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Cancer Gene Therapy via NKG2D and FAS Pathways

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1. Introduction

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 human (Glienke et al., 1998). Like most activating receptors, NKG2D is a multi-subunit receptor complex. Signaling in NKG2D is mediated by specialized signaling adaptors. In mouse NKG2D associates with two distinct adaptors: DAP-10 and DAP-12/KARAP (Diefenbach et al., 2002a), while in human NKG2D exclusively uses DAP-10 (Rosen et al., 2004). Non-covalent interactions are responsible for these associations (Diefenbach et al., 2002b). 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 mouse 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 Expression of NKG2D receptor

The NKG2D receptor is constitutively expressed on most innate immune effector cells of lymphoid origin, including NK cells, most TCR γδ T cells (Jamieson et al., 2002), 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 (Taieb et al., 2006) 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).

	Human	Mouse
NK cells	All NK cells	All NK cells
TCRαβ T cells	Naïve, activated and memory CD8+ T cells Subpopulation of Synovial and circulating CD4+ T cells in rheumatoid arthritis patients	Activated and memory CD8+ T cells Not express on naïve CD8+ T cells Not express on CD4+ T cells
TCRγδ T cells	Most blood and IEL TCRγδ T cells	25% of splenic TCRγδ T cells Large fraction of NKT cells
NKT cells	ND	
DC	ND	IKDC subset
Macrophages	ND	Only mRNA

Table 1. Pattern of NKG2D receptor expression in human and in mouse. IEL: intestinal intraepithelial lymphocytes. IKDC: interferon producing killer dendritic cells. ND: not determined. (Coudert and Held, 2006).

1.2 Function of NKG2D receptor

Human NKG2D signals exclusively via DAP-10, while mouse NKG2D associates 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 mouse 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 mouse 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 phosphatidylinositol 3- kinase (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 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.

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; Maasho 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).

1.3 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. 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 including cytomegalovirus UL16-binding protein (ULBP; also known as RAET1 proteins) consisting of five members (ULBP1–ULBP4 and RAET1G) (Bahram et al., 1994; 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 and the minor histocompatibility protein H60 and MULT (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). 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).

1.4 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) 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 (Hue 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).

1.5 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; Jinushi et al., 2003; Groh et al., 1999; Groh 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 (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).

1.6 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 KD. MULT1 protein is a type I transmembrane protein with an N-terminal signal sequence of 25 aa, 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. Compare to other NKG2D ligands MULT1 lacks α3- like domain and the GPI trans amidation site (Diefenbach et al., 2003).

MULT1 protein is a glycoprotein with two ectodomains containing four N-glycosylation sites and one O-glycosylation site. 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. 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).

1.6.1 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).

1.6.2 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).

MULT1 protein has a K_D of 6 nM and K_{off} of ~0.006 S^{-1} which is several times lower than RAE1є 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, RAE1є 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). 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).

1.7 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 (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 (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 (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.

1.8 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).

1.8.1 Escaping immune recognition

In nature, both host and pathogen are under natural selection pressure to diversify and refine their defense 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 (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). 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 (Zhou et al., 2005; Chalupny et al., 2006).

1.8.2 Evading tumor responses

One of the main functions of NKG2D is to participate in antitumour immune response and immune surveillance (Diefenbach et al., 2001; Smyth 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 TGF-β, effectively anergizing NKG2D-mediated immune recognition, and switching off the expression of NKG2D ligands as they progress (Groh et al., 2002; 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).

1.8.3 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., 1996; 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., 2002b; 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., 2003). 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 specialties that are specific for different cell types and tissues (Eagle and Trowsdale, 2007).

1.9 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 using NKG2D receptor-ligand interactions (Coudert and Held, 2006). Chimeric anti-tumor mAb/NKG2D-ligand, with the antibody portion of the chimeric protein specific to 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 (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 (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 tumor 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., 2002a; Carayannopoulos et al., 2002b; 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).

1.10 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 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 (Mollinedo and Gajate, 2006). CD95 receptors are expressed on the surface of cells as preassociated homotrimers (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) (Siegel et al., 2000). CD95 receptors only function as trimers (Kischkel et al., 1995).

