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The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
The specific aim of the funded research was to develop a TRAIL-based anticancer drug using a gene therapy approach. To accomplish the proposed aim, I have created a novel TRAIL gene composed of three functional elements (secretion signal, trimer-forming domain, and TRAIL gene segment for apoptosis induction), and a naked DNA-type delivery vector. Functional analysis revealed that this novel artificial gene expresses and secretes into the culture supernatant, and efficiently kills various tumor cells but not normal cells tested. Recombinant TRAIL protein was observed to be more potent in target cell killing when combined with other potentiators such as Actinomycin D, Interferon-gamma, and Akt inhibitors. Thus, novel TRAIL gene-based therapy in combination with these potentiators is expected to produce better therapeutic efficacy. Currently, developed gene therapy tool is tested in animal tumor models. If proven to be safe and effective in vivo, this novel gene therapy approach will be tested for human cancers. Successful completion of the current research project will provide a powerful therapeutic tool for treatment of human cancers.
INTRODUCTION
The research project was to develop a TRAIL-based anticancer gene therapy. TRAIL, a tumor-specific and membrane-bound killer molecule in nature was engineered to secrete into the culture supernatant for better target access. This gene therapy tool was expected to be a promising therapeutic for treatment of human cancers.

BODY
As proposed, I accomplished construction of the secretable trimeric TRAIL gene composed of three unrelated elements (secretion signal, trimer-forming domain, and TRAIL gene segment for cell death induction). This TRAIL gene produced trimeric TRAIL protein and showed potent killer activity in various tumor cells. For gene delivery, I proposed to develop an adeno-associated vector (AAV). However, I turned to a naked DNA-based gene delivery system because of recent safety issues in virus-based gene delivery. Recombinant TRAIL protein was observed to effectively kill target tumor cells when combined with other potentiators such as Actinomycin D, Interferon-gamma, and Akt inhibitors. Thus, novel TRAIL-based cancer gene therapy in combination with these potentiators is expected to produce higher therapeutic efficacy. Currently, the developed gene therapy approach composed of the gene delivery system and artificial TRAIL gene is under test in animal tumor models. If proven to be safe and effective in vivo, this novel gene therapy approach will be tested for human cancers. Successful completion of the current research project will provide a powerful therapeutic tool for treatment of human cancers.

KEY RESEARCH ACCOMPLISHMENTS
Construction of secretable trimeric TRAIL expression cassette
Gene delivery vector
Enhancement of TRAIL-induced apoptosis by Actinomycin D
Enhancement of TRAIL-induced apoptosis by Interferon-gamma
Enhancement of TRAIL-induced apoptosis by EGF/Akt inhibitors

REPORTABLE OUTCOMES
Publications: 4
Manuscripts: 1
Abstracts: 1
Grant proposal: 1

CONCLUSIONS
Collected data indicate that the developed TRAIL-based gene therapy tool is a promising anticancer therapeutic. Further feasibility tests in animal tumor models will assess possible application to human cancers.

REFERENCES
N/A

APPENDICES
Publications
The role of caspase-8 in resistance to cancer chemotherapy

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Abstract Toxicity of chemotherapeutic agents against cancer cells is mediated through the initiation of programmed cell death. Apoptosis is one of the most conserved cascade of intracellular proteolytic events propagated by a family of cysteine proteases called caspases. Many receptor- and non-receptor-mediated death signals induce apoptosis via activation of caspase-8 (FLICE/MACH). Mechanisms of tumor resistance to cytotoxic drugs through decreased apoptosis may occur by altered expression of caspase-8, upregulation of caspase-8 inhibitors like FLIP (FLICE-like Inhibitory Protein), or sequestration of caspase-8 by Bcl-2. Modulation of caspase-8 and apoptosis may be a therapeutic strategy for sensitization of drug-resistant malignancies to radiation or combination chemotherapy. © 2002, Elsevier Science Ltd. All rights reserved.

INTRODUCTION

When cancer cells are exposed to effective drug therapy, they respond by activation of cell death pathways including apoptosis and necrosis. Apoptotic signal transduction pathways have been the focus of recent intensive research. Caspases, a family of cysteine-aspartic acid proteases, play a central role in the activation and propagation of death signaling. However, proliferating cancer cells can evade death signals by manipulating the nascent apoptotic machinery to neutralize the activity of proteases. In particular, a mechanism of resistance to cancer chemotherapy revolves around the initiator protease of apoptosis, caspase-8.

TWO TYPES OF APOPTOSIS INVOLVE CASPASE-8

Cell death can be initiated by receptor-dependent and -independent signaling, and different cell types induce apoptosis through one of two pathways (Fig. 1). Procaspase-8 can be activated through either pathway. In receptor-mediated apoptosis, several cytokines, including tumor necrosis factor (TNF), Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL/Apo-2L) bind to specific death receptors which activate the signal transduction pathways of apoptosis. Fas binds to FasL, which, via TNF receptor (TNFR), and TRAIL to the DR4/TRAIL-R1 or DR5/TRAIL-R2/TRICK2 death receptors. Death receptors contain intracellular binding regions called death domains. Ligation of death receptors recruits the adaptor molecules FADD/MORT1 (Fas-associating death domain protein) or TRADD (TNF receptor-associating death domain protein) to the death domain of the death receptor. FADD also contains a death effector domain (DED) that binds to procaspase-8 zymogens via interaction with the two DED on the N-terminus of procaspase-8. This cluster of death receptor, FADD, and procaspase-8 is called the death-inducing signaling complex (DISC). Oligomerization of two inactive procaspase-8 molecules induces autocleavage of procaspase-8 into a large and small subunit that heterodimerize into the active caspase-8 unit. In type I apoptosis, activated caspase-8 then dissociates from the DISC and cleaves downstream substrates including procaspase-3, PARP (poly-ADP-ribosyl polymerase), and ICAD (inhibitor of caspase-associated deoxyribonuclease) which results in generalized protein cleavage, DNA fragmentation, and apoptotic cell death.

In type II apoptosis, death signals are mediated through the mitochondria. Cytochrome c is released from the inner membrane of mitochondria into the cytoplasm and associates with ATP, Apg1, and procaspase-9 in a complex known as the apoptosome. The formation of the apoptosome cleaves and activates caspase-9 that then cleaves caspase-3. In type II apoptosis, procaspase-8 can be activated downstream of activated caspase-3. Activation of caspase-8 may help to amplify the apoptotic signal induced by drugs through a feedforward loop in which proapoptotic molecules like BID cause further mitochondrial release of cytochrome c and activation of caspase-3. Both types I and II apoptosis can be modulated by Bcl-2, a protein localized to the inner membrane of mitochondria that inhibits release of cytochrome c.

CHEMOTHERAPEUTIC AGENTS ACTIVATE CASPASE-8

Docetaxel, methotrexate and cytarabine have been shown to activate the Fas/FasL type I death pathway in leukemia cells. Since procaspase-8 is downstream of FasL/CD95 and TRAIL, caspase-8 is activated when these death receptors are involved. In colon cancer cells, doxorubicin and cisplatin were shown to sensitize cells to TRAIL-mediated, caspase-8-dependent apoptosis. TRAIL was shown to induce apoptosis by binding to the DR4 or DR5 receptors and activating caspase-8 through interaction with the FADD linker molecule. Certain formulations of TRAIL, and antibodies that bind to DR4 or DR5 have shown promising results as therapeutic agents specific for cancer cells with minimal toxicity to normal cells.

Although many death pathways are activated via death receptor/receptor interactions, several studies have shown that chemotherapeutic-induced apoptosis is receptor-independent. Chemotherapy-induced apoptosis in Jurkat T cells was not dependent on Fas/LFas interactions. Caspase-8 can be activated by chemotherapy (e.g. cisplatin, topotecan, gemcitabine, paclitaxel, and etoposide) in a Fas-independent way in several cancer cell types including non-small cell lung cancer (NSCLC) and leukemia. In the NSCLC cell line NCI-H460, caspase-8 was activated during cisplatin-induced apoptosis, independent of FADD and death receptors. Cell death was blocked by a dominant-negative caspase-8 as well as the caspase-8 inhibitor IETD-fmk. In osteosarcoma cells cisplatin activated caspase-8 and subsequently effector caspase-3. In Jurkat T cells, etoposide seemed to induce type II apoptosis that activated caspase-8 downstream of mitochondria and...
caspase-8 activation.8 Arsenic trioxide (As$_2$O$_3$) is an effective treatment for acute promyelocytic leukemia [APL; (x5-17)] resistant to all-trans retinoic acid. In NB4 APL cell lines, As$_2$O$_3$ was shown to function by a CD95-independent, caspase-8-mediated mechanism.80 These studies represent a growing list of in vitro examples where caspase-8 was involved in receptor-dependent and receptor-independent cell death induced by chemotherapeutic agents (see Fig. 1).

CASPASE-8 AND DRUG RESISTANCE

Drug resistance to chemotherapy can develop in cancer cells by several mechanisms that deactivate cytotoxic drugs including expression of P-glycoprotein and the multidrug resistance protein (MRP) efflux pumps.32 Other mechanisms include genetic deletion or inactivation of pro-apoptotic molecules, or conversely, upregulation of anti-apoptotic proteins. Since caspase-8 is central to drug-induced apoptosis, it is not surprising that modulation of apoptosis at caspase-8 has been shown to be an important mechanism for resitance to chemotherapy in several tumors (Fig. 2).

The tumor suppressor CASP8 gene encodes for the procaspase-8 protein, and its expression is altered in several malignancies. In neuroblastoma (NB), a common childhood tumor of the peripheral nervous system, CASP-8 is inactivated in one-third of cases.30 Highly malignant NB tissues with amplification of MYCN frequently lack CASP-8 mRNA expression. Inactivation of the CASP-8 gene occurred by DNA methylation as well as through gene deletion of its locus at human chromosome 2p23.38 This correlated with resistance to Fas- and doxorubicin-mediated apoptosis. Primitive neuroectodermal brain tumor cell lines are resistant to TRAIL-induced apoptosis by loss of caspase-8 expression.83 An MCF-7 line resistant to doxorubicin-induced apoptosis was shown to have decreased expression of caspase-8 and other pro-apoptotic molecules.85 A mutation in CASP-8 that inserts Ala repeats, extending the gene 88 amino acids, was discovered in an oral squamous cell head and neck carcinoma.87 This mutation decreased the ability of the altered protein to trigger apoptosis and also generated a tumor antigen which was recognized by cytolytic T cells.

In addition to gene deletion and mutation, cancer cells can overexpress caspase inhibitors like c-FLIP (PADD-like IL-1β-converting enzyme inhibitory protein; also known as FLAME1, c-FLIP, CASH, CLARP, MR1, and Usurpin).88,89 After ligation of death receptors, c-FLIP can be recruited to the activated receptor complex. c-FLIP subsequently inactivates the DISC by blocking further recruitment of procaspase-8 into the complex, thereby inhibiting the activation of caspase-8.90 FLIP expression has been described in solid tumors including melanoma and late stage Kaposi sarcoma.91,92 In addition, c-FLIP levels inversely correlated with sensitivity to TRAIL-induced apoptosis in melanoma and fibroblast cell lines.93,94 Neuroblastomas, in addition to overexpressing anti-apoptotic c-FLIP, have evolved another mechanism to thwart caspase-8-dependent apoptosis. Certain neuroblastomas can express the anti-apoptotic mitochondrial protein Bcl-2, which has been shown to bind and sequester caspase-8.95 Downregulation of Bcl-2 by antisense oligonucleotides was found to release and activate caspase-8 as well as to induce apoptosis. Similarly, the protein BAR (bifunctional apoptosis regulator), which contains a DED-like domain, interacts with the DED of caspase-8, and was shown to mediate the binding of caspase-8 to Bcl-2. This interaction between BAR, Bcl-2, and caspase-8 suppressed Fas-induced cell death.96 Therefore, the sequestration of caspase-8 by Bcl-2 is another important mechanism
by which caspase-8 can regulate the response of tumors to cytotoxic drugs (Fig. 2).

CLINICAL IMPLICATIONS OF CHEMOTHERAPY RESISTANCE TO APOPTOSIS: MODULATION OF CASPASE-8

A recent debate published in Drug Resistance Updates highlighted the complex and controversial involvement of apoptosis in the clinical response to chemotherapy. In recognition of the importance of apoptosis in certain responses to chemotherapy, attention and new research has turned to the modulation of the apoptotic signaling pathway to overcome resistance to cytotoxic agents. Since caspase-8 is downregulated in certain tumors, therapeutic induction of caspase-8 expression and function may restore or enhance apoptotic sensitivity to chemotherapy of any tumor containing procaspase-8. In MCF-7 and MDA-MB231 breast cancer cells, interferon-γ increased expression of caspase-8 and sensitized them to CD95-mediated cell death. In Fas-resistant adult T-cell leukemia cell lines, the proteasome inhibitors lactacyctin or MG-115 activated caspase-8 and apoptosis. Another study demonstrated that combination therapy with doxorubicin sensitized previously TRAIL-resistant fibroblasts to apoptosis by TRAIL that activated caspase-8. This strategy of combining chemotherapy with biologic or immunotherapy to stimulate apoptosis may prove effective for drug-resistant tumors.

We have described multiple mechanisms at the DNA and protein levels where modification of caspase-8 may have given tumors a selective advantage for growth. Modulation of c-FLIP, Bcl-2, or caspase-8 activity by specific gene transfer, demethylating agents, or methylase inhibitors for silenced caspase-8 genes may be novel ways to abrogate chemotherapy resistance and improve outcomes in cancer treatment. For example, treatment with the demethylating agent 5-Aza-2'-deoxycytidine (5-AzaC) reversed hypermethylation of the caspase-8 mRNA and protein with restoration of caspase-8 function. Furthermore in that study, in cells with absent caspase-8 expression, selective downregulation of FLIP synergized with 5-AzaC to induce death receptor and drug-mediated cell death. Several lines of exciting new data point to caspase-8-dependent tumor response to chemotherapy in vitro. They suggest that biological manipulation of caspase-8 expression may synergize with chemotherapy to enhance tumor killing. The ultimate relevance of these findings, however, depends on demonstration of efficacy in vivo. Use of genetic knockout and transgenic murine models may provide mechanistic insight into therapeutic applications. Future study must examine the significance on outcomes (e.g., tumor responses, metastasis rates, survival) of manipulation of caspase-8 expression.

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References

Hypoxia Inhibition of Apoptosis Induced by Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL)

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Hypoxia is a common environmental stress. Particularly, the center of rapidly growing solid tumors is easily exposed to hypoxic conditions. Thus, tumor cell response to hypoxia plays an important role in tumor progression as well as tumor therapy. However, little is known about hypoxic effect on apoptotic cell death. To examine the effects of hypoxia on TRAIL-induced apoptosis, human lung carcinoma A549 cells were exposed to hypoxia and treated with TRAIL protein. Hypoxia significantly protected A549 cells from apoptosis induced by TRAIL. Western blotting analysis demonstrated that hypoxia increased expression of antiapoptotic proteins such as Bcl-2, Bcl-XL, and IAP family members. The increase of these antiapoptotic molecules is believed to play an hypoxia-mediated protective role in TRAIL-induced apoptosis. Our findings suggest that an increase of antiapoptotic proteins induced by hypoxia may regulate the therapeutic activity of TRAIL protein in cancer therapy.

Key Words: TRAIL; hypoxia; apoptosis; Bcl-2.

Local growth of malignant tumors is largely dependent upon adequate nutrient and oxygen supply. Oxygen is absolutely indispensable for optimal energy metabolism in mammalian cells, and cancer cells are no exception. The center of a rapidly growing solid tumor is easily exposed to hypoxic conditions or even anoxic conditions (1, 2). Thus, tumor cell responses to hypoxia are important for tumor progression as well as tumor therapy. Recent findings showed that tumor cells exposed to hypoxia increase the antiapoptotic potentials via many dysregulations in apoptosis signaling pathways (3, 4).

Apoptosis is an active cell death process that is genetically regulated. This process plays an important role in the development and homeostasis of multicellular organisms (5). Among apoptosis-inducing proteins, the best characterized are the ligand-type cytokine molecules of the TNF family. TNF family member proteins such as TNF-α, Fas ligand and TRAIL are type II transmembrane molecules that trigger the apoptotic signal cascade by binding to cognate receptors displayed on the cell surface (6, 7).

TRAIL is known to induce apoptosis in a variety of tumor cells but not in most normal cells. Recent preclinical studies showed that repeated systemic administration of biologically active recombinant TRAIL protein limited tumor growth without detectable toxicity (8, 9). These results indicate that TRAIL is a promising therapeutic to treat human cancers.

Several studies have demonstrated that solid tumor cells exposed to hypoxia are resistant to both radiotherapy and most commonly used anticancer drugs (3, 10). However, little is known about the effects of hypoxia on TRAIL-induced tumor cell apoptosis. To assess these effects, we exposed A549 cells to hypoxia and treated them with recombinant TRAIL protein. Here we report that hypoxia-treated A549 cells are significantly resistant to TRAIL-induced apoptosis. Hypoxia increased expression of antiapoptotic proteins such as Bcl-2, Bcl-XL, and IAP family members. Our data suggest that hypoxia-induced upregulation of these molecules may modulate the apoptotic activity of TRAIL protein.

MATERIALS AND METHODS

Cell culture. Human lung carcinoma A549 cells were obtained from ATCC and maintained in F-12K culture medium supplemented with 10% (v/v) fetal bovine serum and antibiotics (100 μg/ml genta-
mycin and 100 μg/ml penicillin-streptomycin). Hypoxia (1% O₂) was induced by maintaining cells inside an air-tight chamber with inflow and outflow valves that were infused with a mixture of 1% O₂.

Cell viability. A549 cells plated in 12 wells were exposed to hypoxia. After 24 h, cells were coincubated with recombinant TRAIL protein (11) for 4 h under the same conditions. As a control, A549 cells were exposed to normoxia for 24 h and treated them with recombinant TRAIL protein for 4 h under normoxia. Cell morphology was photographed under the microscope, and cell viability was determined by the crystal violet staining method (11). Briefly, cells were stained for 10 min at room temperature with staining solution (0.5% crystal violet in 30% ethanol and 3% formaldehyde), washed four times with water, and dried. Cells were lysed with 1% SDS solution, and measured at 550 nm. Cell viability was calculated from relative dye intensity and compared to the controls.

Western blotting. To prepare whole-cell lysates, cells were harvested, resuspended in lysis buffer (25 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, 0.1 mM DTT, protease inhibitor mixture) and sonicated. Proteins were separated on an 8–15% SDS gel and analyzed by Western blotting as described previously (11). DR4 (AAP-420) and DR5 (AAP-430) were probed with antibody obtained from Stressgen (Victoria, Canada); Bcl-2 (sc-492), Bcl-XL (sc-7195), and cIAP-2 (sc-7944) were from Santa Cruz (Santa Cruz, CA); cIAP-1 (58533) was from BD Pharmingen (San Diego, CA); and XIAP (AF-822) was from R & D Systems (Minneapolis, MN).

RESULTS AND DISCUSSION

Hypoxia Inhibits TRAIL-Induced Apoptosis

Tumor cells exposed to hypoxia have been shown to be resistant to radiotherapy as well as chemotherapy (3, 10). Studies have demonstrated that TRAIL selectively kills tumor cells without damaging normal tissues in vivo (8, 9). Despite considerable attention on TRAIL as a promising anticancer therapy, no experimental data linking hypoxia and TRAIL-induced tumor cell apoptosis are available.

To investigate the effects of hypoxia on TRAIL-induced apoptosis, we exposed human lung carcinoma A549 cells to hypoxic or normoxic conditions for 24 h and treated them with biologically active recombinant TRAIL protein (11) for 4 h under the same conditions. As shown in Fig. 1, TRAIL induced apoptotic cell death in cells exposed to normoxia, an oxygenated conditions. In contrast, hypoxia-treated cells were significantly resistant to TRAIL-induced apoptosis under a wide range of TRAIL concentrations (50–200 ng/ml). Cell morphology examination also supported an inhibitory role of hypoxic conditions in TRAIL-induced apoptosis (Fig. 2). To rule out the possibility that hypoxic conditions directly affected TRAIL protein activity, we incubated TRAIL protein for 4 h under hypoxic conditions and examined the apoptotic activity of TRAIL protein. Hypoxia-treated TRAIL protein was as efficient as untreated control TRAIL protein in killing HeLa cells, which indicated that hypoxia did not affect the apoptotic activity of TRAIL protein. Therefore, our data suggest that hypoxia may directly regulate apoptotic and/or antiapoptotic signals in A549 cells.

FIG. 1. Effect of hypoxia on TRAIL-induced apoptosis. A549 cells plated in 12 wells were exposed to normoxia or hypoxia for 24 h and coincubated with recombinant TRAIL protein (0–200 ng/ml) for 4 h under the same conditions. Cell viability was determined by crystal violet staining method. Viability of control cells was set at 100%, and viability relative to the control is presented. Experiments were performed in triplicate, at least twice. Bars indicate standard errors.

Hypoxia Increases Expression of Antiapoptotic Proteins

Hypoxia is known to regulate the expression of many genes (12). Nevertheless, little is known about the hypoxia-induced genes that play a direct role in either apoptosis or antiapoptosis. Recently, we (13) and others (14, 15) identified a FADD-activated caspase-8 signaling pathway to be a major signaling pathway in TRAIL-induced apoptosis. Caspase-8 activation cleaved Bid, a Bcl-2 family member, which is involved in mitochondrial events, including cytochrome c release (16). These results suggest that cellular factors involved in activation or inhibition of caspases and/or cytochrome c release may regulate TRAIL-induced apoptosis. We hypothesized that expression of those factors may be regulated by hypoxia.

To address such possibility, we first examined if hypoxia regulates expression of antiapoptotic molecules such as Bcl-2 and Bcl-XL, both of which have been well known to inhibit cytochrome c release from the mitochondria (17, 18). A549 cells were exposed to hypoxia for 1, 6, 12, 24, or 48 h and subjected to Western blotting analysis. As shown in Fig. 3A, hypoxia significantly increased Bcl-2 and Bcl-XL expression. The increase of Bcl-2 expression was detected after 6 h of incubation at hypoxic conditions and sustained up to 24 h in a hypoxic setting. The increase of Bcl-XL expression was slightly delayed as detected during the 12-h incubation. Increased expression also lasted through 48 h hypoxia treatment. Despite different profiles in Bcl-2 and Bcl-XL expression, up-regulation of Bcl-2 and Bcl-XL may account for hypoxia-mediated protection in TRAIL-induced apoptosis, because maximal protection by hypoxia in TRAIL-induced apoptosis was observed after 24 h pretreatment with hypoxia (Fig. 1). Our result is also supported by other investigators who have demonstrated that Bcl-2 and Bcl-XL block TRAIL-induced apoptosis (19). Thus, the overex-
expression of Bcl-2 family members, Bcl-2 and Bcl-XL, may contribute to the protective role of hypoxia.

Next, we examined expression of IAP family members, cIAP-1, cIAP-2 and XIAP, after exposure of A549 cells to hypoxia. IAP family members have been shown to inhibit caspase-9 activation by inhibiting apoptosome formation, which is composed of cytochrome c/Apaf-1/caspase-9 (20, 21). The IAP protein family also blocks caspase activation and activity by directly binding active caspases (22, 23). Thus, increase of IAP family proteins may protect TRAIL-induced apoptosis.

Hypoxia-treated A549 cells showed increased expression of cIAP-1, cIAP-2, and XIAP. The increase of cIAP-1 expression was rapid, as detected within 1 h after exposure to hypoxia, whereas the increase of cIAP-2 and XIAP expression reached a peak after a 12-h exposure to hypoxia. The expression patterns of cIAP-2 and XIAP were identified to be similar to each other. Expression of IAP family proteins with the exception of cIAP-1, also conceded with the function test results (see Fig. 1). Thus, increased expression of IAP family proteins is also believed to play a hypoxia-induced protective role in TRAIL-induced apoptosis.

