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CONSTRUCTION AND CHARACTERIZATION OF A NOVEL FUSION PROTEIN FROM THE EXTRACELLULAR DOMAIN OF MULT1 AND TRANSMEMBRANE AND INTRACELLULAR DOMAINS OF FAS AND ITS THERAPEUTIC EVALUATION FOR CANCER TREATMENT USING AN ADENOVIRAL DELIVERY SYSTEM

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by
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ABSTRACT

One of the strategies that tumor cells adopt to evade immunosurveillance mounted by elements of the innate immune system, such as NK cells, is to down-regulate certain cell surface molecules through a process also called shedding. Mouse UL16-binding protein-like transcript 1 (MULT1), which can activate NK cells through NK cell receptor NKG2D, is one of such molecules. Tumor cells can also avoid Fas mediated apoptosis by down-regulating its expression, secreting antagonistic 'decoy' receptors, or expressing anti-apoptotic molecules. In this study, we report the design and evaluation of the antitumor activity of a novel fusion protein MULT1E/FasTI, consisting of the extracellular domain of MULT1 and transmembrane and intracellular domains of Fas. We hypothesized that this protein, when expressed on a cell, would not only activate NK cells and other NKG2D expressing killer cells through its MULT1E region but also send death signals to induce apoptosis of the cell through the FasTI region. We cloned cDNA encoding the extracellular domain of MULT1 gene from thymus of new born mice and ligated it to the transmembrane and intracellular domains of mouse *fas* cDNA. The resulting fusion cDNA was inserted into a mammalian cell expressing vector under the control of CMV promoter. The vector was then transfected into mouse TC-1 lung epithelial cancer cells; and stable cell lines expressing the fusion protein were established. *In vitro* cell culture studies demonstrated that the binding of NKG2D/Fc, a recombinant protein of mouse NK cell receptor, to MULT1E/FasTI expressed on tumor cells was able to elicit apoptosis as assayed by Annexin V-FITC staining and caspase-3 ELISA and also activated NK cells as indicated by enhanced interferon-gamma expression. *In vivo* subcutaneous tumor studies demonstrated that tumor cells expressing

MULT1E/FasTI grew significantly slower than tumors without the protein. Pulmonary metastasis studies showed that most of the mice completely rejected tumor cells expressing MULT1E/FasTI. We also examined the use of a replication-defective adenovirus as a gene therapy vector to deliver the fusion protein into tumor cells. *In vitro* and *in vivo* studies not only demonstrated that the novel fusion protein can be successfully delivered by adenoviral vectors but also confirmed antitumor activity of the fusion protein. Therefore, the reported fusion protein strategy represents a novel and hopeful new anticancer agent for cancer patients.

DEDICATION

To the three most important people in my life, my father, my mother and my brother

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ABBREVIATIONS

aa,	amino acid
ALPS,	autoimmune lymphoproliferative syndrome
B-CLL,	B cell lymphocytic leukemia
bp,	base pair
cDNA,	complimentary deoxyribonucleic acid
CEA,	carcinoembryonic antigen
cFLIP,	FADD-like interleukin-1ß-converting enzyme inhibitory protein
CMV,	human cytomegalovirus immediate-early
Cyto c,	cytochrome c
DAP10,	DNAX-activating protein of 10kDa
DAP12,	DNAX-activating protein of 12kDa
DD,	death domain
DED,	death effector domain
DISC,	death-inducing signaling complex
DR4,	death receptor 4
DR5,	death receptor 5
FACS,	fluorescent activated cell sorting
FADD,	Fas-associated DD containing protein
FasL,	fas ligand
FasTI,	fas transmembeane and intracellular region
FBS,	fetal bovine serum
Fc,	Fragment of antibody without antigen-binding sites

Gld, generalized lymphoproliferative disease

GPI, glycosyl phosphatidyl inositol

HCMV, human cytomegalovirus

IAP, inhibitor-of-apoptosis protein

IKDC, interferon producing killer dendritic cells

IL, interleukin

IPR, lymphoproliferation

ITAM, immunoreceptor tyrosine-based activation motif

kDa, kilo Dalton

KIR, killer cell Ig-like receptor

LT, limphotoxin

MACH, MORT1-associated CED-3 homolog

MCMV, murine cytomegalovirus

MHC-1, major histocompatability class-1

MICA/B, MHC-class-I-polypeptide related sequence A/B

mRNA, messenger ribonucleic acid

MULT1, murine UL16 binding protein-like transcript 1

NKG2D, natural-killer group 2, member D receptor

NKG2D-L, natural-killer group 2, member D receptor-long

NKG2D-S, natural-killer group 2, member D receptor-short

NKG2D/Fc, NKG2D receptor fused to fragment of antibody without antigen-binding site

NKT cell, natural-killer T cell

ORF,	open reading frame
PCR,	polymerase chain reaction
PI3K,	phosphatidylinositol 3- kinase
PLAD	preligand binding assembly domain
RAE1,	retinoic acid early transcript 1
RT-PCR,	reverse transcriptase polymerase chain reaction,
TGF-ß	transforming growth factor-beta
TILs,	tumor-infiltrating lymphocytes
TNF,	tumor necrosis factor
TNFR1,	TNF receptor 1
TRAIL,	TNF-related apoptosis-inducing ligand
TRAIL-R1,	TRAIL receptor 1
TRAIL-R2,	TRAIL receptor 2
ULBP,	UL16-binding protein
VEGF,	vascular endothelial growth factor

CHAPTER ONE

INTRODUCTION

1.1. NKG2D Receptor

1.1.1. Structure of NKG2D Receptor

NKG2D (natural-killer group 2, member D) belongs to a sub-family of C type lectin-like receptors. NKG2D is a homodimeric, type II transmembrane glycoprotein (Wolan et al., 2001). The *NKG2D* gene is located in the NK gene complex which is on chromosome 6 in the mouse (Ho et al., 1998) and chromosome 12 in humans (Glienke et al., 1998; Renedo et al., 2000). Like most activating receptors, NKG2D is a multi-subunit receptor complex. Signaling in NKG2D is mediated by specialized signaling adaptors. In the mouse NKG2D can associate with two distinct adaptors DAP-10 and DAP-12/KARAP (Diefenbach et al., 2002), while in humans NKG2D exclusively uses DAP-10 (Wu et al., 1999; Rosen et al., 2004). Non-covalent interactions are responsible for these associations (Diefenbach et al., 2002; Wu et al., 1999). One NKG2D homodimer associates with two DAP-10 dimers to form a hexameric complex (Garrity et al., 2005).

Two distinct NKG2D isoforms (NKG2D-S and NKG2D-L) are expressed in mice as a result of alternative exon usage and are responsible for differential adaptor associations. The short (NKG2D-S) and long (NKG2D-L) isoforms differ by their 13 NH_2-terminal amino acids. While DAP-10 associates with both NKG2D isoforms, the extended cytoplasmic domain of NKG2D-L prevents the association with DAP-12 (Diefenbach et al., 2002a; Rosen et al., 2004). NKG2D-L is constitutively expressed in resting NK cells. In contrast, the abundance of NKG2D-S increases considerably upon NK cell stimulation with cytokines (Rabinovich et al., 2006).

NKG2D has the ability to interact with a significant number of distinct ligands with affinities ranging from 4 to 800nM (Carayannopoulos et al., 2002a; O'Callaghan et al., 2001; Li et al., 2001). Both chains of the NKG2D homodimer contribute to the interaction with the different monomeric ligands, making contacts with either the α1 or α2 domain of the ligand. Thus, the symmetric, homodimeric NKG2D receptor binds asymmetric ligands, and the contribution of the individual NKG2D chains is unequal (Radaev et al., 2002; Mc Farland et al., 2003). It is surprising that mouse and human NKG2D, which are only 69% identical in their ectodomains, can recognize most ligands of the other species (Mc Farland et al., 2003).

1.1.2. Expression of NKG2D Receptor

The NKG2D receptor is constitutively expressed on most innate immune effector cells of lymphoid origin, including NK cells (Bauer et al., 1999; Vance et al., 1997; Jamieson et al., 2002) most TCR γδ T cells (Jamieson et al., 2002; Bauer et al., 1999), and a large fraction of NKT cells (Jamieson et al., 2002; Gumperz et al., 2002). Functional NKG2D is also found on murine interferon producing killer dendritic cells (IKDC) which are of myeloid origin (Taib et al., 2006; Chan et al., 2006). On adaptive immune system cells, NKG2D is constitutively expressed on all human CD8+ T cells and on activated and memory (but not on naive) CD8+ αß T cells in the mouse (Jamieson et al., 2002). NKG2D is not normally expressed on CD4+ T cells (Table 1) (Coudert and Held, 2006).

Table 1. NKG2D receptor expression in humans and mice

Pattern of the NKG2D receptor expression in human and in mouse

	Human	Mouse
NK cells	All NK cells [7,11]	All NK cells [12,13]
TCRαβ T cells	Naive, activated and memory $CD8^+$ T cells [11]	Activated and memory $CD8^+$ T cells [13]
	Subpopulations of synovial and circulating $CD4^+$	Not expressed on naive $CD8^+$ T cells [13]
	T cells in rheumatoid arthritis patients [17]	Not expressed on $CD4^+$ T cells [13]
TCRγδ T cells	Most blood and IEL TCRγδ T cells [25]	25% of splenic TCRγδ T cells [13]
NKT cells	ND	Large fraction of NKT cells [13,14]
DC	ND	IKDC subset [15,16]
Macrophages	ND	Only mRNA [6,58]

IEL: intestinal intraepithelial lymphocytes, IKDC: interferon producing killer dendritic cells; ND: not determined.

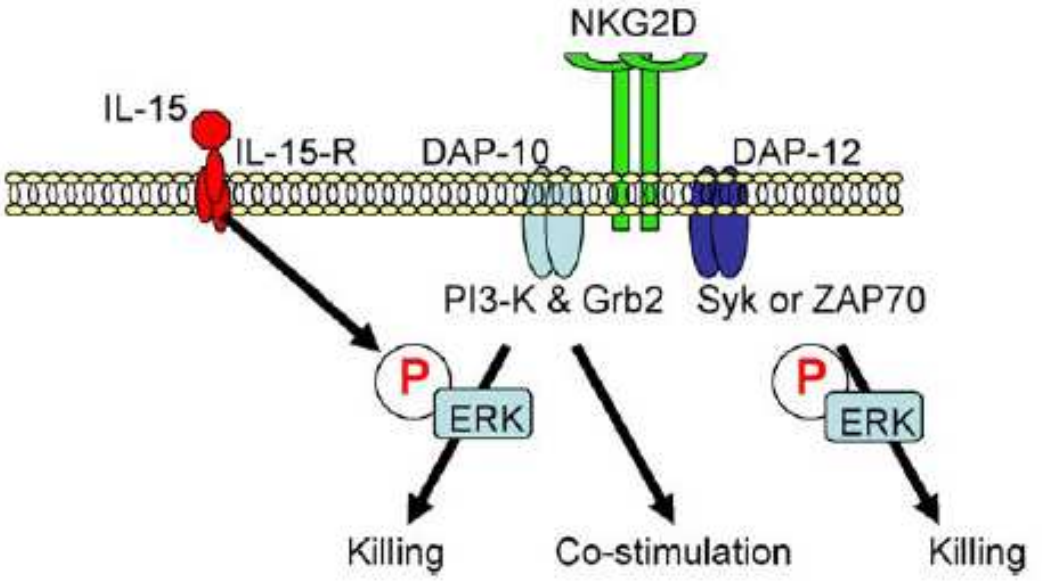
(Coudert and Held. *Sem cancer Biol*. 2006. 16: 333-43)

1.1.3. Function of NKG2D Receptor

Human NKG2D signals exclusively via DAP-10, mouse NKG2D can associate with both DAP-10 and DAP-12. Upon NKG2D engagement, DAP-12 recruits ZAP-70 and Syk protein tyrosine kinases with the help of its immunoreceptor tyrosine-based activation motif (ITAM) (Lanier et al., 1998). It has been observed that mice deficient for DAP-12 retained significant NKG2D-dependent NK cell mediated killing (Diefenbach et al., 2002a; Zompi et al., 2003). Moreover, NK cells from Syk/ZAP-70 deficient mice also retained significant lytic activity. In contrast, DAP-10 lacks an ITAM but instead contains a YINM motif. Upon engagement of human NKG2D, the recruitment of the p85 subunit of PI3-K (Wu et al., 1999) and of Grb2 to DAP-10 occurs (Chang et al., 1999). Both p85 and Grb2 have to be recruited to DAP-10 for full calcium flux and cell-mediated cytotoxicity (Upshaw et al., 2006). The residual lytic activity observed in DAP-12 deficient mice, was abrogated when pharmacological blockade of Src family kinases and phosphatidylinositol 3- kinase (PI3-K), which act down-stream of DAP-10 were used (Colucci et al., 2002), indicating that DAP-10 is crucial for NK cell cytotoxicity. The NKG2D–DAP-10 complex triggers granule release and cytotoxicity following NKG2D crosslinking in human NK cells (Billadeau et al., 2003). Thus, ITAM-independent, DAP-10- dependent signaling triggers NKG2D-dependent cytotoxic function in NK cells (Figure 1.1) (Coudert and Held, 2006).

Besides NK cells, NKG2D receptors are constitutively expressed in human CD8+ T cells and upon activation in mouse CD8+ T cells. Since T cells generally lack DAP-12 expression, NKG2D signaling occurs exclusively via DAP-10 in humans and in mice. In T cells, NKG2D serves as co-stimulatory and in some instances, as primary activation

function. In CD8+ T cells NKG2D engagement enhances T cell activation rather than induces activation (Groh et al., 2001; Verneris et al., 2004; Maasho et al., 2005; Markiewicz et al., 2005). Prolonged exposure of T cells derived from human intestinal epithelium, to high amounts of IL-15 changes NKG2D function and expression by up-regulating DAP-10 (Roberts et al., 2001).



(Coudert and Held. *Sem cancer Biol*. 2006. 16: 333-43)

Figure 1.1. NKG2D function and components of the signaling cascades. NKG2D signals via adaptor protein DAP-10 (human) or DAP-10 and DAP-12/KARAP (mouse). DAP-12 signaling is mediated by ITAMs, which can recruit ZAP-70 or Syk protein tyrosine kinases thereby providing NKG2D with primary activation function. On the other hand, DAP-10 uses a YINM motif to recruit PI3-K and Grb2.

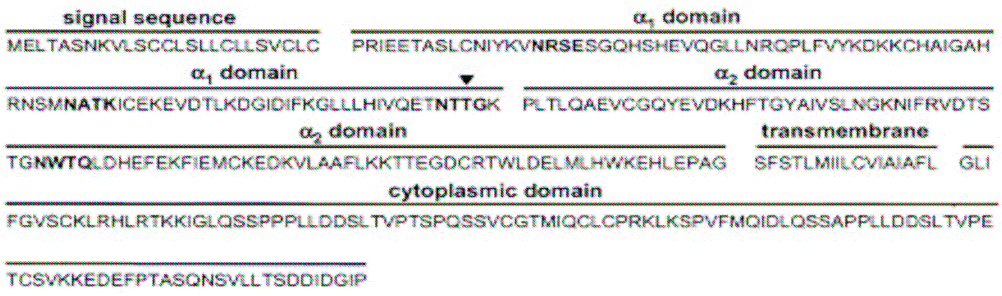
1.2. MULT1 Ligand

1.2.1. Structure of MULT1 Ligand

Murine ULBP-like transcript 1 (MULT1) is a ligand of NKG2D receptor. NKG2D receptors present on the effector cells recognize and bind to MULT1 on target cells. The cDNA sequence of MULT1 consists of a full length open reading frame (ORF) of 1.1 Kb and encoding a protein with a molecular weight of 37.1 kDa. MULT1 protein is a type I transmembrane protein with an N-terminal signal sequence of 25 aa and with two class I MHC like α domains (89 aa and 91 aa, respectively), a transmembrane domain 17 aa and a cytoplasmic domain of 109 aa (Figure. 1.2A). Compared to other NKG2D ligands MULT1 lacks α3- like domain and GPI trans amidation site (Diefenbach et al., 2003; Kawai et al., 2001).

MULT1 protein is a glycoprotein with two ectodomains containing four N-glycosylation sites (Figure. 1.2B, bold) and one O-glycosylation site (Figure. 1.2B, arrowhead). Sequence alignments of MULT1 with other known mouse NKG2D ligands such as H60, Rae1ß and other known human NKG2D ligands such as MICA, ULBP1 and MHC-1, reveals that MULT1 protein is distantly related to known NKG2D ligands, which are in turn distantly related to MHC class I proteins (Figure 1.3C and 1.3D). The sequence identity of MULT1 with known human ligands like MICA and ULBP1, are 16.7% and 29.9% respectively, thus MULT1 is closely related to the members of human ULBP family (Diefenbach et al., 2003).

A



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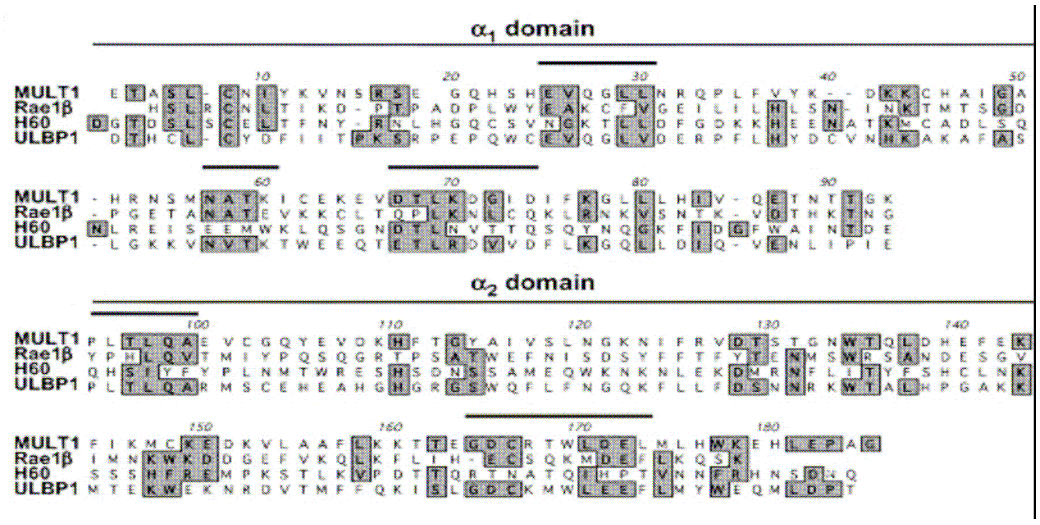


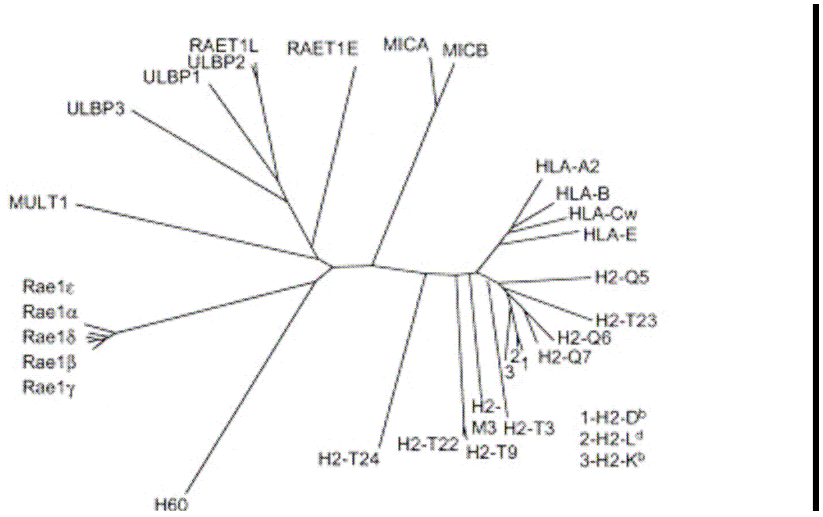
(Diefenbach et al. *Eur J Immunol*. 2003. 33: 381-91)

Figure 1.2. Structure of MULT1 Ligand. (A) MULT1 is a type I transmembrane protein. The domain structure of MULT1 is shown. Potential N-glycosylation sites are printed in bold and the single potential O-glycosylation site is marked by an arrowhead. (B) Comparison of the aa sequences of the ectodomains of MULT1, Rae1ß, H60 and ULBP1 using the ClustalW alignment algorithm. Shaded boxes show similar or identical amino acid residues. The bold lines indicate patches of conserved aa residues between MULT1 and ULBP1.

C

	MULT1				
Rae1β	20.1 (38.2)	**Rae1β**			
H60	22.5 (38.5)	28.3 (44.3)	**H60**		
ULBP1	29.9 (46.7)	22.1 (38.5)	13.9 (29.9)	**ULBP1**	
MICA	16.7 (29.3)	24.0 (37.8)	18.4 (37.3)	26.2 (40.6)	**MICA**
MHC-I	16.7 (30.7)	20.6 (35.2)	24.6 (43.4)	23.8 (40.2)	30.4 (49.4)

D



(Diefenbach et al. *Eur J Immunol*. 2003.33:381-91)

Figure 1.3. Amino acid similarity of MULT1 and other MHC class I-like molecules. (C) Sequence relatedness of MULT1 and other MHC class I-like proteins. The overall percentage of amino acid identity is shown (with similarity shown in parentheses) as determined by alignment using the ClustalW algorithm. (D) The tree dendogram illustrates the relationships between the mouse and human NKG2D ligands and classical and non-classical class I MHC molecules.

1.2.2. Expression of MULT1 Ligand

MULT1 mRNA is detected in a wide variety of tissues such as thymus, spleen, lymph nodes, and to a lesser extent liver and heart, but is not detected in kidney or brain. However, surface expression of MULT1 is not detected in lymph node, liver or kidney cells, suggesting that MULT1 may be regulated post-transcriptionally (Diefenbach et al., 2003).

MULT1 mRNA expression has been observed in multiple tumor cell lines like YAC-1, WEH17.1, A20, P815, S49.1, BW5147 and TRAMP-C1. In WEHI7.1, S49.1 and BW5147 T cell lymphomas, and the P815 mastocytoma, MULT1 is the only known NKG2D ligand expressed in the cells. Other cell lines such as A20 B cell lymphoma and TRAMP-C1 prostate carcinoma coexpress MULT1 and RAE1 ligands (Diefenbach et al., 2003). The finding that MULT1, like RAE1 and H60 family members, is expressed by multiple tumor cell lines suggests that MULT1 contributes to immune surveillance in tumors (Diefenbach et al., 2002b).