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 death-inducing signaling complex (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). 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 (Boldin et al., 1996). Thus, activation of Fas results in receptor aggregation and formation of 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. Once procaspase-8 associates with FADD, the high local concentration of procaspase-8 leads to its autoproteolytic cleavage and activation (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).

1.11 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). Rat FasL has no signal sequence at the N-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. 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).

1.11.1 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 (Bellgrau 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 (Kase et al., 2003) whereas, stronger FasL expression was found in liver metastases of colon cancer relative to the primary tumor (Houston and O'Connell, 2004).

1.11.2 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 (Igney and Krammer, 2002). 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 (Houston and O'Connell, 2004).

1.12 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; 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 (Hohenberger and Tunn, 2003; Ogasawara et al., 1993; 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 (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).

1.13 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). 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. 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. 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 developed to provide cloning sites for transgene insertion (Gomes and Tong, 2006).

1.14 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; 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 tumor cells. The early clinical trial data of the NTR/CB1954 system in patients with liver cancer or prostate cancer are extremely encouraging (Palmer et al., 2004). Adenoviral delivery of TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) showed significant anti-tumor efficacy in animal models of aggressive primary and metastatic cancer (Ma et al., 2005). Clinical trials of a recombinant adenovirus expressing interleukin-12 (IL-12) in patients with advanced digestive tumors have produced evidence of antitumor effects (Sangro et al., 2004).

2. 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 engagement of the fusion protein with NKG2D receptor would send death signals into the cells that express the fusion protein and induce apoptosis of the cell (Fig.1).

2.2 Objectives

The work presented here is a two pronged approach of using a novel fusion protein consisting of MULT1 extracellular domain and Fas transmembrane and intracellular domains for cancer therapy. First, the construction and expression of MULT1E/FasTI fusion protein 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.

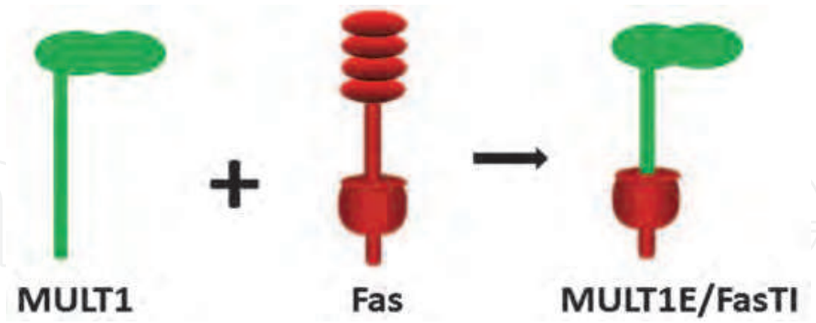


Fig. 1. Schematic representation of proposed mechanism of novel fusion protein MULT1E /FasTI.

3. Construction and evaluation of fusion protein MULT1E/FasTI

3.1 Plasmid construction of pMULT1E/FasTI

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 an 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.

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 CGGGATCCCCC AGAA ATCGCCTATGGTTGTTGTTGACC and the 3′ primer was CGGAATTCTCACTCCAGACA TTGTCCTTCATTTTC. 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.

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 (Fig.2) and used for transfection.

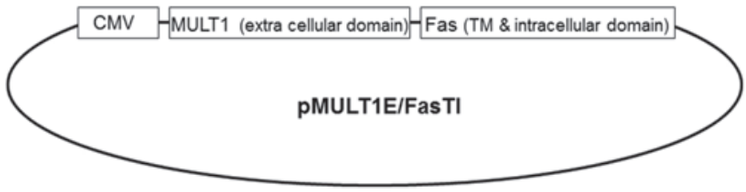


Fig. 2. Construction of the MULT1E/FasTI plasmid.

3.2 Expression of pMULT1E/FasTI

Lung carcinoma TC-1 tumor cells were transfected with pMULT1E/FasTI. Three clones that were zeocin resistant were selected and labeled as L-5, L-7 and L-10. An *in vitro* cell growth study showed that all the clones grew at a similar rate as TC-1 cells. 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 (Fig. 3). To confirm that MULT1E of the fusion protein can indeed bind to NKG2D, the cells were incubated with NKG2D/Fc, a recombinant fusion protein, 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 (Fig. 4) with L-10 cells the strongest. 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.