It is hypothesized that if hypoxia down-regulates TRAIL receptor DR4 and/or DR5, the TRAIL-R-mediated death signal would be attenuated and result in less apoptosis. Thus, we examined whether hypoxia has a regulatory function in TRAIL receptor expression. Hypoxia did not have a significant influence on DR4 expression (Fig. 3B), even though DR4 expression remarkably decreased after 48 h of hypoxic incubation. However, it is unlikely that this decrease is associated with a protective role of hypoxia in TRAIL-induced apoptosis since the effect of hypoxia on TRAIL-induced apoptosis was tested after 24 h of hypoxic incubation and lasted for 4 h longer. In contrast, hypoxia slightly increased DR5 expression (Fig. 3B). However, this increase did not enhance TRAIL-induced apoptotic cell death (see Fig. 1). Thus, DR5 increase may not be sufficient to counteract antiapoptotic functions driven by antiapoptotic molecules upregulated under hypoxic conditions.

Our data demonstrate that hypoxia protects cells from TRAIL-induced apoptosis. Other studies also showed
that tumor cells under hypoxic conditions are resistant to radiotherapy and chemotherapy (10). These results suggest that hypoxia may be a common resistance mechanism by which tumor cells escape tumoricidal activity of cancer therapies or natural defense systems in vivo. Even though the detailed mechanisms are not fully understood, our data suggest that an increase of antiapoptotic proteins may be one of the key mechanisms. Thus, for a successful anticancer therapy, it is imperative to develop a strategy against hypoxic tumor cells. Combination therapies may be more effective than TRAIL protein alone. For example, TRAIL protein plus a chemotherapeutic drug that is selectively toxic to hypoxic tumor cells such as mitomycin C (24), triapazamine (25) or AQ4N (26), may be a good combination therapy for hypoxic tumor cells. TRAIL protein plus an inhibitor of the antiapoptotic proteins increased under hypoxic conditions may also be a possible consideration for use in a combination therapy for hypoxic tumor cell killing. Antisense of Bcl-2 may also increase TRAIL-induced apoptotic activity under hypoxic conditions. Another avenue to consider would include whether SMAC/DIABLO, a protein that directly binds and inactivates IAP family members induced by hypoxia, enhances TRAIL-induced apoptosis under hypoxia.

REFERENCES


IFN-γ Inhibition of TRAIL-Induced IAP-2 Upregulation, a Possible Mechanism of IFN-γ-Enhanced TRAIL-Induced Apoptosis

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Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a type II transmembrane cytokine molecule of TNF family and a potent inducer of apoptosis. The anticancer activities of TNF family members are often modulated by interferon (IFN)-γ. Thus, we investigated whether IFN-γ enhances TRAIL-induced apoptosis. We exposed HeLa cells to IFN-γ for 12 h and then treated with recombinant TRAIL protein. No apoptosis was induced in cells pretreated with IFN-γ, and TRAIL induced 25% cell death after 3 h treatment. In HeLa cells pretreated with IFN-γ, TRAIL-induced cell death to more than 70% at 3 h, indicating that IFN-γ pretreatment sensitized HeLa cells to TRAIL-induced apoptosis. We investigated molecules that might be regulated by IFN-γ pretreatment that would affect TRAIL-induced apoptosis. Western blotting analyses demonstrated that TRAIL treatment increased the level of IAP-2 protein and IFN-γ pretreatment inhibited the upregulation of IAP-2 protein by TRAIL protein. Our data indicate that TRAIL can signal to activate both apoptosis induction and antiapoptotic mechanism, at least, through IAP-2 simultaneously. IFN-γ or TRAIL treatment alone did not change expression of other pro- or antiapoptotic proteins such as DR4, DR5, FADD, Bax, IAP-1, XIAP, Bcl-2, and Bcl-XL. Our findings suggest that IFN-γ may sensitize HeLa cells to TRAIL-induced apoptosis by preventing TRAIL-induced IAP-2 upregulation, and IFN-γ may play a role in anticancer therapy of TRAIL protein through such mechanism.

Key Words: TRAIL; IFN-γ; apoptosis; IAP-2.

Materials and Methods

Cell culture. HeLa cells were obtained from ATCC and maintained in F-12K culture medium supplemented with 10% (v/v) fetal calf serum. IFN-γ, a type II interferon, is primarily secreted from T cells and natural killer cells. It has a variety of functions such as inhibition of viral replication and anti-proliferation of rat vascular smooth muscle cells (6-8). IFN-γ has been shown to make target cells susceptible to various apoptotic stimuli, including Fas-mediated apoptosis. Recently, several studies demonstrated that both IFN-γ and TNF synergism in cancer cell apoptosis and necrosis (9-12). However, little is known about the enhancing or inducing mechanism of IFN-γ on tumor cell apoptosis.

Here we report that IFN-γ enhances TRAIL-induced HeLa cell apoptosis. Treatment of TRAIL protein increased the expression of IAP-2 protein. Most importantly, IFN-γ pretreatment blocked TRAIL-induced IAP-2 upregulation. Our data suggest that IFN-γ inhibition of TRAIL-induced IAP-2 upregulation may enhance TRAIL-induced apoptosis.
IFN-γ Enhancement of TRAIL-induced Apoptosis in HeLa Cells

IFN-γ pretreatment has been shown to sensitize various cells to TNF-α- and FasL-induced apoptosis (15–17). To determine whether IFN-γ regulates TRAIL-induced apoptosis, we examined the effect of IFN-γ on TRAIL-induced apoptosis. HeLa cells were exposed to IFN-γ (100 U/ml) for 12 h and then treated with recombinant TRAIL protein to induce apoptosis for additional 3 h. As shown in Figs. 1A and 1B, TRAIL induced apoptosis in approximately 25% of HeLa cells, but induced apoptosis in 70% of HeLa cells pretreated with IFN-γ. Although IFN-γ induces apoptosis in some cells, pretreatment of IFN-γ for 12 h did not induce apoptosis in HeLa cells at all. These results suggest that IFN-γ enhances the sensitivity of HeLa cells to TRAIL-induced apoptosis.

IFN-γ Inhibition of TRAIL-Induced IAP-2 Upregulation in HeLa Cells

Recently, others (18, 19) and we (20) identified a FADD-activated caspase-8 signaling pathway to be a major signaling pathway in TRAIL-induced apoptosis. Caspase-8 activation cleaved Bid and led to a mitochondrial event including cytochrome c release (21). These results suggest that cellular factors involved in activation or inhibition of caspases and/or cytochrome c release may regulate TRAIL-induced apoptosis. To examine the possible mechanism of enhanced TRAIL-induced HeLa cell apoptosis by IFN-γ pretreatment, we investigated if IFN-γ and/or TRAIL treatment reg-
were pretreated with IFN-γ (100 U/ml) for 12 h and further coincubated with or without TRAIL protein (100 ng/ml) for 1 h. Whole cell lysates were prepared, separated (40 μg), and analyzed for proapoptotic proteins by Western blotting. (B) Expression of antiapoptotic proteins was analyzed by Western blotting as described in A. The NS indicates a nonspecific protein band for ensuring equal protein loading.

FIG. 2. Expression of pro- and antiapoptotic proteins in IFN-γ- and/or TRAIL-treated HeLa cells. (A) HeLa cells plated in 6 wells were pretreated with IFN-γ (100 U/ml) for 12 h and further coincubated with or without TRAIL protein (100 ng/ml) for 1 h. Whole cell lysates were prepared, separated (40 μg), and analyzed for proapoptotic proteins by Western blotting. (B) Expression of antiapoptotic proteins was analyzed by Western blotting as described in A. (C) HeLa cells plated 6 wells were pretreated with IFN-γ (0–200 U/ml) for 12 h and further coincubated with or without TRAIL protein (100 ng/ml) for 1 h. Expression of antiapoptotic proteins was analyzed as described in A. Effect of IFN-γ on TRAIL upregulation of IAP-2 protein was analyzed by Western blotting as described in A. The NS indicates a nonspecific protein band for ensuring equal protein loading.

ulates the expression of pro- and/or antiapoptotic proteins such as DR-4, DR-5, FADD, Bax, Bcl-2, Bcl-XL, and IAP family members. IFN-γ pretreatment did not change the expression level of the examined proteins (Fig. 2). Unexpectedly, TRAIL treatment for 1 h significantly increased the protein level of IAP-2 (Fig. 2B), but not others. These results indicated that, in addition to activating apoptosis induction, TRAIL triggers signals for blocking apoptosis as well. Thus, we hypothesized that IFN-γ may prevent TRAIL-induced IAP-2 upregulation, resulting in enhanced TRAIL-induced HeLa cell apoptosis. To investigate this possibility, HeLa cells were pretreated with IFN-γ for 12 h and further incubated with TRAIL protein for additional 1 h. To be surprised, IFN-γ pretreatment completely blocked TRAIL-induced increase of IAP-2 protein (Fig. 2B). The inhibition of IAP-2 protein expression by IFN-γ pretreatment was observed in the wide range of IFN-γ concentrations (25–200 U/ml) (Fig. 2C). IAP family members inhibit caspase-9 activation by inhibiting apoptosome formation, which is composed of cytochrome c/Apaf-1/caspase-9 (22, 23). IAP-2 protein was shown to block caspase activation as well as activity by directly binding active caspases (24, 25). Thus, blocking of IAP-2 protein expression results in caspase activation and apoptosis enhancement. Therefore, IFN-γ inhibition of TRAIL-induced IAP-2 upregulation is believed to enhance TRAIL-induced apoptosis.

Here we demonstrate that IFN-γ enhances TRAIL-induced apoptosis. Importantly, we first here report that TRAIL increases expression of IAP-2 protein. This result indicates that TRAIL can also activate an antiapoptotic signaling pathway associated with IAP-2 upregulation. Although TRAIL increases IAP-2 protein level, TRAIL is a potent HeLa cell apoptosis inducer. Nevertheless, our findings suggest that efficient attenuation of IAP-2 expression can enhance apoptotic activity of TRAIL protein. Therefore, if combined with an enhancer, TRAIL may produce better therapeutic results in cancer therapy. TRAIL protein plus IFN-γ may be a possible consideration for use in a combination therapy.

REFERENCES


Regulation of Akt by EGF-R inhibitors, a possible mechanism of EGF-R inhibitor-enhanced TRAIL-induced apoptosis

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Abstract

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a type II transmembrane cytokine and a potent inducer of apoptosis. Epidermal growth factor (EGF) signaling is well known to involve in tumor survival and overexpression of EGF receptor (EGF-R) attributes to decreased responsiveness to many available therapies in cancer treatment. We investigated whether EGF-R inhibitors enhance TRAIL-induced apoptosis. We exposed A549 cells to Genistein, PD153035, and PD158780 for 12 h and then treated with recombinant TRAIL protein. TRAIL alone induced 25% cell death after a 3-h treatment, but in cells pretreated with EGF-R inhibitors, TRAIL induced cell death to more than 70% after 3 h treatment. Genistein enhanced TRAIL-induced apoptosis in a time- and dose-dependent manner. Western blot analyses showed that pretreatment with Genistein down-regulated the protein levels of total Akt and phosphorylated active Akt. Genistein also decreased the protein level of Bcl-XL that is regulated by Akt. These molecules are well characterized to act against induction of apoptotic cell death. Therefore, our data suggest that EGF-R inhibitor may sensitize A549 cells to TRAIL-induced apoptosis by regulating expression of these proteins. EGF-R inhibitors may play an important role in the anti-cancer activity of TRAIL protein, especially in TRAIL-resistant tumors that arise by expressing constitutively active Akt. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: TRAIL; EGF-R; Genistein; Apoptosis; Akt

Epidermal growth factor receptor (EGF-R) is a 170 kDa protein composed of an extracellular ligand binding domain, a short transmembrane domain, and an intracellular domain [1]. EGF-R and EGF are expressed in many cancers such as breast, ovary, and lung [2-4]. Upon binding its receptor, EGF activates EGF-R and initiates signal transduction cascades including the cellular components such as phospholipase C, PI-3 kinase, Akt, and MAPK, which are important for cell proliferation and cell survival [5-7].

Apoptosis is an active cell death process that is genetically regulated and plays an important role in the development and homeostasis of multicellular organism [8]. Among apoptosis-inducing proteins, the best characterized are the ligand-type cytokine molecules of the TNF family. TNF family member proteins such as TNF-α, Fas ligand, and TRAIL are type II transmembrane molecules that trigger the apoptotic signal cascade by ligating cognate receptors displayed on the cell surface [9,10].

Although TRAIL is a TNF family member [11,12], it has some notable differences when compared with TNF-α and FasL. Unlike Fas, TRAIL receptors DR4 and DR5 are widely expressed [11]; thus, most tissues and cell types are potential targets to TRAIL [11,12]. TRAIL induces apoptosis in a wide variety of tumor cells but not in most normal cells. Recent preclinical studies demonstrated that repeated systemic administration of recombinant TRAIL protein effectively limited tumor growth without serious side effects [13,14]. Thus, considerable attention has been paid to TRAIL as a promising therapy for human cancers.

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**Abbreviations: EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; TRAIL, TNF-related apoptosis-inducing ligand.

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Recently, several studies indicated that elevated Akt activity protects tumor cells from TRAIL-induced apoptosis and constitutively active Akt is an important regulator of TRAIL sensitivity in tumor cells [15–17]. Activation of EGF-R is also one of the stimuli that constitutively activates Akt [16–19]. To examine whether EGF-R signaling affects TRAIL-induced apoptosis, we blocked EGF-R activation by preincubating A549 cells with EGF-R inhibitors such as Genistein, PD153035, and PD158780 and further exposed to recombinant TRAIL protein. Pretreatment with EGF-R inhibitors enhanced TRAIL-induced apoptosis. The enhancement effect of EGF-R inhibitors on TRAIL-induced apoptosis increased in a time- and dose-dependent manner. We also showed that treatment with Genistein down-regulated total Akt and phosphorylated active Akt protein levels. Our data suggest that, combined with TRAIL, EGF-R inhibitors may be useful drugs in cancer therapy, especially in cancers resistant to TRAIL by expressing constitutively active Akt.

Materials and methods

Cell culture. A549 cells were obtained from ATCC and maintained in F-12K culture medium supplemented with 10% (v/v) fetal bovine serum and antibiotics (100 μg/ml gentamicin and 100 μg/ml penicillin-streptomycin).

Cell viability. Cells plated in 12-wells were pretreated with EGF-R inhibitors, 0-100 μM Genistein, PD153035, or PD158780 (Calbiochem, San Diego, CA) for 0–12 h and further incubated with recombinant TRAIL protein for additional 3 h. Cell morphology was photographed under the microscope and cell viability was determined by crystal violet staining method as described [20]. Briefly, cells were stained for 10 min at room temperature with staining solution (0.5% crystal violet in 30% ethanol and 3% formaldehyde), washed four times with water, and dried. Cells were then lysed with 1% SDS solution and measured at 550 nm. Cell viability was calculated from relative dye intensity and compared to controls.

Western blot. To prepare whole cell lysates, cells were harvested, resuspended in lysis buffer (25 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, 0.1 mM DTT, and protease inhibitor mixture), and sonicated. Proteins were separated on an 8–15% SDS gel and subjected to Western blot analyses as described previously [21,22]. Antibodies used for Western blot analyses are Akt (559028) and phospho-Akt (559029) from BD Pharmingen (San Diego, CA), Bcl-XL (SC-7195) from Santa Cruz (Santa Cruz, CA).

Results and discussion

Enhancement of TRAIL-induced apoptosis by EGF-R inhibitors

Several studies have demonstrated that EGF-R-mediated signals crucially contribute to cancer progression, including angiogenesis, metastatic spread, and the inhibition of apoptosis [3,23–27]. To explore the functional role of EGF signaling in apoptotic cell death, we examined the effect of EGF-R inhibitors on TRAIL-induced apoptosis [28]. A549 cells, that are resistant to apoptotic induction of TRAIL alone, were exposed to EGF-R inhibitors Genistein (50 μM), PD153035 (5 μM), and PD158780 (10 μM) for 12 h and then treated with recombinant TRAIL protein for additional 3 h. TRAIL alone induced apoptosis in approximately 25% of the cells (Figs. 1 and 2). Pretreatment with PD153035 and PD158780 increased TRAIL-induced apoptosis to 50% (Fig. 1) and pretreatment with Genistein increased to 70% (Figs. 1 and 2). Genistein alone had no effect on tumor cell viability (Fig. 2). Genistein also enhanced TRAIL-induced apoptosis in a dose- and time-dependent manner (Fig. 3). Our data suggest that blocking EGF-R activation enhances TRAIL-induced cell death. The targets of this effect may be either pro- or anti-apoptotic signals.

Down-regulation of total Akt and phosphorylated active Akt proteins by Genistein

To address the mechanism by which EGF-R inhibitors enhance TRAIL-induced apoptosis, we first examined phosphorylation of EGF-R after treating with EGF-R inhibitors. Under the normal growth conditions, Western blot did not efficiently detect a phosphorylated active form of EGF-R. Many growth factor receptor-mediated signals are known to promote cell survival through the Akt pathway. Moreover, recent studies demonstrated that elevated Akt protects tumor cells from TRAIL-induced apoptosis and that constitutively active Akt is an important regulator of TRAIL sensitivity [15–18]. Thus, we next examined whether EGF-R inhibitors regulate expression and/or activation of Akt. We employed Western blot analysis to investigate phosphorylation and expression level of Akt. A549 cells showed moderate protein levels of total Akt and active Akt. Interestingly, Genistein treatment decreased
Genistein TRAIL

Fig. 2. Effect of Genistein on TRAIL-induced apoptosis. (A) A549 cells cultured in 12-well were pretreated with Genistein (50 μM) for 12 h, and then further co-incubated with or without recombinant TRAIL protein (100 ng/ml) for additional 3 h. Cell viability was determined by crystal violet staining method. Viability of control cells was set at 100% and viability relative to the control is presented. The experiments were performed at triplicate, at least twice. The bar indicates standard error. (B) Cell morphology under the conditions described in (A) was photographed.

Genistein TRAIL

Fig. 3. Genistein enhanced TRAIL-induced apoptosis in a dose- and time-dependent manner. (A) A549 cells cultured in 12-well were pretreated with Genistein (0, 12, 25, 50, and 100 μM) for 12 h, and then further co-incubated with or without recombinant TRAIL protein (100 ng/ml) for additional 3 h. Cell viability was determined by crystal violet staining method. Viability of control cells was set at 100% and viability relative to the control is presented. The experiments were performed at triplicate, at least twice. The bar indicates standard error. (B) A549 cells cultured in 12-well were pretreated with Genistein (50 μM) for 0, 3, 6, 9, and 12 h, and then further co-incubated with or without recombinant TRAIL protein (100 ng/ml) for additional 3 h. Cell viability was determined as described in (A). The bar indicates standard error.

Fig. 4. Regulation of total Akt, phosphorylated active Akt, and Bcl-XL protein by Genistein. A549 cells cultured in 12-well were pretreated with Genistein (50 μM) for 0, 3, 6, 9, and 12 h. Whole cell lysates were prepared as described in Materials and methods and subjected to Western blot analysis. NS indicates a nonspecific protein band that was used to ensure equal protein loading.

Studies have demonstrated that Akt regulates Bcl-2 family member proteins, potent regulators of apoptosis following growth factor withdrawal [29–32]. Anti-apoptotic Bcl-2 family proteins, such as Bcl-XL, are localized to the outer mitochondrial membrane and function to regulate various apoptosis activities [30,31]. We therefore examined whether Genistein-induced decreased Akt affects Bcl-XL expression. As expected, Western blot analysis showed that Genistein treatment decreased expression of Bcl-XL protein level in a time-dependent manner (Fig. 4). Although we cannot rule out the possibility that Genistein directly regulates Bcl-XL expression, Bcl-XL expression appears to be associated with Akt activation and/or Akt expression level. Thus, our data suggest that enhanced TRAIL-induced apoptosis by EGF-R inhibitors may be accomplished via decreased total and active Akt, and at least in part via down-regulation of Bcl-XL protein, which is believed to be mediated by decreased active Akt. A recent study showed that Bcl-XL protects tumor cells from TRAIL-induced apoptosis [29–33]. Therefore, our data are in good agreement with the previous findings.

In summary, our data demonstrate that EGF-R inhibitors significantly enhance TRAIL-induced A549 cell apoptosis. Genistein, a potent EGF-R inhibitor, decreased total Akt, phosphorylated active Akt, and Bcl-XL in A549 cells. Anti-apoptotic protein Bcl-XL has been shown to play an important role in the maintenance of keratinocyte survival in response to EGF-R signaling [29,33,34]. Our findings suggest that EGF-R inhibitors may enhance the apoptotic activity of TRAIL protein by regulating Akt protein. Therefore, combined with EGF-R inhibitors, TRAIL may become a more
effective anti-cancer therapy, especially, in TRAIL-resistant tumors, whose resistance is mediated by constitutively activation and/or overexpression of Akt.

References

[34] H. Walczak, A. Bouchon, H. Stahl, P.H. Krammer, Tumor necrosis factor-related apoptosis-inducing ligand retains its apoptosis-inducing capacity on Bel-2- or Bel- XL-overexpressing chemotherapy-resistant tumor cells, Cancer Res. 60 (2000) 3051–3057.
IFN-γ Enhances TRAIL-induced Apoptosis through IRF-1

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Running title: IRF-1-mediated IFN-γ enhancement of TRAIL-induced apoptosis

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Abstract

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family and potent inducer of apoptosis. TRAIL has been shown to effectively limit tumor growth \textit{in vivo} without detectable cytotoxic side effects. Interferon (IFN)-\(\gamma\) often modulates the anti-cancer activities of TNF family members including TRAIL. However, little is known about the mechanism. To explore the mechanism, A549, HeLa, LNCaP, Hep3B and HepG2 cells were pretreated with IFN-\(\gamma\), and then exposed to TRAIL. IFN-\(\gamma\) pretreatment augmented TRAIL-induced apoptosis in all these cell lines. A549 cells were selected and further characterized for IFN-\(\gamma\) action in TRAIL-induced apoptosis. Western blotting analyses revealed that IFN-\(\gamma\) dramatically increased the protein level of interferon regulatory factor (IRF)-1, but not TRAIL receptors (DR4 and DR5). To elucidate the functional role of IRF-1 in IFN-\(\gamma\)-enhanced TRAIL-induced apoptosis, IRF-1 was first overexpressed by using an adenoviral vector AdIRF-1. IRF-1 overexpression minimally increased apoptotic cell death, but significantly enhanced apoptotic cell death induced by TRAIL when infected cells were treated with TRAIL. In further experiments using antisense oligonucleotides, a specific repression of IRF-1 expression abolished enhancer activity of IFN-\(\gamma\) for TRAIL-induced apoptosis. Therefore, our data indicate that IFN-\(\gamma\) enhances TRAIL-induced apoptosis through IRF-1.
Introduction

Apoptosis is an active cell death process that is genetically regulated. This process plays an important role in the development and homeostasis of multicellular organisms (Nagata, 1997). Among apoptosis-inducing proteins, the best characterized are the ligand-type cytokine molecules of the TNF family. TNF family member proteins such as TNF-α, Fas ligand and TRAIL are type II transmembrane molecules that trigger the apoptotic signal cascade by ligating cognate receptors displayed on the cell surface (Cha et al., 1999; Hymowitz et al., 1999).