1.2.3. Function of MULT1 Ligand

High-level expression of NKG2D ligand on a tumor cell helps the tumor cell to overcome class 1-mediated inhibition of NK cells resulting in its cell lysis (Carayannopoulos et al., 2002). Tumor cells expressing high levels of MULT1 are highly susceptible to NK mediated lysis and strongly induce IFN-γ production in freshly isolated, as well as IL-2 expanded, NK cells. MULT1 also induces the production of nitric oxide in activated macrophages. When ectopically expressed by tumor cells, MULT1 induces a very potent antitumor response *in vivo* resulting in strong rejection of the transduced tumor cells in syngeneic B6 mice. Interestingly, the MULT1-transduced

tumor cells have been observed to prime the mice, rendering them immune to the tumor antigens of the parental tumor cell (Diefenbach et al., 2003). Tumor cells expressing different NKG2D ligands such as MULT1, RAE1ß and/or H60 can induce protective immunity against multiple tumor cell lines such as RMA, B16-BL6 and EL4 (Hayakawa et al., 2002; Gilfillan et al., 2002).

MULT1 protein has a K_D of 6 nM and K_{off} of ~0.006 S^{-1} which is several times lower than RAE1ϵ and H60 (K_D ~ 10-30 nM). MULT1 has a $t_{1/2}$ of ~ 2 min, longer than either H60 (~ 20 s) or RAE1 α-δ (~ 5s). These results indicate that MULT1 binds NKG2D with the highest affinity of all known ligands and has a half life longer than all known NKG2D ligands (Carayannopoulos et al., 2002a). Thus, three distinct MHC class 1-like molecules in the mouse, H60, RAE1ϵ and MULT1 bind NKG2D with high affinity despite low mutual sequence identity (<20%).

Evolutionary advantage of selecting such a complicated receptor ligand system is two-fold. First, the functional consequences of NKG2D engagement are pleiotropic, involving T cell co-stimulation, NK cell activation, macrophage stimulation, and possibly regulation of fetal development (Diefenbach et al., 2000; Groh et al., 2001; Cerwenka et al., 2000; Zou et al., 1996). Precise execution of these diverse functions requires multiple genes with distinct promoter/enhancer sequences, posttranslational controls, and even kinetics of binding. Second, microbes exert enormous selective pressure to diversify immune-related functions, albeit at differing rates (Klein et al., 1993; Khakoo et al., 2000). Recent evidence suggests that human CMV interferes with the NKG2D system using the *UL16* gene product to bind ULBP1 and ULBP2 (Cosman et al., 2001) also, mouse CMV gp40 downregulates H60 (Krmpotic et al., 2002). Pathogen-encoded factors

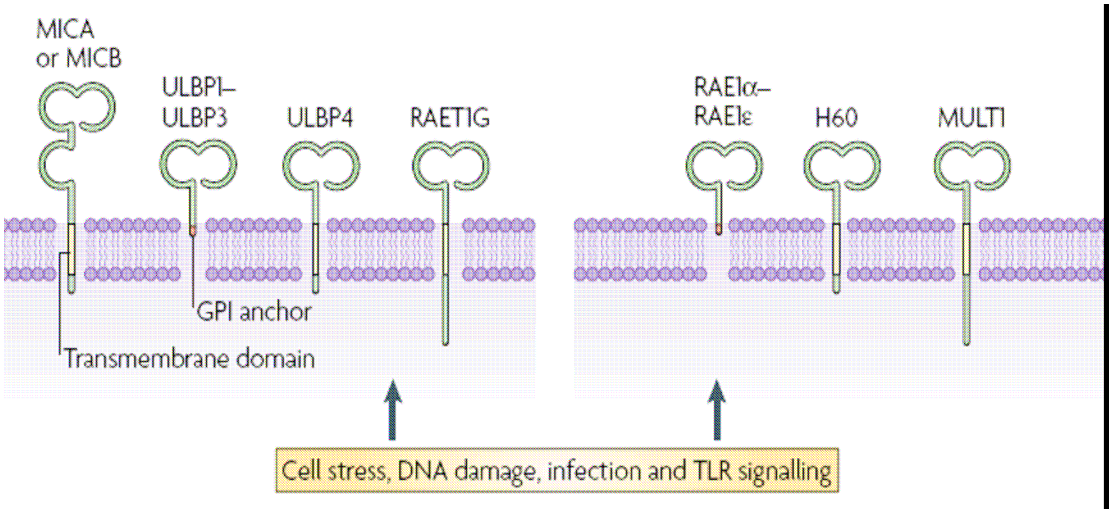
such as these might have selected for NKG2D-binding partners which retain receptor specificity but lack susceptibility to interference or subversion (e.g., ULBP3, which does not bind to UL16), resulting in the current repertoire of dissimilar NKG2D ligands (Carayannopoulos et al., 2002).

1.3. Other NKG2D ligands

NKG2D ligands are structurally similar to MHC class I molecules. The number of NKG2D ligands currently known stands at seven both in humans and mice (Figure 1.4 and Table 1.2). In humans these ligands are grouped into two families; the MHC-class-I-polypeptide related sequence A (MICA) and MICB protein family and the other family includes cytomegalovirus UL16-binding protein (ULBP; also known as RAET1 proteins) consisting of five members (ULBP1–ULBP4 and RAET1G) (Bahram et al., 1994; Bauer et al., 1999; Cosman et al., 2001; Chalupny et al., 2003; Bacon et al., 2004). NKG2D ligands are variable in both their amino acid sequence and domain structure. MICA, for example, only shares 20–25% sequence identity with ULBP molecules (Radosavljevic et al., 2002). In mice there are five retinoic acid early transcript 1 (RAE1) proteins, the minor histocompatibility protein H60 and MULT1 (Figure 1.3) (Deifenbach et al., 2000; Cerwenka et al., 2000; Carayannopoulos et al., 2002; Diefenbach et al., 2003).

All ligands share an MHC-class-I-like α1α2 domain that binds to NKG2D. The MICA and MICB proteins also have an additional α3 domain. The RAE1 proteins in mice and ULBP1, ULBP2 and ULBP3 in humans are glycosylphosphatidylinositol (GPI)- anchored receptors. By contrast, MICA, MICB, ULBP4, RAET1G, H60 and MULT1 possess transmembrane domains and cytoplasmic tails (Eagle and Trowsdale, 2007). The *ULBP* genes are clustered in the telomeric region of human chromosome 6; a corresponding region with NKG2D ligands is found on mouse chromosome 10. The *MICA* and *MICB* genes are localized within the human HLA locus on chromosome 6, which also harbors orthologous MHC class I related *Rae* genes (Radosavlievic et al., 2002). Some NKG2D ligands are polymorphic, over 70 distinct alleles have been

identified in MIC genes (Radosavljevic et al., 2002; Stephens et al., 2001). Even though the number of NKG2D ligands in humans and mice are same, phylogenetic analysis shows that these ligands have almost certainly diversified independently from each other (Raulet et al., 2003; Stephens et al., 2001).



(Eagle and Trowsdale. *Nat rev Immunol*. 2007. 7: 737-44)

Figure 1.4. The seven NKG2D ligands expressed in humans and in mice. All ligands have MHC-class-I-related α1 α2 domains. The MHC-class-I-polypeptide-related sequence A (MICA) and MICB proteins also have an additional α3 domain. The retinoic acid early transcript 1 (RAE1) proteins in mice and cytomegalovirus UL16- binding protein 1(ULBP1), ULBP2 and ULBP3 in humans are glycosylphosphatidylinositol (GPI)-anchored receptors. By contrast, MICA, MICB, ULBP4 (also known as RAET1E), RAET1G, H60 and MULT1 (murine UL16-binding-protein-like transcript 1) possess transmembrane domains and cytoplasmic tails. NKG2D ligands are expressed at the cell surface as a result of cellular stress, such as the response to DNA damage, infection, and as a result of Toll-like receptor (TLR) signaling.

Table 2. NKG2D Ligands

NKG2D ligands	Cell-surface attachment	NKG2D binding affinity
Human		
MICA* (also known as PERB11.1)	Transmembrane domain*	1 μM*
MICB (also known as PERB11.2)	Transmembrane domain	0.8 μM
ULBP1 (also known as RAET1I)	GPI anchor	1.1 μM
ULBP2 (also known as RAET1H and Alcan-α)	GPI anchor	ND
ULBP3 (also known as RAET1N)	GPI anchor	ND
ULBP4 (also known as RAET1E and LETAL)	Transmembrane domain	ND
RAET1G	Transmembrane domain	ND
RAET1L‡	GPI anchor	ND
Mouse		
RAE1α	GPI anchor	690 nM
RAE1β	GPI anchor	345 nM
RAE1γ	GPI anchor	586 nM
RAE1δ	GPI anchor	726 nM
RAE1ε	GPI anchor	ND
H60	Transmembrane domain	26 nM
MULT1	Transmembrane domain	6 nM

(Eagle and Trowsdale. *Nat rev Immunol*. 2007. 7: 737-44)

1.3.1. NKG2D Ligand Expression

The expression of NKG2D ligands are induced by a wide variety of stimuli referred as “cellular stress”, which includes tumorigenesis (Gasser et al., 2005), infection by a variety of pathogens (Lodoen et al., 2006), classic cell-stress stimuli such as heat shock (Venkataraman et al., 2007; Groh et al., 1996) and, also, as a result of Toll-like receptor (TLR) signaling (Nedvetzki et al., 2007). The aberrant expression of NKG2D ligands has also been linked with autoimmune diseases, including rheumatoid arthritis, coeliac disease and autoimmune diabetes (Ogasawara et al., 2003; Meresse et al., 2004; Heu et al., 2004). Not much is know about the precise mechanisms that lead to upregulation of NKG2D ligands. In cancer, NKG2D ligand expression has been associated with activation of the DNA-damage response pathways by genotoxic stress (Gasser et al., 2005). The triggers for switching on of NKG2D-ligand expression during infection have not yet been well defined (Eagle and Trowsdale, 2007).

1.3.2. NKG2D Ligands and Tumor

A large fraction of tumor cells express NKG2D ligands constitutively. MICA/B expression is detected on many types of epithelial tumor cell lines of different tissue origins (Bauer et al., 1999; Gorh et al., 1996; Jinushi et al., 2003; Gorh et al., 1999; Gorh et al., 1998). In contrast, ULBPs are preferentially expressed on T cell leukemia cell lines (Pende et al., 2002) as well as on freshly isolated lymphoid leukemia cells. RAE-1 and H60 are up-regulated in skin treated with carcinogens (Girardi et al., 2001) and are found on skin, renal and lung carcinoma cell lines (Girardi et al., 2001; Smyth et al., 2002). The murine NKG2D ligands H60 and RAE-1 are also found on numerous hematopoietic tumor cell lines (Cerwenka et al., 2000; Lowin-Kropf et al., 2002). In some cases,

NKG2D ligand up-regulation has been observed to be associated with transformation, having both oncogene and tumor suppressor roles. Embryonic fibroblasts deficient for JunB show an enhanced expression of RAE-1ϵ and MULT1. JunB exerts tumor suppressor activity through the negative regulation of c-jun function (Deng et al., 1993). The chronic activity of the DNA damage response pathways have also been implicated to be responsible for the constitutive expression of NKG2D ligands such as RAE-1, MULT1 in mouse lymphoid tumor cell lines (Gasser et al., 2005).

1.3.3. Escape Mechanism by Tumors

Tumors have developed many distinct mechanisms that would allow them to escape the detection by NKG2D expressing effector cells. As cancer progresses, immune pressure on the tumor may lead to selection of cells devoid of NKG2D ligands. It has been observed in cancer patients that most primary tumors seem to express NKG2D ligands, whereas more advanced tumors and metastases express very low level ligand (Vetter et al., 2004; Raffaghello et al., 2005). This leads to selection of variants with low levels of NKG2D ligands.

NKG2D ligand cleavage has been observed in some tumors. Metalloproteinases can cleave MICA/B off the cell surface of tumor cells (Salih et al., 2002; Doubrovina et al., 2003) reducing their cell surface levels and limiting recognition by NKG2D-expressing effector cells. In addition, soluble NKG2D ligands such as MICA in the serum, upon binding to NKG2D, induce the internalization and lysosomal degradation of the NKG2D receptor on CD8+ T cells and NK cells (Doubrovina et al., 2003; Jinushi et al., 2003), reducing the efficiency of NKG2D recognition.

TGF-ß, a major immunosuppressive cytokine produced by tumor cells also decreases the surface expression of MICA, effecting tumor cell recognition by CD8+ T and NK cells (Friese et al., 2004). *In vitro* experiments have shown that NK cells cultured in the presence of TGF-ß down-regulated NKG2D receptor expression (Castriconi et al., 2003; Lee et al., 2004). It has been reported that IFN-γ can render certain susceptible target cells resistant to NK cell responses *in vitro* and *in vivo.* This has been attributed to an up-regulation of MHC class I molecules, which are recognized by inhibitory NK cell receptors (Welsh et al., 1981; Bui et al., 2006).

Research has also shown that sustained NKG2D ligand encounters can promote NK cell dysfunction *in vitro* and *in vivo*. The enforced constitutive expression of NKG2D ligands such as RAE-1ß, RAE-1ϵ and MICA as transgenes in mice impair NKG2D functions *in vivo* (Wiemann et al., 2005). This observed NKG2D dysfunction also raises the possibility that CD8+ T cells and human NK cells may similarly be susceptible to inactivation.

1.3.4. NKG2D Ligands Diversity

Even though NKG2D ligands are not functionally equivalent, their roles are redundant to some extent (Komatsu et al., 1999). The evolutionary advantage for the presence of diverse NKG2D ligands can be explained with 3 possible reasons: 1) escaping immune recognition, 2) evading tumor responses and 3) tissue specific function (Eagle and Trowsdale, 2007).

1.3.4.1. Escaping Immune Recognition

In nature, both host and pathogen are under natural selection pressure to diversify and refine their defence strategies in response to improvements made by their competitor.

It has been observed that viruses, such as human cytomegalovirus (HCMV), MCMV, Influenza A, and Epstein–Barr virus, induce NKG2D ligands in infected cells (Lodoen et al., 2006; Draghi et al., 2007; Pappworth et al., 2007). As an escape mechanism HCMV deploys immunoevasin proteins such as UL16 that can bind to MICB, ULBP1, ULBP2 and RAET1G and prevent the expression of NKG2D ligands, helping the virus to escape immune recognition (Cosman et al., 2001; Chalupny et al., 2003; Bacon et al., 2004; Welte et al., 2003; Wu et al., 2003; Dunn et al., 2003; Vales et al., 2003; Rolle et al., 2003). Since viruses have evolved mechanisms to evade immune system, the host responds by developing variants of NKG2D ligands by gene duplication and going beyond the reach of the virus (Zou et al., 2005; Chalupny et al., 2006).

1.3.4.2. Evading Tumor Responses

One of the main functions of NKG2D is to participate in antitumour immune response and immune surveillance (Diefenbach et al., 2001; Cerwenka et al., 2001; Smuth et al., 2005). Tumors have evolved many mechanisms that would allow them to avoid NKG2D-mediated immune attack. Some of these mechanisms are, shedding soluble NKG2D ligands like MIC from their cell surface or down regulating MICA expression, producing transforming growth factor-β (TGF-β), effectively anergizing NKG2D-mediated immune recognition and switching off the expression of NKG2D ligands as they progress (Groh et al., 2002; Castriconi et al., 2003; Lee et al., 2004; Vetter et al., 2004).

Possessing multiple NKG2D ligands under the control of different cancer-related stress-response would provide the host with a fail-safe alert mechanism. Since expression of an individual NKG2D ligand may be lost as part of a cancer immunoediting process,

the advantage of having more than one NKG2D ligand is that it would be much more difficult for the cancer to switch off multiple NKG2D ligands at once and help a host in detecting tumors (Eagle and Trowsdale, 2007).

1.3.4.3. Tissue Specific Functions

In humans, MICA and RAET1G proteins are expressed constitutively in the polarized epithelial-cell layer of the gut where they are likely to come in contact with pathogens (Groh et al., 1996). MICA and ULBP1–ULBP3 are expressed by normal airway epithelial cells (Borchers et al., 2006). RAE1 transcripts were reported in mouse embryonic tissues such as embryonic brain (Nomura et al., 1996). MICA has a specialized role in the gut, whereas ULBP4 may have a related but equally specialized role in the skin (Groh et al., 1996).

NKG2D ligands, like RAE-1 or MICA/B are not expressed in most tissues in healthy adult mice and humans (Nomura et al., 2996; Groh et al., 1996). ULBP1-3 mRNA is expressed in various healthy tissues (Cosman et al., 2001) and ULBP4 mRNA expression is detected in the skin (Jan Chalupny et al., 2003). Likewise, MULT1 mRNA is expressed in a wide variety of tissues such as thymus, spleen, lymph node, liver and heart (Carayannopoulos et al., 2002; Diefenbach et al., 2003). RAE-1ß and RAE-1δ mRNA expression is detected in the early embryos, particularly in the brain (Nomura et al., 2006). Bone marrow cells express low levels of RAE-1 and H60 but not MULT1 (Ogasawara et al., 2005). Some NKG2D ligands are constitutively expressed in a restricted number of normal cells, indicating that they may have evolved unique tissue-specific functions that are not necessarily relate to their role in immune surveillance. Hence, it seems that NKG2D-ligand diversity may have allowed for the evolution of

individual ligands with functional specialities that are specific for different cell types and tissues (Eagle and Trowsdale, 2007).

1.3.5. NKG2D Dependent Immunotherapy

As NKG2D receptor recognizes ligands that are constitutively expressed on many transformed but not on most normal cells, this provides an opportunity for their use in immunotherapy of cancer. Many different therapeutic strategies are being developed that use NKG2D receptor-ligand interactions (Coudert and Held, 2006).

Chimeric anti-tumor mAb/NKG2D-ligand, with the antibody portion of the chimeric protein specific tumor cell targeting, while the NKG2D ligand re-directs NKG2D-expressing effector cells to the site of tumor have been generated. An anti-CEA (carcinoembryonic antigen)/MICA chimera and H60/anti-CEA specifically bind CEA+ human tumor cells and enhanced the *in vitro* lysis by NK cells in a NKG2D-dependent manner (German et al., 2005; Zhou et al., 2005; Zhou et al., 2005).

NKG2D receptor fused to the cytoplasmic portion of CD3ζ has been expressed in splenic T cells. This chimeric NKG2D receptor/CD3ζ protein confers primary activation function to T cells in response to NKG2D ligand-bearing tumor cells *in vitro* and induces memory response to NKG2D ligand-negative tumor cells (Zhang et al., 2005).

Cytokines, such as IL-21, IL-12 and IFN-α, exert anti-tumor effects by up-regulating NKG2D cell surface expression have been used in some tumor models with positive results. Mice treated with IL-21, have been observed to reject tumors cells more efficiently than control mice. IL-21, up-regulated NK cell mediated NKG2D-dependent tumor cell lysis *in vitro* and the rejection of grafted tumor cells *in vivo* (Takaki et al.,

2005). Similar results were observed with IL-12 and IFN-α (Reiter et al., 1993; Zhang et al., 2005).

Irradiation or alkylating compounds commonly used in chemotherapy treatment of cancer activate the DNA damage response pathway and can induce the expression of NKG2D ligands in mouse and human cells. ULBP3 and MICA are up-regulated by transretinoic acid in patients with chronic B cell lymphocytic leukemia (B-CLL) (Poggi et al., 2004). These treatments rendered cells susceptible to killing by autologous NKG2D expressing effector cells and can be used as part of the combination therapy regime with any of the above discussed approaches (Coudert and Held, 2006).

NKG2D recognition of multiple stress-inducible host proteins is of considerable research interest since this system has potential to be manipulated for therapeutic purposes. Tumor cells expressing NKG2D ligands have been shown to be susceptible to NK cell mediated lysis, to induce a very potent antitumor response, and to provide protective immunity *in vivo* (Carayannopoulos et al., 2002; Carayannopoulos et al., 2002a; Diefenbach et al., 2003; Kotturi et al., 2008; Eagle and Trowsdale, 2007).

NKG2D recognition system has potential as a promising entry point to induce and/or improve immune responses against cancer for the following reasons. First, NKG2D ligands are generally poorly and only transiently expressed on healthy tissues, while they are constitutively expressed at significant levels on tumor cells. Second, NKG2D ligands are expressed on a broad variety of tumor cells of distinct tissue origins. Third, in situations where NKG2D ligands are poorly expressed, it may be possible to enhance their expression using radiation and/or chemotherapies. Fourth, NKG2D is expressed on all NK cells and also on a substantial fraction of T lymphocytes, providing a

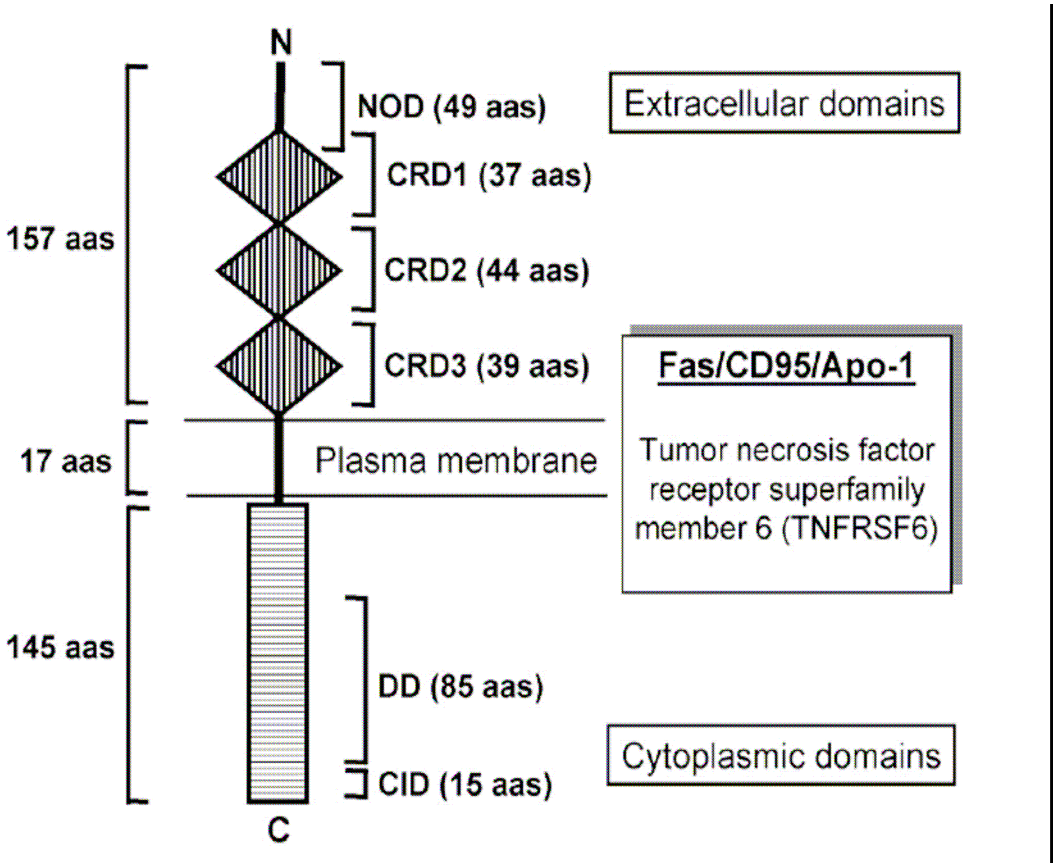
large number of potential effector cells. Fifth, cytokines may be used to improve NKG2D function. Finally, NKG2D-mediated adoptive immunotherapy should, in principal, be applicable to all individuals as the NKG2D receptor is monomorphic (Coudert and Held, 2006).

