3-1: Combinational immunotherapy using Ad vectors expressing human TNF α (Ad-MCMV-TNF) and human IL-2 (Ad-CA-IL-2 (Addison et al., 1995a)).

Vector(s)	Ad-MCMV-TNF ³	Ad-MCMV-TNF / Ad-CA-IL-2 ³	Ad-CA-IL-2 ³
Fraction Cured ¹	0 / 18	4 / 17	2 / 19
Lethality ²	2 / 20	3 / 20	0 / 19

1 Fraction cured refers to the number of mice undergoing complete and permanent disappearance of the transplanted tumour.

2 Lethality refers to the fraction of mice which died as a result of vector administration (typically within 1-4 days post injection).

3 Ad-MCMV-TNF administered at a dose of 2.5×10^8 pfu; Ad-CA-IL-2 administered at a dose of 2.5×10^8 pfu.

Table A3-2: Combinational immunotherapy using Ad vectors expressing a membrane bound mutant of murine TNF α (Ad-mTNF-MEM) and human IL-2 (Ad-CA-IL-2 (Addison et al., 1995a)).

Vector(s)	Ad-mTNF-MEM ³	Ad-mTNF-MEM / Ad-CA-IL-2 ³	Ad-CA-IL-2 ³
Fraction Cured ¹	0 / 6	0 / 6	2 / 6
Lethality ²	1 / 7	2 / 8	2 / 8

1 Fraction cured refers to the number of mice undergoing complete and permanent disappearance of the transplanted tumour.

2 Lethality refers to the fraction of mice which died as a result of vector administration (typically within 1-4 days post injection).

3 Ad-mTNF-MEM administered at a dose of 2.5×10^8 pfu; Ad-CA-IL-2 administered at a dose of 2.5×10^8 pfu.

Table A3-3: Combinational immunotherapy using Ad vectors expressing a p75 TNF receptor specific mutant of murine TNF α (Ad-mTNF-75) and human IL-2 (Ad-CA-IL-2 (Addison et al., 1995a)).

Vector(s)	Ad-mTNF-75 ³	Ad-mTNF-75 / Ad-CA-IL-2 ³	Ad-CA-IL-2 ³
Fraction Cured ¹	----	0 / 5	1 / 5
Lethality ²	----	0 / 5	0 / 5

1 Fraction cured refers to the number of mice undergoing complete and permanent disappearance of the transplanted tumour.

2 Lethality refers to the fraction of mice which died as a result of vector administration (typically within 1-4 days post injection).

3 Ad-mTNF-75 administered at a dose of 2.5×10^8 pfu; Ad-CA-IL-2 administered at a dose of 2.5×10^8 pfu.

Table A3-4: Combinational immunotherapy using Ad vectors expressing a membrane bound mutant of murine TNF α (Ad-mTNF-MEM) and murine IL-12 (Ad-mIL-12 (Bramson et al., 1996b)).

Vector(s)	Ad-mTNF-MEM ³	Ad-mTNF-MEM / Ad-mIL-12 ³	Ad-mIL-12 ³
Fraction Cured ¹	0 / 9	7 / 10	5 / 17
Lethality ²	2 / 11	8 / 18	1 / 18

1 Fraction cured refers to the number of mice undergoing complete and permanent disappearance of the transplanted tumour.

2 Lethality refers to the fraction of mice which died as a result of vector administration (typically within 1-4 days post injection).

3 Ad-mTNF-MEM administered at a dose of 2.5×10^8 pfu; Ad-mIL-12 administered at a dose of 2.5×10^8 pfu.

Table A3-5: Combinational immunotherapy using Ad vectors expressing a membrane bound mutant of murine TNF α (Ad-mTNF-MEM) and murine IL-12 (Ad-MEM-IL-12R).³

Vector(s)	Ad-mTNF-MEM ³	Ad-mTNF-MEM / Ad-MEM-IL-12R ³	Ad-MEM-IL-12R ³
Fraction Cured ¹	----	2 / 5	1 / 4
Lethality ²	----	0 / 5	0 / 4

1 Fraction cured refers to the number of mice undergoing complete and permanent disappearance of the transplanted tumour.

2 Lethality refers to the fraction of mice which died as a result of vector administration (typically within 1-4 days post injection).

3 Ad-mTNF-MEM administered at a dose of 4.9×10^8 pfu; Ad-MEM-IL-12R administered at a dose of 1×10^7 pfu.

Table A3-6: Combinational immunotherapy using Ad vectors expressing a p75 TNF receptor specific mutant of murine TNF α (Ad-mTNF-75) and murine IL-12 (Ad-MEM-IL-12R).

Vector(s)	Ad-mTNF-75 ³	Ad-mTNF-75 / Ad-MEM-IL-12R ³	Ad-MEM-IL-12R ³
Fraction Cured ¹	0 / 10	5 / 13	3 / 13
Lethality ²	0 / 10	2 / 15	2 / 15

1 Fraction cured refers to the number of mice undergoing complete and permanent disappearance of the transplanted tumour.

2 Lethality refers to the fraction of mice which died as a result of vector administration (typically within 1-4 days post injection).

3 Ad-mTNF-75 administered at a dose of 4.9×10^8 pfu; Ad-MEM-IL-12R administered at a dose of 1×10^7 pfu.

Table A3-7: Combinational immunotherapy using Ad vectors expressing a p75 TNF receptor specific mutant of murine TNF α (Ad-mTNF-75), and a membrane bound mutant of murine TNF α (Ad-mTNF-MEM)

Vector(s)	Ad-mTNF-75 ³	Ad-mTNF-75 / Ad-mTNF-MEM ³	Ad-mTNF-MEM ³
Fraction Cured ¹	1 / 5	1 / 5	0 / 5
Lethality ²	0 / 5	0 / 5	0 / 5

1 Fraction cured refers to the number of mice undergoing complete and permanent disappearance of the transplanted tumour.