Although TRAIL is a TNF family member (Pitti et al., 1996; Wiley et al., 1995), it has some notable differences when compared with TNF-α and FasL. For example, unlike Fas, TRAIL receptors DR4 and DR5 are widely expressed (Pitti et al., 1996; Wiley et al., 1995), thus most tissues and cell types are potential targets to TRAIL. Furthermore, TRAIL induces apoptosis in a wide variety of tumor cells but not in most normal cells. Recent preclinical studies demonstrated that repeated systemic administration of recombinant TRAIL protein effectively limited tumor growth without detectable toxicity (Griffith et al., 2000; Walczak et al., 1999). Thus, considerable attention has been paid to TRAIL as a promising therapeutic to treat human cancers.

The transcription factor interferon regulatory factor (IRF)-1 was identified as a regulator of the interferon (IFN)-γ system (Taniguchi et al., 1988). Accumulated evidence shows that IRF-1 functions as a tumor suppressor (Harada et al., 1993; Park et al., 2000; Tamura et al., 1995; Tamura et al., 1997; Tan et al., 1996; Tanaka et al., 1994a; Tanaka et al., 1996; Tanaka et al., 1994b; Yim et al., 1997). IRF-1 suppresses the transformed
phenotype (Harada et al., 1993; Tanaka et al., 1994a; Tanaka et al., 1994b) and is essential for DNA-damage-induced apoptosis in mitogen activated T lymphocytes (Tamura et al., 1995; Tamura et al., 1997). IFN-γ has been also shown to sensitize cells to various apoptotic stimuli including TNF family members (Sasagawa et al., 2000; Shin et al., 2001; Suk et al., 2001a). Recently, several studies demonstrated IFN-γ and TNF synergism in cancer cell apoptosis and necrosis (Sasagawa et al., 2000; Suk et al., 2001a; Suk et al., 2001b). However, little is known about the synergy or enhancing mechanism of IFN-γ on tumor cell apoptosis. Thus, we investigated the role and regulation mechanism of IFN-γ in TRAIL-induced apoptosis. In A549, HeLa, LNCaP, Hep3B and HepG2 cells, IFN-γ-pretreatment augmented TRAIL-induced apoptosis. IFN-γ dramatically increased IRF-1 expression in A549 cells. Overexpression of IRF-1 protein by an adenoviral vector AdIRF-1 increased TRAIL-induced apoptosis upon exposure of infected cells to TRAIL treatment. INF-γ-enhanced TRAIL-induced apoptosis was significantly blocked by antisense oligonucleotide that specifically suppresses IRF-1 protein expression. Therefore, our data indicate that IRF-1 is a key component in the IFN-γ enhancement mechanism in TRAIL-induced apoptosis.
Results

Enhancement of TRAIL-induced apoptosis by IFN-γ

In some cell types, IFN-γ induces cell death and has anti-tumor activities (Kirkwood & Ernstoff, 1990; Kirkwood et al., 1990; Nunokawa & Tanaka, 1992). IFN-γ has also been shown to increase susceptibility of target cells to Fas ligand- or TNF-α-induced apoptosis (Fish et al., 1999; Sasagawa et al., 2000; Suk et al., 2001a). Generally, combination therapies produce a better efficacy than individual therapies in cancer treatment. We previously observed that A549 cells (from human lung carcinoma) are relatively resistant to TRAIL. Thus, we selected A549 cells as our experimental model to determine whether IFN-γ also enhances TRAIL-induced apoptosis. A549 cells were pretreated with IFN-γ (100 U/ml) for 12 hours, and then exposed to recombinant TRAIL protein (Seol & Billiar, 2000) for additional 3 hours. The results of cell viability tests showed that TRAIL alone induced 20% cell death after a 3 hour incubation, but 12-hour IFN-γ pretreatment increased TRAIL-induced cell death to more than 60% (Fig 1A). INF-γ treatment alone did not induce cell death in this cell line. INF-γ was also observed to enhance TRAIL-induced apoptosis in other cell lines such as HeLa, LNCaP, Hep3B and HepG2 (data not shown), indicating that IFN-γ acts in a broad range of tissues to enhance TRAIL-induced apoptosis. Examination of cell morphology also supported enhancer activity of IFN-γ in TRAIL-induced apoptosis (Fig. 1B). Because extra-cellular stimuli activate intra-cellular signal cascades lined with many cellular factors in general, our data suggest that factors modulated by IFN-γ may play a role in an enhancer mechanism in TRAIL-induced apoptosis.
Stimulation of IRF-1 protein expression by IFN-γ

Recently, we reported that TRAIL death-inducing signal transmits from activated receptors through caspase-8, Bid, released cytochrome c, and executioner caspases including caspase-3 (Seol et al., 2001). It was suggested that modulation of any of these signaling components regulate TRAIL-induced apoptosis. Thus, we investigated whether IFN-γ treatment regulates expression of these molecules. A549 cells were treated with IFN-γ for 12 hours and subjected to Western blotting analyses. Analysis results demonstrated that IFN-γ treatment did not change the expression level of TRAIL receptors DR4 and DR5, while expression of IRF-1 was dramatically increased by IFN-γ treatment (Fig. 2). In parallel, we also examined other signaling components that appear to affect mainstream signaling of TRAIL-induced cell death. Similar to TRAIL receptors, IFN-γ treatment did not change the expression level of other pro- and anti-apoptotic proteins such as FADD, Bax, Bcl-2, Bcl-XL, cIAP-1, cIAP-2 and XIAP (data not shown). Our data suggest that IRF-1, a nuclear transcription factor, may play a role in mediating enhancer effects of IFN-γ in TRAIL-induced A549 cell apoptosis.

Enhancement of TRAIL-induced apoptosis by overexpression of IRF-1 protein

IRF-1 has been shown to suppress tumor growth *in vivo* (Park et al., 2000; Yim et al., 1997; Zhou et al., 2000). Thus, we hypothesized that IRF-1 may directly mediate the enhancer effects of IFN-γ in TRAIL-induced apoptosis. To examine this possibility directly, we first overexpressed IRF-1 by taking advantage of AdIRF-1, an adenoviral vector expressing IRF-1. AdIRF-1-infected cells demonstrated a minimal increase in
baseline cell death throughout the experimental setting, whereas additional TRAIL treatment significantly increased cell death in AdIRF-1-infected cells (Fig. 3A). In contrast, TRAIL did not significantly change cell death in AdEGFP-infected or uninfected control cells. Examination of cell morphology also supported the functional role of AdIRF-1 in TRAIL-induced cell death (Fig. 3B). To confirm IRF-1 protein expression by AdIRF-1 infection, infected cells were subjected to Western blotting analysis (Fig. 3C). IRF-1 protein was highly expressed by AdIRF-1 infection in contrast to the AdEGFP control vector that showed no expression. This result indicates that enhanced TRAIL-induced cell death occurred via IRF-1.

*Blockade of IFN-γ-enhancement by IRF-1 suppression in TRAIL-induced apoptosis*

Although overexpression of IRF-1 enhanced TRAIL-induced apoptosis, the role of IRF-1 in mediating IFN-γ enhancer activity in TRAIL-induced apoptosis is unclear. Therefore, to address this question, we utilized an antisense oligonucleotide that specifically suppresses IRF-1 protein expression. A549 cells were transfected with a sense or antisense oligonucleotide, and pretreated with IFN-γ for 12 hours, followed by TRAIL treatment for additional 3 hours. The sense oligonucleotide did not affect TRAIL-induced apoptosis in IFN-γ-pretreated cells. However, the antisense oligonucleotide almost completely protected IFN-γ-pretreated cells from TRAIL-induced cell death (Fig. 4A). Western blotting analysis revealed that IRF-1 protein expression was effectively suppressed by the antisense oligonucleotide (Fig. 4B). Therefore, our data demonstrate that IFN-γ enhances TRAIL-induced apoptosis through IRF-1, and IRF-1 is a key mediator in transmitting IFN-γ enhancer signal in TRAIL-induced cell death.
Discussion

We have demonstrated that IRF-1 directly regulates IFN-γ enhancement of TRAIL-induced apoptosis. Overexpression of IRF-1 protein by AdIRF-1 enhanced TRAIL-induced apoptosis, and a specific suppression of IRF-1 protein expression by an antisense oligonucleotide prevented enhancer activity of IFN-γ in TRAIL-induced apoptosis. This is the first indication that IFN-γ enhancement of TRAIL-induced apoptosis is regulated by IRF-1 protein. Other studies have demonstrated that IFN-γ also synergizes Fas- and TNF receptor-mediated tumor cell death (Fish et al., 1999; Park et al., 2000; Sasagawa et al., 2000; Shin et al., 2001; Suk et al., 2001a). Thus, IFN-γ commonly enhances cell death induced by the three major death-inducing ligands of the TNF family. These results indicate that IFN-γ regulation of death signaling pathways is commonly involved in TRAIL-, Fas ligand- and TNF-α-induced cell death. However, it is poorly understood how IRF-1 regulates IFN-γ enhancement of apoptosis induced by these ligand molecules. As suggested (Fish et al., 1999; Park et al., 2000; Sasagawa et al., 2000; Shin et al., 2001; Suk et al., 2001a), IFN-γ or IFN-γ-induced IRF-1 may inhibit activation of the transcription factor NF-kB which antagonizes activation of various apoptosis-inducing signals. IRF-1 was shown to play a critical role in DNA-damage-induced apoptosis in mature T lymphocytes (Tamura et al., 1995; Tamura et al., 1997), and regulate a cycline-dependent kinase inhibitor p21 and lysyl oxydase genes (Tan et al., 1996; Tanaka et al., 1991). Thus, it is tempting to examine if p21-driven cell cycle arrest is involved in this enhancer mechanism. In addition, as we reported recently (Park et al., 2002b), IRF-1 may regulate expression of cellular factors induced by TRAIL and
enhance TRAIL-induced apoptosis. However, which cellular factors are the targets of IRF-1 has yet to be determined. The protein level of FADD, Bax, Bcl-2, Bcl-XL, cIAP-1, cIAP-2 and XIAP that are known to act in death signaling pathways in TRAIL-induced apoptosis did not change significantly in response to IFN-γ. Thus, other cellular factors involved in death signaling pathways activated by TRAIL are now under investigation.

We do not rule out the possibility that IRF-1 may transmit enhancer activity of INF-γ via a protein-protein interaction in TRAIL-induced apoptosis. As well documented, p53, a tumor suppressor and transcription factor, modulates cell physiology not only by interacting with various cellular factors (Sala et al., 1996; Sorensen et al., 1996), but also by regulating transcription of the target genes (Ball, 1997; Bokoch et al., 1996; Somasundaram & El-Deiry, 1997). Thus, this possibility is also under investigation in this laboratory.

Importantly, a recent study demonstrated that TRAIL plays an essential role in the natural killer (NK) cell-mediated and IFN-γ-dependent tumor surveillance in vivo (Smyth et al., 2001; Takeda et al., 2002). IFN-γ was shown to modulate TRAIL-mediated tumor surveillance, not only by regulating TRAIL expression on NK cells, but also by sensitizing tumor cells to TRAIL-induced cytotoxicity. Although the mechanism by which IFN-γ sensitizes tumor cells to TRAIL-induced apoptosis was not elucidated in the report, our data suggest an active role of IRF-1 in the mechanism. Thus, our data sheds light on better understanding an in vivo tumor surveillance mechanism (Smyth et al., 2001; Takeda et al., 2002). This result and our data also suggest a possible combination therapy of IFN-γ and TRAIL for cancer treatment in humans. Because combination therapies produce a better prognosis than individual therapies in cancer treatment, the
combination of IFN-$\gamma$ and TRAIL may be a very promising anti-cancer therapy to treat human cancers. Furthermore, our data also suggest that in addition to IFN-$\gamma$, IRF-1 may combine with TRAIL protein to induce effective tumor cell death.
Materials and methods

Cell Culture

A549, HeLa, LNCaP, Hep3B and HepG2 cells were obtained from ATCC and maintained in suggested culture medium supplemented with 10% (v/v) fetal bovine serum and antibiotics (100 μg/ml gentamycin and 100 μg/ml penicillin-streptomycin).

Cell Viability

Cells grown in 12-wells were pretreated with IFN-γ (100 U/ml) (Roche Molecular Biochemicals, Mannheim, Germany). After 12 hours, recombinant TRAIL protein (Seol & Billiar, 2000) was added to culture media directly and coincubated for 3 additional hours. Cell viability was determined by the crystal violet staining method as described (Seol & Billiar, 1999), and cell morphology was photographed under the microscope. Briefly, cells were stained for 10 min at room temperature with staining solution (0.5% crystal violet in 30% ethanol and 3% formaldehyde), washed four times with water, and dried. Cells were lysed with 1% SDS solution, and measured at 550 nm. Cell viability was calculated from relative dye intensity and compared to the controls.

Western Blotting

To prepare whole cell lysates, cells were harvested, resuspended in lysis buffer (25mM Hepes (pH 7.4), 100mM NaCl, 1mM EDTA, 5mM MgCl₂, 0.1mM DTT, and protease inhibitor mixture) and sonicated. Proteins were separated on 12 or 15% SDS gel and analyzed by Western blotting as described previously (Park et al., 2002a; Seol et al., 2001). DR4 (AAP-420) and DR5 (AAP-430) were probed with antibody obtained from
Stressgen (Victoria, Canada) and IRF-1 (sc-497) from Santa Cruz (Santa Cruz, CA).

**Adenoviral Vectors**

E1- and E3-deleted AdIRF-1 was constructed through Cre-lox recombination as described (Hardy et al., 1997). Briefly, cDNA for IRF-1 or EGFP, driven by the CMV promoter and terminated by the SV40 polyA signal was inserted into the shuttle vector pAdlox to create pAdlox-IRF-1 or pAdlox-EGFP. Recombinant adenovirus was generated by cotransfection of appropriately digested pAdlox-IRF-1 or pAdlox-EGFP and Ψ5 helper virus DNA into the Ad packaging cell line CRE8 which expresses Cre recombinase. Recombinant adenoviruses were propagated on 293 cells and purified by cesium chloride density gradient centrifugation and subsequent dialysis.

**Adenoviral Infection**

A549 cells were plated in 6 or 12-well plates, and adenoviral infections were performed the next day for 4 hours with virus diluted in Opti-MEM I (Gibco, NY) to the desired multiplicity of infection (MOI 0 to 80). The infected cells were washed 3 times with phosphate-buffered saline and maintained with the F-12K culture medium. After 24 hours, infected cells were exposed to recombinant TRAIL protein for 3 hours. Cell viability was determined by crystal violet staining method (Seol et al., 2001), and morphology was photographed under the microscope. IRF-1 protein expression in AdIRF-1-infected cells was confirmed by Western blotting.

**Transfection of Oligonucleotides**
A549 cells grown in 6 or 12-well were transfected with 2 μg of IRF-1 sense (S) or antisense (AS) phosphothioated oligonucleotide (S, 5'-GCA TCT CGG GCA TCT TTC-3'; AS, 5'-GAA AGA TGC CCG AGA TGC-3') using GenePorter transfection reagent (Gene Therapy Systems, CA). After 6 hours, the cells were exposed to IFN-γ for 12 hours and coincubated with TRAIL protein for additional 3 hours, then assayed for viability. For IRF-1 immunoblotting, transfected cells were treated with IFN-γ for 2 hours before the cells were lysed.
Acknowledgements

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Figure legend

Fig. 1. Effect of IFN-γ on TRAIL-induced apoptosis.

(A) A549 cells plated in 12-well were pretreated with IFN-γ (100 U/ml) for 12 hours, and then coincubated with or without recombinant TRAIL protein (100 ng/ml) for additional 3 hours. Cell viability was determined by crystal violet staining method. Viability of control cells was set at 100%, and viability relative to the control was presented. The experiments were performed at triplicate, at least twice. The bar indicates standard error.

(B) Cell morphology under the conditions as described in (A) was photographed.

Fig. 2. Expression of TRAIL receptors (DR4 and DR5) and IRF-1 protein.

A549 cells were pretreated with IFN-γ (100 U/ml) for 12 hours, and then coincubated with or without recombinant TRAIL protein (100 ng/ml) for 1 hour. Whole cell lysates were prepared as described in Experimental Procedures and subjected to Western blotting analysis.

Fig. 3. Effect of IRF-1 overexpression on TRAIL-induced apoptosis.

(A) A549 cells were infected with AdEGFP or AdIRF-1 for 4 hours, washed, and further cultured. 24 hours later, recombinant TRAIL protein (100 ng/ml) was added to culture medium and incubated for 3 hours. Cell viability was determined by crystal violet staining method. Viability of control cells was set at 100%, and viability relative to the control was presented. The experiments were performed at triplicate, at least twice. The bar indicates standard error.
(B) Cell morphology under the conditions as described in (A) was photographed.

(C) A549 cells were infected with AdEGFP or AdIRF-1 for 4 hours, washed, and further cultured. 24 hours later, whole cell lysates were prepared and subjected to Western blotting analysis for IRF-1 expression. The NS indicates a nonspecific protein band that was used to ensure equal protein loading.

**Fig. 4. Effect of oligonucleotides on IFN-γ enhancement of TRAIL-induced apoptosis.**

(A) 6 hours after transfection of IRF-1 sense or antisense oligonucleotide, A549 cells were pretreated with IFN-γ (100 U/ml) for 12 hours, and further exposed to TRAIL protein (0 or 100 ng/ml) for 3 hours. Cell viability was determined by crystal violet staining method. Viability of control cells was set at 100%, and viability relative to the control was presented. The experiments were performed at triplicate, at least twice. The bar indicates standard error.

(B) 6 hours after transfection of IRF-1 sense or antisense oligonucleotide, A549 cells were pretreated with IFN-γ (100 U/ml) for 2 hours. Whole cell lysates were prepared and subjected to Western blotting analysis for IRF-1 expression. The NS indicates a nonspecific protein band that was used to ensure equal protein loading.
REFERENCES


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ABSTRACT
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#3479 IFN-γ enhances TRAIL-induced apoptosis through IRF-1. Sang-Youel Park, John H. Yim, Steven J. Hughes, Timothy R. Billiar, and Dai-Wu Seol.
Department of Surgery, University of Pittsburgh, Pittsburgh, PA.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the TNF family and potent inducer of apoptosis, has been shown to limit tumor growth without cytotoxic side effects in vivo. In combination with IFN-γ, TRAIL was shown to more efficiently kill a variety of cancer cell lines. The nuclear transcription factor interferon regulatory factor-1 (IRF-1) is thought to mediate many of the cytotoxic effects of IFN-γ. The purpose of this study was to determine if IRF-1 mediates IFN-γ enhancement of TRAIL-induced apoptosis. Cell death was induced in cultured A549 human lung carcinoma cells with TRAIL. Pretreatment with IFN-γ for 12 hrs was added to enhance TRAIL-induced killing. Cell death was assessed by crystal violet assay. Protein lysates from cell culture were assessed by Western blotting for IRF-1 expression. TRAIL induced 20% cell death after 3 hrs treatment. Pretreatment with IFN-γ significantly increased TRAIL-induced cell death to more than 60% at 3 hrs. Western blotting analysis revealed significantly increased expression of IRF-1, but did not demonstrate differences in TRAIL receptor (DR4 and DR5), caspase (caspase-3 and -8), adapter protein (FADD), Bcl-2 family (Bax, Bcl-2 and Bcl-XL) or IAP family (IAP-1, IAP-2 and XIAP) levels. To examine the functional role of IRF-1, IRF-1 was overexpressed using an adenoviral gene vector strategy (AdIRF-1) or blocked using antisense oligonucleotide. AdIRF-1-infected cells demonstrated a minimal increase in baseline cell death of 10% at 3hrs, however, treatment with TRAIL increased cell death to > 60%. Accordingly cells treated with antisense oligonucleotides to IRF-1 significantly inhibited TRAIL-induced killing to <25%. Our data demonstrate that IFN-γ enhances TRAIL-induced apoptosis through an IRF-1-mediated mechanism. Because IRF-1 is a transcription factor, it may regulate genes involved in apoptosis enhancing function of IFN-γ. Currently, the genes regulated by IFN-γ through IRF-1 are under investigation.
May 30, 2002

Division of Research Grant  
National Institutes of Health  

To whom it may concern:

Enclosed is my NIH grant submission, “TRAIL: Death Signaling Analysis and Cancer Gene Therapy”. I respectfully request that this application be assigned to the Cancer Molecular Pathology Study Section (CAMP).

This proposal has studies on apoptotic cell death and cancer gene therapy using a death-inducing gene. The CAMP study section contains a number of experts in these areas who can provide a thorough review of this proposal.

Thank you for your consideration of my request.

Sincerely,

Dai-Wu Seol, Ph.D.  
Assistant Professor of Surgery  
University of Pittsburgh School of Medicine
1. **TITLE OF PROJECT**

   TRAIL: Death Signaling Analysis and Cancer Gene Therapy

2. **RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION**

   □ NO □ YES

   (If "Yes," state number and title)

   Number: Title:

3. **PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR**

   New Investigator □ No □ Yes

   3a. NAME (Last, first, middle)

   Seol, Dai-Wu

   3b. DEGREE(S)

   PhD

   3c. POSITION TITLE

   Assistant Professor

   3d. MAILING ADDRESS (Street, city, state, zip code)

   W1513 Biomedical Science Tower

   University of Pittsburgh

   200 Lothrop Street

   Pittsburgh PA 15261

   E-MAIL ADDRESS:

   seold@pitt.edu

4. **HUMAN SUBJECTS RESEARCH**

   □ No □ Yes

   4a. Research Exempt

   □ No □ Yes

   If "Yes," Exemption No.

   4b. Human Subjects Assurance

   M1259

   4c. NIH-defined Phase III Clinical Trial

   □ No □ Yes

   5. **VERTEBRATE ANIMALS**

   □ No □ Yes

   5a. If "Yes," IACUC approval Date

   4/3/02

   5b. Animal welfare assurance no

   A3187-01

6. **DATES OF PROPOSED PERIOD OF SUPPORT**

   From 04/01/03 Through 03/31/08

   7. **COSTS REQUESTED FOR INITIAL BUDGET PERIOD**

   7a. Direct Costs ($) 175,000

   7b. Total Costs ($) 260,082

   8. **COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT**

   8a. Direct Costs ($) 875,000

   8b. Total Costs ($) 1,296,025

9. **APPLICANT ORGANIZATION**

   Name University of Pittsburgh

   Address Office of Research

   350 Thackeray Hall

   Pittsburgh, PA 15260

   Institutional Profile File Number (if known) 2059802

10. **TYPE OF ORGANIZATION**

    Public: □ Federal □ State □ Local

    Private: □ Private Nonprofit

    For-profit: □ General □ Small Business

    □ Woman-owned □ Socially and Economically Disadvantaged

11. **ENTITY IDENTIFICATION NUMBER**

    25-0965591

    DUNS NO. (if available)

    00-451-4360

    Congressional District 14

12. **ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE**

    Name Michael M. Crouch

    Title Director, Office of Research

    Address 350 Thackeray Hall

    University of Pittsburgh

    Pittsburgh, PA 15260

    Telephone 412-624-7400

    FAX 412-624-7409

    E-Mail ornih@orserver.off-res.pitt.edu

13. **OFFICIAL SIGNING FOR APPLICANT ORGANIZATION**

    Name Michael M. Crouch

    Title Director, Office of Research

    Address 350 Thackeray Hall

    University of Pittsburgh

    Pittsburgh, PA 15260

    Telephone 412-624-7400

    FAX 412-624-7409

    E-Mail offres@orserver.off-res.pitt.edu

14. **PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE:**

    I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

15. **APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE:**

    I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

Signature of PI/PD Named in 3a.

Signature of Official Named in 13.