A great deal has yet to be understood about the involvement of NKG2D ligands in disease. A lot is known about the function of MICA; however, investigation of the expression and function of other NKG2D ligands with transmembrane domains and cytoplasmic tails is needed. A better understanding of the differences in the functional properties of NKG2D ligands and the pathways that regulate NKG2D ligand expression could help us develop better therapeutic interventions that could induce NKG2D-mediated immune responses and more efficient therapeutic strategies in the future (Eagle and Trowsdale, 2007).

1.4. Fas/CD95

1.4.1. Structure of Fas/CD95

CD95/APO-1/Fas receptor is a member of the tumor necrosis factor (TNF) superfamily of receptors. Its main function in signaling is the induction of apoptosis (Schulze-Osthoff et al., 1998). CD95/Fas receptor is expressed on various human cells, including myeloid cells, T lymphoblastoid cells, and diploid fibroblasts. Fas (Figure 1.5) is a 48-kDa type I transmembrane receptor of 319 amino acids with a single transmembrane domain of 17 amino acids, an N-terminal cysteine-rich extracellular domain and a C-terminal cytoplasmic domain containing 145 amino acids relatively abundant in charged amino acids. The cytoplasmic portion of Fas contains a domain called "*death domain*" of about 85 amino acids. The "*death domain*" is very crucial as it plays a role in transmitting the death signal from the cell's surface to intracellular pathways and mediates signaling through protein–protein interactions (Nagata, 1997). The tertiary structure of the Fas death domain consists of six antiparallel, amphipathic α helices. Helices α1 and α2 are centrally located and flanked on each side by α3/α4 and α5/α6. This leads to an unusual topology in which the loops connecting α1/α2 and α4/α5 cross over each other. The presence of a high number of charged amino acids in the death domain is responsible for interactions between death domains (Huang et al., 1996; Mollinedo and Gajate, 2006). CD95 receptors are expressed on the surface of cells as preassociated homotrimers (Papoff et al., 1999; Siegel et al., 2000). These interactions were found to be mediated by a domain in the N-terminus, within the first of the cysteine-rich domains called PLAD (preligand binding assembly domain) (Papoff et al., 1999; Siegel et al., 2000). CD95 receptors only function as trimers (Kischkel et al., 1995).



(Mollinedo and Gajate. *Drug Resist Updates*. 2006.9:51–73)

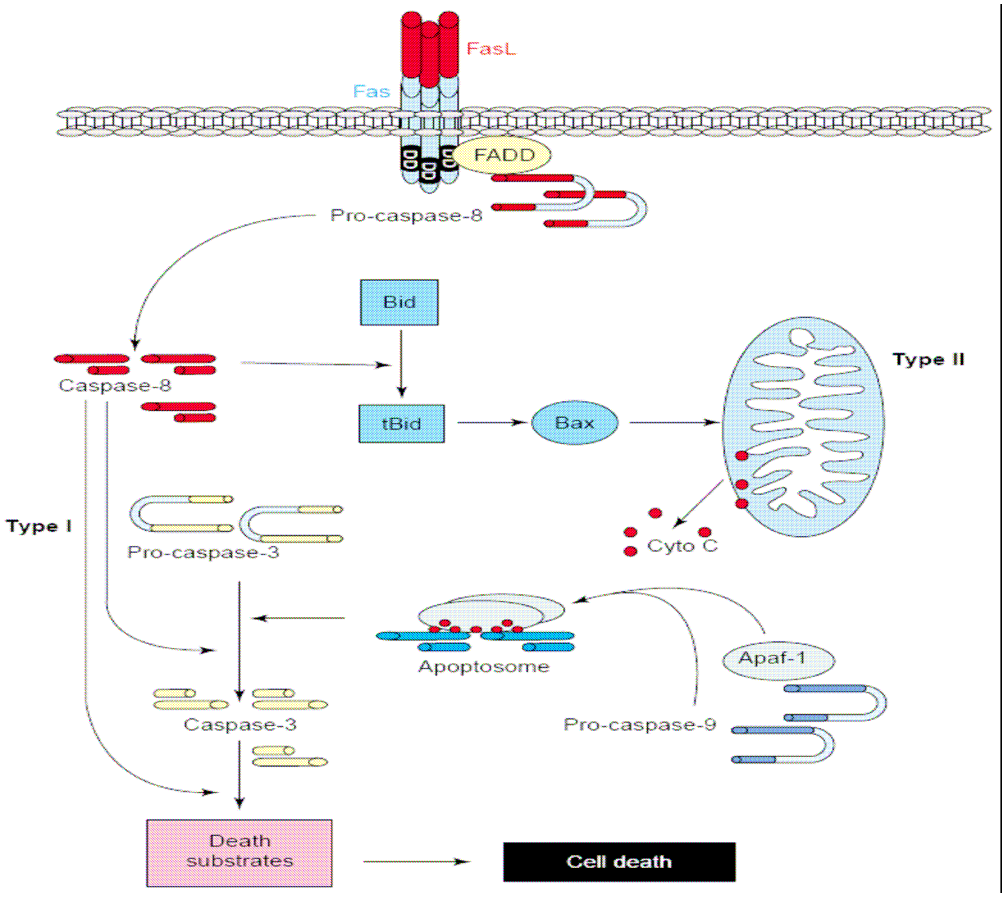
Figure 1.5. Schematic diagram of the human Fas death receptor. Mature human Fas protein consists of 319 amino acids (aas) with an N-terminal extracellular domain of 157 aas, a short transmembrane region (17 aas) and a C-terminal cytoplasmic domain of 145 aas. An N-terminal extracellular oligomerization domain (NOD) of 49 aa (Arg-1 to Pro-49) responsible for the FasL-independent oligomerization of the receptor. Three cysteine-rich domains (CRD1-Gln31 to Val-67, CRD2-Pro-68 to Cys-111-, and CRD3-Arg-112 to Lys150-) containing four, six and eight Cys residues in each domain, respectively. A cytoplasmic death domain (DD) of 85 aas (Ser-214 to Ile-298) is crucial for apoptotic signaling. The last 15 amino acids (Asp-305 to Val-319) of the Fas amino acid sequence represent a C-terminal inhibitory domain (CID).

The *lpr* gene in mouse encodes the structural gene for Fas receptor and is located on chromosome 19. Mice carrying the lymphoproliferation (lpr) point mutation convert Ile-225 to Asn-225 in the cytoplasmic region of the mouse Fas antigen. Mice expressing the defective Fas antigen are subjected to a lymphoproliferation syndrome showing lymphadenopathy and a systemic lupus erythematosus-like autoimmune disease (Watanabe-Fukunaga et al., 1992). In humans, patients with autoimmune lymphoproliferative syndrome (ALPS) type 1A have heterozygous germ line mutations in the APT-1 *Fas* gene. The mutation in human Fas (V238N) leads to inhibition of apoptosis, together with a dramatic inhibition in Fas death domain self-association, binding to FADD (Huang et al., 1996) and lymphocytes are resistant to Fas-induced apoptosis (Siegel et al., 2000; Frederiksen et al, 2000). In mice and humans, these point mutations in Fas alter protein structure of the death domain affecting its function and indicating that the intracellular portion of the Fas molecule is critical for death receptor oligomerization and required for apoptotic activity (Mollinedo and Gajate, 2006).

The molecular ordering of the general events in Fas-mediated signaling after binding of Fas to its cognate ligand include four successive steps (Algeciras-Schimnich et al., 2002; Mollinedo and Gajate, 2006). 1) FasL-induced formation of Fas microaggregates at the cell surface. 2) Recruitment of FADD to form a DISC in an actin filament dependent manner. 3) Formation of large Fas surface clusters positively regulated by DISC-generated caspase-8. 4) Actin filament-dependent internalization of activated Fas through an endosomal pathway.

As discussed above, CD95 contains a protein–protein interaction domain in its cytoplasmic region termed the death domain (DD) (Peter et al., 1999). When the

preassociated receptor is ligated, CD95 becomes competent to form the DISC. In the DISC, the adaptor molecule Fas-associated DD containing protein (FADD) binds to CD95 through homotypic interaction of its DD with the DD of CD95 (Kischkel et al., 1995; Chinnaiyan et al., 1996). In addition to its DD, FADD contains another protein–protein interaction domain at its N-terminus, termed the death effector domain (DED). This domain recruits caspases containing these DED domains to the DISC. Both the DD and DED enable proteins containing the same domains to interact with one another. FADD interacts with procaspase-8 through its DED (Muzio et al., 1996; Boldin et al., 1996). Thus, activation of Fas results in receptor aggregation and formation of "death-inducing signaling complex" (DISC) (Kischkel et al., 1995), containing trimerized Fas, FADD and procaspase-8. The apoptotic caspases perform different roles. The effector caspases, which include caspases 3, 7, and 6 are responsible for most of the cleavage of proteins characteristic of apoptosis and are responsible for cleavage of proteins which induce the major morphological changes observed during programmed cell death (Ernshaw et al., 1999). Caspase-8 is a main initiator caspase and transduces the first signals of apoptosis in CD95 signaling and is expressed as two isoforms, caspase-8/a and -8/b, which are both recruited to the activated CD95 receptor (Scaffidi et al., 1997). Two molecules (FADD and caspase-8) are the key components of the CD95 DISC (Figure 1.6). Once procaspase-8 associates with FADD, the high local concentration of procaspase-8 leads to its autoproteolytic cleavage and activation (Medema et al., 1997; Salvesan and Dixit, 1999). Following the autoproteolytic cleavage of the enzyme, caspase-8 is released from the DISC as an active heterotetramer (Peter and Krammer, 2003).



(Houston and O'Connell. *Curr opin pharma*. 2004. 4; 321-24)

Figure 1.6. Apoptosis signaling via the Fas receptor. Binding of FasL to Fas induces the recruitment of FADD and pro-caspase-8 to the cytoplasmic tail of Fas, and the formation of the DISC. At the DISC, caspase-8 is activated. In type I cells, sufficient caspase-8 is generated to activate pro-caspase-3 directly. However, in type II cells, activation of pro-caspase-3 occurs indirectly through cleavage and activation of Bid. Truncated Bid (tBid) triggers the release of pro-apoptotic molecules from the intermembrane space of mitochondria. Released cytochrome c (cyto c) clusters with Apaf-1 and pro-caspase-9 in the presence of dATP to activate caspase-9. Activated caspase-9 cleaves and activates caspase-3, triggering a caspase cascade, which ultimately results in the death of the cell.

Studies have shown that Fas together with FADD and procaspase-8 forming DISC are translocated into lipid rafts following activation with FasL (Hueber et al., 2002; Scheel-Toellner et al., 2002). The importance of lipid rafts in Fas-mediated apoptosis was supported by finding that expression of membrane sphingomyelin enhanced Fas-mediated apoptosis through increasing DISC formation, activation of caspases, efficient translocation of Fas into lipid rafts, and subsequent Fas clustering (Miyaji et al., 2005).

Another component of the DISC observed in the first description of this complex is Cap3. Cap3 serves to establish the correct conformation of the DISC (Kischkel et al., 1995). Caspase-10 is another caspase that associates with FADD through the homotypic association with its DED, and is involved in DISC formation (Kischkel et al., 1995). c-$FLIP_L$ is another protein that is part of DISC, which depending on its expression level, can either, activate caspase-8/10 in the DISC (at low concentrations) or block it (at high concentrations) (Chang et al., 2002; Peter and Krammer, 2003).

Depending on the quantity of caspase-8 produced at the DISC, apoptosis can occur through two different pathways (Scaffidi et al., 1998). Type I cells have a high production of caspase-8 at the DISC and process caspase-3 directly, leading to activation of DISC and ultimate apoptosis of the cell. In Type II cells, only a small amount of caspase-8 is produced in the DISC. The DISC in these cells is formed quite poorly, little FADD is recruited and little active caspase-8 induced. Apoptosis in these cells is dependent on the cleavage of the BH3 domain containing Bcl-2 family member BID (Li et al., 1998; Luo et al., 1998) and results in a proapoptotic fragment tBID. This fragment induces the proapoptotic functions of the mitochondria by causing aggregation of Bax or Bak (Korsmeyer et al., 2000). This aggregation results in loss of cytochrome c from the

mitochondrial intermembrane space. The adaptor APAF-1, cytochrome c, and dATP then form a large protein complex, the apoptosome, and activate caspase-9 (Li et al., 1997). Caspase-9, thus formed, activates caspase-3 which then results in apoptosis of the cell. Thymocytes and T cells are Type I cells and hepatocytes are Type II cells (Figure 1.6) (Yin et al., 1999; Lindsten et al., 2000; Wei et al., 2001; Lacronique et al., 1996; Rodriguez et al., 1996; Strasser et al., 1995; Peter and Krammer, 2003).

1.5. Fas Ligand (FasL)

FasL belongs to the TNF family and can be found as a 40-kDa membrane-bound or a 26-kDa soluble protein (Nagata, 1997; Suda et al., 1993). Rat FasL has no signal sequence at the NH_2 terminus, but has a domain of hydrophobic amino acids in the middle of the molecule, indication that it is a type 11 membrane protein with the COOH-terminal region outside the cell. Mouse and human FasL are 76.9% identical at the amino acid sequence level and are functionally interchangeable. A stretch of about 150 amino acids in the extracellular region of FasL show significant homology to the corresponding region of other members of the TNF family which includes TNF, lymphotoxin (LT), CD40 ligand, CD27 ligand, CD30 ligand and OX40 ligand. A single FasL gene is located on human and mouse chromosome 1 in the neighborhood of the OX40 ligand gene (Baum et al., 1994). Generalized lymphoproliferative disease (Gld) mice carry autosomal recessive mutation on mouse chromosome 1. There is a point mutation near the COOH-terminus of the coding region. This mutation changes a phenylalanine to a leucine in the extracellular region and abolishes the ability of FasL to bind to Fas receptor (Nagata, 1997; Suda et al., 1993).

Fas/FasL system is the major regulator of apoptosis at the cell membrane in mammalian cells through a receptor/ligand interaction (Mollinedo and Gajate, 2006). Stimulation of Fas by FasL results in receptor aggregation (Chan et al., 2000) of previously assembled trimers (Papoff et al., 1999; Siegel et al., 2000) and recruitment of the adaptor molecule FADD (Chinnaiyan et al., 1995; Mollinedo and Gajate, 2006).

1.5.1 Expression of FasL

FasL has been found to be expressed on cells of the lymphoid/myeloid lineage, including activated T cells and natural killer (NK) cells, where it plays an important role in immune homeostasis, T cell and NK cell-mediated toxicity (Brunner et al., 2003). FasL is also found to be expressed in sites such as the eye (Griffith et al., 1995) and testis (Bellgran et al., 1995) contributing to immune privilege by inducing apoptosis of infiltrating proinflammatory immunocytes (Houston and O'Connell, 2004).

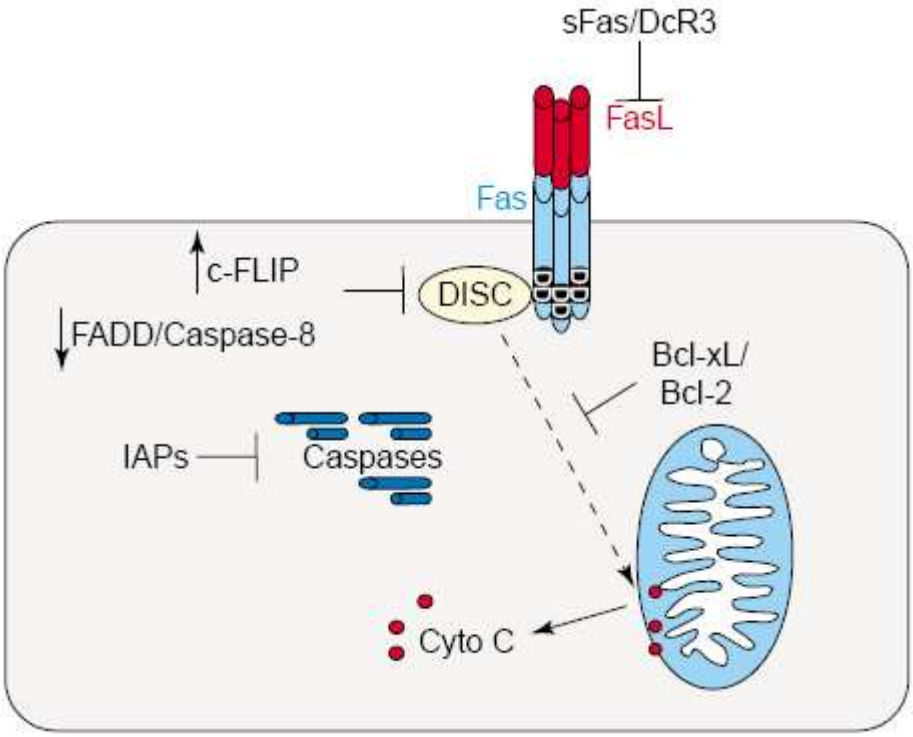
FasL expression has also been observed in a variety of tumor cells indicating a possibility that FasL could mediate immune privilege in human tumors by inducing apoptosis of anti-tumor lymphocytes and also, stimulate proliferation of tumor cells (Houston and O'Connell, 2004). Tumor expression of FasL was first demonstrated in the colon carcinoma cell line SW620, where it could induce apoptosis of Fas-sensitive lymphoid cells *in vitro* (O'Connell et al., 1996). A functional FasL expression has also been reported on numerous tumors of varying origin including colon (Okada et al., 2000), gastric (Zheng et al., 2003), lung (Niehans et al., 1997) carcinoma, and astrocytoma (Saas et al., 1997). Tumor cells expressing FasL demonstrated the ability to kill Fas-sensitive target cells when co-cultured *in vitro*. Apoptosis of tumor-infiltrating lymphocytes (TILs) has also been detected *in situ* within FasL-expressing human tumors such as esophageal carcinoma (Houston and O'Connell, 2004; Okada et al., 2000; Zheng et al., 2003; Niehans et al., 1997).

FasL expression was found to be higher in metastatic tumors than in primary ones. In breast and cervical tumors, high FasL expression was significantly associated with lymph node metastases (Mottolese et al., 2000; Kase et al., 2003) whereas, stronger

FasL expression was found in liver metastases of colon cancer relative to the primary tumor (Mann et al., 1999; Belluco et al., 2002; Chopin et al., 2003; Houston and O'Connell, 2004).

1.5.2. Inhibition of Apoptosis

One of the hallmarks of cancer is resistance to apoptosis (Hanahan and Weinberg, 2000). Most cancer cells are relatively resistant to apoptosis mediated through Fas. Fas-mediated apoptosis can be inhibited at different points in the apoptotic signaling pathway. Cells may secrete soluble 'decoy' receptors, such as sFasL or DcR3, which can bind to FasL and inhibit FasL-induced apoptosis (Pitti et al., 1998). FADD-like interleukin-1ß-converting enzyme inhibitory protein (cFLIP) binds to the DISC and prevents the activation of caspase-8 (Irmler et al., 1997). Reduced expression of FADD (Tourneur et al., 2003) or caspase-8 (Fulda et al., 2001) can also inhibit Fas signaling. IAPs present in the cytosol can bind to and inhibit caspases and upregulation of Bcl-2 or Bcl-xL can render type II cells resistant to Fas-mediated apoptosis. Cytochrome c and inhibitor-of-apoptosis protein (IAP) can inhibit apoptosis (Figure 1.7) (Igney and Krammer, 2002; O'Connell et al., 2000). Thus, because of their insensitivity to Fas-mediated apoptosis, tumor cells can express FasL without undergoing apoptosis (Houston et al., 2003). It has been observed that resistance to Fas-mediated apoptosis protects tumor cells not only from tumor-expressed FasL but also from FasL expressed as a cytotoxic mediator by infiltrating anti-tumor T cells and NK cells (Elsasser-Beile et al., 2003; Houston and O'Connell, 2004).



(Houston and O'Connell. *Curr opin pharma*. 2004.4; 321-24)

Figure 1.7. Mechanisms of resistance to Fas-mediated apoptosis. Fas-mediated apoptosis can be inhibited at different points in the apoptotic signalling pathway. Cells may secrete soluble 'decoy' receptors, such as sFasL or DcR3, which can bind to FasL and inhibit FasL-induced apoptosis. FADD-like interleukin-1b-converting enzyme inhibitory protein (FLIP) binds to the DISC and prevents the activation of caspase-8; reduced expression of FADD or caspase-8 can also inhibit Fas signalling. IAPs present in the cytosol can bind to and inhibit caspases, whereas upregulation of Bcl-2 or Bcl-xL can render type II cells resistant to Fas-mediated apoptosis.

1.5.3. Immunotherapy

Death receptors are members of the tumor necrosis factor (TNF) receptor gene superfamily, consisting of more than 20 proteins with a broad range of biological function, including regulation of cell death, survival, differentiation or immune regulation (Debatin and Krammer, 2004). Death receptors share regions of high homology including cysteine-rich extracellular domains and a cytoplasmic domain of about 80 amino acids called death domain (DD), which plays a crucial role in transmitting the death signal from the cells surface to intracellular signaling pathways (Mollinedo and Gajate, 2006).

The death receptors which have potential to induce apoptosis are Fas, TNF receptor 1 (TNFR1), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1), TRAIL-R2, death receptor 4 (DR4) and death receptor 5 (DR5). Due to their potential to induce apoptosis, ligands such as TNF, Fas ligand (FasL) and TRAIL are interesting candidates for antitumor therapy (Shankar and Srivastava, 2004; van Geelen et al., 2004; Mollinedo and Gajate, 2006). However, ligands of the TNF family and their cognate receptors have been found to play a key role in liver pathogenesis and have become a major challenge for the clinical application of death receptor-targeted therapy (Faubion and Gores, 1999; Hohenberger and Tunn, 2003; Ogasawara et al., 1993; Tanaka et al., 1997; Jo et al., 2000; Leverkus et al., 2000; Nesterov et al., 2002).

Conventional chemotherapy is based on the perception that malignant cells have uncontrolled proliferation. The rather modest impact of antiproliferative drugs in the clinic is not surprising since many tumors have a low growth capacity. In addition, exposure of normal tissues that have a high rate of cellular proliferation, such as the bone marrow, the gastrointestinal epithelial cells and the cells of the hair follicles, to anti-

proliferative drugs leads to major toxicities. The effectiveness of anticancer drugs reflects the ability of tumor cells to detect and respond to the perturbation induced by the drug (Gajate and Mollinedo, 2002; Mashima and Tsuruo, 2005). The failure of some tumor cells to die following drug treatment and their resistance to drugs is due to their resistance to apoptosis as tumors cells have defects in triggering their own death by apoptosis (Mollinedo and Gajate, 2006). If liver toxicity could be circumvented, Fas would be a worthy anticancer target due to its potent proapoptotic activity and widespread expression in tumor cells (Mollinedo and Gajate, 2006).