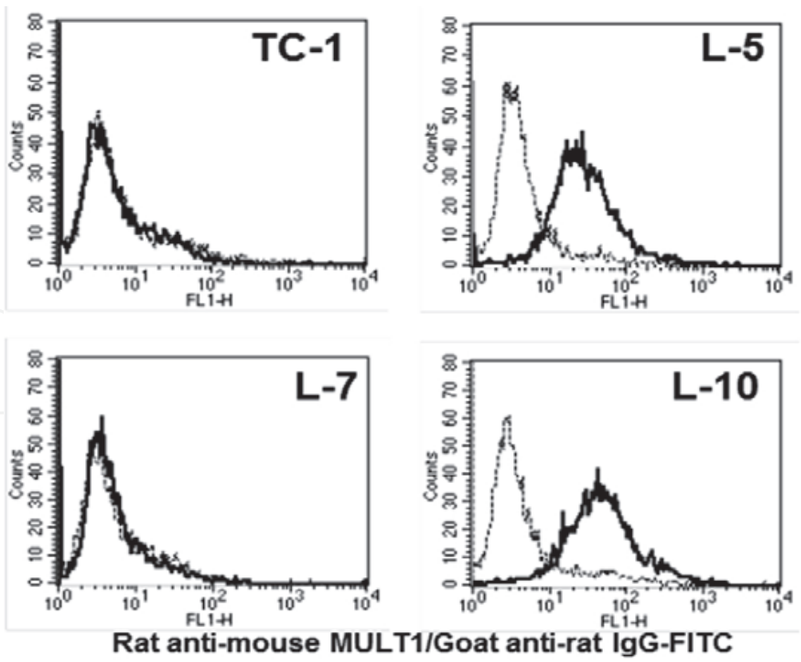


Fig. 3. FACS analysis of MULT1E/FasTI expression. A total of $5x10^5$ cells of TC-1 and clones L5, L7 or L10 were stained with purified rat anti-mouse MULT1 antibody followed by goat anti-mouse IgG F(ab')-FITC. The dashed lines are isotype controls.

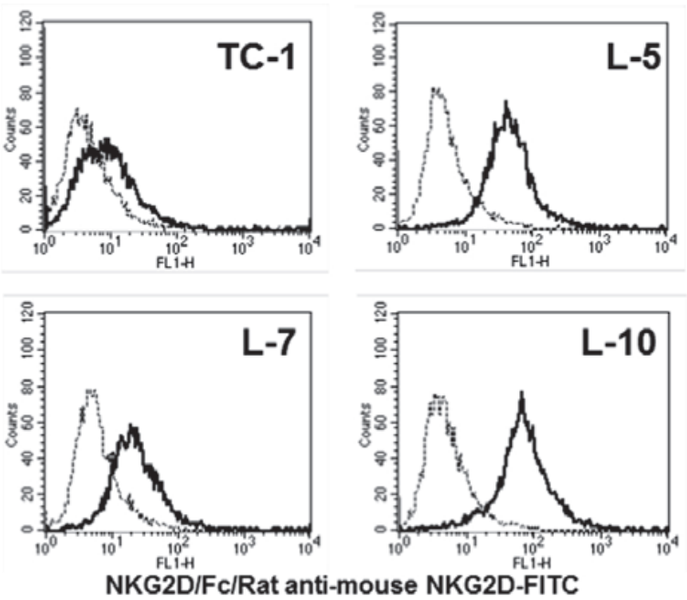


Fig. 4. FACS analysis of MULT1E/FasTI expression. A total of 1x10^6 cells of TC-1 and clones L-5, L-7 or L-10 were first treated with NKG2D/Fc and then stained with anti-mouse NKG2D antibody conjugated with FITC. The cells were analyzed on FACS Calibur with CellQuest software. Dashed lines are controls without NKG2D/Fc incubation.

3.3 Fusion protein MULT1E/FasTI induces apoptosis of cells

To confirm the concept that when bound to its ligand NKG2D 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 (Fig. 5). 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 (Fig. 6A and 6B). 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 (Fig. 6C). The treatment of NKG2D/Fc induced more apoptotic cells in clone L-10 than clone L-5 (Fig. 6A and 6C).

3.4 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 (Fig. 7A). 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 (Fig. 7B).