Date 5/24/02

Date 5/24/12
Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a membrane-bound cytokine molecule. Soluble TRAIL protein forms a homotrimer and is a potent apoptosis inducer in many cancer cell lines. TRAIL was shown to have tumor-specific killing activity without damaging normal tissues in vivo. Thus, TRAIL has been considered to be a promising cancer therapy. Nonetheless, relatively little is known about TRAIL-induced death signaling. In our preliminary studies, we found that many extracellular stimuli and expression of cellular factors modulate TRAIL-induced apoptosis. Therefore, we hypothesize that analysis of TRAIL signaling and the down-stream signal components involved will give some insights into tumor selectivity of TRAIL, leading to better development of TRAIL as an anti-cancer drug. In view of high efficacy and limited cytotoxic side effects, we have created a novel TRAIL expression cassette for cancer gene therapy. Using this DNA expression cassette, we succeeded in producing a secretable trimeric TRAIL (stTRAIL) protein with potent apoptotic activity in a wide variety of cancer cell lines in vitro but not normal cells. Under AIM I, we will characterize the cellular factors activated by inhibitors and enhancers of TRAIL-induced apoptosis. This aim will be accomplished by Western blotting analysis using TRAIL inhibitor- and/or enhancer-treated cells. We will analyze the identified components by overexpressing or blocking the genes encoding the corresponding components, or blocking the functions of the components. Under AIM II, we will develop TRAIL-based cancer gene therapies. This aim will be accomplished by employing molecular, cellular and surgical techniques. A recombinant plasmid DNA expression construct and an adenoviral vector harboring the secretable trimeric TRAIL sequence will be tested with or without death enhancers in vitro and in vivo. Completion of these aims will significantly advance our understanding of TRAIL-induced death signaling and, more importantly, lead to the development of therapies that can be applied to treat human cancers.
# RESEARCH GRANT

## TABLE OF CONTENTS

| Description, Performance Sites, and Personnel | 2-  
| Table of Contents | 2  
| Detailed Budget for Initial Budget Period (or Modular Budget) | 3  
| Budget for Entire Proposed Period of Support (not applicable with Modular Budget) | N/A  
| Budgets Pertaining to Consortium/Contractual Arrangements (not applicable with Modular Budget) | N/A  
| Biographical Sketch—Principal Investigator/Program Director (Not to exceed four pages) | 5-7  
| Other Biographical Sketches (Not to exceed four pages for each—See instructions) | 8-20  
| Resources | 21-22  

## Research Plan

- Introduction to Revised Application (Not to exceed 3 pages) | N/A  
- Introduction to Supplemental Application (Not to exceed one page) | N/A  
- A. Specific Aims | 23  
- B. Background and Significance | 23-27  
- C. Preliminary Studies/Progress Report/Phase I Progress Report (SBIR/STTR Phase II ONLY) | 27-36  
- D. Research Design and Methods | 36-45  
- E. Human Subjects | N/A  
- Protection of Human Subjects (Required if Item 4 on the Face Page is marked “Yes”) | N/A  
- Inclusion of Women (Required if Item 4 on the Face Page is marked “Yes”) | N/A  
- Inclusion of Minorities (Required if Item 4 on the Face Page is marked “Yes”) | N/A  
- Inclusion of Children (Required if Item 4 on the Face Page is marked “Yes”) | N/A  
- Data and Safety Monitoring Plan (Required if Item 4 on the Face Page is marked “Yes” and a Phase I, II, or III clinical trial is proposed) | N/A  
- F. Vertebrate Animals | 46  
- G. Literature Cited | 47-52  
- H. Consortium/Contractual Arrangements | N/A  
- I. Letters of Support (e.g., Consultants) | 53-56  
- J. Product Development Plan (SBIR/STTR Phase II and Fast-Track ONLY) | N/A  

## Checklist

- Other items (list):  

## Appendix

Appendices NOT PERMITTED for Phase I SBIR/STTR unless specifically solicited.

Number of publications and manuscripts accepted for publication (not to exceed 10)

Other items (list):
### BUDGET JUSTIFICATION PAGE

**MODULAR RESEARCH GRANT APPLICATION**

<table>
<thead>
<tr>
<th>Initial Budget Period</th>
<th>Second Year of Support</th>
<th>Third Year of Support</th>
<th>Fourth Year of Support</th>
<th>Fifth Year of Support</th>
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<td>$ 175,000</td>
<td>$ 175,000</td>
<td>$ 175,000</td>
<td>$ 175,000</td>
<td>$ 175,000</td>
</tr>
</tbody>
</table>

**Total Direct Costs Requested for Entire Project Period**: $ 875,000

---

### Personnel

Dai-Wu Seol, Ph.D. (50% effort), is an Assistant Professor in the Department of Surgery at the University of Pittsburgh. Dr. Seol has been actively involved in TRAIL research since early 1998. Dr. Seol will be responsible for the overall direction of the project.

Steven J. Hughes, M.D. (5% effort), is an Assistant Professor in the Department of Surgery at the University of Pittsburgh. Dr. Hughes has actively collaborated with PI to investigate death receptor signaling. Dr. Hughes is also an expert in quantitative RT-PCR. He will serve as a coinvestigator.

John H. Yim, M.D. (5% effort), is an Assistant Professor in the Department of Surgery at the University of Pittsburgh. Dr. Yim is an expert in *in vivo* gene delivery and animal surgery. Dr. Yim has been an active collaborator on PI’s projects. He will directly oversee the gene transfer studies in animal tumor models. He will serve as a coinvestigator.

### Consortium

---

**Fee (SBIR/STTR Only)**
BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2. Photocopy this page or follow this format for each person.

NAME
Dai-Wu Seol, Ph.D.

POSITION TITLE
Assistant Professor

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

<table>
<thead>
<tr>
<th>INSTITUTION AND LOCATION</th>
<th>DEGREE</th>
<th>YEAR(s)</th>
<th>FIELD OF STUDY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Korea University, Seoul, Korea</td>
<td>B.S.</td>
<td>1990</td>
<td>Molecular Biology</td>
</tr>
<tr>
<td>University of Pittsburgh School of Medicine, Pittsburgh, PA</td>
<td>Ph.D.</td>
<td>1998</td>
<td>Molecular Oncology</td>
</tr>
</tbody>
</table>

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. DO NOT EXCEED TWO PAGES.

A. POSITIONS AND AWARDS

POSITIONS
1986-1988 Military Service, Korea
1990-1991 Research Assistant, Cancer Research Institute, Seoul National University School of Medicine, Korea
1991-1991 Research Assistant, Virus Institute (supported by WHO for Hanta Virus), Korea University School of Medicine, Korea
1992-1998 Graduate student, University of Pittsburgh School of Medicine, Pittsburgh, PA
1998-2000 Research Fellow, Dept. of Surgery, University of Pittsburgh School of Medicine, Pittsburgh, PA
2000-present Assistant Professor, Dept. of Surgery, University of Pittsburgh School of Medicine, Pittsburgh, PA

HONORS AND AWARDS
1989 Nominated as Honored Student of Korea University, Korea
1994 1st Place Award, 5th Annual Dept. of Pathology Research Presentations, University of Pittsburgh School of Medicine, Pittsburgh, PA

B. PUBLICATIONS

PEER-REVIEWED ARTICLES


PATENTS

ABSTRACTS and PROCEEDINGS
2. Chen, Q., Seol, D. W. and Zarnegar, R. Activation of hepatocyte growth factor receptor (c-met) gene expression by AP-1. 90th Annual Meeting, AACR (April 10-14, 1999)
ACTIVE

CMRF (Competitive Medical Research Fund) (Seol) 1/15/00-6/30/02 N/A
PCI (Pittsburgh Cancer Institute) $25,000

TRAIL signaling and anti-cancer drug development
To determine the cellular factors involved in TRAIL death signaling
To develop TRAIL gene therapy vector

DAMD17-01-1-0607 (Seol) 6/15/01-7/14/02 N/A

DOD $74,837

TRAIL-based anti-cancer drug development
To develop TRAIL gene therapy vector
BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2. Photocopy this page or follow this format for each person.

NAME

Steven J. Hughes, M.D.

POSITION TITLE

Assistant Professor

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

<table>
<thead>
<tr>
<th>INSTITUTION AND LOCATION</th>
<th>DEGREE</th>
<th>YEAR(s)</th>
<th>FIELD OF STUDY</th>
</tr>
</thead>
<tbody>
<tr>
<td>University of Minnesota, Duluth, MN</td>
<td>B.A.</td>
<td>1988</td>
<td>Chemistry</td>
</tr>
<tr>
<td>Mayo Medical School</td>
<td>M.D.</td>
<td>1993</td>
<td>Medicine</td>
</tr>
</tbody>
</table>

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

Professional Employment and Experience:

1993-1994 University of Michigan Hospitals, Ann Arbor, MI, Surgical Internship
1994-1996 University of Michigan Hospitals, Ann Arbor, MI, General Surgery Resident
1996-1998 University of Michigan Medical School, Ann Arbor, MI, Surgical Oncology Research Fellow
1997-1998 University of Michigan Hospitals, Ann Arbor, MI, Surgical Critical Care Fellow
1998-1999 University of Michigan Hospitals, Ann Arbor, MI, General Surgery Resident
1999-2000 University of Michigan Hospitals, Ann Arbor, MI, General Surgery Chief Resident
2000-present University of Pittsburgh Medical School, Pittsburgh, PA, Assistant Professor of Surgery
2001-present VA Pittsburgh Healthcare System, Pittsburgh, PA, Chief of General Surgery

Honors, Awards, Fellowships:

<table>
<thead>
<tr>
<th>Title of Award</th>
<th>Year</th>
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<tbody>
<tr>
<td>Undergraduate Research Opportunities Program Scholarship</td>
<td>1986</td>
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<tr>
<td>Departmental Honors Department of Chemistry, University</td>
<td>1988</td>
</tr>
<tr>
<td>Bulldog Award Outstanding contributions to the University</td>
<td>1987</td>
</tr>
<tr>
<td>Bulldog Award Outstanding contributions to the University</td>
<td>1988</td>
</tr>
<tr>
<td>Bronze Beeper Award Excellence as a teacher &amp; mentor</td>
<td>1996</td>
</tr>
<tr>
<td>Frederick A. Coller Surgical Society Research Fellowship</td>
<td>1996</td>
</tr>
<tr>
<td>Resident Research Award Frederick A. Coller Surgical</td>
<td>1997</td>
</tr>
<tr>
<td>Young Investigator Award Department of Surgery, University</td>
<td>1998</td>
</tr>
<tr>
<td>Robert V. Danto Memorial Cancer Research Award</td>
<td>1998</td>
</tr>
<tr>
<td>Resident Research Award Department of Surgery, University</td>
<td>1998</td>
</tr>
<tr>
<td>Michigan Society of Thoracic and Cardiovascular Surgeons</td>
<td>1998</td>
</tr>
<tr>
<td>Administrative Chief Resident Department of Surgery,</td>
<td>1999</td>
</tr>
</tbody>
</table>

Relevant Publications:


### OTHER SUPPORTS – STEVE HUGHES, M.D.

#### ACTIVE

(Hughes)  
American College of Surgeons Faculty Research Fellowship  
**7/1/01-6/30/03**  
N/A  
$80,000  
The trafficking and function of Fas (CD95) protein in esophageal adenocarcinoma  
$80,000  
The major goal of this grant is to determine the effects of bile salts and PKC activity on Fas cell-surface expression.

(Godfrey)  
Cepheid  
**7/1/01-7/1/03**  
5%  
$25,000  
Novel marker development for intra-operative QRT-PCR detection for lymph node metastasis  
The major goal of this project is the identification of mRNA targets and development of primer sets and chemistries for QRT-PCR detection of lymph node metastases.

#### COMPLETED

(Hughes)  
CMRF  
**2/1/01-2/1/02**  
N/A  
$25,000  
The major goal of this grant was to investigate the role of wild-type p53 on Fas cell-surface expression.
**BIOGRAPHICAL SKETCH**

Provide the following information for the key personnel in the order listed on Form Page 2.

Photocopy this page or follow this format for each person.

<table>
<thead>
<tr>
<th>NAME</th>
<th>POSITION TITLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>John H. Yim, M.D.</td>
<td>Assistant Professor</td>
</tr>
</tbody>
</table>

**EDUCATION/TRAINING** (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

<table>
<thead>
<tr>
<th>INSTITUTION AND LOCATION</th>
<th>DEGREE</th>
<th>YEAR(s)</th>
<th>FIELD OF STUDY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yale University, New Haven, CT</td>
<td>B.S.</td>
<td>1986</td>
<td>Mol. Biophys. &amp; Biochem.</td>
</tr>
<tr>
<td>Stanford University School of Medicine, Palo Alto, CA</td>
<td>M.D.</td>
<td>1992</td>
<td>Medicine</td>
</tr>
</tbody>
</table>

**RESEARCH AND PROFESSIONAL EXPERIENCE:** Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

**Previous Employment**

1992-94 Intern and Resident in General Surgery, Barnes Hospital, St. Louis, MO
1994-97 Fellow, Surgical Oncology Training Grant, Washington University, St. Louis, MO
1997-00 Senior and Chief Resident in General Surgery, Barnes-Jewish Hospital, St. Louis, MO
2000- Assistant Professor, Department of Surgery, University of Pittsburgh, Pittsburgh, PA

**Research Experience**

1982 American Heart Association Student Fellow, National Heart, Lung, and Blood Institute, Bethesda, MD
1985 Foundation for the Advancement of Education in Science (FAES) Summer Student Fellow, National Institute of Dental Research, Bethesda, MD
1986 Thesis Research, Department of Biology, Yale University, New Haven, CT, Thesis: Conservation of the Notch locus sequence in different strains of wild-type Drosophila melanogaster
1986-87 Research Assistant, Laboratory of Microbiology and Immunology, National Institute of Dental Research, Bethesda, MD
1988-90 Research Assistantship, Department of Surgery, Stanford University School of Medicine, Palo Alto, CA
1994-97 Fellow, Surgical Oncology Training Grant, Laboratory of Biological Therapy, Department of Surgery, Washington University School of Medicine, St. Louis, MO

**Honors and Awards**

1982 National Merit Finalist
1982 Maryland Distinguished Scholar
1982 American Heart Association Fellowship
1985 FAES Summer Student Fellowship
1986 Cum laude, Yale University
1988 Stanford Medical Scholars Award, Stanford University School of Medicine
1997 James Ewing Foundation Trainee Award, Society of Surgical Oncology
1998 Samuel A. Wells, Jr. Resident Research Award, Washington University Department of Surgery

**Posters:**


Presentations:


Peer-Reviewed Articles
Principal Investigator/Program Director (Last, first, middle): Seol, Dai-Wu

OTHER SUPPORTS – JOHN H. YIM, M.D.

PENDING

American College of Surgeons (YIM) 7/1/02-6/30/04 60%
Faculty Research Fellowship 2002 $80,000
IRF-1 Induced Tumor Suppression of Breast Cancer

To determine the mechanism of IRF-1 induced breast cancer specific tumor suppression in vitro in human and mouse breast cells
BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2. Follow this format for each person. DO NOT EXCEED FOUR PAGES.

NAME

Timothy R. Billiar

POSITION TITLE

Professor and Chair, Department of Surgery

EDUCATION

(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

<table>
<thead>
<tr>
<th>INSTITUTION AND LOCATION</th>
<th>DEGREE</th>
<th>YEAR (S)</th>
<th>FIELD OF STUDY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doane College, Crete NE</td>
<td>BA</td>
<td>1979</td>
<td>Natural Sciences</td>
</tr>
<tr>
<td>University of Chicago Pritzker School of Medicine, Chicago IL</td>
<td>MD</td>
<td>1983</td>
<td>Medicine</td>
</tr>
</tbody>
</table>

A. POSITIONS AND HONORS

1983-1987  Surgery Intern, Resident, Research Fellow, Department of Surgery, University of Minnesota
1987-1990  Research Fellow, Department of Surgery, University of Pittsburgh
1989-1992  Surgery Resident & Research Assistant Professor, Department of Surgery, University of Pittsburgh
1992-1997  Asst & Assoc Professor, Department of Surgery, University of Pittsburgh
1997-1999  Watson Professor, Department of Surgery, University of Pittsburgh
1999-Present Professor and Chair, Department of Surgery, University of Pittsburgh

HONORS AND APPOINTMENTS: Alpha Omega Alpha; Samuel P. Harbison Endowed Assistant Professor of Surgery 1992-1994; George HA Clowes Jr Memorial Research Career Development Award, American College of Surgeons 1992-1997; President - Society of University Surgeons 2000; Editorial Boards: Shock; Nitric Oxide: Biology and Chemistry; Journal of the American College of Surgeons; Journal of Surgical Research; Sepsis; American Journal of Physiology; Archives of Surgery

MEMBERSHIP ON FEDERAL GOVERNMENT PUBLIC ADVISORY COMMITTEES:

1993: Ad Hoc Review Committee, NIH-ALCB-1
1994: Ad Hoc Review Committee, NIH-NIAID

B. SELECTED PUBLICATIONS


The aims of this project are: 1) To determine how NO/cGMP/G-kinase inhibits TNFα signaling in hepatocytes; 2) To determine the pathways leading to efficient S-nitrosylation of caspases in hepatocytes; 3) To identify iNOS-induced protective genes in hepatocytes.

The aims of this project are: 1) To determine how CD14 is regulated and processed in hepatocytes; and 2) To determine the functions of hepatocyte-derived CD14.

The postdoctoral training program is designed to train fellows, principally surgeons, for a period of three years each, in basic science research relevant to trauma and sepsis. Three subfoci of research have been targeted: 1) the role of cytokines in regulation of nitric oxide production; 2) role of cytokines in microbial control; and 3) role of cytokines in regulation of immune response.
The aims of this project are: 1) To determine the mechanisms of the upregulation of iNOS and COX-2 during hemorrhagic shock; and 2) To determine how induced NO promotes the inflammatory response during resuscitated hemorrhagic shock.

P50-GM-53789 (Billiar)  7/1/00-6/30/04  3%
NIH/NIGMS
$42,704
Molecular Biology of Hemorrhagic Shock
Core A: Administrative Core
This core will provide a central administrative office for management of the trauma center grant.

P50-GM-53789 (Billiar)  7/1/00-6/30/04  3%
NIH/NIGMS
$158,394
Molecular Biology of Hemorrhagic Shock
Core B: Animal Models Core
This core will provide the animal tissues to be studied in the various projects, ensuring a uniform source of material.

R01-HL-63426-02 (Pham)  1/1/99-12/31/02  5%
NIH/NHLBI
$22,194 (subcontract)
Nitric Oxide in Allograft Vasculopathy
This is a subcontract to an R01 held by Dr. Si Pham at the University of Miami, to provide expertise in vector development.

U01-HL-66948 (Glorioso)  9/30/00-8/31/05  10%
NIH/NHLBI
$157,846
Cardiovascular Gene Therapy Center
Pre-Clinical Project: iNOS Gene Therapy to Prevent Allograft Vasculopathy (Simmons)

The aims of this project are: 1) To determine the efficacy of several viral vectors containing the human iNOS gene and their respective control vectors for suppression of cardiac transplant arteriosclerosis in the rat model; 2) To determine toxicity of Ad-iNOS, AAV-iNOS, and Lt-iNOS and their respective control vectors in the rat model; 3) To determine efficacy and toxicity in a porcine chronic rejection model of the ideal viral vector and its respective control vector, as determine in Aim 2; and 4) To determine the toxicity of iNOS gene transfer in human cardiac myocytes.

U01-HL-66948 (Glorioso)  9/30/00-8/31/05  5%
NIH/NHLBI
$54,640
Cardiovascular Gene Therapy Center
Administrative Core (Glorioso)
The core will provide administrative support and guidance for the Cardiovascular Gene Therapy Center.
Principal Investigator/Program Director: Seol, Dai-Wu

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2. Photocopy this page or follow this format for each person.

NAME
Paul D. Robbins

POSITION TITLE
Professor

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

<table>
<thead>
<tr>
<th>INSTITUTION AND LOCATION</th>
<th>DEGREE (if applicable)</th>
<th>YEAR(s)</th>
<th>FIELD OF STUDY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haverford College, PA</td>
<td>B.S.</td>
<td>1980</td>
<td>Biology</td>
</tr>
<tr>
<td>University of California/Berkeley, CA</td>
<td>Ph.D.</td>
<td>1985</td>
<td>Molecular Biology</td>
</tr>
<tr>
<td>Whitehead Institute (MIT), MA</td>
<td>Post-doc</td>
<td>1986-90</td>
<td>Molecular Biology</td>
</tr>
</tbody>
</table>

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. DO NOT EXCEED TWO PAGES.

APPOINTMENTS AND POSITIONS

Assistant Professor, Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, 1990-1995
Director, Vector Core Facility, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, 1991-
Associate Professor (with tenure), Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, 1996-1999
Interim Director, Molecular and Cellular Oncology Program, University of Pittsburgh Cancer Institute, 1997-2001
Professor (with tenure), Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, January, 2000
Professor, Department of Orthopaedic Surgery, University of Pittsburgh School of Medicine, 2001-
Director of Basic Research, Molecular Medicine Institute, University of Pittsburgh, 2001-

HONORS AND AWARDS (SELECTED)

Magill-Rhodes Scholarship, Haverford College, 1976-1980
High Honors in Biology, Haverford College, 1980
Jane Coffin Childs Memorial Fund for Medical Research Fellowship, 1986-1989
Ad Hoc Member of Molecular Biology and Genetics Study Section, ACS (1991).
Temporary Member, Cell Biology and Physiology (CBY2) Study Section (June, 1996)
Editorial Board, Molecular Biotechnology, Gene Therapy and Molecular Biology, Journal of Gene Medicine, Cancer Gene Therapy, Arthritis Research, Genes and Immunity
Associate Editor, Gene Therapy, Cancer Research
Co-Organizer, 1st and 2nd Meeting on Gene Therapy for Arthritis and Related Musculoskeletal Diseases, Co-Organizer and Session Chair, Viral Gene Vectors: Molecular Biology, Design and Application to Gene Therapy, Banff, Canada (April, 2001), Chairman and organizer, 2001 ASGT Symposium on Musculoskeletal Disorders, Scientific Review Board, National Gene Vector Laboratory, (1998-)
SELECTED RECENT REFEREED ARTICLES (from a total of 210)


ACTIVE

5R01 CA55227-10 (Robbins) 08/01/98-06/30/03 20%
NIH/NCI $154,503
Regulation of Transcription by the Retinoblastoma Anti-oncogene
Examine the regulation of transcription by Rb and cyclin D1 mediated through an interaction with TAFII250

SP01 CA73743-03 (Lotze) 01/01/98-12/31/03 5%
NIH/NCI $75,398
Dendritic Cell Biology and Therapy
Vector Core

2P01 DK44935-06A1 (Glorioso) 07/01/99-06/30/04
NIH/NIDDK
Model Systems Toward Development of Human Gene Therapy
Project 2 $112,000 10%
Virus Vector Core $65,806 5%
Administrative Core $37,000 5%
Examine the immune mechanism through which local gene transfer to joints confers a systemic therapeutic effect.

JDFI (Trucco) 10/01/99-09/30/04
Juvenile Diabetes Foundation $130,044 (core only) 5%
$99,668 (project) 10%

ROBBIN00G0 (Robbins) 04/01/00-03/31/02 5%
Cystic Fibrosis Foundation $55,272
Identification of Peptides that Facilitate Internalization into Airway Epithelial Cells

MDA (Huard) 07/01/00-06/30/03 5%
Muscular Dystrophy Assn. $91,486
Development of Approaches to Facilitate Expansion and Transplantation of Allogeneic Muscle Derived Stem Cells

1U01 HL66949-01 (Glorioso) 09/28/00-08/31/05 5%
NIH/NHLBI $240,086
Supplement to Cardiovascular Gene Therapy Center Grant for Preclinical Vector Core
Construct adenoviral vectors for Heart, Lung and Blood Institute funded investigators

NIH (Rinaldo) 09/30/98-08/31/03 5%
SP01 AI43664-03 $51,329 (Core only)
Antigen delivery for adjuvant HIV immunotherapy – Vector core
Develop adenoviral vectors expressing SIV and HIV antigens.