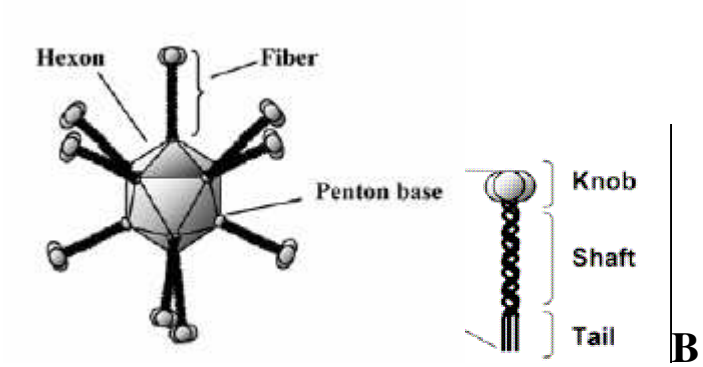
1.6. Adenovirus and Cancer Gene Therapy

Different viral vectors like lentivirus, retrovirus, pox virus, herpes simplex virus-1, vaccinia virus, adeno-associated virus (AAV) and adenovirus have been used for experimental cancer gene therapy (Young et al., 2006). These viral vectors have been used individually and in combination with conventional therapies to treat cancers that are refractory to just conventional therapy (surgery, radiation, and chemotherapy). Of all the vectors used, adenoviruses are one of the most widely accepted viral agents for cancer gene therapy. The features of adenovirus that make them well suited for gene therapy are: its capacity for gene transfer (up to 7-8 Kb), *in vivo* stability, inability to integrate into host genome, ability to transduce dividing and non dividing cells, a well characterized genome and relative ease of production, purification and manipulation. From a clinical point of view, adenovirus is endemic in the human population and its natural pathogenicity is associated with mild respiratory infections, and therefore, manifests a well defined safety profile (Gomes and Tong, 2006; Young et al., 2006).

Adenovirus was first isolated and cultured from human tonsils and adenoid tissues (Garnett et al., 2002; Neumann et al., 1987; Zubko et al., 1976). Currently, 51 human adenovirus serotypes have been identified and grouped into six subgroups (A-F) of which the most widely studied serotype are group C types 2 and 5. Adenovirus is a non-enveloped icosohedral particle which carries a 36 Kb double stranded DNA genome. The capsid consists of three main components: hexon, penton and fiber (Figure 1.8). Hexon is the most abundant structural protein which appears to play a role in coating the virus. The pentameric structure called penton is known to mediate viral internalization. The fiber protrudes from the penton bases and appears to play a role in viral attachment to the

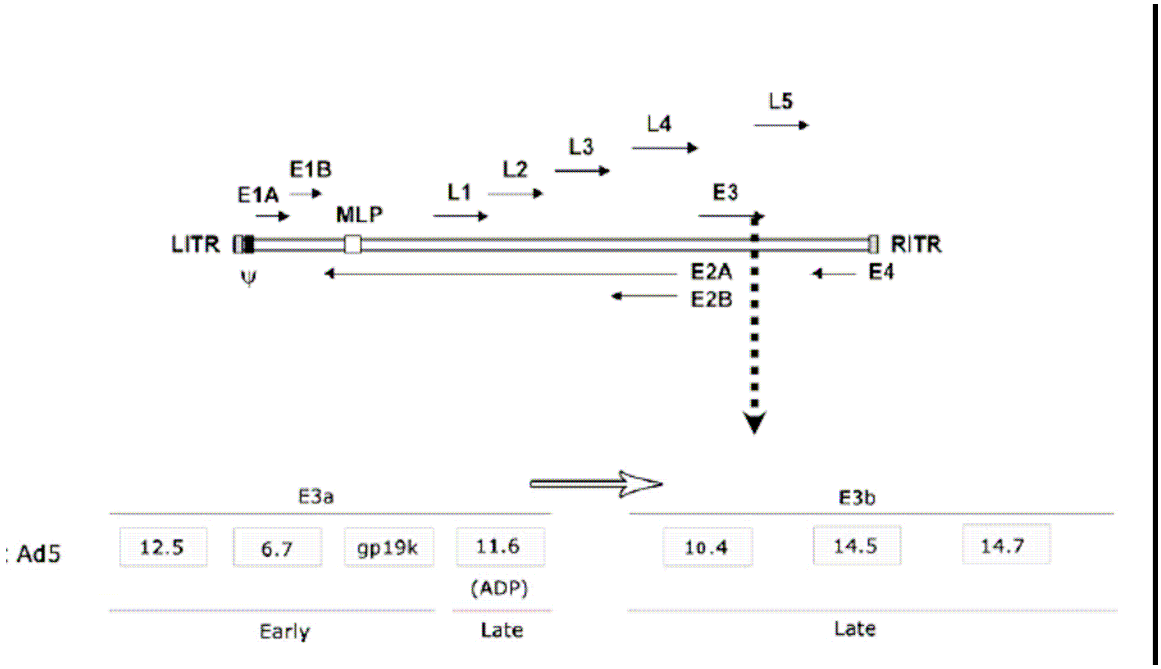
cellular receptor namely coxsackie adenovirus receptor (CAR). Attachment via knob-CAR interactions is followed by interactions between cellular integrins and an arginine-glycine aspartic acid motif (RGD-motif) located at the penton base. This binding leads to the formation of endosomes, viral internalization, disassembly and the release of viral nucleic acid. Thereafter viral DNA is transported to the nucleus where the genes are expressed and viral replication occurs. The adenoviral genome can be divided into immediately early (*E1A*), early (*E1B, E2, E3, E4*), intermediate (*IX, IVa2*) and late genes (Figure 1.9A). The early genes are expressed prior to viral replication consisting of mainly regulatory proteins that prepare the host cell for virus DNA replication and block antiviral mechanisms. The late viral genes encode for viral structural proteins. Importantly, E3 region encodes a variety of proteins involved in immune response evasion. Adenoviruses with deletions in E1 and/or E3 regions have been deleted to provide cloning sites for transgene insertion. These deletions have been found to not compromise adenoviral replicative function (Figure 1.9B) (Gomes and Tong, 2006; Xia et al., 2000; McConnell and Imperiale, 2004).

A



(Kanerva and Hemminki, Ann Med. 2005.37;33-43)

Figure 1.8. Adenovirus structural components. (A) Adenoviral virion showing the main structural components of the virus. (B) Adenoviral fiber consists of the tail, shaft and knob. The knob is involved in CAR mediated high affinity interactions required for cellular attachment. The tail mediates CAR independent low affinity interactions through cellular integrins and is involved mainly in viral penetration and internalization.



(Kanerva and Hemminki, Ann Med. 2005.37; 33-43)

Figure 1.9. Adenoviral early and late genes. A) Schematic representation of the adenoviral genome representing the viral early and late genes. The early genes E1, E2, E3 and E4 encode for regulatory proteins that prepare the host cell for virus DNA replication and block antiviral mechanisms. The late genes encode viral structural proteins. B) The adenoviral E3 genes that have used for cloning of transgenes along with their cellular functions.

1.6.1. Conditional Replicative and Oncolytic Adenovirus for Cancer Therapy

Research has shown that adenovirus can be safely used for gene delivery. Adenoviruses have been modified by replacing early genes, E1A and E1B or E3 with the gene of interest. Since the E1 unit is essential for viral replication, the recombinant vector is replicative defective and its replication requires helper functions provided by a packaging cell line with complementing E1 genes. However, these recombinant constructs have been useful mainly at local/ regional stage. Their therapeutic limitation has been the incomplete infection of tumor cells, transient expression of the transgene, and a lack of systemic efficacy. Recently, conditional replicative oncolytic adenoviruses have been shown to replicate and kill tumor cells without harming normal cells. The tumor specificity of these viruses has been manifested through the incorporation of tissue or tumor specific promoters that limit viral gene (Tong, 2006; Ralph et al., 2005; Gomes and Tong, 2006).

A recent study indicates that the use of Adenoviral vectors for clinical gene therapy is widespread. As of July 2006, adenoviral vectors are used in 26% of the 1,192 current worldwide gene therapy clinical trials. Of the 301 clinical trials involving the use of Ad vectors, 76% are for the treatment of cancer followed by vascular disease and monogenic disorders at 14% and 7%, respectively (http://www.wiley.co.uk/ genetherapy /clinical/) (Campos and Barry. 2007).

Adenoviral gene therapy approaches have shown promising results in clinical trials. Adenovirus mediated delivery of NTR (nitroreductase enzyme) from *E.coli* by direct intratumoural injection in patients with primary or secondary liver cancer showed appropriate levels of NTR expression in tumour cells. The early clinical trial data of the

NTR/CB1954 system in patients with liver cancer or prostate cancer are extremely encouraging (Plamer et al., 2004). Adenoviral delivery of TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) showed significant anti-tumour efficacy in animal models of aggressive primary and metastatic cancer (Jacob et al., 2004; Ma et al., 2005). Clinical trials of a recombinant adenovirus expressing interleukin-12 (IL-12) in patients with advanced digestive tumours have produced evidence of antitumour effects (Soiffer et al., 1998; Sangro et al., 2004).

1.6.2. Combination Gene Therapy

For some cancers, the best approach is a combination of surgery, radiation therapy, and chemotherapy. Surgery or radiation therapy can treat cancers that are confined locally, while chemotherapy can kill cancer cells that have spread to distant sites. Sometimes radiation therapy or chemotherapy is administered before surgery to shrink a tumor, thereby improving the opportunity for complete surgical removal. Radiation therapy and low-dose chemotherapy after surgery can also help destroy any remaining cancer cells. The stage of cancer often determines whether single therapy or a combination is needed. The rationale for combination therapy is to use methods that work by different mechanisms of action, thereby decreasing the likelihood of developing resistant cancer cells (Chabner and Roberts, 2005).

Adenoviral gene therapy methods are being used with other combination therapy approaches. Clinical trials using Ad containing the wild-type p53 tumor suppressor gene for the treatment of non-small-cell lung cancer, suggested some clinical activity when combined with the chemo-agent cisplatin (Nemunaitis et al., 2000), and led to the clinical development of ING201, ADVEXIN, and Gendicine (Peng, 2005). Ad-OC-TK, an

adenoviral vector with osteocalcin (OC) promoter and Herpes Simplex Virus-thymidine kinase (HSV-tk) was tested in clinical trials with Docetaxel and estramustine (DE) combination chemotherapy has shown encouraging results (Shirakawa et al., 2007; Shirakawa, 2008).

CHAPTER TWO

HYPOTHESIS AND OBJECTIVES

2.1. Hypothesis

A novel fusion protein consisting of MULT1 extracellular domain and Fas transmembrane and intracellular domains (MULT1E/FasTI) when expressed on a cell, would activate NKG2D expressing cells such as NK cells from its MULT1E extracellular region, upon binding of MULT1E to NKG2D receptor on NK cells. At the same time the interaction between MULT1E/NKG2D systems should induce apoptosis into the cell through transmembrane and intracellular region of Fas.

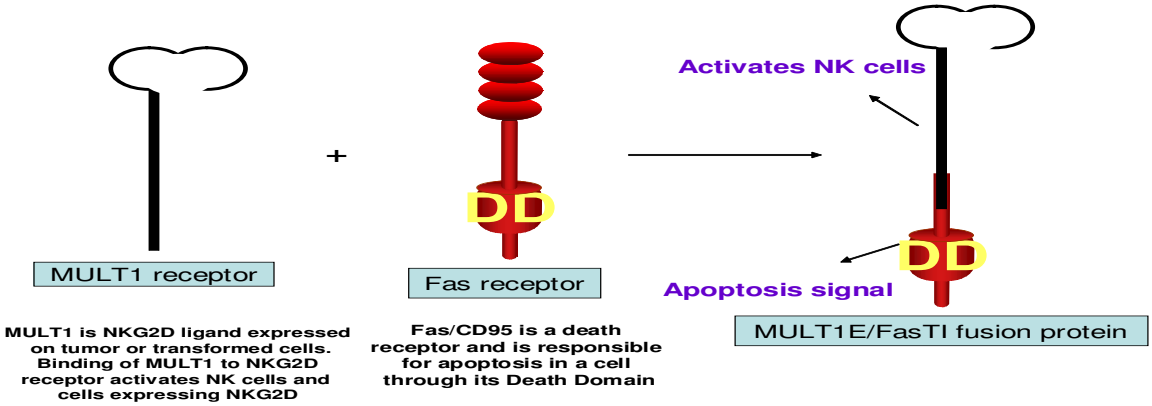


Figure 2. Schematic representation of proposed mechanism of novel fusion protein MULT1E/FasTI. When MULT1 receptor extracellular region whose function is activating NKG2D expressing cells such as NK cells is fused with Fas transmembrane and intracellular region whose function is transmitting apoptotic signal into cells are combined into one functional receptor, the resulting fusion receptor will possess the function of both the receptors.

2.2. Objectives

The work presented here is a two pronge approach of using a novel fusion protein consisting of MULT1 extracellular domain and Fas transmemberane and intracellular domain for cancer therapy. First, construction and expression of MULT1E/FasTI fusion is examined. Second, *in vitro* and *in vivo* activity of the fusion protein is tested. Finally, the adenoviral vector mediated delivery and *in vivo* therapeutic effect of the novel fusion protein is evaluated.

CHAPTER THREE

Tumor cells expressing a fusion protein of MULT1 and Fas are rejected *in vivo* by apoptosis and NK cell activation

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Tumor cells expressing a fusion protein of MULT1 and Fas are rejected *in vivo* by apoptosis and NK cell activation

Running Title: MULT1E/FasTI induces tumor cell rejection via apoptosis and NK cell activation

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Key Words: MULT1, Fas, NKG2D, Apoptosis, NK cell

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3.1. Abstract

Tumor cells evade immunosurveillance by elements of the innate immune system, such as natural killer (NK) cells, by downregulating or 'shedding' certain cell-surface molecules like mouse UL16-binding protein-like transcript 1 (MULT1) that can activate NK cells through NK cell receptors such as NKG2D; they also avoid Fas-mediated apoptosis by down regulating its expression. In the present study we report the design and evaluation of the antitumor activity of a novel fusion protein, MULT1E/FasTI, consisting of the extracellular domain of MULT1 and the transmembrane and intracellular domains of Fas. The fusion construct (pMULT1E/FasTI) was transfected into the mouse pulmonary carcinoma cell line TC-1; and stable cell clones expressing the fusion protein were established. *In vitro* cell culture studies demonstrated that the binding of the NKG2D/Fc, a recombinant protein of mouse NK cell receptor, to MULT1E/FasTI expressed on tumor cells was able to elicit apoptosis as assayed by Annexin V–fluorescein isothiocyanate staining and caspase- 3 enzyme-linked immunosorbent assay and to activate NKG2D-expressing cells, such as NK cells. *In vivo* subcutaneous tumor studies demonstrated that tumor cells expressing MULT1E/FasTI grew significantly slower than tumors without the protein. Pulmonary metastasis studies showed that most of the mice completely rejected tumor cells expressing MULT1E/FasTI. This approach may generate a new therapeutic agent for tumor treatment when combined with tumor cell-specific gene delivery vehicles such as oncolytic adenovirus vectors.

3.2. Introduction

Natural killer (NK) cells are large granular bone marrow derived lymphocytes serving as an important component of the innate immunity. They act as a 'rapid force' that attack virally infected, transformed and/or tumor cells (Trinchieri, 1989; Diefenbach and Raulet, 2001; Moretta et al., 2001). NK cells respond faster than T and B cells as they do not have to rearrange the T-cell receptor or the immunoglobulin genes to create a highly diverse repertoire of specificities. Instead, NK cells recognize the target cells by employing 'missing-self recognition' (Ljunggren and Karre, 1990). NK cell function is controlled by a balance of NK cell inhibitory and activating signals. Three inhibitory receptor families have been identified in mice and humans (Yokoyama et al., 1995; Wilson et al., 2000; Takei et al., 2001). NK cell inhibitory receptors bind to self-MHC (major histocompatibility complex) class I molecules, and this binding results in profound inhibition of the NK cell (Raulet et al., 2001). A wide variety of NK cell-activating receptors have also been found, such as NKG2A, NKG2C, NKG2D and NKG2E. NKG2A/C/E, which are type-2 transmembrane receptors with sequence similarities to C-type lectins with highly related sequence, may be present as heterodimers with another protein (CD94) and recognize a non classical MHC class 1 molecule known as human leukocyte antigen-E (in humans) or Qa1 (in mice) (Braud et al., 1999). NKG2D, in contrast, is a homodimeric C-type lectin-like protein that is expressed by all NK cells, subsets of NKT and γδ T cells (Bauer et al., 1999; Jamieson et al., 2002; Diefenbach et al., 2000). After stimulation, virtually all mouse CD8+ T cells and macrophages also express NKG2D (Jamieson et al., 2002; Diefenbach et al., 2000).

Several distinct ligands for NKG2D have been identified. Human NKG2D ligands include MHC class 1 chain-related protein A (MICA), MICB, ULBP and RAET1 (Bauer et al., 1999) Mouse NKG2D ligands include retinoic acid early transcript 1 (Rae1), histocompatibility 60 (H60) (Malarkannan et al., 1998) and mouse UL16-binding protein-like transcript 1 (MULT1) (Carayannopoulos et al., 2002; Diefenbach et al., 2003).

Fas (CD95) is a transmembrane cell-surface death receptor that belongs to the tumor necrosis factor (TNF) receptor superfamily. Fas contains three cysteine-rich extracellular domains at the N-terminus, which are responsible for ligand binding, the transmembrane domain, and an intracellular death domain (DD) of about 80 amino acids that is essential for transducing the apoptotic signals (Peter and Krammer, 2003). Binding of FasL (CD95L) to Fas receptor results in aggregation of the receptor molecules and recruitment of the adapter molecule, Fas-associated death domain (FADD), through DD–DD interactions. FADD also has another domain called the death-effector domain, which recruits pro-caspase-8 and/or procaspase- 10 to the receptor, resulting in formation of a multimeric protein complex called the death-inducing signaling complex (DISC) (Houston and O'Connell, 2004). DISC activates effector caspases such as caspase-3 that in turn cleave a restricted set of target proteins and are responsible for apoptosis in the cell (Sahh et al., 2002).

Tumor cells have developed many strategies for escaping immune surveillance. One of these strategies is to shed NKG2D ligands such as MICA (Gorh et al., 2002; Sahh et al., 2002). Shedding of these ligands reduces their surface levels and affects the susceptibility to NKG2D-mediated cytolysis by NK cells. Tumor cells can also escape from Fas-mediated apoptosis by decreasing surface expression of Fas (Moller et al.,

1994; Ivanov et al., 2003) secreting an antagonistic 'decoy' receptor (Pitti et al., 1998) expressing anti-apoptotic molecules such as BCL2 family members (Boise et al., 1993; Sarid et al., 1997) down regulating and mutating pro-apoptotic genes such as BAX, APAF1 or Fas (Strand et al., 1996; Ionov et al., 2000; Teitz et al., 2000; Soengas et al., 2001).

In the present study, we intended to combine the NK cell-mediated cytolysis and Fas-mediated apoptosis into one fusion protein by using the extracellular domain of MULT1 and the transmembrane and intracellular domains of Fas in a mouse model. We hypothesized that the engagement of NK cells and/or other immune cells with tumor cells expressing the fusion protein will not only send an apoptotic signal to the tumor cells but also activate the NK cells through the NKG2D receptor so that not only the engaged tumor cells will be killed via Fas-induced mechanisms but also may be lysed directly by the activated NK cells. The results demonstrate that this fusion protein is functioning as expected.

3.3. Materials and Method

3.3.1. Mice and cells

C57BL/6J mice (male or female) at 6–8 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in our pathogen-free animal facilities. The animal experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH Publication Number 85-23) and the institutional guidelines. The mouse lung carcinoma cell line TC-1 (ATCC # JHU-1) was cultured in RPMI 1640 medium containing 10% FBS and 100 μg/ml gentamicin at 37 ^{o}C with 5% CO_2.

3.3.2. Cloning of MULT1 extracellular domain into pcDNA3.1 (+)/Zeo

Thymus glands from 4-day old newborn C57BL/6J mice were removed and stored in liquid nitrogen. The glands were homogenized using a tissue homogenizer and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). Primers were designed for amplification of the extracellular domain of MULT1 (Genebank accession # NM_029975) from 236bp to 868bp. The sequence of the 5' primer is CCCAAGCTTATGGAGCTG ACTGCCAGTAACAAGGTCC and that of the 3' primer is CGGGATCCGGTACTGAAA GATCCTGCAGGCTCCAG. At the 5' end of the upstream primer, a HindIII enzyme site was created and at the 5' end of downstream primer, a BamHI site was created. cDNA was synthesized from the extracted total RNA using RT-PCR kit (Promega, Madison, WI). The fragment was excised and gel purified using a Qiagen gel purification kit (Valencia, CA). Double enzyme digestion was performed on the purified fragment using HindIII and BamHI. The enzyme digested

fragment was then ligated into a pcDNA3.1 (+) vector (Invitrogen, CA). The full MULT1 cDNA sequence in the new vector, pMULT1E, was confirmed by DNA sequencing.

3.3.3. Cloning of Fas transmembrane and intracellular domains into pcDNA3.1 (+)/Zeo

The cDNA clone of the Fas receptor in pDNR-LIB (ATCC # 10088798) was purchased from American Type Collection Centre (ATCC, Manassas, VA). A pair of primers was designed for amplification of the transmembrane and intracellular domains of Fas from 524 bp to 1013bp (Genebank accession# BC061160). The 5' primer used was CGGGATCCCCCAGAAATCGCCTATGGTTGTTGTTGACC and the 3' primer was CGGAATTCTCACTCCAGACATTGTCCTTCATTTTC. At the 5' end of upstream primer, a BamHI enzyme site was created and at the 5' end of downstream primer, an EcoRI enzyme site was created. DNA PCR was performed to amplify the Fas transmembrane and intracellular domains from pDNR-LIB. The gel purified fragment was treated with BamHI and EcoRI enzymes and ligated into the pcDNA3.1 (+)/Zeo vector to create pFasTI. The DNA sequence of the transmembrane and intracellular domains of Fas in vector pFasTI was confirmed by DNA sequencing.

3.3.4. Creation of the vector pMULT1E/FasTI

The cDNA fragment encoding the MULT1 extracellular domain was cut out from pMULT1E by HindIII and BamHI enzyme digestion and ligated into the pFasTI. The resulting vector was named pMULT1E/FasTI (Figure 3.1) and used for transfection.

3.3.5. Transfection of TC-1 cells

TC-1 mouse lung carcinoma cells were transfected with linearized pMULT1E/FasTI vector using Lipofectamine (Invitrogen, Carlsbad, CA) as directed by

the manufacturer. In order to obtain stable clones expressing the fusion protein, the transfected cells were cultured in medium containing 250μg/ml zeocin. Zeocin resistant clones were obtained and subcultured in the presence of zeocin.