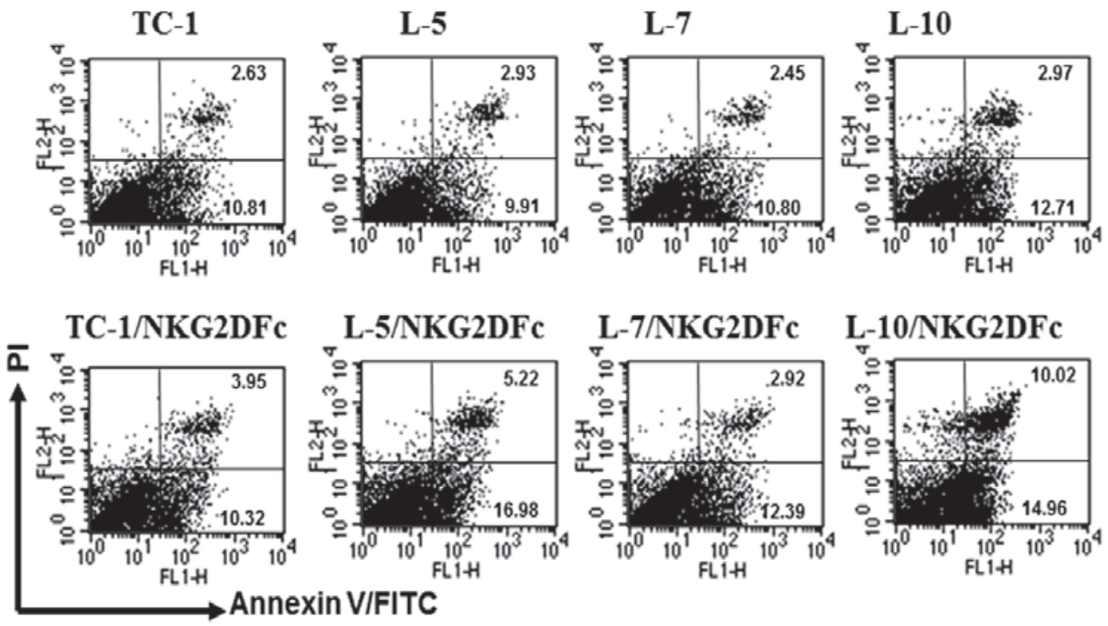


Fig. 5. MULT1E/FasTI induces apoptosis. A total of 1x10^6 cells of TC-1 and clones L-5, L-7 and L-10 were treated with 1 μg/ml NKG2D/Fc for 16 h. The cells were then analyzed for apoptosis and necrosis using Annexin V staining and PI. This figure is an example of the FACS data.

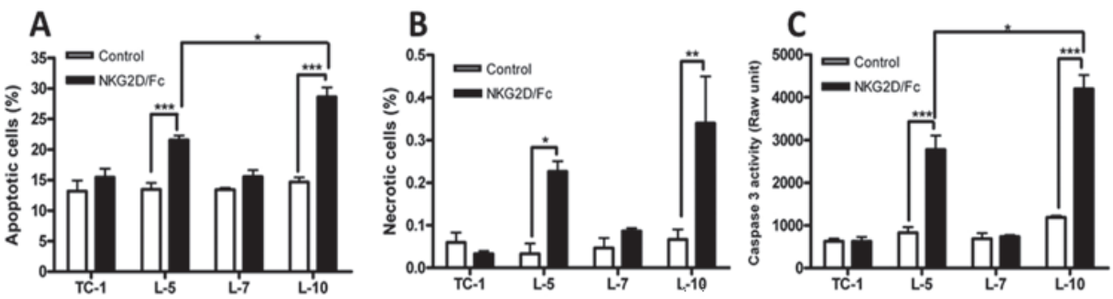


Fig. 6. MULT1E/FasTI induces apoptosis. A total of 1x10^6 cells of TC-1 and clones L-5, L-7 and L-10 were treated with 1 μg/ml NKG2D/Fc for 16 h. The cells were then analyzed for apoptosis and necrosis using Annexin V staining (A, B) or caspase-3 assay (C). The statistical analyses were conducted between 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.