NIH (Herberman) 08/01/99-07/31/04 5%
SP30 CA47904-13 $60,087
Cancer Center Support Grant – Vector Core
Construct and provide vectors for members of the University of Pittsburgh Cancer Center

Maxygen Incorporated 08/01/01-07/31/02 $100,000
Test the biological activity of mutant B7.1 molecules in vivo.
RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory: Attached

Clinical: N/A

Animal: Attached

Computer: Attached

Office: Attached

Other: Attached

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Attached
RESOURCES AND ENVIRONMENT

Laboratory space for this research is provided by the Department of Surgery on the 15th floor of the Biomedical Science Tower. It currently consists of approximately 44,000 square feet. There are facilities available for organ perfusion studies, microvascular surgery, tissue culture, biochemistry, and molecular biology. Current permanent equipment located in the labs on the 15th floor of the Biomedical Science Tower includes:

- Automat LB lumonometer
- Slot blot apparatus
- Sorvall RC5B centrifuge
- Beckman XL90 ultracentrifuge
- DNA sequence apparatus and power supply
- Hoeffer PAGE system and PAGE transfer apparatus
- 3 Ludlum Geiger counter
- 4°C cold room
- Brinkman polytron
- 4 microcentrifuges
- 3 light microscopes
- Drying ovens
- Mettler analytical balance
- 5 refrigerators
- 4 -70°C freezers
- pH meter
- 4 tissue culture CO2 incubators
- 1 PRJ international refrigerated centrifuge
- 1 dry air incubator
- 1 Beckman table-top refrigerated centrifuge
- 5 tissue culture hoods
- 1 laminar floor hood
- Cytospin centrifuge
- Beckman LS-scintillation counter
- Beckman gamma 5500 counter
- Milton Roy spectrophotometer, Spectronic 601
- Multiple sample microtest harvester
- 2 water baths
- V-Max microplate reader, Molecular Devices
- 1 Shimadzu spectrofluorophotometer
- 1 ZEOS 486 computer, on-line with GenBank
- 2 Macintosh SE computers
- 1 Macintosh II computer
- 1 Macintosh laser printer
- 6 horizontal gel electrophoresis systems
- Waters HPLC system with automatic injector
- Beckman model J6M centrifuge elutriator
- Perkin-Elmer DNA thermocycler
- UV stratalinker
- Shaking water bath
- 32P station with shield and cansisters
- SpeedVac vacuum dryer
- Microwave
- 4 Platform rockers
- 5 Thermoline heat bloks
- LKB Ultraspec II
- 2-LAB line incubator/shaker
- Dish washing and sterilizing facility

Normal and nude mice will be purchased from Harlan Sprague Dawley. The animals will be housed in the Biomedical Science Tower animal facility. There is ample office space in Scaife 12th floor with computer secretarial staff for preparing abstracts, papers, protocols, etc.
A. SPECIFIC AIMS

Despite dramatic advances in our understanding about pathophysiological mechanisms by which human cancers arise, cancer remains a major cause of mortality worldwide. Thus, more effective therapies are needed. Studies have shown that TRAIL kills a variety of cancer cell lines in vitro, and specifically induces tumor cell apoptosis without damaging normal tissues in vivo (3, 4). However, relatively little is known about TRAIL death signaling, especially on modulation of the death signal under pathophysiological conditions or co-existence of other stimuli. Previously, we demonstrated that caspase-8 is a necessary signal component for TRAIL-induced apoptosis, and TRAIL receptor-mediated death signal transmits through caspase-8, Bid, cytochrome c release, activation of executioner caspses in order. TRAIL-induced apoptosis was significantly attenuated when the function of any of these signaling components was blocked. Normal cell physiology is the consequence of integration of many signals at a given time. Any signal or strength change of an existing signal, therefore, can affect other existing signals and cell physiology as a result. Many cell signaling pathways may affect TRAIL-induced cell death, and come to act by modulating cellular components lined in the main stream of TRAIL death signaling. For example, as we reported recently, hypoxia significantly inhibited TRAIL-induced apoptosis. This hypoxia inhibition appears to be due to the increased levels of anti-apoptotic proteins such as Bcl-2, Bcl-XL, and inhibitor of apoptosis protein (IAP) family members. Conversely, interferon (IFN)-γ enhanced TRAIL-induced apoptosis. IFN-γ increased the level of interferon responsive factor (IRF)-1. We also found that Akt activation, also known as a major cell survival and anti-apoptotic signal, is associated with the increased level of XIAP protein. Therefore, we hypothesize that extracellular stimuli regulate cellular signaling components, which modulate TRAIL-induced apoptosis.

In order to maximize the therapeutic potential of TRAIL, efficient means to deliver this protein in vivo must be established. Protein therapy in general has many limitations, including elimination of impurities and high cost burden in cancer treatment. In our experience in TRAIL protein purification from bacterial cells, protein production yield was very poor. Thus, to alleviate such limitations, we established a gene therapy approach, by which TRAIL protein is produced and supplied by the delivered gene. This gene therapy approach is presumed to sustain TRAIL protein levels for longer periods of time than direct protein delivery. In our preliminary studies, we have created a DNA expression cassette composed of the three unrelated DNA sequences encoding a secretion signal, a trimer-forming domain and apoptotic TRAIL sequence (amino acid 114-281). Using this DNA cassette, we succeeded in producing a potent secretable trimeric TRAIL (stTRAIL) protein. This stTRAIL was shown to have higher tumoricidal activity than reported recombinant TRAIL protein, but no death-inducing activity in normal cells isolated from human patients. We found that TRAIL-induced apoptosis does not depend upon p53 status, typically considered a critical factor in cancer therapies using chemotherapeutic agents or radiation. Therefore, it is imperative to examine our TRAIL gene therapy approach in vivo, alone or in combination with enhancers. Based on our preliminary studies, we will pursue two interrelated research aims:

AIM I: To characterize the cellular factors activated by inhibitors and enhancers of TRAIL-induced apoptosis. We hypothesize that factors inhibiting or enhancing TRAIL-induced apoptosis may modulate proapoptotic and/or antiapoptotic proteins involved in TRAIL death signaling. Research under this aim will characterize the cellular factors involved in TRAIL-induced apoptosis and how these molecules and their interactions are regulated by stimuli that inhibit or enhance TRAIL-induced apoptosis.

AIM II: To optimize TRAIL gene transfer as an anticancer therapy. In our preliminary studies, we have created a promising artificial gene that can express stTRAIL protein. The efficacy and specificity of the two gene therapy vectors (a naked plasmid DNA and an adenoviral vector) harboring the stTRAIL gene will be established.

B. BACKGROUND AND SIGNIFICANCE

Apoptosis is a biological process that plays a pivotal role in the development and homeostasis of multicellular organisms (1-3). Aberrations of this process can be detrimental to organisms. Excessive apoptosis causes damage to normal tissues in certain autoimmune disorders whereas a failure of apoptosis allows cells to grow unlimitedly, resulting in cancers. A wide variety of molecules have been identified to induce apoptosis. Among these molecules, the apoptosis-inducing signaling mechanism is best characterized in ligand-type cytokine molecules including the tumor necrosis factor (TNF) family members. The TNF family members such
as TNF-α, Fas ligand (FasL) and TRAIL are membrane-bound polypeptide molecules. The C-termini of the molecules protrude outwards and contain receptor-binding domains.

Upon binding to cognate receptor, TNF-α trimerizes the receptor molecule (TNF-R1), eliciting the recruitment of a cellular adaptor protein, TNF-R1-associated death domain-containing protein (TRADD) (4), to the activated TNF-R1. TRADD further recruits other cellular factors such as FADD (5), TNF-R-associated factor (TRAF)2 (5), and receptor-interacting protein (RIP) (6). FADD interacts directly with procaspase-8, leading to its proteolytic activation (7-9). TRAF2 and RIP stimulate the signal pathways leading to activation of nuclear factor kB (NF-kB) and Jun kinase, which have been shown to inhibit apoptosis in some cell types (10, 11). Activation of NF-kB also leads to induction of the genes involved in proinflammatory and immune reactions (12-15). These observations indicate that TNF-α induces limited apoptosis in a number of cell systems unless the anti-apoptotic signal pathway is blocked (16-18).

Unlike TNF-α/TNF-R1 system, the activation of Fas by Fas ligand (FasL) recruits only FADD as an adaptor molecule (19-22). As a result, Fas mainly activates the apoptotic signaling pathways. This probably explains the observation that FasL has stronger apoptotic activity than TNF-α.

TRAIL also activates cognate receptor molecules through trimerization (23, 24), similar to other TNF family members. Four different TRAIL receptors have been thus far identified, including DR4/TRAIL-R1 (25), DR5/TRAIL-R2 (26-28) and two decoy receptors (DcR1 and DcR2) (25, 27-29). DR4 and DR5 are intact functional TRAIL receptors through which the apoptosis-inducing activity of TRAIL is transmitted into the cytoplasm, whereas DcR1 and DcR2 are truncated TRAIL receptors where the cytoplasmic regions containing the death domains are deleted. Thus, overexpression of DcR1 and DcR2 blocks the function of DR4 and DR5 (25, 27-29), probably by competing with DR4 or DR5 for TRAIL. Recent studies (23, 24) on the crystal structure of TRAIL protein revealed that soluble TRAIL (amino acid 114-281) forms a homotrimer similar to the FasL/Fas system rather than the TNF-α/TNF-R1 system, activation of DR4 and DR5 recruits FADD as a cellular adaptor protein.

Once activated by TNF-α, FasL or TRAIL, caspase-8 initiates caspase cascades leading to cleavage of many cellular components. Studies have demonstrated that death receptor-mediated signal diverges at the point of caspase-8 (Fig. 1). One death signaling pathway (type I signal pathway) involves direct activation of executioner caspases (caspase-3 and -7). The other death signaling pathway (type II signal pathway) activates executioner caspases following the involvement of so-called mitochondrial events. The type II signal pathway is initiated by Bid, a Bcl-2 family member. Bid cleaved by caspase-8 targets the mitochondria and induces cytochrome c release (30, 31). Once released, cytochrome c binds to Apaf-1 and participates in caspase-9 activation (32, 33). The activated caspase-9 is then able to activate caspase-3 (32). The mitochondrial events also include the release of Smac/DIABLO from mitochondria (34, 35). The release of Smac/DIABLO from mitochondria appears to be induced by cleaved Bid, and occurs simultaneous to the cytochrome c release. IAP family members (c-IAP1, c-IAP2, XIAP) have been known as potent inhibitors of caspase activation and activity (36-38). These proteins interact with caspases (-9, -3, and -7) and inhibit caspase activation/activity. Once released,
Smac/DIABLO binds to IAP family members, thus releasing bound XIAP from caspases (39-43). Caspases free of IAPs are more easily activated by caspase activation signals. The type I and II apoptotic signal pathways meet at the point of activation of executioner caspases. Many studies demonstrated that mitochondrial events amplify apoptotic signal and promote apoptosis. In fact, ‘amplification’ refers to ‘enhancement’. Major factors of ‘enhancement’ are likely to be Smac/DIABLO and cytochrome c. Without engagement of type II signal, activation of the executioner caspases by only the type I signal is inadequate to induce apoptosis in many cell types. Once activated (by type I and/or II signal pathway), executioner caspases cleave and liberate a DNase termed CAD (caspase-activated DNase) from an inhibitor of CAD (ICAD/DFF-45) by cleaving the ICAD protein (44-46). This process leads to DNA degradation, a hallmark event in apoptosis. In addition, activation of executioner caspases lead to cleavage of numerous cytosolic, cytoskeletal, and nuclear proteins.

Although TRAIL is a TNF family member (47, 48), it has some notable differences from TNF-α and FasL. TRAIL-Rs are more widely expressed in various tissues than Fas (47, 48), thus most tissues and cell types may be TRAIL targets. TRAIL has a unique selectivity for triggering apoptosis in tumor cells and may be less active against normal cells. Hence, in contrast to FasL or agonistic Fas antibody, which induces fulminant massive liver damage (49, 50) when introduced systemically, TRAIL exhibited no detectable cytotoxicity in mice (51) and non-human primates (52). HIV-1-infected T cells were also shown to be more susceptible than uninfected T cells to TRAIL (53). Recent results using TRAIL knock-out mice demonstrated that the mice are prone to spontaneous tumorigenesis (54). Another line of evidence has demonstrated that TRAIL is an active NK cell-mediated blocker of tumor metastasis (54, 55). These features have not only focused considerable attention on TRAIL as a potential therapeutic to treat human cancers and AIDS, but also as an important cellular factor of natural defense mechanisms.

Despite the significant importance of TRAIL in physiology and potential for cancer therapy, relatively little is known about TRAIL death signaling events, compared to other family members such as TNF-α and FasL. Studies have demonstrated that TNF-α and FasL signals are transmitted through a common cellular adaptor protein FADD (19-21). Recent studies suggest that apoptotic signals induced by TRAIL are also relayed by FADD (56-59). Recently, we identified caspase-8 as a cellular signaling component required for TRAIL-induced apoptosis (60). These results suggest that initial signals triggered by TNF-α, FasL and TRAIL may be similar. However, how the unique selectivity of TRAIL against tumors is exerted in vivo is poorly understood. Some insights into the selectivity of TRAIL-induced apoptosis are likely to come from studies delineating the mechanisms by which certain factors can modulate cell susceptibility to TRAIL. For example, we found that IFN-γ significantly enhances TRAIL-induced apoptosis (61). IFN-γ was identified to enhance TRAIL-induced apoptosis through the transcription factor IRF-1. We postulate that IFN-γ regulates expression of the genes involved in TRAIL death signaling via IRF-1. Rapidly growing solid tumors commonly contain regions of cells existing in hypoxic conditions in their centers (62, 63). Hypoxia under this pathophysiological condition has been known to render human cancers resistant to various cancer therapies including radiotherapy and chemotherapy (64-66). Thus, effective blockade of hypoxic effects will result in improved TRAIL efficacy in cancer treatment. Our analyses on antiapoptotic molecules revealed that hypoxia significantly increased expression of Bcl-2, Bcl-XL and IAP family members (67). We propose to study the signaling mechanism by which hypoxia regulates expression of these molecules. This study will lead to develop a successful strategy of treating hypoxic cancers. We found that constitutively active Akt (protein kinase B) also potently blocks TRAIL-induced apoptosis. Studies have reported constitutive activation of Akt in cancer cell lines including LNcaP that originated from human prostate cancer (68, 69). Akt is known as a key molecule for transmitting cell survival signals (70-72). Normally cellular fate is controlled by balance of cell death and cell survival signals. However, how cell survival signals modulate cell death signals, and vice versa, is poorly understood. Past studies demonstrated that Akt acts its antiapoptotic function by phosphorylating caspase-9 (73) and Bad (74). In our preliminary studies on hypoxia, we came to realization that IAP family members are potent antiapoptotic molecules, and actively involved in cell death signaling. In fact, we found Akt activation influences XIAP expression. IAP family members do not inhibit only caspase-9 activation through blocking formation of apoptosome (a complex of Apaf-1, caspase-9, and cytochrome c), but also activation of caspases (caspase-3, -7) and activity of active caspases. Thus, it is intended to examine how Akt signal modulates IAP family members. Study on phosphorylation, protein-protein interactions, and degradation of IAP family
members will delineate underlining mechanisms by which Akt signal regulate cell death signal, and could lead to the development of more effective TRAIL therapy. In our preliminary studies, we found Actinomycin D (ActD) as an incomparably potent apoptosis enhancer for TRAIL-induced apoptosis. Combined with ActD, TRAIL efficiently induced apoptosis in many cancer cell lines at much lower dose than TRAIL alone. Other chemotherapeutic agents such as Mytomycin C, Cis-platinum, and Taxol did not significantly enhance apoptotic activity of TRAIL. Importantly, the combination of ActD and TRAIL effectively killed tumor cells independent of the p53 status. Because p53 has been known as a critical factor for radiotherapy and chemotherapy, and is mutated in more than 50% human cancers (75), this combination approach should be an effective strategy to treat p53 mutant human cancers. ActD is a well-known transcription inhibitor, but how ActD enhances TRAIL-induced apoptosis is poorly understood. Blockade of general transcription by ActD in a default fashion has been believed to play a role in cell death enhancement. However, we identified that ActD did not significantly change major signal components involved in TRAIL-induced apoptosis. Those molecules examined include TRAIL-Rs (DR4, DR5), FADD, Bid, caspases (-3, -8, -10), Bcl-2 family members (Bcl-2, Bcl-XL, Bax), cytochrome c released, and heat shock proteins. Nevertheless, we do not rule out the possibility that ActD may regulate other factors. We also postulate that ActD may directly modulate TRAIL death signaling and/or protein-protein interaction of death signal components including increased TRAIL receptor aggregation, and propose to study the mechanism in this proposal. It is also needed to examine the effects of ActD on hypoxia. Therefore, it is evident why our studies on characterization of the cellular factors activated by inhibitors and enhancers of TRAIL-induced apoptosis are so important. Our studies will significantly advance our understanding on TRAIL-induced apoptosis in general and lead to the development of a better TRAIL-based cancer therapy.

Preclinical study results reported recently (76) demonstrated that TRAIL protein is a potent anticancer agent that is safe in mice, rat and nonhuman primates. However, there are many limitations to using TRAIL protein as an anticancer drug. First, production of sufficient quantities of purified recombinant TRAIL protein for clinical use is a challenging task. Even in a bacterial expression system commonly used for recombinant protein production, the production yield is not adequate (based on our own and others experience). Removal of contaminants such as endotoxin from TRAIL protein is expensive and challenging. Furthermore, no studies have determined whether bacteria-produced TRAIL protein is biologically identical to the protein produced by mammalian cells. Thus, many of the above concerns could be eliminated if TRAIL protein is produced from mammalian cells. TRAIL protein produced from transfected mammalian cells has been used to test its safety and efficacy in previous studies (51). However, their approach poses serious cost burdens that may limit use for cancer treatment. An alternative way to supply TRAIL protein is to use a gene therapy approach, where a DNA sequence can be a source for TRAIL protein. Because soluble proteins have better access to the targets in general, we designed TRAIL protein to be secreted into the blood circulation in vivo. To accomplish this goal, we have tested many secretion signals including those derived from TRAIL receptor DR5, Ig kappa chain, albumin, interleukine (IL)-1b, fibrillin-1, and growth hormone. In our tests, two secretion signal sequences from human fibrillin-1 (77) and growth hormone (78) were identified to work well for our purpose. When fused to TRAIL, they effectively secreted TRAIL protein into the culture media. We then searched for a polypeptide sequence for trimerization. We hypothesized that the addition of a trimerization domain into TRAIL protein may stabilize the functional structure of TRAIL protein resulting in enhanced apoptotic activity because the functional structure of TRAIL protein is a homotrimer, but the intrinsic trimerization activity of TRAIL protein is too weak to ensure long-term stable trimeric structure. Functional tests of the isoleucine zipper (ILZ) (mutant derivative of the leucine zipper) (79) demonstrated that ILZ is a strong trimerization domain. According to our hypothesis that secreted TRAIL protein with deletion of secretion signal in the process of protein secretion may have reduced immune response in vivo, we searched for a protease-specific sequence. Furin-specific sequence (80) came to our attention, and was identified to be appropriate for this purpose. As a result, we have created two DNA expression cassettes (with or without Furin-specific sequence) composed of a secretion signal (from fibrillin-1 or growth hormone), a trimerization domain and an apoptotic TRAIL sequence (amino acid 114-281) (48). Using these DNA cassettes, we have succeeded in producing two forms of secretable trimeric TRAIL (stTRAIL) proteins. Our functional tests demonstrated that stTRAIL is more potent in apoptosis-inducing activity than the reported recombinant TRAIL protein (amino acid 114-281). stTRAIL proteins did not induce apoptosis in normal cells such as sinivial cells, chondrocytes, and dendritic
cells isolated from human patients. Thus, our data indicate that our novel stTRAIL proteins could be superior anticancer drugs. Creation of these stTRAIL expression cassettes prompts us to move forward to develop a stTRAIL-based cancer gene therapy and optimize the approach in vivo. We postulate that our novel stTRAIL-based gene therapy approach may be safe, and cost-effective tool for cancer treatment. To optimize in vivo gene delivery, we will test two approaches (naked DNA-based and virus-based). In this way, the body would become a virtual factory for supplying the quantities of stTRAIL. stTRAIL gene therapy alone and combination with death enhancers such as ActD and INF-γ will be also tested for efficacy and safety in vivo. If proven successful, our novel stTRAIL-based gene therapy can be applied to treat many human cancers. Therefore, our studies are very significant for cancer therapy in general.

C. PRELIMINARY STUDIES/PROGRESS REPORT

TRAIL was first identified and characterized between late 1995 and early 1996 (47, 48). Since early 1998, we have focused on TRAIL as our major research (81). Currently, we possess most of the key reagents for TRAIL and apoptosis research in general. These reagents include the expression constructs for wild-type and mutant TRAIL, TRAIL-R and caspases, purified recombinant TRAIL and caspase proteins, as well as various cell lines stably transfected with the gene involved in apoptosis.

Requirement of caspase-8 and death signaling pathway in TRAIL-induced apoptosis

For the past several years, many research groups have focused on determining what cellular signaling components are important in TRAIL-induced apoptosis. When we started this study, both adaptor and initiator caspase responsible for transmitting apoptotic signal of TRAIL were not determined. We concentrated on identification of the initiator caspase rather than the adaptor molecule.

Caspases are activated by two general mechanisms in physiological conditions. Initiator caspases such as caspase-8, -9 and -10 with a long prodomain are activated by upstream non-caspase activators such as adaptor protein FADD (caspase-8 and -10) or cellular protein complex such as Apaf-1 and cytochrome c (caspase-9), whereas executioner caspases with a short prodomain are activated by upstream active caspases. Based on coimmunoprecipitation analysis and understanding of biochemical mechanism in activation, caspase-8 and -10 have been suspected as the responsible initiator caspases for TRAIL signaling. In fact, we found that caspase-8 is rapidly activated (within one hour) in response to treatment with TRAIL (Fig. 2). This activation was associated with Bid cleavage, cytochrome c release, caspase-3 activation and DNA fragmentation factor (DFF)-45 cleavage, all of which are well-characterized cellular events observed during apoptotic process. Our data indicate that TRAIL-induced apoptosis transmits apoptotic signals through both type I and II pathways. The z-IETD-fmk, a caspase-8 peptide inhibitor, blocked caspase-8 activation and downstream caspase-3 activation, resulting in inhibition of TRAIL-induced apoptosis (Fig. 3). Overexpression of a caspase-8-
Jurkat cells, susceptibility to both TRAIL and Fas was fully restored (Fig. 6). Based on molecular structure and coimmunoprecipitation analysis, studies suggested that caspase-10 might also play a role as an initial caspase for TRAIL action. Nonetheless, RT-PCR and sequence analyses have revealed that this caspase-8-deficient Jurkat cells express wild-type caspase-10, pointing that the expression level of caspase-10 does not complement with caspase-8 deficiency in Jurkat cells. Previous studies have demonstrated that the cowpox serpin CrmA effectively blocks TRAIL-R- (25, 26) and Fas-mediated apoptosis (82, 83). CrmA was also shown to effectively block caspase-8 activity but not caspase-10 activity, although both caspase-8 and caspase-10 were able to activate/cleave various downstream executioner caspases (9). Therefore, it is clear that caspase-8 but not caspase-10 is required for TRAIL-induced apoptosis. Recent studies also identified FADD as an adaptor molecule (56-59). The involvement of FADD and caspase-8 suggests that death signaling triggered by TRAIL is similar to that triggered by FasL rather than TNF-α. Our data delineating the cellular components and the flow of death signal activation by TRAIL facilitate the functional analysis of individual cellular factors in TRAIL-induced apoptosis. Based on the data obtained from this preliminary study (60), we have investigated effects of many extracellular stimuli on TRAIL-induced apoptosis. We hypothesize that extracellular stimuli modulate functions of cellular factors involved in cell death and/or cell survival, which in turn determine cell fate.