3.3.6. Fusion protein expression by transfected TC-1 clones

For the analysis of MULT1E/FasTI surface expression, cells from TC-1 and zeocin-resistant clones were gently detached using TrypLE Express (Invitrogen) and washed with staining buffer twice. Five hundred thousand of each clone were incubated with 1μg monoclonal rat anti-mouse MULT1 antibody (R&D Systems, Minneapolis, MN) for 30 minutes at 4 ^{o}C. After washing twice with staining buffer, the cells were stained with FITC-labeled goat F(ab') anti rat IgG antibody for 30 minutes at 4 ^{o}C. After washing twice with staining buffer, cells were re-suspended in 0.5 ml staining buffer and analyzed on FACS Calibur (Becton Dickinson, San Jose, CA) using CellQuest software (Becton Dickinson, San Jose, CA). To confirm that MULT1E expressed as the fusion protein in the transfected cells can indeed bind to NKG2D, cells were first treated with 1μg/ml of NKG2D/Fc, a recombinant protein of mouse NKG2D (R&D Systems) for 30 min at RT, stained with FITC-labeled rat anti-mouse NKG2D antibody, and analyzed by FACS . Three clones, L-5, L-7, and L-10 together with TC-1 cells were used for further studies.

3.3.7. RT-PCR

Two million cells of TC1, L-5, L-7 and L-10 were used to extract total RNA using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA samples were DNase 1 treated prior to reverse transcription using RQ1 RNase free DNase (Promega, Madison, WI). The treated RNAs were then used for RT-PCR using Access Quick^{TM} RT-PCR system

(Promega, Madison, WI) according to the manufacture's protocol. Two different RT-PCR reactions were performed, one for amplifying only MULT1 extracellular fragment using MULT1 forward and reverse primers (5' CCCAAGCTTATGGAGCTGAC TGCCAGTAACAAGGTCC3'and 5'CGGGATCCGGTACTGAAAGATCC TGCAGGC TCCAG 3'), the other for amplifying the complete MULT1-Fas fusion protein mRNA with MULT1 forward and Fas reverse primers (5' CCCAAGCTTATGGAGCTG ACTGCCAGTAACAAGGTCC 3' and 5'CGGAATTCTCACTCCAG ACATTGTCCTT CATTTTC 3'). RT-PCR for β-actin was also performed as control.

3.3.8. Induction of apoptosis in cells expressing the fusion protein

To determine if cells expressing the fusion protein can be induced to undergo apoptosis, one million cells of TC-1 and clones L-5, L-7, and L-10 were treated with 1μg/ml of NKG2D/Fc for 16 hrs. The apoptosis of the cells were measured using two systems: a TACS Annexin V-FITC apoptosis kit (R&D Systems) and a caspase-3 fluorometric assay (R&D Systems). For the Annexin-V assay, $2x10^5$ cells in triplicate were stained according to the instructions provided by the manufacturer. Briefly, cells were trypsinized, washed twice with staining buffer, and incubated with Annexin V-FITC and PI in binding buffer at room temperature for 15 min in the dark. Stained cells were analyzed by FACS. For caspase-3 analysis, $8x10^5$ cells in triplicates were used for analysis of caspase-3 activity according to the instructions provided by the manufacturer. Briefly, cells were trypsinized, washed with PBS, and lysed. The lysates were incubated with caspase-3 substrate for 1 hour at 37 ^oC and the fluorescent signal was detected using a SpectraMax Gemini XS microplate reader (Molecular Devices, Sunnyvale, CA).

3.3.9. Activation of NK cells by MULT1E/FasTI

To test whether the MULT1E/FasTI fusion protein can activate NK cells, $1x10^6$ cells of TC-1 and clones L-5, L-7, and L-10 were co-cultured for three hours at a ratio of 1:2 with NK cells isolated from the spleens of female C57BL/6J mice using a mouse NK cell isolation kit (Miltenyi Biotec, Auburn, CA). The NK cells were then recovered and stained with anti-mouse NK1.1 antibody conjugated with FITC (BD Biosciences, San Diego, CA). The cells were then permeabilized and fixed using the Cytofix/Cytoperm Plus (BD Biosciences), stained with anti-mouse interferon-γ antibody-PE (BD Biosciences) and analyzed by FACS using the CellQuest software for intracellular IFN-γ production.

3.3.10. *In vivo* tumor studies

Female C57BL/6J mice at 6–8 weeks of age were used in two tumor model studies: a subcutaneous tumor model and a pulmonary metastasis model with cells of TC-1 and clones L-5, L-7, and L-10. For the subcutaneous study, $2x10^5$ cells of the above clones in 0.2 ml HBSS were injected subcutaneously in the right flank of each of the 4 animals. Tumors were measured twice weekly. Tumor size was calculated as $1/2LW^2$, where W and L are the shortest and longest diameters of the tumor, respectively. For pulmonary metastasis studies, $2x10^5$ cells in 0.5ml of HBSS were injected intravenously. Four weeks after tumor injection, the mice were euthanized and their lungs were excised. The tumor nodules on each lung were counted using a dissecting microscope, tumor weight was also determined by weighing the lungs.

3.3.11. Statistical analysis

GraphPad software (Prism, San Diego, CA) was used to make graphs. One-way or Two-way ANOVA with Bonferoni post-tests were used to perform the statistical analyses of the data. Student *t* test was used to analyze the subcutaneous tumor growth data (Figure 5). The significance was represented as $p<0.05$, *; $p<0.01$, **; $p<0.001$, ***.

3.4. Results

3.4.1. Expression of MULT1E/FasTI

TC-1 cells were transfected with pMULT1E/FasTI (Figure 3.1). Clones that were zeocin resistant were selected and labeled as L-5, L-7 and L-10. An *in vitro* cell growth study shows that all the clones grow at a similar rate (data not shown). The cells of these clones were stained with anti-mouse MULT1 antibody and analyzed by fluorescence-activated cell sorting (FACS). The result showed that TC-1 cells and clone L-7 cells were negative, whereas clones L-5 and L-10 cells were strongly positive (Figure 3.2A). To confirm that MULT1E of the fusion protein can indeed bind to NKG2D, the cells were incubated with NKG2D/Fc and then stained with anti-mouse NKG2D antibody conjugated with fluorescein isothiocyanate (FITC). TC-1 cells and clone L-7 cells were dimly positive, whereas clones L-5 and L-10 cells were strongly positive (Figure 3.2B) with L-10 cells the strongest. To further confirm the fusion protein expression, reverse transcriptase (RT)–PCR was performed on RNA samples from these clones using two pairs of primers: the first pair covered only the extracellular domain of MULT1; whereas the second pair covered the entire fusion protein. As Figure 3.2C shows, although all the clones are positive for the first pair of primers (646 bp), signals of clones L-5 and L-10 are much stronger than those of TC-1 cells and clone L-7. Only clones L-5 and L-10 are positive for the second pair of primers (1134 bp, Figure 3.2D). The results indicate that clones L-5 and L-10 are MULT1E/FasTI-positive clones, whereas TC-1 and clone L-7 are negative for the fusion protein, but express some endogenous MULT1 protein.

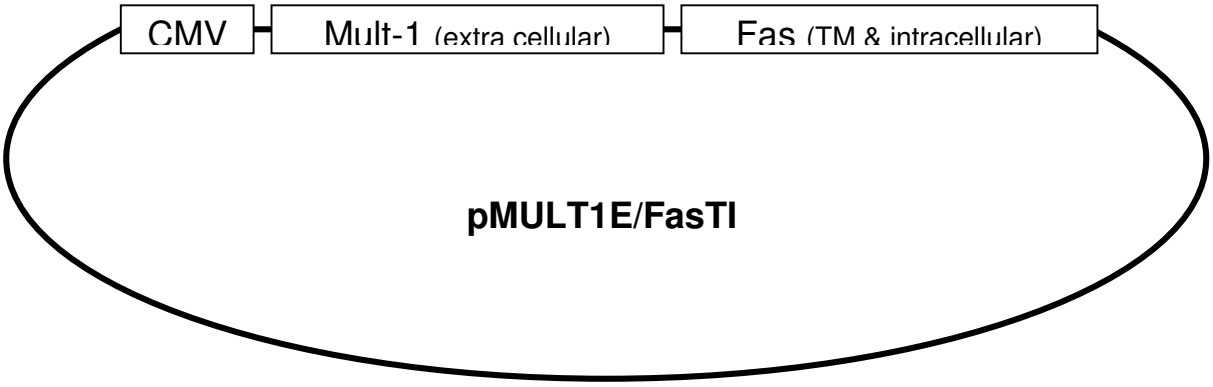


Figure 3.1. Construction of the MULT1E/FasTI plasmid. The extracellular domain of mouse UL16-binding protein-like transcript 1 (MULT1) cDNA and the transmembrane (TM) and intracellular domains of mouse Fas cDNAwere cloned by PCR. The two pieces of cDNAs in the order of MULT1E/FasTI were inserted into plasmid pcDNA3.1/Zeo.

A

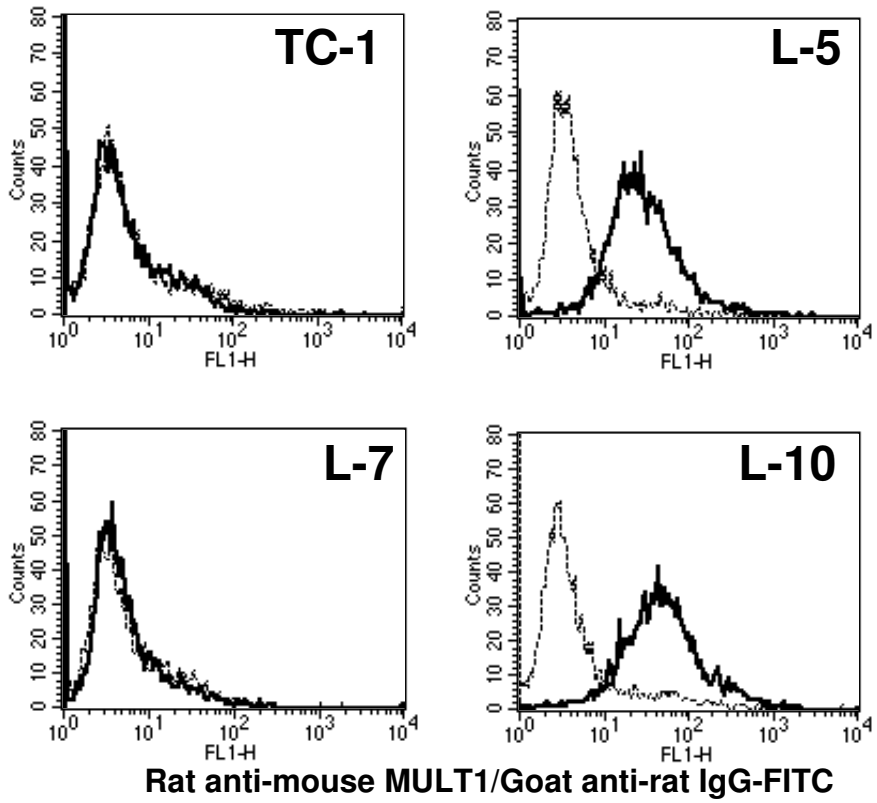


Figure 3.2. Fluorescence-activated cell sorting (FACS) analyses of MULT1E/FasTI expression. (A) A total of $5x10^5$ cells of TC-1 and clones L5, L7 or L10 were stained with purified rat anti-mouse MULT1 antibody followed by goat anti IgG F(ab')-fluorescein isothiocyanate (FITC). Purple peak is isotype controls

B

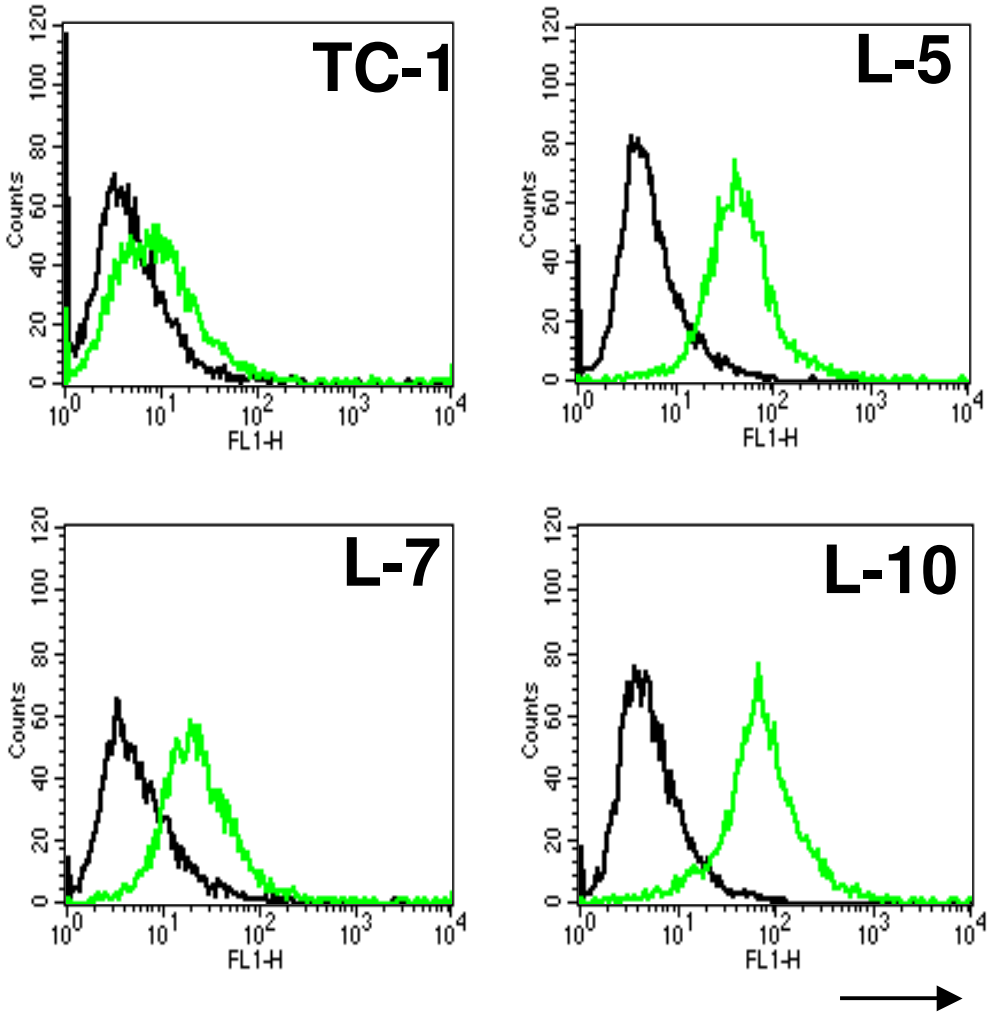


Figure 3.2. Fluorescence-activated cell sorting (FACS) analyses of MULT1E/FasTI expression. (B) A total of $1x10^6$ cells of TC-1 and clones L-5, L-7 or L-10 were first treated with NKG2D/Fc and then stained with anti-mouse NKG2D antibody conjugated with FITC. The cells were analyzed on FACS Calibur with CellQuest software. Black lines are controls without NKG2D/Fc incubation.

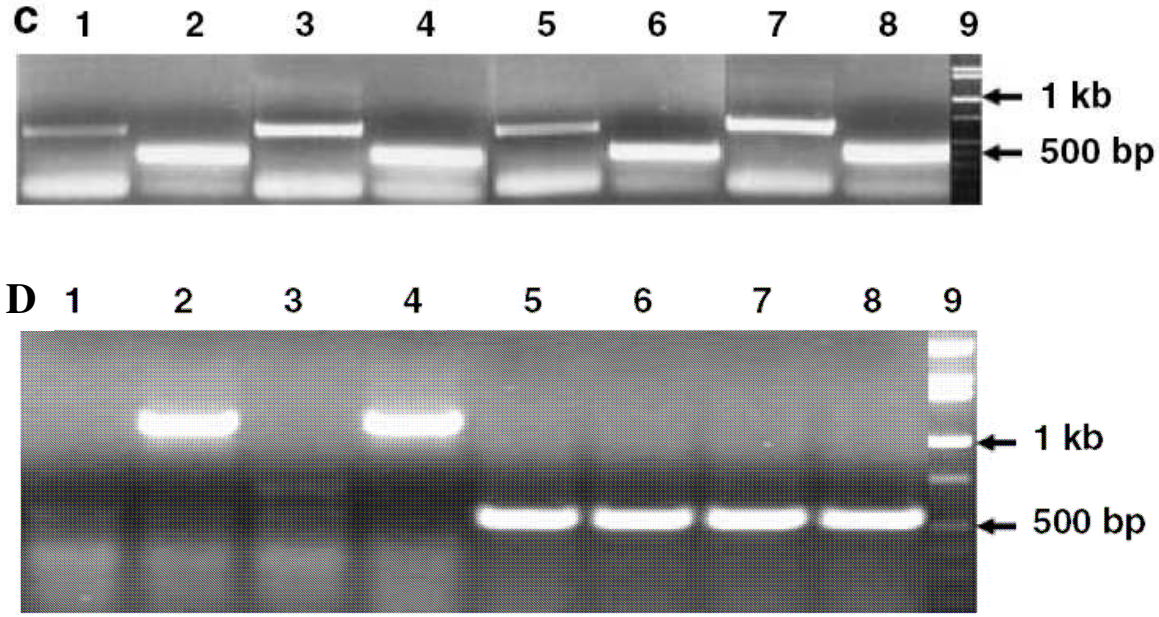


Figure 3.2. Reverse transcription (RT)–PCR analyses of MULT1E/FasTI expression. (c) RT–PCR. Lanes 1, 3, 5 and 7: RT–PCR products from RNAs of TC-1 cells and clones of L-5, L-7 and L-10 using MULT1 primers, which product is 646 bp; lanes 2, 4, 6 and 8: RT–PCR products from RNAs of TC-1 cells and clones of L-5, L-7 and L-10 using primers for ß-actin as controls; line 9: 1 kb marker. (d) RT–PCR. Lanes 1–4: RT–PCR products from RNAs of TC-1 cells and clones of L-5, L-7 and L-10 using primers covering the entire fusion gene sequences, which product is 1134 bp; lanes 5–8: RT–PCR products from RNAs of TC-1 cells and clones of L-5, L-7 and L-10 using primers for ß-actin as controls; lane 9: 1 kb marker.

3.4.2. Fusion protein MULT1E/FasTI induces apoptosis of cells

To confirm the concept that when bound by its ligand fusion protein MULT1E/FasTI can send death signals through its Fas portion into the cells, TC-1 cells and clones L-5, L-7, L-10 were treated with recombinant protein NKG2D/Fc and analyzed by Annexin V staining and caspase-3 activation assay. The treatment of NKG2D/Fc increased both Annexin V-positive cells and Annexin V/propidium iodide (PI) double-positive cells in clones L-5 and L-10, but not in TC-1 cells or clone L-7 (Figure 3.3A). After the NKG2D/Fc treatment, not only apoptotic cells (Annexin V-positive cells and Annexin V/PI double-positive cells), but also the necrotic cells (PI-positive/Annexin V-negative cells) in clone L-5 and L-10 were significantly higher than those of TC-1 or clone L-7 (Figures 3.3B and C). Similarly, caspase-3 activities in cells of clones L-5 and L-10 were significantly higher than those of TC-1 or clone L-7 (Figure 3.3D). The treatment of NKG2D/Fc induced more apoptotic cells in clone L-10 than clone L-5 (Figures 3.3B and D).

A

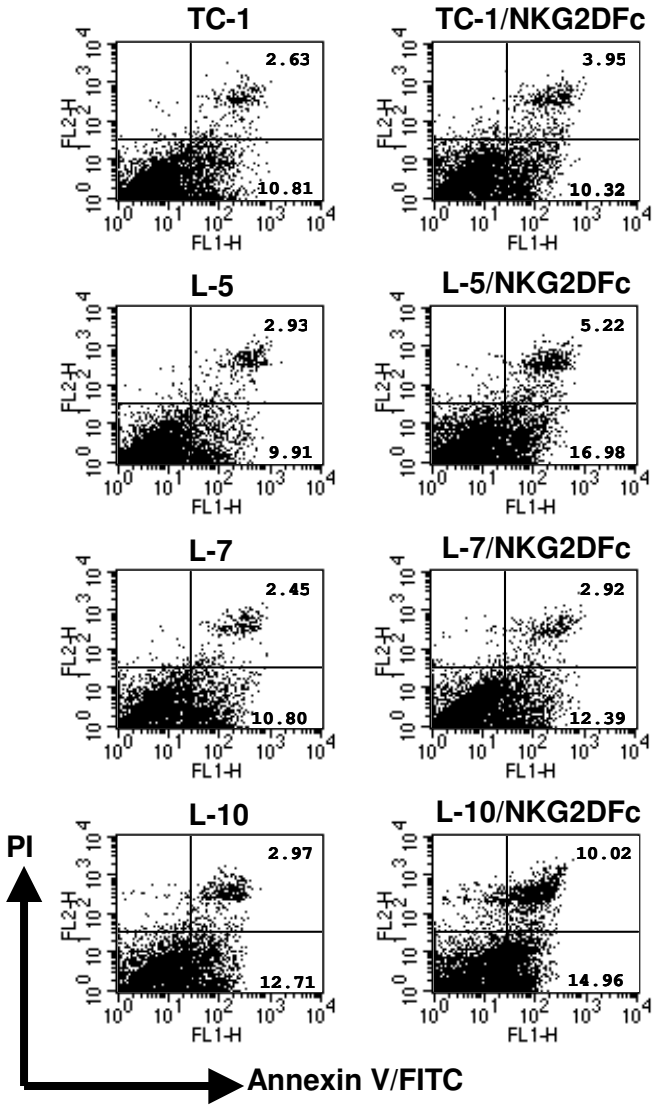
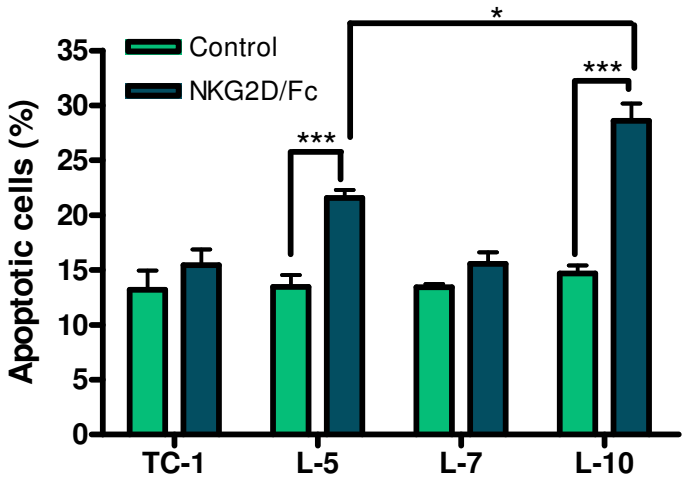


Figure 3.3. Mouse UL16-binding protein-like transcript 1 E (MULT1E)/FasTI induces apoptosis. A total of 1x106 cells of TC-1 and clones L-5, L-7 and L-10 were treated with 1 µg/ml NKG2D/Fc for 16 h. The cells were then analyzed for apoptosis and necrosis using Annexin V staining and PI (A) An example of the fluorescence-activated cell sorting (FACS) data.