2 Lethality refers to the fraction of mice which died as a result of vector administration (typically within 1-4 days post injection).

3 Ad-mTNF-75 administered at a dose of 2.5×10^8 pfu; Ad-mTNF-MEM administered at a dose of 2.5×10^8 pfu.

APPENDIX IV

Table A4-I: Cells and cell lines used for this thesis

<i>Cell / Cell Line</i>	<i>Description</i>	<i>Culture Medium</i>
293	human embryonic kidney cells transformed with the Ad E1 region (Graham et al., 1977)	MEM
293 N3S	human embryonic kidney cells transformed with the Ad E1 region (Graham et al., 1977) - contact independent	Joklik's
MRC5	human fibroblasts (ATCC CCL 171)	α -MEM
B16BL6	murine melanoma (Fidler, 1975)	α -MEM
MT1A2	murine mammary adenocarcinoma line derived from primary PyMidT transgenic tumours (Guy et al., 1992)	MEM
PT ₀₅₁₆	murine fibroblasts from FVB/N kidneys (Addison, 1997a)	α -MEM
MT3	PT ₀₅₁₆ transformed with PyMidT (Addison, 1997a)	α -MEM (400ng/mL G418)
L929	murine fibroblast cell line (Thomas et al., 1975)	RPMI-1640 (20mM HEPES)
A673/6	human rhabdomyosarcoma cell line (Iwata et al., 1985)	α -MEM (5% FBS)
NDL	murine mammary adenocarcinoma line derived from mutant transgenic neu tumours (Putzer et al., 1997)	Dulbecco's (30ng/mL EGF)
CT6	IL-2 dependent T cell line (Ranges et al., 1989)	RPMI-1640 (10 μ M β -mercaptoethanol, 20U/mL IL-2)
1° PyMidT	Primary tumour cells from transgenic PyMidT mice	MEM

APPENDIX V: List of Abbreviations

Ab- antibody
Ad - adenovirus
ADCC - antibody-dependent cell-mediate cytotoxicity
bFGF - basic fibroblast growth factor
BSA - bovine serum albumen
CAM - cellular adhesion molecule
CAR - coxackie virus adenovirus receptor
CD - cytosine deaminase
cDNA - coding deoxyribonucleic acid
CF - cystic fibrosis
CTL - cytotoxic lymphocyte
DBP - DNA binding protein
dCTP - deoxycytosine triphosphate
DNA - deoxyribonucleic acid
ECM - extracellular matrix
EDTA - ethylene diamine tetra acetic acid.
EGF - epidermal growth factor.
ELAM - endothelial-leukocyte adhesion molecule 1
ELISA - enzyme linked immunosorbent assay.
FBS - fetal bovine serum
FTIC - fluoresceine
GDP - guanine diphosphate
GM-CSF - granulocyte/macrophage - colony stimulating factor
GTP - guanine triphosphate
HCMV - human cytomegalovirus
H & E - haematoxylon and eosin
HIV - human immunodeficiency virus
HRP - horse raddish peroxidase
HSV-TK - herpes simplex virus - thymidine kinase
I-CAM-I - intercellular adhesion molecule - I
IFN γ - interferon gamma
IL-1 - interleukin 1
IL-2 - interleukin 2
IL-4 - interleukin 4
IL-6 - interleukin 6
IL-8 - interleukin 8
IL-10 - interleukin 10
IL-12 - interleukin 12
ILP - isolate limb perfusion
IOP - isolated organ perfusion

APPENDIX V: List of Abbreviations (continued)

IP - immunoprecipitation
IRES - internal ribosomal entry site
ITRs - inverted terminal repeats
LAK - lymphokine activated killer cell
LB - Luria-Bertani broth
LBA - Luria-Bertani broth agar
LDL - low density lipoprotein
MAPK - mitogen activate protein kinase
MCMV - murine cytomegalovirus
MEM - minimal essential medium
MHC - major histocompatibility complex
MLP - major late promoter
MMTV - mouse mammary tumour virus
MOI - multiplicity of infection.
MTT - 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NBF - neutral buffered formalin
NF - nuclear factor
NK - natural killer
OD₅₇₀ - optical density (at 570nm)
OVA - ovalbumin
PBS - phosphate buffered saline
PCR - polymerase chain reaction
pfu - plaque forming unit
PI - propidium iodide
PyMidT - polyoma middle - T
RCA - replication competent adenovirus
RNA - ribonucleic acid
RT - room temperature
RTK - receptor tyrosine kinase
SB - super broth
SBTI - soyabean trypsin inhibitor
SDS - sodium docecyl sulfate
SDS PAGE - SDS polyacrylamide gel electrophoresis
STD - standard
SV40 - simian vacuolating virus 40
TACE - TNF α converting enzyme
TBP - TATA binding protein
TCR - T cell receptor
TE - tris - EDTA
TIL - tumour infiltration lymphocyte

APPENDIX V: List of Abbreviations (continued).

TGF β - transforming growth factor beta

TNF α - tumour necrosis factor alpha

TNFR - TNF receptor

TP - terminal protein

VA RNA - viral associated RNA

VCAM-I - vascular cell adhesion molecule

VEGF - vascular endothelial growth factor