**IFN-γ enhancement of TRAIL-induced apoptosis through IRF-1**

In general, combination therapies produce better efficacy than individual therapies in cancer treatment. Combination therapies can also significantly reduce side effects in many cases. IFN-γ has been shown to sensitize target cells to various apoptosis stimuli including TNF-α (84) and FasL (85). IFN-γ alone has been used as an anti-cancer drug. However, no intensive studies have been conducted for IFN-γ on TRAIL-induced apoptosis. To investigate effect of IFN-γ on TRAIL action, we pretreated A549 cells that originated from human lung carcinoma and are resistant to TRAIL alone with IFN-γ for 12 hours and further incubated with TRAIL protein for additional 3 hours. The IFN-γ pretreatment enhanced TRAIL-induced apoptosis (Fig. 7). IFN-γ treatment significantly increased IRF-1, but not other protein levels including DR4 and DR5 under the same condition as determined by Western blotting (Fig. 8). We used an
IRF-1 is a key factor mediating IRF-γ enhancement activity in TRAIL-induced apoptosis. Although IFN-γ is expected to activate many cellular signaling pathways, our data indicate that IFN-γ enhancement of TRAIL-induced apoptosis is mediated by IRF-1. IRF-1 is a transcription factor (86, 87) of which expression is stimulated by type I IFN (α and β) as well as type II IFN (γ). IRF-1 binds to the virus-inducible “enhancer-like” element (IRF-E) and regulates the genes containing such motif including the human IFN-β gene (86, 88). Currently, we do not know how IRF-1 enhances TRAIL-induced apoptosis. We hypothesize that IRF-1 may regulate the cellular factor(s) having pro- and/or anti-apoptotic function, and propose to identify such factors in this proposal.

**Hypoxia inhibition of TRAIL-induced apoptosis**

Hypoxia is a commonly observed pathophysiological condition in the center of solid tumors (62, 63). Hypoxia is known to render tumor cells resistant to many cancer therapies including radiotherapy and chemotherapy (64-66). Furthermore, tumor hypoxia enhances genetic mutations in tumors (89, 90) and facilitates tumor metastasis (63). In general, tumor cell resistance to various therapies is one of the most significant problems in cancer treatment. TRAIL has been considered to be a promising anti-cancer therapy, thus we examined whether hypoxia affects TRAIL-induced apoptosis.

We exposed A549 cells to hypoxic or normoxic condition for 24 hours and exposed the cells to TRAIL (11) for additional 4 hours. TRAIL induced apoptosis in normoxia-treated cells, but not in hypoxia-treated cells over a wide range of TRAIL concentrations (50-200 ng/ml) (Fig. 12). Cell morphology examination also supported the inhibitory role of hypoxia in TRAIL-induced apoptosis (Fig. 13). Thus, we examined antiapoptotic proteins such as Bcl-2 and Bcl-XL by Westen blotting analysis. A549 cells exposed to hypoxia for 1, 6, 12, 24 or 48 hours significantly increased Bcl-2 and Bcl-XL level (Fig. 14A). The Bcl-2...
increase was detected after 6 hours of hypoxia and sustained for up to 24 hours in the hypoxic setting. The Bcl-XL increase was slightly delayed as detected during the 12 hour incubation, but lasted through 48 hours of hypoxia treatment. Despite difference in timing that Bcl-2 and Bcl-XL increase, upregulation of Bcl-2 and Bcl-XL may account for hypoxia protection in TRAIL-induced apoptosis, because maximal hypoxia protection in TRAIL-induced apoptosis was observed after 24 hour hypoxia pretreatment (Fig. 12). Bcl-2 and Bcl-XL have been shown to block TRAIL-induced apoptosis (19), hence their increased levels may be associated with a protective mechanism of hypoxia in TRAIL-induced apoptosis.

Next, we examined IAP family member proteins such as c-IAP1, c-IAP2 and XIAP, after exposure to hypoxia. Hypoxia-treated A549 cells also increased the protein levels of c-IAP1, c-IAP2 and XIAP (Fig. 14A). The c-IAP1 increase was rapid, as detected within 1 hour after exposure to hypoxia, whereas the c-IAP2 and XIAP increase reached a peak after 12 hour of exposure to hypoxic conditions. The patterns of c-IAP2 and XIAP increase were similar to each other. The increased levels of IAP family member proteins with the exception of c-IAP1 also correlated with the results of function tests (Fig. 12). Thus, in addition to Bcl-2 and Bcl-XL increase, increased IAP family member proteins appear to play a protective role of hypoxia in TRAIL-induced apoptosis. Hypoxia did not have a significant influence on DR4 level (Fig. 14B), even though DR4 level remarkably decreased after 48 hours of hypoxic incubation. However, this decrease is unlikely to be associated with a protective role of hypoxia in TRAIL-induced apoptosis since the effect of hypoxia on TRAIL-induced apoptosis was tested after 24 hours of hypoxic incubation and lasted for 4 hours longer (thus, up to 24 hour time point).

In contrast, hypoxia slightly increased DR5 levels (Fig. 14B). However, this increase did not significantly enhance TRAIL-induced apoptosis (Fig. 12). Thus, DR5 increases may not be sufficient to counteract antiapoptotic functions driven by antiapoptotic proteins increased under hypoxia. Based on our data, we believe that the increased level of antiapoptotic proteins such as Bcl-2 and IAP family members is a key in hypoxia inhibition of TRAIL-induced apoptosis. Our data also suggest that hypoxia is a common resistance mechanism by which tumor cells escape tumoricidal activity of cancer therapies including TRAIL. Studies have demonstrated that hypoxia is a major stimulus for the increased expression of a large number of genes relevant to the growth and survival of cancer cells (91). As also shown below, cell growth and survival signals including Akt activation potently inhibit TRAIL-induced apoptosis.
Dissecting hypoxia-activated signals and identification of cellular factors involved will increase therapeutic potential of TRAIL in cancer treatment. Therefore, we will investigate, in this proposal, how hypoxia regulates antiapoptotic molecules.

**Constitutively active Akt, a potent inhibitor of TRAIL-induced apoptosis**

It is believed that the cellular response is determined by balance of cell-survival signals and cell-death signals. However, how cell survival signals modulate cell death signals (also vise versa) is poorly understood. Many cell survival stimuli transmit their signals through the cellular factors. Akt (protein kinase B) is one of the key factors of cell survival signal transduction. Many growth factor receptors (e.g. EGF-R) on the cell surface receive cell survival signals and activate Akt via phosphorylation. Once activated, Akt inhibits apoptotic cell death induced by many proapoptotic stimuli. Studies have demonstrated that Akt phosphorylates caspase-9 (73) and Bad (74), which inhibits apoptotic cell death. As others reported, we also observed that a prostate cancer cell line LNCaP with constitutively active Akt is completely resistant to TRAIL-induced apoptosis. Because caspase-9 and Bad are the cellular factors of the signaling pathway specifically associated with mitochondrial events (type II signal), it is questioned how constitutive activation of Akt inhibits type I signal transduction and completely blocks TRAIL-induced apoptosis. PTEN, a negative regulator of PI-3K that activates Akt, is deleted in LNCaP cells (92, 93), and results in constitutive activation of Akt. Thus, LNCaP is a good experimental system to investigate Akt function in apoptotic cell death. To address the question raised, we treated the cell line with Wartmannin (200nM), a specific inhibitor of PI-3K, for 6h and analyzed cellular factors by Western blotting. As expected, treatment of the cell line with Wartmannin blocked Akt activation. Surprisingly, inhibition of Akt activation in this cell line resulted in dramatic reduction of XIAP but not c-IAP1 and C-IAP2 level (Fig. 15). Under conditions of reduced level of XIAP, the cell line was very susceptible to TRAIL. Our data suggest that Akt activation correlates with the expression level of XIAP. XIAP is a factor functioning in a converging point of type I and II signal. Therefore, there is no doubt that overexpression of XIAP effectively blocks apoptotic cell death. Moreover, XIAP is known to be most potent caspase inhibitor among IAP family members (36). Studies have shown that XIAP is ubiquitinated and regulated by ubiquitin-proteasome pathway (94), thus we hypothesize that Akt activation may regulate XIAP levels via modulation of ubiquitin-proteasome pathway. Furthermore, we identified a consensus phosphorylation site for Akt (RXRXXS/T) (73, 74) in XIAP protein (Fig. 16). Currently, it is not known whether XIAP is phosphorylated by Akt, and phosphorylation regulates turn-over of XIAP protein. Therefore, we propose to study how Akt regulates XIAP expression.

**Actinomycin D (ActD), a potent enhancer of TRAIL-induced apoptosis**

The distribution of TRAIL-Rs in tissues suggested a wider scope of targets than TNF-α and FasL in apoptosis. Although TRAIL is a potent apoptosis inducer without damaging normal tissues in vivo, we found that TRAIL alone has limited killing capacity in some cancer cell lines (Table 1). In general, combination therapies produce better efficacy in cancer treatment than individual therapies. Many chemotherapeutic agents are known to cause cytotoxic side effects at an effective dosage. If a low dosage of a chemotherapeutic agent can sensitize cells to TRAIL-induced killing (especially against TRAIL-resistant cancers), this combination therapy would be superior to TRAIL alone. Thus, we have tested chemotherapeutic agents in two TRAIL-resistant cell lines (A549 with wild-type p53 and HepG2 with mutant p53) to examine whether they can enhance the apoptotic activity of TRAIL. ActD greatly enhanced TRAIL-induced apoptosis in both cell lines, whereas Taxol, Cis-Platinum and Mitomycin C did not (Fig. 17). These reagents did not kill either cell line in
the absence of TRAIL. In other cell lines, ActD was also a potent enhancer of TRAIL-induced apoptosis (Table 1).

![Graph comparing relative potentiation activity](image)

**Fig. 17 Comparison of relative potentiation activity**

![Graph showing ActD potentiation of TRAIL-induced apoptosis](image)

**Fig. 18 ActD potentiates TRAIL-induced apoptosis**

ActD is a known inhibitor of RNA polymerase II for mRNA synthesis. Significant enhancer activity of ActD was obtained at 40 ng/ml (Fig. 18), a concentration that is 1/25 of the dosage commonly used to block mRNA synthesis. Nevertheless, our data asked whether ActD acts its enhancer activity through blocking transcription in TRAIL-induced apoptosis. To address this question, we examined whether ActD affects global mRNA transcription. A549 cells were exposed to ActD (100 or 1000 ng/ml) for 0-12 hours and subjected to Northern blot analysis for whole mRNA expression. In more detail, we used RNAzolB solution to prepare the RNA samples from ActD-treated cells. RNAzolB solution separates total RNA (aqueous phase) from proteins. If ActD affects mRNA transcription, total mRNA amount in each ActD-treated sample should change. Therefore, we prepared the total RNA samples on the “preparation volume-basis”, in which a given volume out of total aqueous phase was taken for each sample and processed into precipitation of total RNA. This processing would allow us to obtain different amount of mRNA in each sample if ActD blocks gene transcription. Each total RNA sample was size-fractionated by agarose gel electrophoresis and transferred to nitrocellulose membranes followed by probing with oligo dT that hybridizes with poly A-containing RNA species (mainly mRNA). Our data demonstrated that mRNA but not total RNA expression was inhibited by ActD in a time-dependent manner (Fig. 19). Therefore, we next investigated whether global inhibition of mRNA expression by ActD is correlated with protein expression. ActD had no significant effect on expression of TRAIL signaling components such as DR4, DR5, FLIP, FADD, caspase-8, Bcl-2 and Bcl-XL (Fig. 20). In contrast, ActD dramatically increased p53 levels, despite globally reduced mRNA expression. Thus, our data suggest that ActD may specifically regulate a factor(s) critically involved in cell death mechanisms. Importantly, regardless of p53
status, which is considered a critical factor in chemotherapy and radiotherapy, TRAIL/ActD killed target cells effectively (Table 1). This TRAIL/ActD-induced cell death was completely blocked by pan-caspase inhibitor z-VAD-fmk. This indicates that ActD-enhanced TRAIL induced cell death via apoptosis. Because it is well known that more than 50% of human cancers contain various p53 mutations (75), our findings could prove to be very significant for cancer therapy in general. Furthermore, our data clearly indicate that many cancer cell lines resistant to TRAIL alone can be effectively killed by the combination of TRAIL and ActD. Considering the limited cytotoxic side effects of TRAIL in vivo, TRAIL fortified by low dose ActD may become an improved cancer therapy. Signaling and enhancing mechanisms of ActD are subjects of this proposal.

Homotrimeric TRAIL protein, an essential structure for apoptosis induction

Comparison of family members and crystal structure of TRAIL protein revealed that biologically active soluble human TRAIL protein (amino acids 114-281) forms a homotrimer (23, 24). Previously, we purified recombinant human TRAIL protein (amino acids 114-281) from bacteria and showed that this TRAIL protein is a potent inducer of apoptosis (60, 81, 95). Biochemical analysis revealed that major form of this TRAIL protein is trimer. However, this TRAIL protein generated two bands (21 kDa and 42 kDa) on non-reducing SDS-gels (Fig. 21). Since the estimated molecular size of amino acids 114-281 is 21 kDa, the appearance of a 42 kDa band suggested the formation of a dimer. The 42 kDa band disappeared following treatment with DTT, a reducing agent. However, boiling had no effect. These observations suggested that the dimeric TRAIL might be generated by an intermolecular chemical bond formation. To examine whether the dimer form of TRAIL protein binds to TRAIL receptors, TRAIL protein was incubated with TRAIL receptor DR5 or a negative control antibody (FLAG-Ab) and pulled-down with the protein (A+G)-agarose beads. The
monomer TRAIL protein (this monomer resulted from trimer disrupted by SDS) efficiently bound to DR5 (lanes 1 and 2), but did not bind to control FLAG-Ab (lanes 1 and 6) (Fig. 22). The dimer TRAIL protein also bound to DR5 (lanes 1 and 2). Affinity of trimer and dimer TRAIL proteins to DR5 was estimated to be similar (lanes 1 and 2). The capacity of the reducing agent DTT to abolish the form of TRAIL suggested that an intermolecular disulfide bridge might account for dimer formation. Inspection of the amino acid sequence of human TRAIL protein identified a unique cysteine residue at position 230. This cysteine residue was conserved in mouse TRAIL protein (47), pointing to a key functional role for this amino acid residue in the biological function of TRAIL protein. To examine the contribution of this cysteine residue, the cysteine was mutated to glycine, and TRAIL (C230G) protein was purified. The mutant TRAIL (C230G) protein appeared only as a monomer on SDS-gel (Fig. 23A), indicating that the cysteine-230 is responsible for forming dimeric TRAIL protein. To examine the role of the cysteine-230 in the apoptotic function of TRAIL, HeLa cells were treated with TRAIL or mutant TRAIL (C230G) protein. Interestingly, TRAIL (C230G) protein exhibited no apoptotic activity in the same time frame where wild-type TRAIL induced massive apoptosis (Fig. 23B). To assess the functional role of the dimeric TRAIL, TRAIL protein was treated with DTT to disrupt the dimer form of TRAIL followed by dialysis to allow the protein to form a trimer. This conversion process slightly increased the apoptotic activity of TRAIL protein (Fig. 24), indicating that cysteine-230 affects apoptotic activity of TRAIL protein through oxidation-reduction, and that oxidized dimeric TRAIL protein has lower (or null) apoptotic activity than that of trimeric TRAIL protein. Therefore, our data indicate that cysteine-230 is required for apoptotic function of TRAIL protein. Subsequent structure analysis of TRAIL protein revealed that free cysteine-230 is required for interacting with zinc ion to form a homotrimer and stabilize the trimeric structure of TRAIL protein (23, 24), and confirmed our findings. Failure of TRAIL protein to form a homotrimer resulted in loss of apoptotic activity, suggesting that trimer structure of TRAIL protein is essential for proper function. Our data prompted us to create a TRAIL DNA cassette that expresses trimeric TRAIL protein, and made possible the gene therapy strategy that will be assessed in this proposal.

Construction of secretable trimeric TRAIL (stTRAIL) expression cassettes

Natural TRAIL is expressed as a transmembrane protein (47, 48). The receptor-binding domain of TRAIL protrudes outwards. Soluble recombinant TRAIL protein (amino acid 114-281) purified from bacterial cells forms mainly a

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**Table 2** Amino acid sequences for the secretion signals, trimer-forming domain and Furin-specific cleavage site

<table>
<thead>
<tr>
<th>Secretion signal sequences</th>
<th>Trimer-forming domain: isoleucine zipper (ILZ)</th>
<th>Furin-specific cleavage site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrillin-1 (SEC2 sequence)</td>
<td>RMKQIEDKIEILSKYIHIEARIKKLGER</td>
<td>SARNRQKR</td>
</tr>
<tr>
<td>Growth Hormone (SEC(CV) sequence)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRRGRLEIALGFTVLLASYTSHGADA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MATGSRSTLLAFGLCLCPWLPQEGSA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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homotrimer, but also a dimer in a certain degree (95) as described above. In our preliminary studies, we found that recombinant TRAIL protein (amino acid 114-281) has weak intrinsic trimerization activity, and that TRAIL protein fused to an unrelated trimerization domain has more stable trimeric structure and higher apoptotic activity. Based on these observations, we have constructed a TRAIL expression cassette composed of three functional elements. First, we designed TRAIL protein to be secreted into the blood circulation because secreted protein has advantage over non-secreted protein in target access in general. Since TRAIL is a cytokine that functions by binding to cell surface receptor of target cells, TRAIL delivered through the blood circulation may have better effects. Among the secretion signals from DR5, Ig kappa chain, albumin, IL-1b, fibrillin-1 and growth hormone we have tested, the SEC2 and SEC(CV) sequences (Table 2) derived from human fibrillin-1 (77) and human growth hormone (78) respectively, were most reliable for our purpose. The SEC(CV) sequence has a furin-specific cleavage site (80) at its C-terminus, and Furin cleavage removes the SEC(CV) secretion signal in the process of protein secretion. Second, we identified an isoleucine zipper (ILZ) sequence (79) as a potent trimerization domain that stabilizes secreted TRAIL. ILZ is a derivative of a leucine zipper and induces homotrimerization. Third, we used the DNA sequence encoding amino acid 114-281 of human TRAIL (47, 48). Others and we have demonstrated that this TRAIL moiety binds to TRAIL receptors and induce apoptosis effectively in a wide variety of cancer cell lines. Using these three functional elements, we constructed stTRAIL cassettes and expression vectors for mammalian cells. A FLAG sequence in the pCMVdwFLAGILZTRAIL(114-281) construct was used to provide the ATG codon for the initiation of translation. In our preliminary studies, we observed that 293T and 293 human embryonic kidney cells were resistant to our soluble TRAIL protein. After transiently transfecting 293T cells with the expression constructs, we prepared whole cell lysates and conditioned media, analyzed stTRAIL expression and secretion by Western blotting. All cells transfected with the expression vectors produced comparable amounts of corresponding cellular stTRAIL protein (Fig. 25, ‘cell lysate’ panel). As expected, both stTRAIL proteins with a secretion signal were detected in culture supernatant (Fig. 25, ‘medium’ panel). The SEC(CV)-containing expression vector, however, secreted much less stTRAIL protein into the culture medium. The molecular size of the secreted SEC(CV)ILZTRAIL protein was identified to be smaller than native cellular protein. This indicates that the SEC(CV) sequence containing a furin-specific cleavage site was restricted by furin and removed during the secretion process. To assess whether the secreted TRAIL proteins form homotrimers, the proteins in the conditioned media containing secreted TRAIL proteins were resolved in native polyacrylamide gel and analyzed by Western blotting. stTRAIL proteins formed a band size consistent with homotrimer (Fig. 26).
Importantly, no dimeric form of stTRAIL proteins was detected. The conditioned media containing stTRAIL proteins were tested in HeLa cells for apoptotic activity (Fig. 27). The control medium prepared by transfecting the FLAGILZTRAIL expression construct did not show any apoptotic activity. In contrast, the medium prepared by transfecting the SEC2ILZTRAIL or SEC(CV)ILZTRAIL construct greatly induced apoptosis even within 90 min of incubation. Functional assay results clearly demonstrated that, despite much lower levels in the conditioned medium (Fig. 25), SEC(CV)ILZTRAIL killed a greater percentage of cells than SEC2ILZTRAIL. This result indicates that SEC(CV)ILZTRAIL has greater apoptotic activity than SEC2ILZTRAIL. Thus, the removal of the fused secretion signal seems to produce stTRAIL protein with higher apoptotic activity. It has yet to be determined if the fused secretion signal affects trimeric structure and results in decreased apoptotic activity. In apoptotic activity test, secreted SEC(CV)ILZTRAIL also showed higher apoptotic activity than our soluble recombinant TRAIL protein (amino acid 114-281) containing a histidine-tag sequence reported previously (Fig. 25). This result suggests that the ILZ sequence stabilizes trimeric structure and enhances apoptotic activity. Apoptosis induced by stTRAIL proteins was completely blocked by pan-caspase inhibitor z-VAD-fmk. Interestingly, unlike in HeLa cells and established cancer cell lines, stTRAIL proteins induced no apoptotic cell death in normal cells such as sinovial cells, chondrocytes, and dendritic cells from patients. Our data indicate that stTRAIL protein is more homogeneous in structure and may be more physiologically relevant than bacteria-produced TRAIL. Furthermore, our data indicate that stTRAIL proteins can become a safer cancer therapy despite enhanced apoptotic activity. Therefore, it is needed to test feasibility of stTRAIL in a cancer gene therapy, and we propose to do so in this proposal.

D. RESEARCH DESIGN AND METHODS

Major points of this proposal involve identification and characterization of the cellular factors for TRAIL action and feasibility test of a TRAIL-based gene therapy. Successful completion of the proposed experiments will require a number of sophisticated molecular, cellular and surgical techniques as well as accessibility to many key reagents. As attested to by our publication record and preliminary data, we have all the key reagents and enough experimental experience. Our laboratory also has the necessary experience to perform the proposed experiments. Strong support from the collaborators ensures successful completion of our proposal. The research strategies are designed under the specific aims.

AIM I: To characterize the cellular factors activated by inhibitors and enhancers of TRAIL-induced apoptosis.