B



C

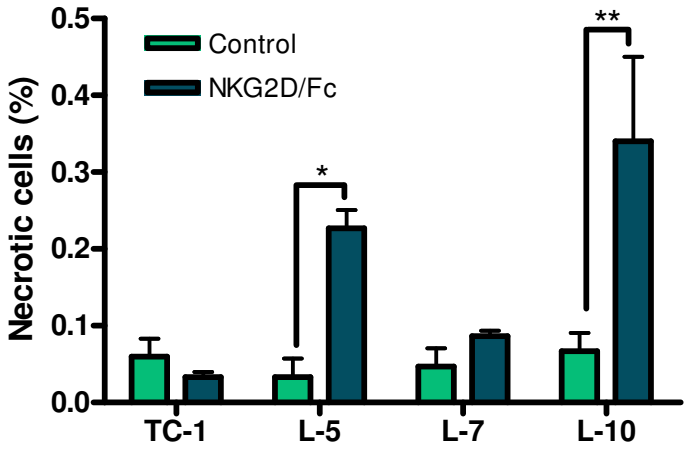


Figure 3.3. Mouse UL16-binding protein-like transcript 1 E (MULT1E)/FasTI induces apoptosis. A total of $1x10^6$ cells of TC-1 and clones L-5, L-7 and L-10 were treated with 1 µg/ml NKG2D/Fc for 16 h. The cells were then analyzed for apoptosis and necrosis using Annexin V staining (B, C) Summaries of data from three separate experiments. The statistical analyses were conducted between the controls (open bars) and NKG2D/Fc-treated cells (solid bars) using two-way analysis of variance (ANOVA). The difference between NKG2D/Fc-treated L-5 cells and NKG2D/Fc-treated L-10 cells was also compared using Student's t-test. *P<0.05; **P<0.01 and ***P<0.001.

D

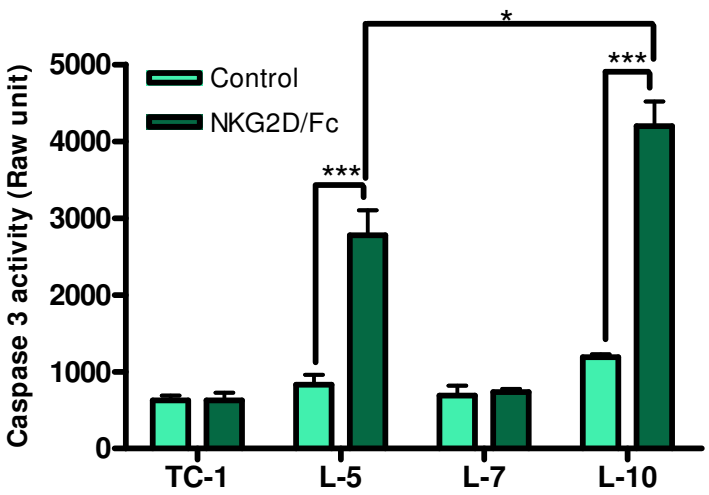


Figure 3.3. Mouse UL16-binding protein-like transcript 1 E (MULT1E)/FasTI induces apoptosis. A total of 1x106 cells of TC-1 and clones L-5, L-7 and L-10 were treated with 1 µg/ml NKG2D/Fc for 16 h. The cells were then analyzed for apoptosis and necrosis using caspase-3 assay (D) according to the manufacturers' protocols. (D) Summaries of data from three separate experiments. The statistical analyses were conducted between the controls (open bars) and NKG2D/Fc-treated cells (solid bars) using two-way analysis of variance (ANOVA). The difference between NKG2D/Fc-treated L-5 cells and NKG2D/Fc-treated L-10 cells was also compared using Student's t-test. *P<0.05; **P<0.01 and ***P<0.001.

3.4.3. Cells expressing MULT1E/FasTI activate NK cells

It is critical to know whether fusion protein MULT1E/ FasTI can activate NKG2D-expressing cells, such as NK cells. Cells from TC-1 or clones L-5, L-7 and L-10 were co-cultured with NK cells isolated from mouse spleen. Intracellular interferon-γ (IFN- γ) was detected by FACS analysis (Figure 3.4A). The percentage of the NK cells that express IFN- γ was significantly increased in wells that contained cells of clone L-5 or L-10 compared to those co-cultured with TC-1 ($P<0.05$). Although the percentage of NK cells expressing IFN-γ in wells that contained cells of clone L-7 increased slightly compared to those co-cultured with TC-1 cells, it was not statistically significant (Figure 3.4B).

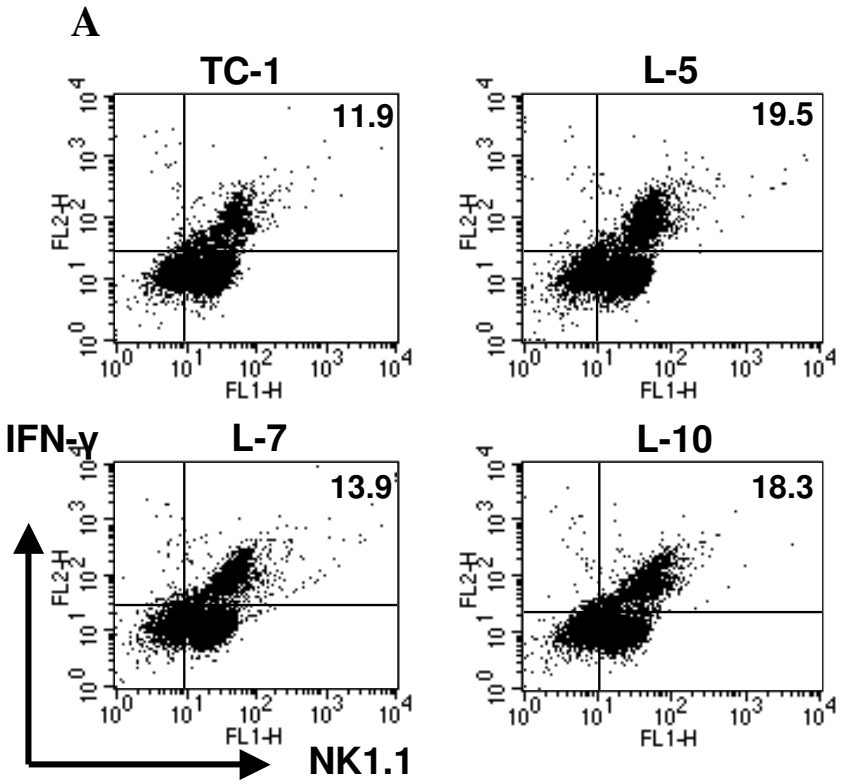


Figure 3.4. Mouse UL16-binding protein-like transcript 1 E (MULT1E)/FasTI activates natural killer (NK) cells. A total of 1x106 cells of TC-1 and clones L-5, L-7 and L-10 were co-cultured with NK cells for 3 h. The cells were stained with anti-NK1.1-FITC and were then permeabilized and fixed, and stained with antimouse interferon-γ (IFN-γ)-phycoerythrin (PE). The cells were analyzed on FACS Calibur with CellQuest software. An example of the fluorescence-activated cell sorting (FACS) data (A)

B

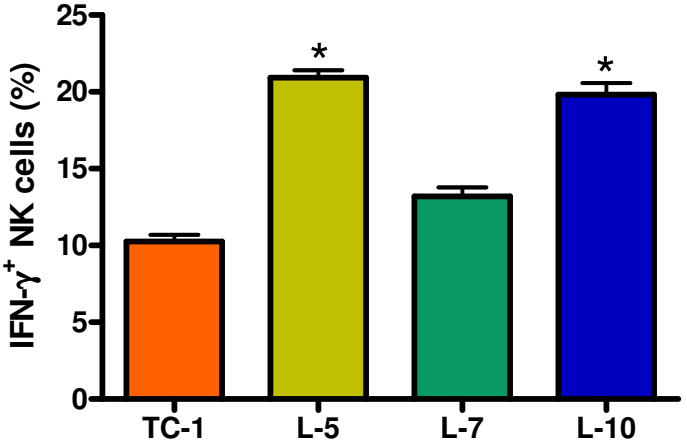


Figure 3.4. Mouse UL16-binding protein-like transcript 1 E (MULT1E)/FasTI activates natural killer (NK) cells. A total of $1x10^6$ cells of TC-1 and clones L-5, L-7 and L-10 were co- cultured with NK cells for 3 h. The cells were stained with anti-NK1.1-FITC and were then permeabilized and fixed, and stained with antimouse interferon-g (IFN-g)-phycoerythrin (PE). The cells were analyzed on FACSCalibur with CellQuest software (B). Summary of data from three separate experiments. *P<0.05.

3.4.4. *In vivo* antitumor effect of fusion protein MULT1E/FasTI

The *in vivo* therapeutic effect of the fusion protein was evaluated in a subcutaneous tumor model as well as a pulmonary metastasis model. Two hundred thousand cells of TC-1 and clones L-5, L-7 and L-10 in 0.2 ml Hank's balanced salt solution (HBSS) were injected subcutaneously into 6- to 8-week-old mice and tumor size was measured twice weekly with a caliper and tumor volume was calculated. The tumor growth of clone L-7 was slightly, but not significantly ($P>0.05$) slower when compared to that of TC-1 cells. At day 18, the growth of clones L-5 and L-10 was significantly slower ($P<0.01$, $P<0.01$) when compared to that of TC-1 cells. At day 24, the difference of tumor growth between TC-1 and clone L-10 was even more significant ($P<0.001$), whereas the difference of tumor growth between TC-1 and clone L-5 remained the same ($P<0.01$; Figure 3.5). An even better antitumor effect of the fusion protein was observed in the pulmonary metastasis model. Four weeks after i.v. tumor cell injection, the mice were euthanized and lungs were excised (Figure 3.6A). The total weight of the lungs with the tumors was measured (Figure 3.6B) and the tumor nodules on the surface of the lungs were counted (Figure 3.6C). The lungs isolated from mice injected with TC-1 cells were fully covered with tumors and weighed an average 0.82 g. All the four lungs have more than 200 tumor nodules each. The lungs isolated from mice injected with clone L-7 cells are covered with many tumors as well and weighed averagely 0.48 g. There are 118, 89, 67, 125 tumor nodules on the lungs. The lungs isolated from mice injected with clones L-5 and L-10 were almost tumor free and weighed much less (0.15 and 0.14 g, respectively) than those of mice injected with either TC-1 cells or clone L-7 cells. The average weight of lungs from normal mice was 0.14 g.

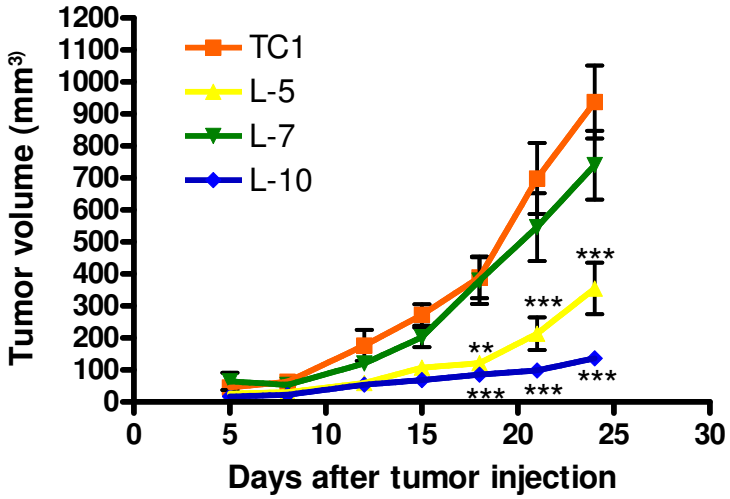


Figure 3.5. Subcutaneous tumor study. A total of 2x105 tumor cells of TC-1 or clones L-5, L-7 and L-10 in 0.2 ml HBSS were subcutaneously injected into C57BL/6J mice (four mice per group). Tumor growth was measured and presented as $1/2LW^2$. **P<0.01; ***P<0.001.

A

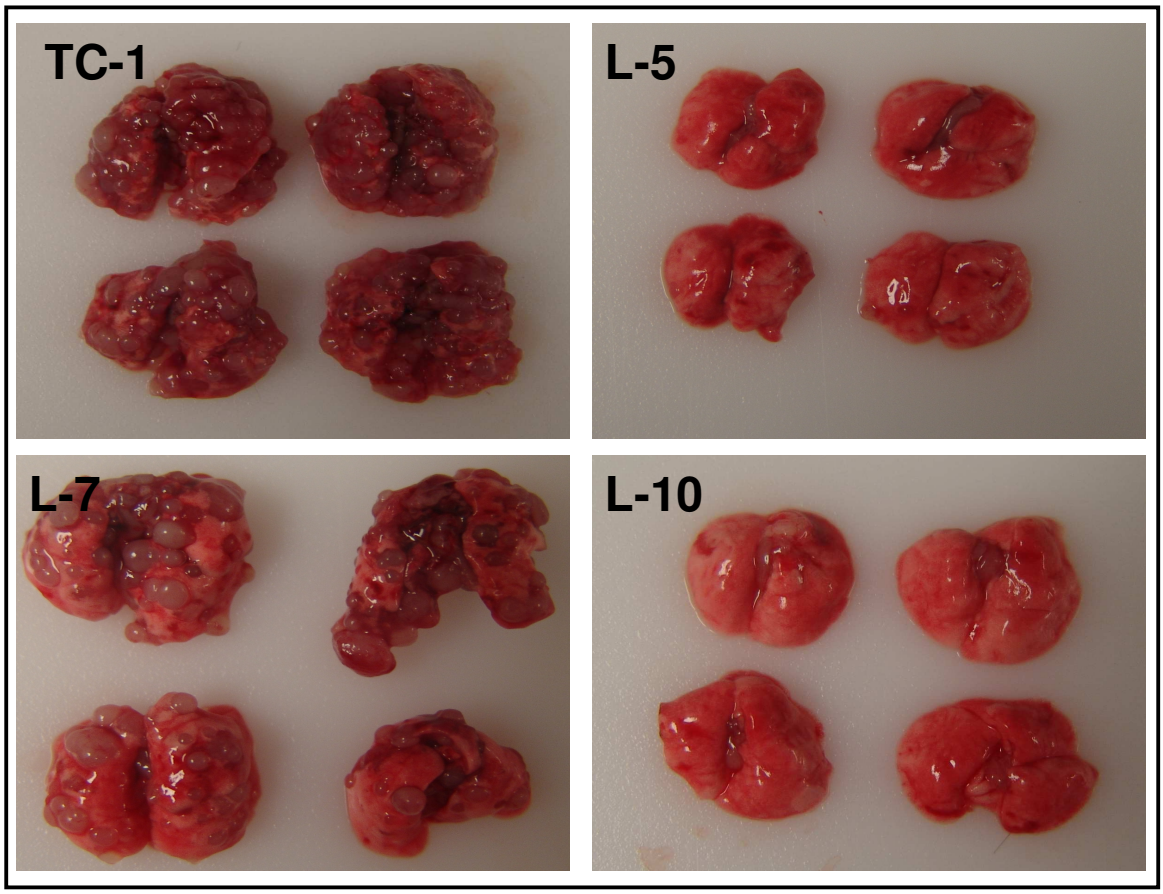
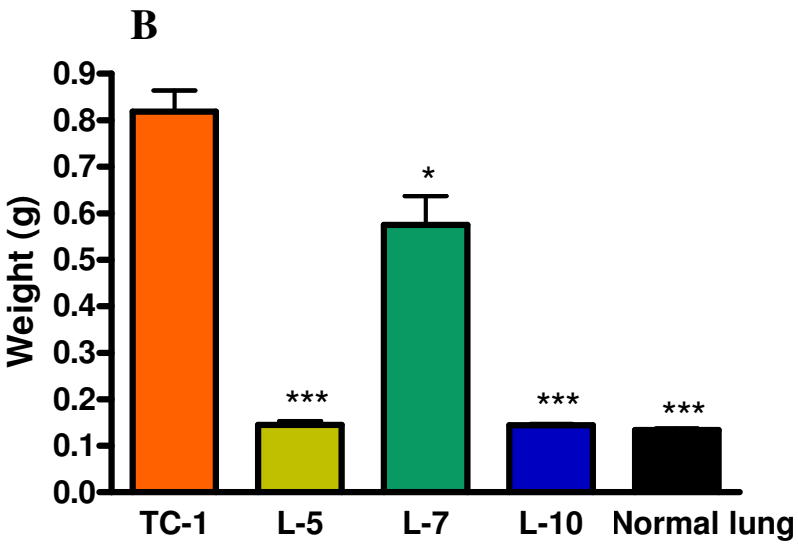


Figure 3.6. Pulmonary metastatic tumor study. A total of $2x10^5$ tumor cells of TC-1 or clones L-5, L-7 and L-10 in 0.5 ml HBSS were i.v. injected into C57BL/6J mice (four mice per group). Four weeks after tumor cell injection, mice were killed and their lungs were dissected (A).



C

Clones	Pulmonary Metastases
TC-1	>200, >200, >200, >200
L-5	0, 0, 1, 1
L-7	118, 89, 67, 125
L-10	0, 0, 0, 2

Figure 3.6. Pulmonary metastatic tumor study. A total of $2x10^5$ tumor cells of TC-1 or clones L-5, L-7 and L-10 in 0.5 ml HBSS were i.v. injected into C57BL/6J mice (four mice per group). Four weeks after tumor cell injection, mice were killed and their lungs were dissected. The lungs were weighted (B) and the tumor nodules on the lungs were counted (C). *P<0.05; ***P<0.001.

3.4.5. Fusion protein MULT1E/FasTI is more potent than MULT1 in tumor rejection.

Stable clones expressing MULT1E protein anchored on the cell membrane were obtained by transfecting TC-1 cells with vector pDispaly/MULT1E and selection. Two of the clones, C-4 and C5, were chosen in this study and the expression of MULT1E protein on the cell surface was confirmed by FACS analysis (data not shown). Clones C-4 and C-5 together with clone L-10 were used in an *in vivo* tumor study. Two hundred thousand cells of each clone were i.v. injected into C57BL/6J mice (four mice per group). Four weeks later, the mice were killed and tumor nodules on lungs were counted. As expected, the lungs of mice received TC-1 cells were fully covered by tumors. Although tumor cells expressing MULT1E protein on their surface were significantly rejected, the rejection was not as complete as tumor cells expressing MULT1E/FasTI (Table 3.1).

Table 3.1. MULT1E/FasTI is more potent than MULT1 in tumor rejection

Clones	*Lung tumor nodules*
TC-1	>200, >200, >200, >200
C-4	7, 1, 6, 5
C-5	4, 7, 2, 4
L-10	0, 0, 0, 0

3.5 Discussion

Different strategies using NK cells for cancer therapy such as *ex vivo* activation of NK cells (Rosenberg et al., 1985) and *in vivo* cytokine therapy (Law et al., 1995) to expand and activate NK cells against cancer cells (Meropol et al., 1998) have been used. However a majority of these studies did not demonstrate significant clinical benefit. As tumors have developed multiple mechanisms to subvert and suppress immune responses by regulating cell surface expression of Fas (Ivanov et al., 2003; Pitti et al., 1998) and Fas ligand (Niehans et al., 1997; Okada et al., 2000; Zheng et al., 2003) resulting in reduced sensitivity of tumor cells to Fas-mediated apoptosis, this resistance to Fas mediated apoptosis protects tumor cells from killing by infiltrating antitumor NK cells and T cells (Elsasser-Beile et al., 2003). Shedding of NKG2D ligands by tumor cells has been described as a novel immune escape mechanism (Raffaghello et al., 2004). Since NKG2D ligand surface levels critically determine the susceptibility to NKG2D-mediated NK cell lysis, stable expression of NKG2D ligands on tumors would help increase NK cell lysis of tumors (Diefenbach and Raulet, 2001). In this study, we enhanced the cell-surface expression of MULT1, one of the mouse NKG2D ligands, by anchoring the extracellular domain of MULT1 on tumor cells using a transmembrane sequence of Fas. We also introduced the DD of Fas in the intracellular domain of the fusion protein MULT1E/FasTI, hoping to develop a bifunctional chimeric protein that can send an apoptosis signal to the tumor cells and at the same time activate NKG2D-expressing immune cells such as NK cells.

The transcription of the fusion protein in the transfected cells was confirmed by RT–PCR and the expression of the fusion protein was characterized by surface FACS

analysis. When anti-mouse MULT1 antibody was used, clones L-5 and L-10 were positive, whereas TC-1 cells and clone L-7 cells were negative (Figure 3.1A). However, when the cells were first incubated with NKG2D/Fc to allow binding to MULT1 and then stained with anti-mouse NKG2D antibody, cells of clones L-5 and L-10 were strongly positive, whereas cells of TC-1 and clone L-7 were dimly positive (Figure 3.2B). These results suggest that TC-1 tumor cells express low levels of endogenous MULT1. The level is so low that a direct staining with anti-MULT1 antibody cannot recognize it. When the cells are incubated with NKG2D/Fc and then stained with anti-NKG2D antibody, the signal may be amplified and therefore can be detected by FACS analysis. In the RT–PCR experiment, when a pair of primers that covers only the extracellular region of MULT1 is used, all the cells of TC-1 and clones L-5, L-7, and L-10 are positive with cells of clones L-5 and L-10 strongly positive (Figure 3.2C). When a pair of primers that covers the entire sequence of the fusion gene is used, only cells of clones L-5 and L-10 are positive. The RT–PCR results confirm that only clones L-5 and L-10 express the fusion protein.

One of the key features of the designed fusion protein is to send apoptosis signals into the cells expressing the protein after binding to its ligand, NKG2D. Although we do not have direct evidence supporting that the binding of NKG2D can form DISC inside tumor cells, a clear apoptosis signal is sent to the cells as indicated by the increased caspase-3 activity and increased Annexin V-positive cells after treatment of recombinant NKG2D/Fc (Figure 3.3). Data from both Annexin V study and caspase-3 study showed a difference between clone L-5 and L-10 with the level of apoptotic cells in clone L-10 higher than that of clone L-5 (Figures 3.3B and D), suggesting that the induction of

apoptosis by the fusion protein may be dose dependent because the FACS analyses demonstrate a slightly higher-level expression of the fusion protein in clone L-10 than that in clone L-5. In this study, we combined high Annexin V cells and high Annexin V/high PI cells as apoptotic cells. We also analyzed the high PI/low Annexin V cells as necrotic cells (Figure 3.3C). Although overall, clone L-10 cells showed more apoptotic cells and more necrotic cells than clone L-5 cells after NKG2D/Fc treatment, the two clones behaved differently before and after NKG2D/Fc treatment. The difference of low PI/high Annexin V cells in clone L-10 before and after treatment was only about 2%, whereas it was about 7% in clone L-5. Perhaps there was more secondary necrosis in clone L-10 than clone L-5.