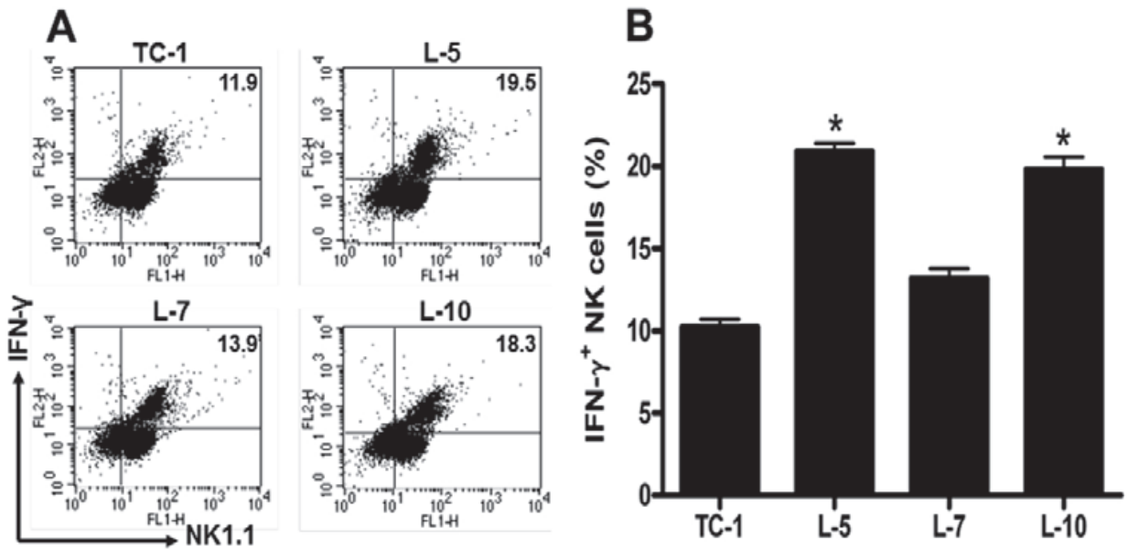


Fig. 7. MULT1E/FasTI activates natural killer (NK) cells. A total of $1x10^6$ cells of TC-1 and clones L-5, L-7 and L-10 were co-cultured with NK cells for 3 h. The cells were stained with anti-NK1.1-FITC and were then permeabilized and fixed, and stained with antimouse IFN-γ-PE. The cells were analyzed on FACS Calibur with CellQuest software. (A) represents an example of the FACS data and (B) is the summery of the data from three separate experiments. *P<0.05.

3.5 *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; Fig. 8). 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 (Fig. 9A). The total weight of the lungs with the tumors was measured (Fig. 9B) and the tumor nodules on the surface of the lungs were counted (Fig. 9C). 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.