Identification and characterization of cellular factors modulated by inhibitors and enhancers of TRAIL-induced apoptosis are important for better understanding of TRAIL action and the development of
TRAIL as an anti-cancer therapy. Furthermore, these studies can address unanswered questions related to a unique selectivity of TRAIL to tumor cells. Under this aim, we will carry out the following experiments;

**EXP 1A: Analysis of death enhancement signals of IFN-γ in TRAIL-induced apoptosis**

Generally, combination therapies produce better efficacy than individual therapies in cancer treatment. In our preliminary studies, we found IFN-γ significantly enhanced TRAIL-induced apoptosis and increased IRF-1 concomitantly. IFN-γ has been used as an anti-cancer drug. The use of antisense oligonucleotide and an adenoviral vector AdIRF-1 identified IRF-1 as a major factor in IFN-γ-enhancing action. However, the mechanisms by which IRF-1 mediates IFN-γ-enhancer functions remain to be determined. Because IRF-1 is a transcription factor (86, 87), we hypothesize that IRF-1 may regulate the genes involved in TRAIL signaling. Recent studies (56-59) including our own findings (60) demonstrated that major TRAIL-induced apoptosis signal flows through TRAIL-R, FADD, caspase-8, executioner caspases, and DNase in order. Thus, we will examine regulation of the proapoptotic genes encoding these cellular components. IRF-1 is likely to upregulate these genes. Studies also demonstrated that TRAIL-induced apoptosis is modulated by antiapoptotic proteins such as Bcl-2 family members (Bcl-2, Bcl-XL), IAP family members (67) and cell survival signals including Akt (73, 74, 93). Thus, these antiapoptotic factors will also be examined. In the context, IRF-1 is expected to downregulate these genes. To exclude the possible activation of other signals, we will use AdIRF-1 vector transduction instead of IFN-γ treatment. RNA isolated from A549 cells that were infected with AdIRF-1 or AdLacZ will be subjected to real-time quantitative RT-PCR and/or Northern blotting for the measurement of expressed message of each gene. The genes identified to be significantly changed in response to IRF-1 will be further analyzed by Western blotting. This analysis will reveal whether gene transcription correlates with the protein levels. The genes showing a significant change in expression will be introduced into cells for functional analysis. For blocking the function of the gene(s) identified, we will use antisense oligonucleotides or a specific chemical inhibitors if available. In the presence or absence of the inhibitors, TRAIL-induced apoptosis will be assessed. This analysis will reveal the functional role of the identified factor in TRAIL-induced apoptosis.

**EXP1B: Analysis of death enhancement signals of ActD in TRAIL-induced apoptosis**

In our preliminary studies, we found that ActD is a potent enhancer of TRAIL-induced apoptosis. ActD-fortified TRAIL killed a wider variety of cancer cell lines *in vitro* than TRAIL alone. More importantly, the combination of TRAIL and ActD effectively killed cancer cell lines independent of p53 status at very low dose (~40ng/ml). Although ActD inhibited mRNA expression, ActD treatment (100 or 1000ng/ml for up to 12 hours) in HepG2 and A549 cells did not significantly change expression of cellular factors examined (Fig. 20). Nevertheless, as shown by p53 and p21, our data suggest that ActD may regulate a specific factor(s) instead of acting in a default fashion of transcription blockade to involve in TRAIL-induced apoptosis. In this context, we will examine more cellular factors by Western blotting. We will expand our target factors to IAP family member proteins, Akt, and the cellular factors involved in free radical generation. The factors identified will be subjected to Northern blotting for the analysis of mRNA message level. The results will make clearer the mechanism by which ActD enhances TRAIL-induced apoptosis.

We will examine the functional role of any cellular factors identified to be significantly regulated by ActD. The factors upregulated by ActD are simply expected to enhance TRAIL killing function. To test this hypothesis, the expression plasmid encoding the gene of the factor will be prepared, introduced into HepG2 and/or A549, and tested for TRAIL susceptibility. In contrast, the factors downregulated by ActD is expected to negatively act on TRAIL-induced apoptosis. We will test this hypothesis by employing antisense oligonucleotides to block expression of the factors. After introducing the antisense oligonucleotide for a specific factor, cells will be exposed to TRAIL and subjected to the cell viability assays. The results from these experiments will advance our understanding on ActD action in general as well as signaling events involved in enhancement of TRAIL-induced apoptosis.

**Potential pitfalls and alternative approaches**

We believe that ActD regulates cellular factor(s) that plays an important role in the mechanism, and identify the factor(s) involved in enhancement of TRAIL-induced apoptosis. However, if we fail to identify such a factor(s), we will focus our research on the functional characterization of p53 in p53 wild-type cells.
Because p53 has been shown to increase DR5 levels, we will examine if wild-type p53 regulates the levels of other pro- and anti-apoptotic molecules in the cells.

**EXP IC: Analysis of hypoxia-induced death inhibitory signaling in TRAIL-induced apoptosis**

Analysis of hypoxic effects on TRAIL-induced apoptosis

Tumor cell resistance is a main factor that reduces therapeutic capacity of the therapies in cancer treatment. As shown in many studies, one of the important mechanisms involved in such resistance is hypoxia (64-66). Tumors under hypoxia have been shown to be resistant to radiotherapy as well as chemotherapy. In our preliminary studies using A549 cells, we found that hypoxia significantly inhibited TRAIL-induced apoptosis. Because TRAIL is a potent tumor-specific killer with undetectable side effects (51, 76), it is an important question whether inhibitory function of hypoxia on TRAIL-induced apoptosis is a cell-type specific or a global phenomenon. To address this question, we will test hypoxia effects on TRAIL-induced apoptosis in more cell lines originated from different tissues such as liver, lung, cervix, breast, pancreas, brain, and skin. Our preliminary studies also demonstrated that hypoxia increased the protein levels of Bcl-2 family member proteins (Bcl-2 and Bcl-XL) and IAP family member proteins (cIAP-1, cIAP-2, and XIAP). Thus, we will examine whether hypoxia regulates these proteins at similar fashion in other cell lines. We will also compare the level of each protein by Western blotting in these cell lines. The information obtained from this study will be valuable to understand the general role of hypoxia on TRAIL-induced apoptosis and lead to the development of better TRAIL therapy.

**Examination of Akt in hypoxia inhibition of TRAIL-induced apoptosis**

Despite its importance, the signaling mechanisms responsible for hypoxia inhibition in cancer treatments are poorly understood. As shown in our preliminary studies, constitutively active Akt is a potent inhibitor of TRAIL-induced apoptosis. We also observed that hypoxia increased many anti-apoptotic proteins. Moreover, activation of Akt resulted in dramatically increased XIAP level. Therefore, we hypothesize that Akt may actively involve in hypoxia signaling and propose to examine its inhibitory function in TRAIL-induced apoptosis. To address this possibility, we will examine the protein levels of phospho-Akt (active Akt) and total Akt by Western blot analysis in the cells exposed to hypoxia. We will also establish the cell lines stably transfected with myristoylated Akt (a form of constitutively active Akt) and K179M-Akt (a null mutant form of Akt) and examine whether these Akts affect expression of anti-apoptotic proteins identified in our preliminary studies.

**Examination of VEGF in hypoxia inhibition of TRAIL-induced apoptosis**

Hypoxia is known to induce many genes including transcription factors (hif-1, AP-1, p53) and growth factors (epo, vegf, nos, et-1) (91). Hypoxia is also known as a potent stimulus to promote angiogenesis in tumor tissues (96). To ensure efficient growth, it is necessary for tumor cells in solid tumor tissues to establish continuous nutrient and oxygen supply. Thus, tumor angiogenesis has been intensively studied and considered as a potential target for cancer treatment. Many factors have been identified to induce angiogenesis in normal and pathophysiological conditions (91). Among those, VEGF (vascular endothelial growth factor) is probably the most important angiogenic growth factor in tumors. VEGF induces endothelial cell proliferation, and critically involved in tumor growth and tumor metastasis. Because VEGF stimulates cell growth in a certain cell type, we cannot rule out the possibility that VEGF also does so in other cell types as other growth factors and cytokines do. If this is the case, VEGF may not only stimulate tumor angiogenesis, but also may protect tumor cells from host natural defenses. However, such a possibility has not yet been intensively examined. We will treat different cancer cell lines from colon, breast, pancreas, and lung with recombinant VEGF, and expose them to TRAIL. If VEGF shows any protective activity, we will analyze the potential signaling mechanism(s). Because many growth factors transmit their survival signals commonly through Akt, we will examine Akt as a major cellular factor for VEGF signaling. We will also examine whether Akt activation by VEGF is associated with regulation of anti-apoptotic proteins we identified. We will employ Western blotting analysis to examine these factors. The outcome of examining our hypothesis will shed light on a functional role of VEGF in tumor cells. Dissecting and identification of signaling pathways and components will advance understanding of VEGF in tumor biology in general and lead to better development of anti-cancer drugs.
Examination of IFN-γ in hypoxia inhibition of TRAIL-induced apoptosis

In our preliminary studies, we observed that IFN-γ potently enhances TRAIL-induced apoptosis. Thus, it is imperative to examine if IFN-γ sensitizes hypoxic tumor cells to TRAIL-induced apoptosis. Outcome of this study could provide a therapeutic tool for hypoxic tumors. To examine this possibility, we will expose cancer cell lines (originated from colon, breast, pancreas, lung, liver) to hypoxia in the presence or absence of IFN-γ, and then treat with TRAIL protein followed by cell viability assays. If IFN-γ sensitizes hypoxic tumor cells to TRAIL, we will examine whether IFN-γ induces any changes of the cellular factors identified to be upregulated by hypoxia, and analyze the function of the identified factor by blocking its activity or overexpression.

Examination of ActD in hypoxia inhibition of TRAIL-induced apoptosis

In contrast to hypoxia, we observed that ActD markedly enhanced TRAIL-induced apoptosis. Thus, it is interesting to examine if ActD-fortified TRAIL can efficiently kill hypoxic tumor cells. To address this question, we will expose tumor cell lines to hypoxia and further incubate with the combination of ActD (50-100ng/ml) and TRAIL under hypoxia. In our preliminary studies, we found that hypoxia significantly increased Bcl-2 family member proteins (Bcl-2 and Bcl-XL) and IAP family member proteins (cIAP-1, cIAP-2, and XIAP). Because these antiapoptotic proteins are believed to play a key role in hypoxia, we will also examine whether ActD regulates the level of these proteins.

EXP ID: Analysis of Akt-mediated death inhibitory signaling in TRAIL-induced apoptosis

In our preliminary studies using LNCaP cells as an experimental system, we observed constitutively active Akt is a potent inhibitor of TRAIL-induced apoptosis and its activation is associated with increased expression of XIAP. Activation of Akt is known to inhibit apoptosis induced by many stimuli. Major factors so far identified to be involved in inhibition mechanism are caspase-9 (73) and Bad (74). However, the involvement of these factors still does not explain how type I death signaling is also blocked by Akt. Thus, based on our preliminary data, we hypothesize that XIAP increased by Akt may be a key factor to block both type I and II death signaling, which results in potent inhibition of TRAIL-induced apoptosis. Therefore, we propose to study the functional role of Akt and its signal targets including XIAP. Data obtained from this study will provide valuable information for apoptosis in general and cancer therapies using apoptosis inducers including TRAIL.

Regulation of XIAP by Akt

IAP family members are known as potent apoptosis inhibitors (36-38, 97). Among the member proteins, XIAP is known to most potently inhibit caspase activation and activity (36). We observed that blocking Akt activation in LNCaP cells dramatically decreased XIAP protein levels. Our data indicate that Akt signaling regulates XIAP expression. This finding is novel, hence the mechanism remains to be determined. To explore the mechanism, we will first examine de novo synthesis of XIAP transcript. In general, the increased message results in the increased protein level. We will employ nuclear run-on experiment to examine this possibility. LNCaP cells treated with or without Wartmannin (200nM) for 6h will be processed and subjected to nuclear run-on experiment. This method will measure de novo synthesized XIAP message. Next, we will measure the message stability in response to Akt activation. Increased message stability is also well known mechanism to increase the protein level. LNCaP cells treated with ActD (1μg/ml) for 3h will be further incubated with or without Wartmannin (200nM) for 0, 1, 2, 3, 4, 5, or 6h, and subjected to Northern blotting for detecting XIAP message level. The data obtained from this experiment will reveal if post-transcriptional regulation (enhancement of message stability) is an underlining mechanism. To examine another post-transcriptional regulation (protein stability/turn-over), we will investigate the involvement of ubiquitination-proteosome degradation mechanism in XIAP since many fast turn-over proteins are ubiquitinated and ubiquitination-proteosome degradation is considered as a main mechanism. Previous studies demonstrated that XIAP is ubiquitinated (94). Thus, we will treat LNCaP cells with or without Wartmannin (200nM) for 6h and process for immunoprecipitation with the antibody that recognizes ubiquitin. After the proteins are separated and transferred to Nitrocellulose membranes, they will be probed with XIAP-recognizing antibody. This experiment will reveal if blocking Akt activity affects ubiquitination of XIAP. Using proteasome inhibitors such as Lactacystin and MG-132, we will also examine if ubiquitin-proteasome is directly involved in XIAP turn-over. LNCaP cells treated with Wartmannin and proteasome inhibitor will be compared with control groups for
XIAP level. To investigate more directly functional role of XIAP upregulation, we will establish stable cell lines transfected with myristoylated Akt (a constitutively active form) and K179M-Akt (a null mutant form) and examine XIAP expression in each cell line. If XIAP levels are regulated by Akt, myristoylated Akt will increase XIAP levels. Our data obtained from these experiments will determine the mechanism by which the Akt cell-survival signal regulates XIAP, a potent apoptosis inhibitor.

Phosphorylation of XIAP by Akt

XIAP protein contains three tandem BIR (baculovirus IAP repeat) domains followed by a RING zinc finger domain (38, 98-105) (Fig. 28). Studies have demonstrated that, in addition to full-length XIAP, the linker-BIR2 fragment of XIAP is also a potent inhibitor of caspases (100). In contrast, other individual BIR or RING domains of XIAP are not likely to be involved in inhibitory function (100). Because XIAP directly inhibits caspase activation and activity, the inhibitory function of XIAP is directly related with the XIAP levels. We have identified a consensus phosphorylation sequence (RXRXXS/T, \( ^{82}RHRKVS^{87} \)) for Akt (73, 74) in the BIR1 domain of XIAP (Fig. 29). Thus, whether this potential site is a target for Akt is important question. To address this question, we will treat LNcaP cells with or without Wartmannin, pull-down proteins with phospho-serine antibody, and probe with XIAP antibody in Western blotting. If Akt phosphorylates at Serine 87 of XIAP protein, this pull-down experiment will enrich XIAP protein. In parallel, we will also conduct an in vitro phosphorylation experiment (in vitro kinase assay) with isolated Akt protein. If Akt phosphorylates XIAP protein, the Serine residue will be mutated to Alanine, and the experiments described above will be conducted to verify the Serine residue as the phosphorylation site. The most important question in this phosphorylation issue is how phosphorylation affects the function of XIAP if it is the case. First, we hypothesize that phosphorylation may affect XIAP stability (half-life). To examine this possibility, we will construct FLAG-tag fused XIAP expression constructs that express wild-type XIAP and mutant XIAP where the Serine 87 was changed to Alanine. Transfection of these constructs into LNcaP cells and measurement of XIAP levels with FLAG antibody following Wartmannin treatment will reveal whether Akt phosphorylation stabilizes XIAP protein. Second, we hypothesize that phosphorylation may affect protein structure of XIAP, which results in stronger binding to caspases (caspase-9, -3 and -7). As a model, we will investigate this possibility using caspase-3. For this experiment, we will use the FLAG-tag-fused wild-type XIAP expression construct described above. We will isolate XIAP protein from transfected LNcaP cells with FLAG antibody-conjugated Agarose beads, separate the isolates into two groups, and treat one group with phosphatase. Phosphatase-treated and -untreated XIAP isolates will be used for binding assays for recombinant caspase-3 protein. If phospho-XIAP has higher affinity for caspase-3, it will capture more caspase-3 protein.

Functional role of XIAP in TRAIL-induced apoptosis

To assess the functional role of XIAP in TRAIL-induced apoptosis, we will establish stable cell lines transfected with full-length wild-type XIAP expression construct. To investigate correlation of XIAP expression and inhibitory role in TRAIL-induced apoptosis, we will establish several clonal cell lines that express different amount of XIAP. These cell lines will be exposed to TRAIL recombinant protein and analyzed for susceptibility.

Potential pitfalls and priorities

Despite presence of a potential Akt phosphorylation site in XIAP protein, whether XIAP protein is phosphorylated by Akt has not yet been determined. If the folded structure of XIAP protein does not allow Akt to access to the site, Akt would fail to phosphorylate the site. In this case, we will focus our research on the mechanisms by which constitutively active Akt regulates the protein levels of XIAP.

AIM II: To optimize TRAIL gene transfer as an anti-cancer therapy.
TRAIL potently kills a wide variety of cancer cell lines in vitro. Recent preclinical studies (76) demonstrated that TRAIL is very safe and efficiently limits tumor growth in vivo. Therefore, TRAIL has a strong potential to be developed as a cancer therapy. In our preliminary studies, we have created an artificial gene encoding secretable trimeric TRAIL (stTRAIL) protein. In functional tests, our stTRAIL was identified to be more potent in cancer cell killing in vitro than other reported forms of TRAIL protein. More importantly, this stTRAIL did not kill normal cells isolated from patients. Our data about safety and potency suggest that stTRAIL may prove superior to wild-type TRAIL as a cancer therapy. Under this research aim, we will pursue two different gene therapy approaches (naked DNA and adenoviral approach). To accomplish this aim, we will develop two gene therapy vectors (a naked plasmid DNA and an adenoviral vector) harboring the stTRAIL gene, and test them in vitro and in vivo for efficacy and cytotoxic side effects. Under this aim, we will carry out the following experiments;

**EXP IIA: Cancer gene therapy using naked DNA**

In general, gene therapy is classified into two approaches such as viral and non-viral. A representative non-viral gene therapy approach is to use naked DNA. The naked DNA (plasmid) can be combined with other transfection enhancers including lipids and polymers. As many studies have demonstrated, naked DNA-based gene therapy approach is safer than viral approaches because the approach does not provoke a severe immune response. The naked DNA approach is also cost-effective in preparation, and easy to handle. Nevertheless, inefficiency in gene delivery is the most notable drawback of naked DNA-based approaches. However, recent improvements in gene delivery methods make the naked DNA-based gene therapy a feasible approach. Based on these previous studies, we will develop and test naked DNA-based gene therapy approach.

**Construction of a plasmid gene transfer vector**

As a gene delivery vector for naked DNA-based gene therapy approach, we will develop a plasmid vector. Although we already constructed pcDNA3-based stTRAIL expression plasmid, we do not think this plasmid vector is appropriate for gene therapy because this plasmid vector contains irrelevant genes (Neo and ampicillin resistance gene) and DNA segment (f1 origin). The Neo resistance driven by SV40 promoter will express in mammalian cells, and may provoke an undesirable immune response. Ampicillin was also shown to cause an allergic response in some individuals (106, 107). Thus, kanamycin has been considered as a more desirable selection marker for plasmid propagation. The f1 origin for generating single stranded DNA is an unnecessary DNA segment for gene therapy vector development. Therefore, we will first develop a plasmid vector comprising minimal DNA segments necessary for gene delivery (Fig. 29). The DNA segment containing CMV immediate early promoter (pCMV), multi-cloning sites (MCS), bovine growth hormone poly A (BGH-polyA) and ColEl replication origin will be obtained from pcDNA3. The DNA segment encoding a bacterial promoter and kanamycin resistance gene will be prepared by PCR from the pShuttle vector of pAdEasy system (Stratagene). Ligation of these two DNA fragments will create pGT2 vector. This high copy number plasmid vector will be easily prepared in large quantities from bacterial cells.

**In vitro analysis of the pGT2-stTRAIL vector**

Our preliminary data demonstrated that SEC(CV)ILZTRAIL(114-281) has greater apoptotic activity than SECIIILZTRAIL(114-281). Therefore, we focus on the SEC(CV)ILZTRAIL(114-281) expression DNA cassette for further studies. To construct the pGT2-stTRAIL plasmid as a stTRAIL delivery vector, pGT2 will be digested with Kpn I and Not I enzymes, and ligated to the SEC(CV)ILZTRAIL(114-281) DNA prepared by digestion with the same enzymes. This plasmid will be amplified in XL-1 Blue cells (Stratagene) and purified using endotoxin-free plasmid preparation kit (Qiagen). For in vitro expression experiments, we will use 293T...
cells that were observed to be resistant to stTRAIL in our preliminary studies. We will transiently transfect
293T cells with the pGT2-stTRAIL plasmid, and examine protein expression and secretion by Western blotting.
The conditioned medium prepared from pGT2-stTRAIL-transfected 293T cells will be tested in HeLa cells for
apoptotic activity. To verify that apoptosis is induced by stTRAIL, we will use soluble DR5 (Alexis) as a
specific stTRAIL inhibitor.

**EXP IIB: Test of the pGT2-stTRAIL plasmid in vivo**

**Analysis of tumorcidal activity**

Before applying to humans, all new therapies must be first tested in animals. Our gene therapy
approach using the pGT2-stTRAIL is intended to treat human cancers. Although animal studies are not
completely paralleled with clinical application for humans, test results in animal models will provide valuable
information for dose determination, injection route, delivery method and so on.

To assess tumorcidal activity of the pGT2-stTRAIL plasmid, we will inject MDA-MB-231, a human
breast cancer cell line established from the grade IV tumor tissue of a cancer patient, into nude mice. Our
preliminary data demonstrated that MDA-MB-231 is susceptible to TRAIL. 1.0X10^6 cells of MDA-MB-231
will be injected subcutaneously into the nude mice. Seven days after injecting the cells, nude mice will be given
20μg pGT2-stTRAIL or pGT2 plasmid through tail vain by the hydrodynamic injection method. Many studies
have demonstrated that hydrodynamic injection method efficiently delivers the plasmid DNA to the liver, and
gives rise to high expression of the gene delivered (108-110). Therefore, this delivery method has been used to
easily test the function of a gene in vivo. In this experimental setting, tumor-killing function of pGT2-stTRAIL
will be assessed by tumor size measurement.

Even though the hydrodynamic injection method is a good gene delivery method for small animals
such as mice, this method is not applicable to human. Recent studies have demonstrated that the plasmid DNA
can be directly injected into the tumor mass (intra-tumoral injection) (111, 112) for expression of the gene
delivered. Thus, we will inject 20μg pGT2-stTRAIL or pGT2 plasmid into tumor graft on nude mice and assess
tumor-killing activity of pGT2-stTRAIL. Lipids and polymers have been shown to strongly enhance
transfection efficiency of plasmid DNA. Studies have demonstrated that lipid- or polymer-plasmid DNA
combination has better transfection efficiency following local injection than systemic delivery (113, 114). We
will inject pGT2-stTRAIL or pGT2 plasmid in combination with lipid (liposome) or polymer (hydrophobic
polymer such as polyethyleneimine (PEI)) into tumor graft on nude mice and assess tumor-killing function of
pGT2-stTRAIL. In these experimental settings, we will assess tumor-killing function of pGT2-stTRAIL by
tumor size measurement.

**Pharmacokinetic analysis of stTRAIL protein in vivo**

Our stTRAIL protein is designed to be secreted and reach cancer targets through the blood stream.
The dose and longevity of blood stTRAIL will be likely to affect efficacy and possibly cytotoxic side effects.
Since these factors are important to improve delivery methods and determine dose of plasmid, pharmacokinetic
analyses will be performed. The pGT2-stTRAIL plasmid (0.1, 0.5, or 1mg/kg body weight) will be injected into
normal mice by hydrodynamic method, and the longevity and induction level of serum stTRAIL will be
measured by quantitative Western blot analyses.