Increased expression of IFN-γ is a key indicator of NK cell activation (Bryceson et al., 2006; Tassi et al., 2006). To confirm the idea that fusion protein MULT1E/FasTI not only sends apoptotic signals into cells expressing it but also activates immune cells that express receptors for MULT1, NK cells isolated from spleen were co-incubated with cells of TC-1 and clones L-5, L-7 and L-10 and the IFN-γ expression was detected by FACS analysis. The IFN-γ expression by NK cells co-cultured with cells of clones L-5 and L-10 significantly increased compared to those of NK cells co-cultured with cells of TC-1 or clone L-7 (Figure 3.4).

To demonstrate whether fusion protein MULT1E/ FasTI has antitumor activity *in vivo*, a subcutaneous tumor model and a pulmonary metastatic tumor model were used in this study. In the subcutaneous tumor study, clones L-5 and L-10 grew significantly slower than TC-1 cells or clone L-7 cells ($P<0.05$ and $P<0.001$, respectively; Figure 3.5). The reason why clone L-10 grew slower than clone L-5 may be due to the level of the

fusion protein expression. The pulmonary metastasis study also confirmed the antitumor activity of fusion protein MULT1E/FasTI. The lungs of mice received i.v. injections of cells of clones L-5 and L-10 were almost clear, whereas lungs from mice received cells of TC-1 or clone L-7 showed many tumors (Figure 3.6C). Compared to TC-1 cells, clone L-7 developed less tumors and the total weight of their lungs was smaller. Again this may be due to the slightly higher-level expression of endogenous MULT1 (Figure 3.2B). The results show that the fusion protein had a much stronger antitumor effect in the pulmonary metastasis setting than in the subcutaneous setting (Figures 3.5 and 3.6). The possible explanation for this difference could be due to the less accessibility of NK cells in subcutaneous tumor tissue. This is in agreement with a study demonstrating that NK cells are more effective against blood borne metastasis (Smyth et al., 2002).

Previous studies have shown that tumor cells ectopically expressing NKG2D ligands such as MULT1 are potently rejected by NKG2D-expressing lymphocytes (Diefenbach et al., 2001; Cerwenka et al., 2001). It is worth to know whether the addition of Fas to the MULT1 protein has additional antitumor effect. Clones expressing MULT1E protein on cell surface were created and studied together with clone L-10. Although the expression of MULT1E on cell surface induced a significant tumor rejection compared to control TC-1 cells, a clear additional antitumor effect was observed when Fas was added to the MULT1 protein (Table 3.1).

The pulmonary metastasis data presented cannot distinguish the following two possibilities: tumor cells expressing the fusion protein are destroyed by NK cells or other NKG2D-expressing cells before implantation or the tumor cells are eradicated by NK cells or other NKG2D-expressing cells after implantation. However, the fact that the

pulmonary metastasis results is better than subcutaneous study results may suggest that tumor cells expressing the fusion protein may be easier to be destroyed by NK cells before implantation. Our planned fusion gene delivery by adenovirus vectors would answer the question whether fusion protein MULT1E/FasTI can eradicate implanted tumors.

A significant challenge facing cancer gene therapy is how to specifically deliver tumor-killing genes into tumor cells efficiently. The recent development of oncolytic adenovirus opens a new window for cancer gene therapy (Cohen and Rudin, 2001; Ries and Korn, 2002). It is not only a virus therapy that can specifically target tumor cells, but also a useful gene delivery system (Lui, 2006). The next step of this study is to use oncolytic adenovirus vectors to systemically deliver the fusion protein MULT1E/FasTI into tumor cells.

In conclusion, a bi-functional chimeric protein containing the extracellular domain of MULT1 and the transmembrane and intracellular domains of Fas is created. Both in vitro and in vivo studies demonstrate its antitumor activity. By combining with an oncolytic adenovirus gene delivery system or other gene delivery systems, this fusion protein may represent a novel and effective approach for cancer therapy.

3.6. Acknowledgement

We thank Eric Holle and his staff for the professional care of the mice used in this study and Lakendra Workman for her administrative contribution in this study. This study was supported in part by the GHS Oncology Foundation and the New Hope fund.

CHAPTER FOUR

In vitro and *in vivo* delivery of novel anticancer fusion protein MULT1E/FasTI via adenoviral vectors

(This chapter represents work submitted to Gene Therapy, March 25, 2009)

In vitro and _in vivo_ delivery of novel anticancer fusion protein MULT1E/FasTI via adenoviral vectors

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Key Words: MULT1, Fas, NKG2D, Apoptosis, NK cell, Adenovirus Gene Therapy

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4.1. Abstract

We previously demonstrated that a novel fusion protein MULT1E/FasTI expressed by TC-1 tumor cells inhibited tumor growth by simultaneously activating NKG2D expressing cells, such as NK cells, through the MULT1E portion and sending a death signal into cells through the Fas portion (Kotturi, *et al*. Gene Therapy, 2008). In this study an adenoviral gene delivery system was used to deliver this fusion protein. Our data indicate that adenoviral vector can efficiently deliver the MULT1E/FasTI fusion protein into TC-1 cells both *in vitro* and *in vivo* as assayed by RT-PCR, FACS analysis, caspase 3 activity and decreased *in vivo* tumor growth. This study further confirms that MULT1E/FasTI represents a powerful bi-functional, therapeutic protein for the treatment of cancers.

4.2. Introduction

NK cells and/or other killer cells recognize and destroy virally infected cells and tumor cells through their surface receptors (Trinchieri, 1989; Diefenbach and Raulet, 2001; Ljunggren and Karre, 1990). NKG2D is a stimulatory receptor expressed by NK cells, subsets of NKT cells and subsets of γδ T cells (Bauer et al., 1999; Diefenbach et al., 2000). Mouse NKG2D is also expressed by virtually all $CD8^+$ T cells and macrophages after stimulation (Jamieson et al., 2002; Diefenbach et al., 2002). Mouse NKG2D ligands include retinoic acid early transcript 1 (Rae1), histocompatibility 60 (H60) (Malarkannan et al., 1998), and mouse UL16-binding protein-like transcript 1 (MULT1) (Carayannopoulos et al., 2002; Diefenbach et al., 2003). Fas (CD95) is a transmembrane cell surface death receptor that belongs to the tumor necrosis factor (TNF) receptor superfamily. Binding of FasL (CD95L) to Fas receptor sends death signals to the cells and induces apoptosis (Peter and Krammer, 2003; Houston and O'Connell, 2004).

One of the strategies that tumor cells have developed to escape NKG2D mediated cytolysis by NK cells or cells expressing NKG2D is down-regulating NKG2D ligands such as MICA (Groh et al., 2002; Sahh et al., 2002), a process also called shedding. Tumor cells also escape Fas-mediated apoptosis by decreasing surface expression of Fas (Moller et al., 1994; Ivanov et al., 2003), secreting an antagonistic 'decoy' receptor (Pitti et al., 1998), expressing anti-apoptotic molecules such as BCL2 family members (Pitti et al., 1998; Boise et al., 1993), down-regulating and mutating pro-apoptotic genes such as BAX, APAF1, or Fas (Sarid et al., 1997; Strand et al., 1996; Ionov et al., 2000; Teiz et al., 2000; Soengas et al., 2001).

In our previous study (Kotturi et al., 2008) we combined the function of NKG2D mediated cytolysis and Fas mediated apoptosis into one functional fusion protein by using the extracellular domain of MULT1 and the transmembrane and intracellular domains of Fas in a mouse model. We created a bi-functional chimeric protein that not only sends apoptotic signal to the tumor cell expressing the fusion protein, but also activates NKG2D expressing cells such as NK cells. Our previous *in vitro* and *in vivo* experimental data demonstrates that MULT1E/FasTI has clear antitumor properties. Here, we used a replication-defective adenovirus as a gene therapy vector to deliver this novel fusion protein into tumor cells. Our results indicate that MULT1E/FasTI delivered by the adenoviral vector is functional and effective *in vitro* and *in vivo*.

4.3. Materials and Methods

4.3.1. Mice and cells

C57BL/6J mice (female) at 6–8 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in our pathogen-free animal facilities. The animal experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH Publication Number 85-23) and the institutional guidelines. The mouse lung epithelial cancer cell line TC-1, obtained from the American Type Culture Collection (Manassas, VA), was cultured in RPMI 1640 medium with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 0.1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10% fetal bovine serum and 0.1mg/ml gentamicin (GIBCO BRL Life Technologies, Gaithersburg, MD). 293A cells were maintained in DMEM (Medaitech, Herndon, VA) supplemented with 10% fetal bovine serum and 0.1mg/ml gentamicin. All cell cultures were incubated at 37 ^{o}C under 5% CO_2.

4.3.2. Construction of the adenoviral vectors

Ad-MULT1E/FasTI, Ad-MULT1E and Ad-Lac-Z adenovirus were generated using the ViraPower Adenoviral Expression System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The full-length cDNAs of MULT1E/FasTI or MULT1E were obtained as previously reported (Kotturi et al., 2008) and cloned into the pCR ®8/GW/TOPO® vector. The pCR ®8/GW/TOPO® vector with MULT1E/FasTI or MULT1E inserts were used as entry clone vectors and transferred into the destination vector pAd/CMV/V5-DEST (Invitrogen, Carlsbad, CA) using the Gateway LR Clonase II enzyme mix according to the manufacturer's directions (Invitrogen, Carlsbad, CA) to generate pAd/CMV/MULT1E/FasTI/V5 and pAd/CMV/MULT1E/V5. The vectors were

linearized with *PacI* enzyme and transfected into 293A cell line using Lipofectamine™ 2000 reagent as per manufacturer's directions. The 293A cells were maintained in DMEM medium until a cytophathic effect was apparent 5–7 days post-transfection. Cells were collected and lysed by subjecting them to four freeze/thaw cycles. The cell debris was pelleted at 3000 x g for 15 min and the supernatant was stored at -80 °C as crude viral lysate. Fifty microliters of crude viral lysate were added into each 293A cell culture dish and incubated for 2-3 days until an 80-100% cytophathic effect was observed. Two recombinant adenoviruses (Ad-MULT1E/FasTI and Ad-MULT1E) were harvested and purified using the Adeno-X ™ virus Mini Purification Kit according to the manufacturer's directions (Clontech, Mountain View, CA) and stored at -80^0 C. Ad-Lac-Z was purchased directly from the manufacturer (Clontech) and amplified as per the above method. Titers of Ad-MULT1E/FasTI, Ad-MULT1E and Ad-Lac-Z stocks were determined using an Adeno-X ™ Rapid Titer Kit as per manufacturer's directions (Clontech).

4.3.3. RT-PCR

TC-1 cells were placed into 6 well plates ($2x10^6$cells/well) and infected with different multiplicities of infection (MOIs) of Ad-MULT1E/FasTI: 500, 250, 100, 50, 25, and 0. Cells were incubated at 37^0C for 24hrs. Total RNA were extracted from the cells using an RNeasy® Plus Mini Kit as per the manufacturer's directions (Quiagen, Germantown, MD). Five hundred nanograms of total RNA from each sample was used to perform the RT-PCR reactions using the MasterAmp™ High Fidelity RT-PCR Kit (Epicentre® Biotechnologies, Madison, WI) with primers amplifying the full length of MULT1E/FasTI: 5' CCCAAGCTTATGGAGCTGACTGCCAGTAACAAGGTCC 3'

and 5' CGGAATTCTCA CTCCAGACATTGTCCTTCATTTTC. RT-PCR of the house keeping gene β-actin was also performed as a control. The RT-PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining.

4.3.4. FACS analysis

TC-1 cells infected with different MOIs of Ad-MULT1E/FasTI (500, 250, 100, 50, 25, and 0) for 24 hours were harvested and 5 x 10^5 cells from each sample were washed twice with staining buffer and incubated with 1μg monoclonal rat anti-mouse MULT1 antibody (R&D Systems, Minneapolis, MN) for 30 minutes at 4 ^oC. After washing twice with staining buffer, the cells were stained with FITC-labeled goat F(ab') anti rat IgG antibody for 30 minutes at 4^oC. After washing twice with staining buffer, cells were re-suspended in 0.5 ml of staining buffer and analyzed on a FACS Calibur (Becton Dickinson, San Jose, CA) using CellQuest software (Becton Dickinson, San Jose, CA).

4.3.5. *In vitro* induction of apoptosis

To determine if MULT1E/FasTI fusion protein delivered by adenoviral vector can send apoptotic signals into infected TC-1 cells *in vitro*, 5x10^5 TC-1 cells were infected with 100 MOI of Ad-MULT1E/FasTI, Ad-MULT1E, or Ad- Lac-Z for 24 hours. The cells were then treated with 1μg/ml of recombinant NKG2D/Fc (R&D Systems, Minneapolis, MN) for 16 hrs. Caspase-3 activity was measured using Caspase-3 Fluorometric Assay Kit (R&D Systems, Minneapolis, MN) according to the instructions provided by the manufacturer. In order to determine if there is a dose effect of pfu on apoptosis, different MOIs of Ad-MULT1E/FasTI (500, 250, 100, 50, 25 and 0) were also tested. Briefly, 8x10^5 cells from each sample were harvested, washed with PBS, and

lysed. The lysates were incubated with caspase-3 substrate for 1 hour at 37^{o}C and the fluorescent signal was detected using a SpectraMax Gemini XS microplate reader (Molecular Devices, Sunnyvale, CA). The assay was performed in triplicates.

4.3.6. *In vivo* tumor study

To study the therapeutic effect of Ad-MULT1E/FasTI in subcutaneous tumors, 2 x 10^5 TC-1 cells were injected subcutaneously into the flank of 6-8 week old C57BL/6J mice according to the animal care (NIH Publication Number 85-23) and institutional guidelines. When the tumors reached 40mm^3 (about a week post-injection), Ad-MULT1E/FasTI, Ad-MULT1E, Ad-Lac-Z or HBSS were injected into individual tumors using a 0.5ml U-100 insulin syringe with 281/2G needle. The virus particles ($1x10^9$pfu/tumor/dose) were given every other day for 4 doses. To monitor tumor progression in the mice, the longest (L) and shortest diameter (W) of the tumors were measured every two days with a linear caliper. The tumor volume was calculated as per formula 1/2 LW^2.

4.3.7. *In vivo* apoptosis detection

In vivo apoptosis in tumors treated with 4 injections of Ad-MULT1E/FasTI, Ad-MULT1E, Ad-Lac-Z or HBSS was detected using the FLIVO^{TM} *in vivo* apoptosis detection reagent. One hundred microlitters of FLIVO^{TM} *in vivo* apoptosis detection reagent was injected into tail vein of mice with tumors 48 hours after the last intra-tumoral injection. Thirty minutes later, the mice were euthanized and tumor tissue was exercised and frozen in liquid nitrogen. 7µm frozen sections were prepared and the apoptosis in the tumor slides was visualized and photographed using an Olympus 1X70

fluorescent microscope (Olympus, Center Valley, PA). The data presented are the sum of apoptotic cells from 12 random fields of each section.

4.3.8. Statistical analysis

Results were expressed as means ± SD. Statistical significance between groups was assessed by the unpaired Student's t-test, two-way analysis of variance (ANOVA) and one way analysis of variance (ANOVA). Data are considered statistically significant if p values were 0.05 or lower.

4.4. Results

4.4.1 Adenoviral vector effectively delivers MULT1E/FasTI into cultured TC-1 cells

Three adenoviral vectors were contructed: 1) Ad-MULT1E/FasTI containing the full fusion protein sequence of MULT1E extracellular domain and FasTI transmembrane and intracellular domain designated; 2) Ad-MULT1E containing only the MULT1E extracellular domain; 3) Ad-Lac-Z containing the Lac-Z gene as control adenoviral vector. The adenoviral vectors were linearized using *Pac*1 and transfected into 293A cells to generate adenoviral stocks. The titer of these adenoviral stocks are in the range of 10^{10} PFU/ml.

TC-1 tumor cells were infected with Ad-MULT1E/FasTI viral particles with different multiplicities of infection (MOI): 500, 250, 100, 25, and 0 for 24 hours. The fusion gene expression was detected by RT-PCR and FACS analysis. Both assays not only demonstrate the fusion gene expression in the infected cells, but also show a clear dose dependent expression manner (Figure 4.1A and 4.1B).

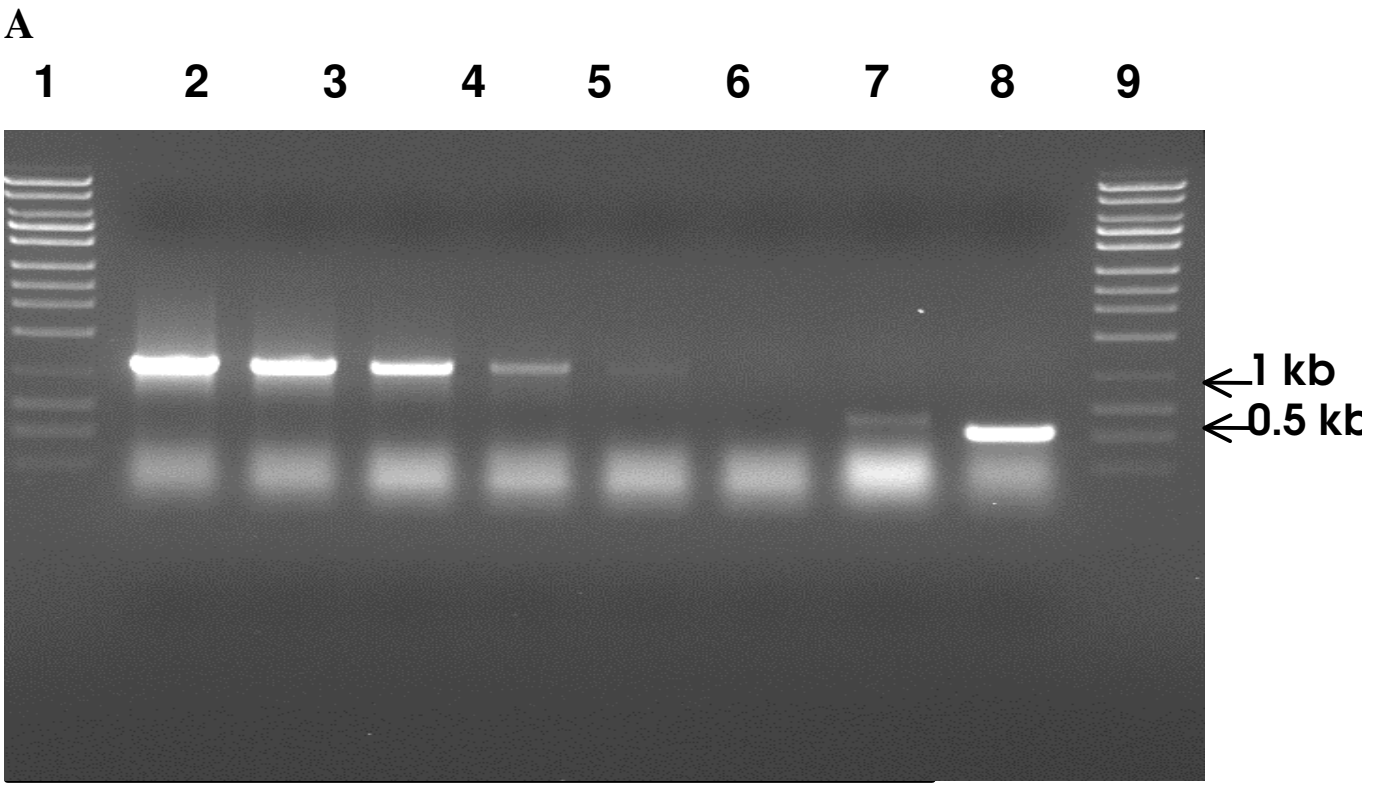

Figure 4.1. Reverse transcriptase (RT)-PCR analysis of AD-MULT1E/FasTI expression at different MOIs. A) RNAs were isolated from infected cells and RT-PCR was performed. Lanes 2, 3, 4, 5, 6 and 7 are 1134bp RT-PCR product of total RNA from TC1 cells infected with Ad-MULT1E/ FasTI at MOIs 500, 250, 100, 50, 25 and 0, amplified with MULT1E forward and Fas reverse primers, Lane 8:ß-actin control and lane 1and 9 are 1 kb markers.

B

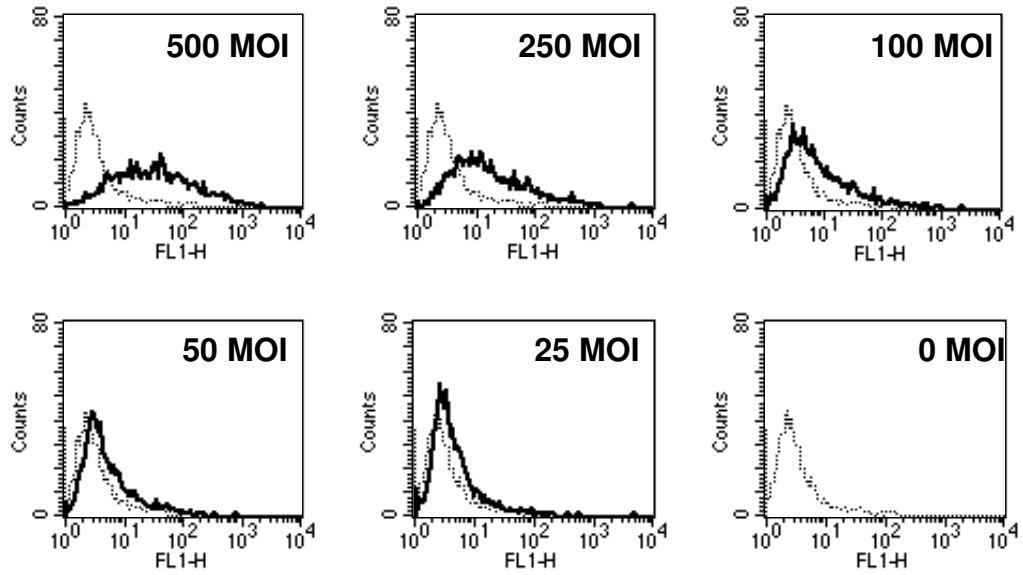

Figure 4.1. Fluorescence-activated cell sorting (FACS) analyses of AD-MULT1E/FasTI expression at different MOIs. (B) 5 x 10^5 TC1 cells were harvested from each group that were infected with Ad-MULT1E/FasTI at MOIs 500, 250, 100, 50, 25, 0 and stained with purified rat anti-mouse MULT1 antibody followed by goat anti IgG F(ab')-fluorescein isothiocyanate (FITC). Dashed lines represent un-infected TC-1 cells.