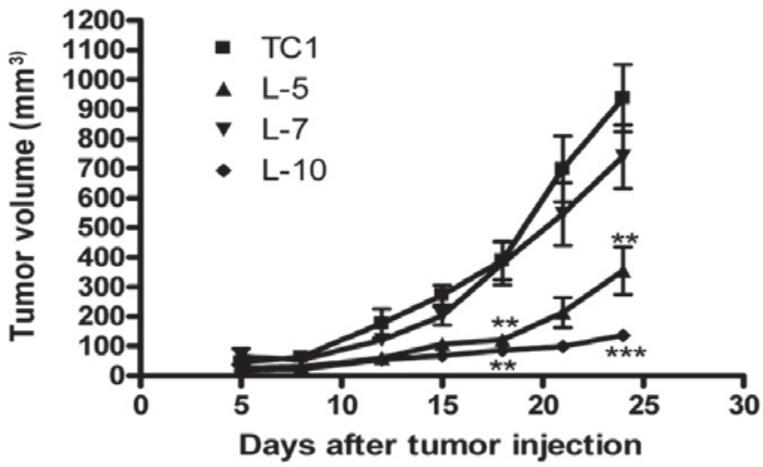
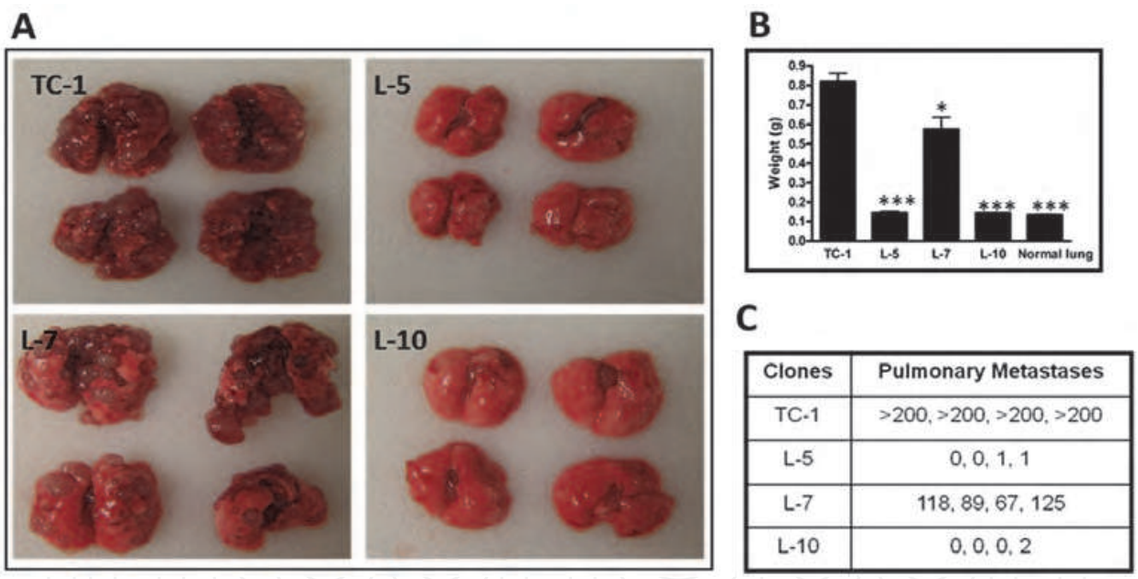


Fig. 8. Subcutaneous tumor study. A total of $2x10^5$ tumor cells of TC-1 or clones L-5, L-7 and L-10 in 0.2 ml HBSS were subcutaneously injected into C57BL/6J mice (four mice per group). Tumor growth was measured and presented as $1/2LW^2$. **P<0.01; ***P<0.001.



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

Fig. 9. 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). The lungs were weighted (B) and the tumor nodules on the lungs were counted (C). *P<0.05; ***P<0.001.

3.6 Construction of adenoviral vectors

In order to effectively deliver the fusion protein into cells, especially tumor cells, adenovirus vectors were chosen. 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 (Section 3.1) were 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 cells 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 collected and 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).

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

Three adenoviral vectors were constructed: 1) Ad-MULT1E/FasTI containing the full fusion protein sequence of MULT1E extracellular domain and FasTI transmembrane and intracellular domains; 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 (Fig. 10) and FACS analysis (Fig. 11). Both assays not only demonstrate the fusion gene expression in the infected cells, but also show a clear dose dependent expression manner.

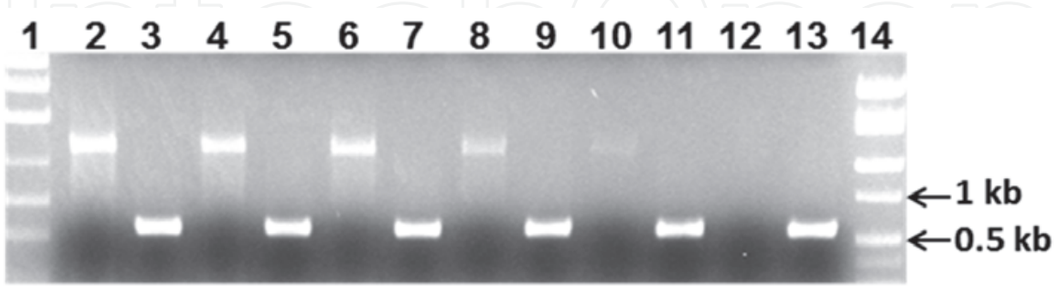


Fig. 10. RT-PCR analysis of AD-MULT1E/FasTI expression at different MOIs. RNAs were isolated from infected cells and RT-PCR was performed. Lanes 2, 4, 6, 8, 10 and 12 are 1134bp RT-PCR product of total RNA from TC1 cells infected with Ad-MULT1E/ FasTI at MOIs 500, 250, 100, 50, 25 and 0, amplified with MULT1E forward and Fas reverse primers; Lanes 3, 5, 7, 9, 11 and 13 are ß-actin controls; and lanes 1 and 14 are 1 kb markers.

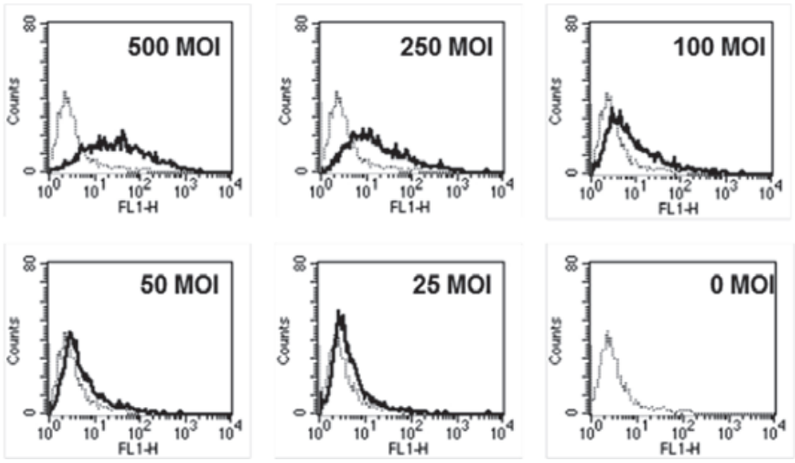


Fig. 11. FACS analyses of AD-MULT1E/FasTI expression at different MOIs. 5 x 10^5 TC1 cells were harvested from each group that were infected with Ad-MULT1E/FasTI at MOIs 500, 250, 100, 50, 25, 0 and stained with purified rat anti-mouse MULT1 antibody followed by goat anti IgG F(ab')-FITC. Dashed lines represent un-infected TC-1 cells.

3.8 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 (Fig. 12A). The caspase activity in Ad-MULT1E/FasTI infected and NKG2D treated TC-1 cells is adenoviral particle dose

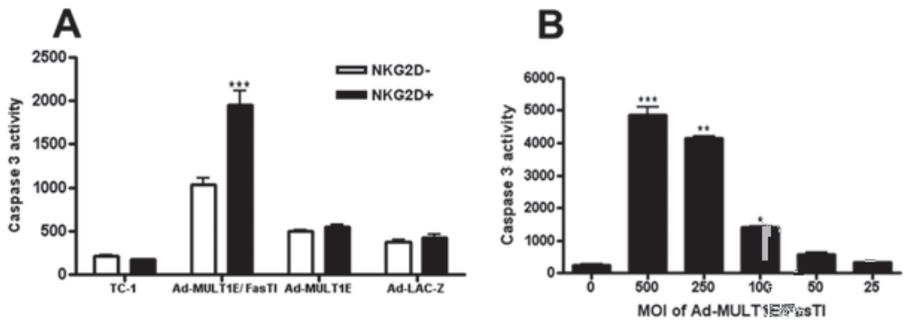


Fig. 12. Ad-MULT1E/FasTI infection induces apoptosis *in vitro*. A) TC-1 (5x10^5) cells were infected with Ad-MULT1E/FasTI, Ad-MULT1E and Ad-Lac-Z at 100 MOI. Twenty-four hour after infection, cells were treated with 1μg/ml NKG2D/Fc for 16 hrs. The cells were then analyzed for apoptosis by caspase-3 assay. 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 ***$p< 0.001$.

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 (Fig. 12B).

3.9 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 or Ad-Lac-Z at a dose of $1x10^9$ pfu/tumor in 0.05ml HBSS was injected into the tumors. Control mice received HBSS only. 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 (Fig. 13A). The end point tumor measurement confirmed the conclusion that Ad-MULT1E/FasTI treatment significantly slowed the tumor growth ($P<0.01$, Fig. 13B).