4.4.2. MULT1E/FasTI delivered by adenoviral vector induces apoptosis in TC-1 cells

To confirm the activity of adenoviral vector delivered MULT1E/FasTI fusion protein in TC-1 cells, recombinant NKG2D/Fc ligand was added to the infected cells. As MULT1E binds to its ligand NKG2D, the binding would send apoptotic signal through its FasTI region into TC-1 cells (Kotturi et al., 2008). When TC-1 cells were infected with 100 MOI of Ad-MULT1E/FasTI and treated with NKG2D/Fc, their caspase 3 activity was significantly higher ($p<0.001$) than the cells that were also infected by Ad-MULT1E/FasTI but not treated with NKG2D/Fc. Ad-MULT1E or Ad-Lac-Z infection showed slightly increased caspase 3 activity ($p>0.05$) compared with non-infected TC-1 cells (Figure 4.2A). The caspase activity in Ad-MULT1E/FasTI infected and NKG2D treated TC-1 cells is adenoviral particle dose dependent: 500 MOI infection generated the highest caspase activity, while a 25 MOI infection did not show any increased caspase 3 activity compared with un-infected TC-1 cells (Figure 4.2B).

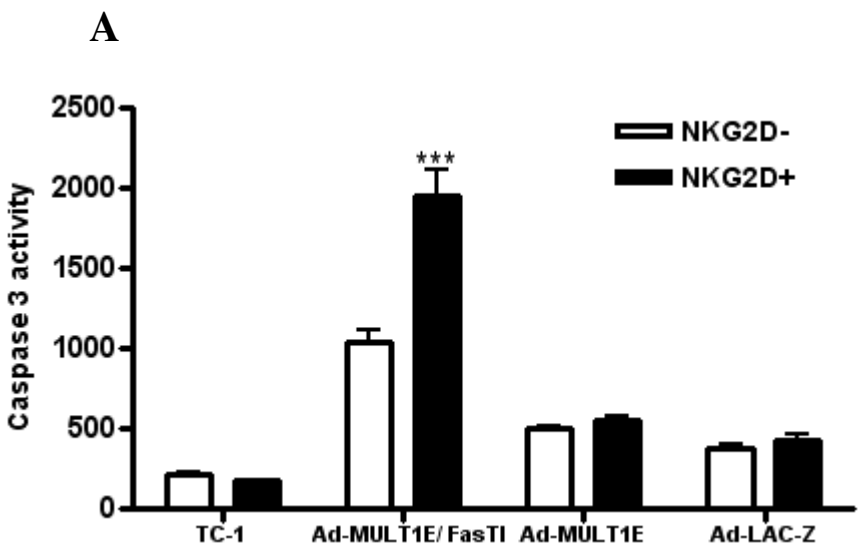

Figure 4.2. Ad-MULT1E/FasTI infection induces apoptosis *in vitro*.

A) TC-1 ($5x10^5$) cells were infected with Ad-MULT1E/FasTI, Ad-MULT1E and Ad-Lac-Z at 100 MOI. Twenty-four hour after infection, cells were treated with 1µg/ml NKG2D/Fc for 16 hrs. The cells were then analyzed for apoptosis by caspase-3 assay. The data represented are summaries of three separate experiments. *p< 0.05; **p< 0.01 and ***p< 0.001.

B

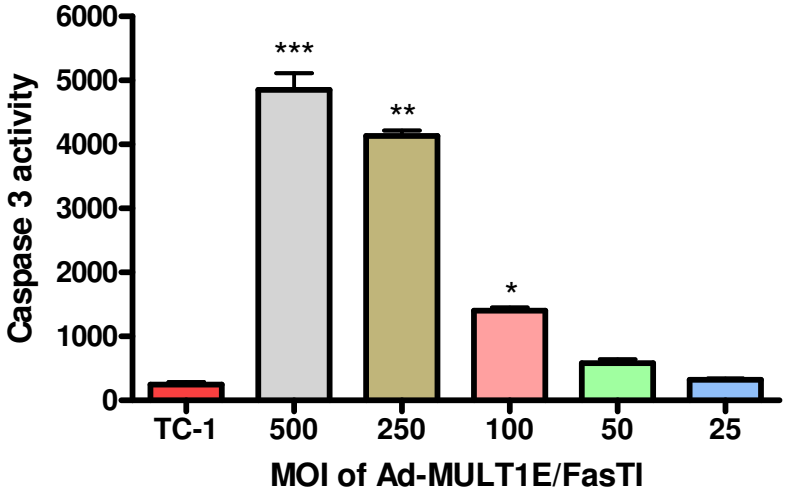

Figure 4.2. Ad-MULT1E/FasTI fusion construct infection induces apoptosis *in vitro*. B) TC-1 ($5x10^5$) cells were infected with Ad-MULT1E/FasTI at different MOIs: 500, 250, 100, 50, 25. Twenty-four hour after infection, cells were treated with 1µg/ml NKG2D/Fc for 16 hrs. The cells were then analyzed for apoptosis by caspase-3 assay. The data represented are summaries of three separate experiments. *p< 0.05; **p< 0.01 and ***p< 0.001.

4.4.3. Intratumor delivery of MULT1E/FasTI by adenoviral vector

To observe the antitumor activity of MULT1E/FasTI delivered by adenoviral vector, subcutaneous TC-1 tumors were grown in C57BL/6J mice. When the tumor reached about a size of 40 mm^3, Ad-MULT1E/FasTI, Ad-MULT1E, Ad-Lac-Z, or HBSS at a dose of $1x10^9$ pfu/tumor in 0.05ml HBSS was injected into the tumors. The injections were repeated every other day for 4 times. The size of the tumors was measured every two days. At day 22 after tumor cell injection, the mice were sacrificed and the tumors were collected and measured. Although tumors received Ad-MULT1E or Ad-Lac-Z grew slightly slower than tumors received only HBSS, tumors received Ad-MULT1E/FasTI showed the slowest growth rate (Figure 4.3A). The end point tumor measurement confirmed the conclusion that Ad-MULT1E/FasTI treatment significantly slowed the tumor growth ($P<0.01$, Figure 4.3B).

A

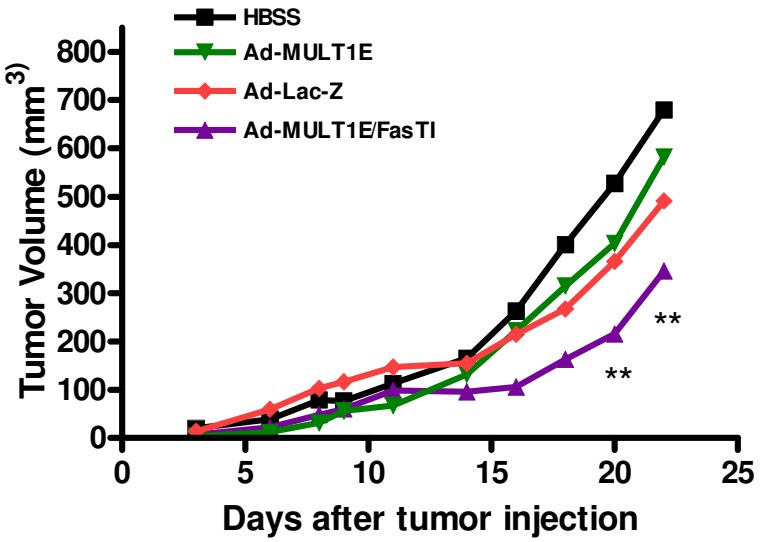

Figure 4.3. MULT1E/FasTI delivered by adenoviral vector inhibits *in vivo* tumor growth. (A) TC1 cells (2 x 105) in 0.2ml HBSS were subcutaneously injected into flanks of C57BL/6J mice (n = 6). One week after tumor cell injection, various viral vectors at a dose of 1 x 10^9 pfu/tumor were intratumorally injected on every other day for a total of 4 injections. Control animals received injections of 50µl HBSS. Tumor growth was measured and presented as $1/2LW^2$.

B

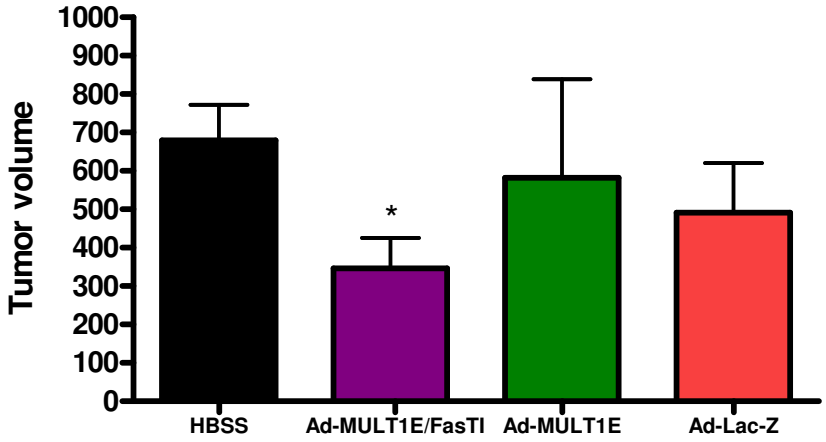

Figure 4.3. MULT1E/FasTI delivered by adenoviral vector inhibits *in vivo* tumor growth. (B) TC1 cells (2 x 10^5) in 0.2ml HBSS were subcutaneously injected into flanks of C57BL/6J mice (n = 6). One week after tumor cell injection, various viral vectors at a dose of 1 x 10^9 pfu/tumor were intratumorally injected on every other day for a total of 4 injections. Control animals received injections of 50µl HBSS. Tumor growth was measured and presented as $1/2LW^2$. At the end of this experiment, the mice were sacrificed and tumors were harvested and measured. *P< 0.05

4.4.4. MULT1E/FasTI delivered by adenoviral vector induces apoptosis in tumor

In order to confirm that MULT1E/FasTI delivered by adenoviral vector slows tumor growth by inducing tumor cell to undergo apoptosis, two days after last adenoviral particle injection, some mice were i.v. injected with FLIVO^{TM} *in vivo* apoptosis detection reagent. Thirty minutes later, the tumor tissues were collected and 7 μm frozen sections were produced. The slides were examined under fluorescent microscope and the green fluorescent cells were counted. The number of apoptotic cells in tumors receiving Ad-MULT1E/FasTI is significantly higher than that of tumors receiving either Ad-MULT1E or Ad-Lac-Z. There are no significantly more apoptotic cells in tumors receiving Ad-MULT1E or Ad-Lac-Z when compared with tumors that received just HBSS (Figure 4.4).

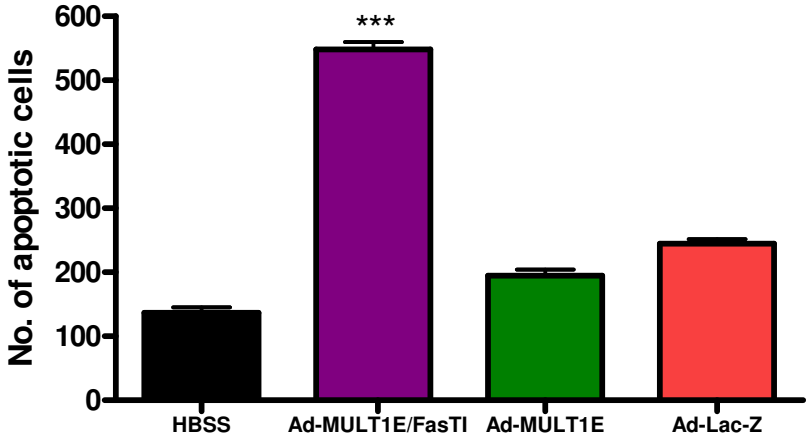

Figure 4.4. MULT1E/FasTI delivered by adenoviral vector induces apoptosis *in vivo*. Two days after the last intratumor virus injection, 100µl of FLIVO^{TM} *in vivo* apoptosis detection reagent (green) was injected into mice. Thirty minutes later, the mice were sacrificed and tumors collected. Frozen sections were made from the tumor tissues and examined under fluorescent microscope. The data presented are the sum of apoptotic cells from 12 random fields of each section. *** $P < 0001$

4.5. Discussion

The findings of our present study show the therapeutic effect of adenovirus-mediated gene therapy delivering the novel fusion protein MULT1E/FasTI. At present, adenoviruses are still an attractive vector to deliver desirable genes for the treatment of cancer because of its high infectivity *in vivo*, which allows for direct vector injection in the clinic and avoids additional manipulations *in vitro* as required for other vector systems such as the retrovirus (Young et al., 2006). The most encouraging finding from our study is that mice that received treatment with Ad-MULTE/FasTI fusion construct showed more apoptosis *in vivo*, formed smaller tumors, and survived longer.

We first examined the efficiency of adenoviral transduction and the effect of MOI on the expression level of fusion protein in TC1 cells *in vitro* using RT-PCR and FACS staining. We observed that the expression of fusion protein MULT1E/FasTI delivered by adenoviral vector is clearly viral particle dose dependent. Higher MOI resulted in marked increase in the expression level of fusion protein, whereas lower MOI reduced expression level (Figure 4.1A and 4.1B). Then, we showed that MULT1E/FasTI fusion protein delivered by the adenoviral vectors was fully functional. Consistent with our previous work (Kotturi et al., 2008), NKG2D/Fc treatment of Ad-MULT1E/FasTI infected TC-1 cells significantly increases the caspase 3 activity of the cells when compared with Ad-MULT1 or Ad-Laz-Z infected cells. Furthermore, the caspase 3 activity of MULT1E/FasTI infected cells is adenovirus dose dependent (Figure 4.2).

We also investigated the *in vivo* therapeutic effect of adenovirus-mediated MULT1E/FasTI gene therapy by intra-tumoral injections of viruses. The results show that the average tumors treated with Ad-MULT1E/FasTI were ~50% smaller compared to

HBSS treated tumors. Tumor volume of MULT1E/FasTI treatment group was smaller on 22nd day compared to other groups (Figure 4.3). A repeated experiment with up to nine viral injections showed similar results (data not shown). This reduction in size of the tumors can be attributed to more apoptosis in tumors treated with Ad-MULT1E/FasTI which is confirmed by FLIVO^{TM} *in vivo* apoptosis detection reagent (Figure 4.4).

NKG2D recognition of multiple stress-inducible host proteins is of considerable research interest and the unique application of the NKG2D/ stress proteins has potential to be manipulated for therapeutic purposes. Tumor cells expressing NKG2D ligands have been shown to be susceptible to NK cell mediated lysis and induce a very potent antitumor response and protective immunity *in vivo* (Diefenbach et al., 2000; Carayannopoulos et al., 2002; Kotturi et al., 2008). The failure of some tumor cells to die following drug treatment and their resistance to drugs is due to their resistance to engage apoptosis, as tumors cells have defects in triggering mechanisms of their own death by apoptosis (Mollinedo and Gajate, 2006). The toxic side effects observed from the use of FasL or agonistic anti-Fas antibodies *in vivo*, resulting in a fatal hepatic damage with symptoms similar to fulminant hepatitis (Ogasawara et al., 1993; Tanaka et al., 1997) has encouraged a shift of focus to therapies that would enhance apoptosis by using Fas ligand independent activation of Fas (Mollinedo and Gajate, 2006). Therefore, our fusion protein MULT1E/FasTI may be both novel and useful as it combines two different functionalities together into one functional protein. It activates NKG2D expressing immune cells through its MULT1E extracellular region and induces apoptosis through its FasTI region.

In summery, our findings confirm that adenoviral mediated delivery and expression of MULT1E/FasTI fusion protein represents a powerful new approach to destructively target tumors. Our approach, therefore, may provide a potential avenue for new cancer therapies and supports further investigation MULT1E/FasTI therapeutic strategy for treating cancer.

4.6. Acknowledgement

We thank Eric Holle and his staff for the professional care of the mice used in this study, Lakendra Workman for her administrative contribution in this study and Angela Howing for her help in preparation of frozen tissue sections. This study was supported in part by the GHS Oncology Foundation and the New Hope fund.

CHAPTER FIVE

SUMMARY

Tumor cells have developed multiple mechanisms to subvert and suppress immune responses by regulating cell-surface expression of Fas (Ivanov et al., 2003; Pitti et al., 1998) and Fas ligand (Niehans et al., 1997; Okada et al., 2000; Zheng et al., 2003) and shedding NKG2D ligands such as MULT1 (Raffaghello et al., 2004), resulting in escape from killing by infiltrating antitumor NK cells and T cells (Elsasser-Beile et al., 2003). As NKG2D ligand surface levels critically determine the susceptibility to NKG2D-mediated NK cell lysis, stable expression of NKG2D ligands on tumors would help increase NK cell lysis of tumors (Diefenbach and Raulet, 2001). In this study, we enhanced the cell-surface expression of MULT1, one of the mouse NKG2D ligands, by anchoring the extracellular domain of MULT1 on tumor cells using a transmembrane sequence of Fas. We also introduced the DD of Fas in the intracellular domain of the fusion protein MULT1E/FasTI, hoping to develop a bifunctional chimeric protein that can send an apoptosis signal to the tumor cells and at the same time activate NKG2D-expressing immune cells such as NK cells.

We cloned the cDNA encoding the extracellular domain of MULT1 gene from thymus of new born mice and ligated it to the transmembrane and intracellular domains of mouse *fas* cDNA. The resulting fusion cDNA was inserted into a mammalian cell expressing vector under the control of CMV promoter. The vector was then transfected into mouse TC-1 lung epithelial cancer cells, and stable cell lines expressing the fusion protein were established. The transcription of the novel fusion protein MULT1E/FasTI in

the transfected cells was confirmed by RT–PCR and its expression was characterized by surface FACS analysis.

One of the key features of the designed fusion protein is to send apoptosis signals into the cells expressing the fusion protein, upon binding to its ligand, NKG2D. Although we do not have direct evidence supporting that the binding of NKG2D can form DISC inside tumor cells, a clear apoptosis signal is sent to the cells as indicated by the increased caspase-3 activity and increased Annexin V-positive cells after treatment with recombinant NKG2D/Fc. Our data shows that fusion protein MULT1E/FasTI, when expressed on cells, not only sends apoptotic signals into cells expressing it, but also activates immune cells that express receptors for MULT1 like NK cells. When co-culutred with NK cells isolated from spleen, fusion proteins expressing clones activated NK cells by producing IFN-γ.

This study shows that fusion protein MULT1E/FasTI has antitumor activity *in vivo*. We used a subcutaneous tumor model and a pulmonary metastatic tumor model in this study. In the subcutaneous tumor study, MULT1E/FasTI expressing clones formed smaller tumors compared to controls; and in the pulmonary metastasis study, mice completely rejected tumor cells expressing the fusion protein. We showed that the fusion protein had a much stronger antitumor effect in the pulmonary metastasis setting than in the subcutaneous setting. This is in agreement with a study demonstrating that NK cells are more effective against blood borne metastasis (Smyth et al., 2002). Previous studies have shown that tumor cells ectopically expressing NKG2D ligands such as MULT1 are potently rejected by NKG2D-expressing lymphocytes (Diefenbach et al., 2001;

Cerwenka et al., 2001). Our data shows that adding Fas to the MULT1 has a clear additional antitumor effect.

A significant challenge facing cancer gene therapy is how to specifically deliver tumor-killing genes into tumor cells efficiently. The recent development of adenovirus as gene delivery vectors opens a new window for cancer gene therapy (Cohen and Rudin, 2001; Ries and Korn, 2002). We used adenoviruses to deliver the fusion protein MULT1E/FasTI into tumor cells. The findings of this study demonstrate the therapeutic effect of adenovirus-mediated gene therapy of novel fusion protein MULT1E/FasTI. The most encouraging finding from a preclinical viewpoint is that mice receiving treatment with Ad-MULTE/FasTI showed more apoptosis *in vivo*, formed smaller tumors, and survived longer.

5.1. Proposed Mechanism of MULT1E/FasTI Fusion Protein

NK cell function is regulated by a balance between activating and inhibiting receptor signals (Trinchieri, 1989; Diefenbach and Raulet, 2001; Moretta et al., 2001). Several types of inhibitory NK cell receptors recognize MHC class I molecules on target cells and prevent NK cell cytotoxicity toward normal cells (Yokoyama et al., 1995). The expression of ligands for activating receptor on target cells tips the balance toward activation of NK cells and induces NK cell cytotoxicity by formation of NK cell lytic synapse. NK cell cytotoxicity involves the secretion of cytolytic effector molecules known as lytic granules. The induction of NK cell effector functions, such as cytotoxicity, requires the contact between the NK cell and its target cell. The events that occur following the interaction between a cytolytic cell and its target cell, and the formation of the NK cell lytic synapse can be divided into three main stages: 1) initiation

stage 2) effector stage and 3) termination stage. Initiation stage includes adhesion and initial signaling for cell activation. Effector stage involves actin reorganization, receptor clustering, raft formation, polarization of the microtubule-organizing centre (MTOC) and lytic granule fusion with the plasma membrane. Termination stage includes a period of inactivity and detachment.

Fas receptor activation can occur through different mechanisms. Binding of homotrimers of FasL to Fas can homotrimerize Fas receptor (Papoff et al., 1999; Siegel et al., 2000). A death domain-independent oligomerization domain in the extracellular region of the Fas, mapping to the N-terminal 49 amino acids, can also mediate homo- and heterooligomerization of the death receptor (Papoff et al., 1999). Apoptosis can be triggered in the absence of FasL by overexpression of the Fas cytoplasmic domain or Fas lacking the N-terminal 42 amino acids (Papoff et al., 1999), suggesting that the extracellular oligomerization domain of Fas is not required to initiate signaling and that self-association of the death domain is necessary and sufficient to induce cell death. The intracellular death domains of death receptors show a high tendency to self-associate, and when overexpressed by gene transfer in eukaryotic cells, trigger apoptotic signaling (Boldin et al., 1995). These findings indicate that the Fas receptor plays an active role in its own clustering and that its oligomerization can be achieved in the absence of FasL.

We hypothesize that, when NKG2D expressing cells such as NK cells come in contact with TC1 cells expressing the MULT1E/FasTI fusion protein, an NK cell lytic synapse would be formed as a result of the receptor-ligand interaction between NK cells and fusion protein expressing target cells. At this NK cell lytic synapse, activating NKG2D receptors bind to MULT1 ligands, cluster together and form lipid rafts.

Formation of lipid rafts consisting of receptor-ligand complexes would result in activation of NK cells and NK cell cytotoxicity with the release of lytic granules consisting of granzymes and IFN-γ at the immunological synapse. Binding of NKG2D to the MULT1E region of the fusion protein causes clustering of the fusion protein, and through death domain interactions would trigger formation of microaggregates resulting in larger clusters of FasTI, formation of DISC, caspase-8 activation and apoptosis in cell. Hence, our fusion protein approach is a two pronged approach for activating NK cells as well as inducing apoptosis, when the fusion protein binds to NKG2D receptors. Even though we do not have evidence of the formation of lytic synapses with NK cells and DISC formation in fusion protein expressing cells, our IFN-γ assay using NK cells and caspase-3 ELISA assay confirm the functionality of both MULT1E and FasTI regions in our fusion protein and the dual role of MULT1E/FasTI.

In summary, a bi-functional chimeric protein containing the extracellular domain of MULT1 and the transmembrane and intracellular domains of Fas is created. It may provide a potential avenue for new cancer therapies and supports further investigation of therapeutic strategies using other NKG2D ligands combined with Fas transmembrane and intracellular domains for treating cancer.

CHAPTER SIX

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