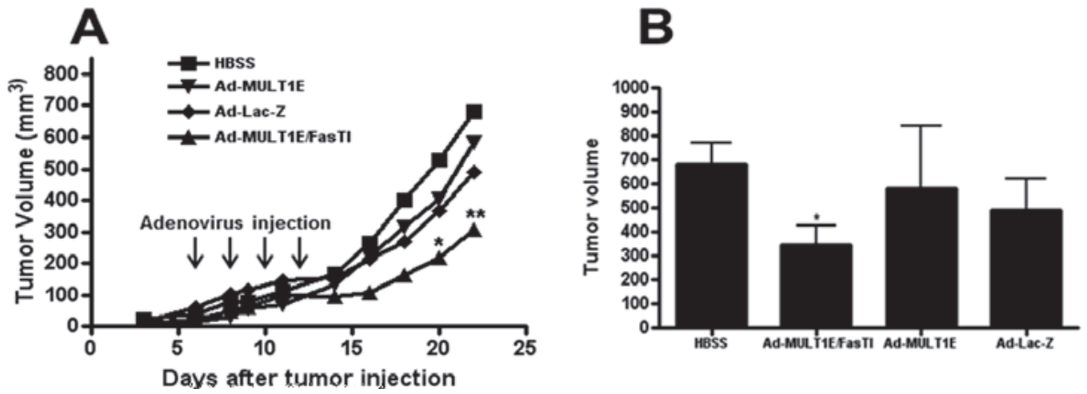


Fig. 13. MULT1E/FasTI delivered by adenoviral vector inhibits *in vivo* tumor growth. TC-1 cells (2×10^5) in 0.2ml HBSS were subcutaneously injected into flanks of C57BL/6J mice (n = 6). One week after tumor cell injection, various viral vectors at a dose of 1×10^9 pfu/tumor were intratumorally injected on every other day for a total of 4 injections. Control animals received injections of 50µl HBSS. Tumor growth was measured and presented as $1/2LW^2$ (A). At the end of this experiment, the mice were sacrificed and tumors were harvested and measured (B). *P< 0.05

3.10 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™ *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 (Fig. 14).

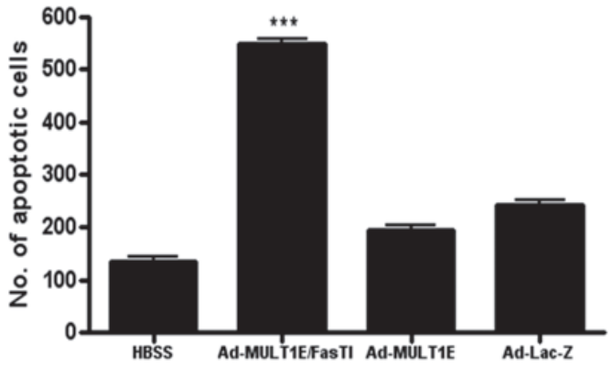


Fig. 14. MULT1E/FasTI delivered by adenoviral vector induces apoptosis *in vivo*. Two days after the last intratumor virus injection, 100µl of FLIVO™ *in vivo* apoptosis detection reagent (green) was injected into mice. Thirty minutes later, the mice were sacrificed and tumors collected. Frozen sections 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. Conclusion

Tumor cells have developed multiple mechanisms to subvert and suppress immune responses by regulating cell-surface expression of Fas (Ivanov et al., 2003) and Fas ligand (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, the 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). 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.

NK cell function is regulated by a balance between activating and inhibiting receptor signals (Trinchieri, 1989; Diefenbach and Raulet, 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., 1996). 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 TC-1 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, activated 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. When combined with adenovirus gene delivery vectors, especially the oncolytic adenovirus vectors, the fusion protein will provide a robust anti-cancer agent.

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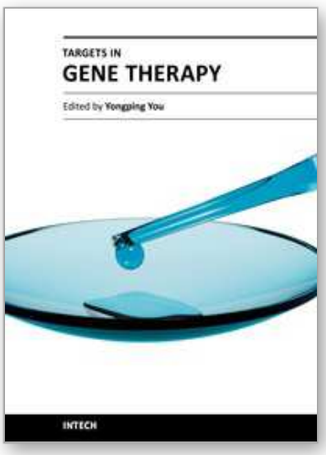

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This book aims at providing an up-to-date report to cover key aspects of existing problems in the emerging field of targets in gene therapy. With the contributions in various disciplines of gene therapy, the book brings together major approaches: Target Strategy in Gene Therapy, Gene Therapy of Cancer and Gene Therapy of Other Diseases. This source enables clinicians and researchers to select and effectively utilize new translational approaches in gene therapy and analyze the developments in target strategy in gene therapy.

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