**Effects of ActD in a combination therapy**

In our preliminary studies, we found ActD significantly enhanced TRAIL-induced apoptosis in vitro.
Thus, we will attempt to test a combination of pGT2-stTRAIL plus ActD on tumorcidal function in vivo. Nude
mice with tumor graft will be given 20μg pGT2-stTRAIL plasmid by hydrodynamic injection. 24 hours later,
the mice will receive ActD (0, 5, 50, or 500μg/kg body weight) intra-peritoneally (IP). The control group,
in which nude mice with tumor graft received ActD (0, or 500μg/kg body weight) without pGT-stTRAIL, will be
compared with the test group for tumorcidal activity and cytotoxic side effects. In the same experimental
settings, we will also test efficacy of local injection. The pGT2-stTRAIL plasmid DNA will be directly injected
into tumor in a third group of nude mice. 24 hours later, the mice will receive ActD through IP. To test lipid- or
polymer-mediated plasmid transfection, we will inject pGT2-stTRAIL DNA combined with lipid or polymer
into tumor on another group of nude mice. Subsequently, ActD at different doses will be delivered to the mice
as described above. In these experimental settings, tumor-killing function of pGT2-stTRAIL plasmid DNA will
be assessed by tumor size measurement.
**Analysis of cytotoxic side effects**

The most important issue in a novel therapy is cytotoxic side effects. In our naked plasmid DNA-based gene therapy, the pGT2-stTRAIL plasmid is delivered systemically (hydrodynamic injection) or locally (intra-tumoral injection). The pGT-stTRAIL plasmid is also tested alone or in combination with lipid or polymer, or further combined with ActD. In each experimental setting, we will assess cytotoxic side effects. Cytotoxic side effects will be examined simply based on movement, behavior, and weight loss of mice. After sacrifice, major organs will be examined for apoptosis by TUNEL assay.

**Potential pitfalls and priorities**

It has not yet been tested if TRAIL/ActD combination is toxic to normal mouse tissues. If this combination induces any severe side effects, we will test “TRAIL only” regimen.

**EXP IIC: Cancer gene therapy using adenoviral TRAIL**

Despite known risk of immune response, virus-based gene therapy thus far been the major gene therapy approach. This popularity is due to the high gene transfer efficiency of viruses. The viral vectors frequently used in gene therapy include the ones originated from adenovirus (Ad), adeno-associated virus (AAV), retrovirus, and herpes virus (115). Among these vectors, the most frequently used viral vector for *in vitro* gene delivery as well as clinical applications is the adenoviral vector. Most of the adenoviral vectors have a deletion in E1 region to ensure replication deficiency in host cells. This replication defect minimizes a possible systemic failure caused by unlimited amplification of virus particles. For our experiments, we will use a Cre-lox recombination-based Ψ5 adenoviral vector system in which the viral E1 and E3 regions are deleted.

**Construction and production of a recombinant viral vector Ad-stTRAIL**

The Ψ5 adenoviral system comprises two steps for viral vector construction. The first step is a shuttle vector construction in which the gene of interest is put into the pAdlox shuttle vector. To construct pAdlox-stTRAIL, we will ligate the expression cassette encoding stTRAIL into the pAdlox shuttle vector. We will then electroporate the Ψ5 helper virus DNA containing most viral components and linearized pAdlox-stTRAIL plasmid together into the Ad packing cell line CRE8 that expresses Cre recombinase, and screen for right clones. Appropriate clones will be selected, further amplified in 293 cells, and processed for purification of a recombinant pAd-stTRAIL virus. Virus particles will be purified by conventional CsCl ultra centrifugation under the technical guidance of our collaborator Dr. Paul Robbins. Importantly, our preliminary data demonstrated that 293 cells are resistant to stTRAIL. Therefore, this conventional adenoviral production method will work for Ad-stTRAIL.

**In vitro analysis of the Ad-stTRAIL vector**

For *in vitro* analysis of stTRAIL expression, we will use 293 cells. Whole cell lysate and conditioned medium of 293 cells infected with Ad-stTRAIL particles (0, 10, 50, or 100MOI) will be subjected to Western blot analysis. For *in vitro* apoptotic function analysis, we will infect Ad-stTRAIL particles into several cell lines (HeLa, MCF-7, HepG2, A549, Panc-1) and assess cell viability. To verify that apoptosis is induced by stTRAIL, we will use soluble DR5 (Alexis) as a specific stTRAIL inhibitor.

**EXP IID: Test of the Ad-stTRAIL vector in vivo**

**Analysis of tumoricidal activity**

Our long-term objectives in developing Ad-stTRAIL particles as a novel cancer therapy are evident - to use the therapy to treat human cancers. Since the results obtained from animal tests can be used for clinical tests in humans, the experiments performed in animals will be designed considering the settings for clinical trials. Systemic delivery of adenoviral particles in humans is not appropriate because it may provoke severe immune response and result in systemic failure. Thus, we will test only locally delivered Ad-stTRAIL particles for tumoricidal activity in animal tumor models. As an animal tumor model, we will use nude mice harboring MDA-MB-231 tumor grafts. To assess tumoricidal activity *in vivo*, 1.0X10^5 or 1.0X10^7 Ad-stTRAIL or Ad-LacZ particles will be directly injected into the tumor site (intra-tumoral injection) when the tumor size measures 0.2-0.5cm in diameter. In this experimental setting, tumor-killing activity of Ad-stTRAIL will be assessed by tumor size measurement. If this experimental setting does not produce sufficient tumoricidal activity, we will increase viral titer to 1X10^10.

Our Ad-stTRAIL particles injected into tumor site are expected to produce stTRAIL protein from infected tumor cells. stTRAIL protein seems likely to be localized to the tumor site and mainly involved in...
killing adjacent tumor cells, but may also get into animal’s circulation. The dose and longevity of blood stTRAIL appear to affect cytotoxic side effects and the dose determination of Ad-stTRAIL particles. For the analyses of the longevity and serum stTRAIL levels, we will deliver Ad-stTRAIL or Ad-LacZ particles ($1.0 \times 10^5$ or $1.0 \times 10^7$) into the tumor. At different time points, we will take blood from tail vain and measure stTRAIL protein levels by quantitative Western blotting.

Effects of ActD in a combination therapy

As proposed for pGT2-stTRAIL plasmid therapy, we will also test Ad-stTRAIL and ActD in a combination. MDA-MB-231 tumor graft on nude mice will receive $1.0 \times 10^5$ or $1.0 \times 10^7$ Ad-stTRAIL or Ad-LacZ particles. 24 hours later, the mice will be given ActD (0, 5, 50, 500ug/kg body weight) through IP. In these experimental settings, tumor-killing activity of combination therapy will be assessed by tumor size measurement.

Analysis of cytotoxic side effects

Local injection of Ad-stTRAIL particles is expected to be relatively safe. Nevertheless, we will assess any cytotoxic side effects generated. Cytotoxic side effects will be assessed based on movement, behavior, and weight loss of mice. After sacrifice, major organs will be examined for apoptosis by TUNEL assay.

RESPONSE

We expect that the reviewers may find our proposal overambitious. In response, we would point out that we submit this application for the first time, and importantly this application represents our ongoing work, thus it does not need start-up time. Furthermore, most of the key reagents are in place. Finally, we have established the necessary collaboration to carry out the complicated assays and experiments not currently available in the PI’s laboratory, thus avoiding the trial and error of the development of new assays and experiments.

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MATERIALS AND METHODS

Cell culture: Individual cell lines are cultured in an appropriate culture medium containing 10% FBS supplemented with antibiotics (100μg/ml Gentamycin and 100μg/ml Penicillin-Streptomycin).

Northern blot analysis: Total RNA extracted from cells using RNAzolB (Biotech Laboratories, Houston TX) is electrophoresed on 1% agarose gel containing 3% formaldehyde. RNA is transferred to Nitrocellulose membranes and UV-crosslinked. Membranes are hybridized at 60°C with DNA probes generated by the randomly primed probe synthesis method. Membranes are washed at the same temperature and visualized flowing exposure to X-ray film.

Western blotting: The nitrocellulose membranes to which proteins were size-fractionated and transferred are pre-incubated with a PBS-Tween solution (0.5% Tween-20 in PBS) containing 5% milk for 1-2 h at room temperature. The membranes are incubated with a hybridization solution (0.5% Tween-20 and 1% milk in PBS) and containing a primary antibody for 1-2 h at RT. After washing once with wash solution (0.5% Tween-20 in PBS), the membranes are incubated with secondary antibody. Following extensive washing, target proteins will be visualized using ECL solutions.

Immunoprecipitation (pull-down) assay: Cells are lysed with RIPA buffer (1% NP-40, 0.1% SDS, 100μg/ml PMSF, 2μg/ml protease inhibitor mixture, and 1mM sodium orthovanadate in PBS). Cell lysates precleared with
agarose beads are further incubated with a primary antibody and protein (A+G)-conjugated agarose beads at 4°C. Precipitated proteins are analyzed by standard Western blotting.

Transfection (transient and stable): Cells plated in 6-wells are transfected with 3μg plasmid DNA using GenePorter transfection reagent (Gene Therapy Systems, CA). For transient transfection, cells are analyzed 24 h after transfection. For stable transfections, 72 h after transfection cells are treated with G418, and selected cells are cloned or pooled for further analysis.

Hypoxia treatment: Hypoxia (1% O2) is induced by maintaining cells inside an air-tight chamber with inflow and outflow valves that are infused with a mixture of 1% O2.

Site-directed mutagenesis: QuickChange site-directed mutagenesis kit (Stratagene) is used to mutate a specific nucleotide.

Viral infection: Cells plated in 6- or 12-wells are infected for 4 h with adenovirus diluted in Opti-MEM I (Gibco, NY) to the desired multiplicity of infection (MOI 0 to 100). The infected cells are washed 3 times with PBS and maintained with culture medium. After 24 h, infected cells are analyzed.

Measurement of cell viability: Cells Crystal violet method is employed as described (29). Cells are stained for 10 min at room temperature with staining solution (0.5% crystal violet in 30% ethanol and 3% formaldehyde), washed 4 times with water, and dried. Cells are lysed with 1% SDS solution and subjected to spectrophotomeric analysis at 550nm.

Quantitative Real-Time RT-PCR (QRT-PCR): QRT-PCR is performed using the 5' nuclease assay and an Applied Biosystems 7700 Sequence Detection Instrument (TaMan) or Cepheid Smart Cycler. mRNA expression is measured relative to the endogenous control gene, β-Gus, using the comparative Ct method. All QRT-PCR assays are carried out at two RNA inputs, 400 and 100 ng, and duplicate reactions are set up for each concentration. Thus, the reported mRNA expression levels are an average of four independent QRT-PCR reactions. RT-negative controls are run for all samples using 400 ng of RNA but omitting the reverse transcriptase. Template-negative controls are also run on each PCR plate. A calibrator RNA sample is amplified in parallel on all plates to allow comparison of samples run at different times.

Quantitative Western blot analysis: The serum TRAIL protein is measured by Western blot analysis. The comparison of the serum TRAIL protein with the standard (purified recombinant TRAIL protein) (29) (Fig. 2 and 5) will make it possible to measure the amount of recombinant trimeric TRAIL protein in serum.

TUNEL assay: This assay identifies cleavage of genomic DNA into oligonucleosomes during apoptosis by labeling 3'-OH termini using terminal deoxynucleotidyl transferase and fluorescein-labeled dUTP substrate (TUNEL assay). Cells are fixed with 2% paraformaldehyde, washed 3 times with PBS, permeabilized with 0.1% Triton X-100 on ice, and incubated with the TUNEL reaction mixture (TUNEL assay kit, Roche) in a humidified chamber (1 h, 37 °C). Cells incorporating labeled dUTP are identified by fluorescence microscopy and photographed.

Nuclear run-on assays: Nuclei are isolated from cells using a Dounce tissue homogenizer in 0.3M sucrose containing 2mM Mg(OAc)2, 3mM CaCl2, 10mM Tris-Cl (pH 8.0), 0.1% Triton X-100, and 0.5mM DTT. One ml of solution is used for 107 cells. The homogenate is then layered over 2ml of the 2mM sucrose buffer and centrifuged. The nuclear pellet is then resuspended in the transcription buffer with a 50-90% yield of nuclei. The labeled run-on transcripts are hybridized with the equal amounts of linearized control and target DNAs that were spotted on Nitrocellulose membranes.

In vitro Akt kinase assay: 293T cells transfected with pCMV6-myrAKT-HA or pCMV6-K179M-Akt-HA are lysed with RIPA buffer. The lysates prepared are then precipitated with anti HA-conjugated agarose beads. Immunoprecipitates are washed three times with RIPA buffer and two times with kinase buffer (20mM HEPES (pH 7.2), 10mM MgCl2, 10mM MnCl2, 1mM DTT, and 3μM ATP) followed by kinased reaction for 30min at 30°C with 5μg GST-XIAP protein in kinase buffer where 3μM ATP is replaced with 3μM [γ-32P]ATP. Reaction products are separated on 12% SDS-gels and autoradiographed.

E. HUMAN SUBJECTS

N/A
F. VERTEBRATE ANIMALS

1. To establish an animal tumor model, we will inject $1.0 \times 10^6$ MDA-MB-231 breast cancer cells subcutaneously into nude mice. When the tumor size reaches 0.2-0.5mm, gene delivery experiments will start.

2. For in vivo gene delivery experiments using the naked TRAIL DNA (plasmid), we will inject 20µg of DNA through mouse tail vein or directly into tumor mass. The same amount of DNA combined with liposome or polymer or ActD (5, 50, or 500µg/kg body weight) will be also tested.

3. For in vivo gene delivery experiments using adenoviral TRAIL vector, the replication-defective AdTRAIL particles ($1.0 \times 10^5$, $1.0 \times 10^7$, or $1.0 \times 10^{10}$) will be injected into tumor mass. AdTRAIL in combination with ActD (5, 50, or 500µg/kg body weight) will be also tested.

4. The Central Animal Facility of the University of Pittsburgh will provide all animal care.

5. Tumor growth will be monitored by size measurement at various time points. If the mice become morbid, the tumor size reaches 2 cm in diameter, or sacrifice is needed to examine cytotoxic side effects in major organs, the mice will be euthanized with CO₂ asphyxiation. This method is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

6. The numbers of animals proposed for these studies are the minimum for analysis.

G. LITERATURE CITED

Attached

H. CONSORTIUM/CONTRACTUAL ARRANGEMENTS

N/A

I. CONSULTANTS

Letters attached


41. Liu, Z., Sun, C., Olejniczak, E. T., Meadows, R. P., Betz, S. F., Oost, T., Herrmann, J., Wu, J. C., and
Fesik, S. W. Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. Nature, 408:
Fernandes-Alnemri, T., Shi, Y., and Alnemri, E. S. A conserved XIAP-interaction motif in caspase-9
44. Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. A caspase-activated
45. Sakahira, H., Enari, M., and Nagata, S. Cleavage of CAD inhibitor in CAD activation and DNA
fragmentation factor induces DNA fragmentation and chromatin condensation during apoptosis. Proc
47. Wiley, S. R., Schooley, K., Smolak, P. J., Din, W. S., Huang, C. P., Nicholl, J. K., Sutherland, G. R.,
Smith, T. D., Rauch, C., Smith, C. A., et al. Identification and characterization of a new member of
of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J Biol Chem,
50. Galle, P. R., Hofmann, W. J., Walczak, H., Schaller, H., Otto, G., Stremmel, W., Krammer, P. H., and
Runkel, L. Involvement of the CD95 (APO-1/Fas) receptor and ligand in liver damage. J Exp Med,
51. Walczak, H., Miller, R. E., Ariail, K., Gliniak, B., Griffith, T. S., Kubin, M., Chin, W., Jones, J.,
Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat Med, 5:
52. Ashkenazi, A., Pai, R. C., Fong, S., Leung, S., Lawrence, D. A., Marsters, S. A., Blackie, C., Chang, L.,
McMurtrey, A. E., Hebert, A., DeForge, L., Koumenis, I. L., Lewis, D., Harris, L., Bussiere, J.,
Koeppen, H., Shahrokh, Z., and Schwall, R. H. Safety and antitumor activity of recombinant soluble
53. Jeremias, I., Herr, I., Boehler, T., and Debatin, K. M. TRAIL/Apo-2-ligand-induced apoptosis in human
susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. J


May 28, 2002

Dai-Wu Seol, Ph.D.
Research Assistant Professor of Surgery
University of Pittsburgh
15 BST
Pittsburgh, PA 15213

Re: "TRAIL: Death Signaling Analysis and Cancer Gene Therapy."

Dear Dai-Wu,

I am delighted to collaborate with you on your proposed grant investigating the modulation of TRAIL signal transduction and its potential use as a therapeutic agent. As you know, my laboratory focuses on membrane trafficking and signal transduction of the death receptor FAS in esophageal adenocarcinoma. We are particularly well-versed in analyzing death receptors signal transduction and my laboratory would be happy to assist you in the quantitative, real-time PCR, and analysis of TRAIL signaling proposed in your grant.

Your project has clear significance, is of general interest, and I look forward to a productive collaboration.

Warmest regards,

Steven Hughes, MD
Assistant Professor of Surgery
University of Pittsburgh
May 20, 2002

Dai-Wu Seol, Ph.D.
Assistant Professor
Department of Surgery
BST W1513
University of Pittsburgh School of Medicine
Pittsburgh, PA 15261

Dear Dai-Wu,

I would be happy to participate as coinvestigator in your project entitled: TRAIL: death signaling analysis and cancer gene therapy. As you know, my role will primarily involve using mouse tumor models to assess the efficacy of injection of TRAIL DNA or Ad-TRAIL into the mice. This will primarily involve assessment of human cancers in nude mice, especially breast cancer.

Furthermore, my staff and I would be happy to assist in other aspects of your research since we have extensive experience in the study of tumor suppressors, apoptosis, viral vectors and gene therapy for cancer.

I wish you every success in this exciting project.

Sincerely,

[Signature]

John H. Yim, M.D.
497 Scaife Hall
3550 Terrace Street
Pittsburgh, PA 15261
(412) 648-3173
Dear Dai-Wu:

I would be very pleased to serve as an unpaid consultant for your grant entitled “TRIAL; Death Signaling Analysis in Cancer Gene Therapy.” As you know, we have had a longstanding collaboration in the area of apoptosis research. I am very pleased to serve as a consultant to your grant and provide any guidance and support that may be useful in the area of apoptosis signaling or in the clinical application of TRIAL as an anti-cancer therapy.

Sincerely yours,

Timothy R. Billiar, M.D.
George Vance Foster Professor and Chair

TRB:lp
January 17th, 2002

Dai-Wu Seol, Ph.D.
Assistant Professor
Department of Surgery
University of Pittsburgh School of Medicine
BST W1513
Pittsburgh, PA 15261

Dear Dai-Wu:

I would be pleased to provide any assistance necessary for your NIH grant proposal entitled "TRAIL: death signaling analysis and cancer gene therapy". As you know, I am Director of the Vector Core Facility that constructs and propagates recombinant viral vectors for gene transfer. As always, we would be willing to provide recombinant adenoviral vectors expressing the proteins of interest needed for the successful completion of your proposed studies. I look forward to continuing to work with you on this exciting project.

Sincerely,

Paul D. Robbins, Ph.D.
CHECKLIST

TYPE OF APPLICATION (Check all that apply.)

[ ] NEW application. (This application is being submitted to the PHS for the first time.)

[ ] SBIR Phase I  [ ] SBIR Phase II: SBIR Phase I Grant No.
[ ] STTR Phase I  [ ] STTR Phase II: STTR Phase I Grant No.

[ ] SBIR Fast Track  [ ] STTR Fast Track

[ ] REVISION of application number:
(This application replaces a prior unfunded version of a new, competing continuation, or supplemental application.)

INVENTIONS AND PATENTS

(Competing continuation appl. and Phase II only)

[ ] No
[ ] Yes. If “Yes,” [ ] Previously reported
[ ] Not previously reported

[ ] COMPETING CONTINUATION of grant number:
(This application is to extend a funded grant beyond its current project period.)

[ ] SUPPLEMENT to grant number:
(This application is for additional funds to supplement a currently funded grant.)

[ ] CHANGE of principal investigator/program director.

Name of former principal investigator/program director:

[ ] FOREIGN application or significant foreign component.

1. PROGRAM INCOME

(See instructions.)

All applications must indicate whether program income is anticipated during the period(s) for which grant support is requested. If program income is anticipated, use the format below to reflect the amount and source(s).

<table>
<thead>
<tr>
<th>Budget Period</th>
<th>Anticipated Amount</th>
<th>Source(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. ASSURANCES/CERTIFICATIONS

(See instructions.)

The following assurances/certifications are made and verified by the signature of the Official Signing for Applicant Organization on the Face Page of the application. Descriptions of individual assurances/certifications are provided in Section III. If unable to certify compliance, where applicable, provide an explanation and place it after this page.

- Human Subjects
- Research Using Human Pluripotent Stem Cells
- Research on Transplantation of Human Fetal Tissue
- Women and Minority Inclusion Policy
- Inclusion of Children Policy
- Vertebrate Animals
- Debarment and Suspension
- Drug-Free Workplace (applicable to new Type I or revised Type I applications only)
- Lobbying
- Non-Delinquency on Federal Debt
- Research Misconduct
- Civil Rights (Form HHS 441 or HHS 690)
- Handicapped Individuals (Form HHS 641 or HHS 690)
- Sex Discrimination (Form HHS 639-A or HHS 690)
- Age Discrimination (Form HHS 680 or HHS 690)
- Recombinant DNA and Human Gene Transfer Research
- Financial Conflict of Interest (except Phase I SBIR/STTR)

STTR ONLY: Certification of Research Institution Participation.

3. FACILITIES AND ADMINISTRATIVE COSTS (F&A)/INDIRECT COSTS.

(See specific instructions.)

[ ] DHHS Agreement dated: 03/30/01  [ ] No Facilities And Administration Costs Requested.

[ ] DHHS Agreement being negotiated with ______________________ Regional Office.

[ ] No DHHS Agreement, but rate established with ______________________ Date

CALCULATION* (The entire grant application, including the Checklist, will be reproduced and provided to peer reviewers as confidential information. Supplying the following information on F&A costs is optional for for-profit organizations.)

<table>
<thead>
<tr>
<th>Period</th>
<th>Amount of base</th>
<th>Rate applied</th>
<th>% = F&amp;A costs</th>
<th>Total F&amp;A Costs</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Initial</td>
<td>$43,299/129,895</td>
<td>49.5/49.0</td>
<td>$21,433/63,649</td>
<td></td>
</tr>
<tr>
<td>b. 02 year</td>
<td>$43,285/129,855</td>
<td>49.0/48.5</td>
<td>$21,210/62,980</td>
<td></td>
</tr>
<tr>
<td>c. 03 year</td>
<td>$173,084</td>
<td>48.50</td>
<td>$83,946</td>
<td></td>
</tr>
<tr>
<td>d. 04 year</td>
<td>$173,027</td>
<td>48.50</td>
<td>$83,918</td>
<td></td>
</tr>
<tr>
<td>e. 05 year</td>
<td>$172,968</td>
<td>48.50</td>
<td>$83,889</td>
<td></td>
</tr>
</tbody>
</table>

TOTAL F&A Costs $421,025

*Check appropriate box(es):

[ ] Salary and wages base  [ ] Modified total direct cost base  [ ] Other base (Explain)

Explanation (Attach separate sheet, if necessary):

4. SMOKE-FREE WORKPLACE [ ] Yes  [ ] No (The response to this question has no impact on the review or funding of this application.)
PERSONAL DATA ON PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

The Public Health Service has a continuing commitment to monitor the operation of its review and award processes to detect—and deal appropriately with—any instances of real or apparent inequities with respect to age, sex, race, or ethnicity of the proposed principal investigator/program director. To provide the PHS with the information it needs for this important task, complete the form below and attach it to the signed original of the application after the Checklist. Do not attach copies of this form to the duplicated copies of the application.

Upon receipt of the application by the PHS, this form will be separated from the application. This form will not be duplicated, and it will not be a part of the review process. Data will be confidential, and will be maintained in Privacy Act record system 09-25-0036, "Grants: IMPAC (Grant/Contract Information)." The PHS requests social Security numbers for accurate identification, referral, and review of applications and for management of PHS grant programs. Provision of the Social Security number is voluntary. No individual will be denied any right, benefit, or privilege provided by law because of refusal to disclose his or her Social Security Number. The PHS requests the Social Security Number under Sections 301 (a) and 487 of the PHS Act as amended (42 USC214a and USC288). All analyses conducted on the date of birth and race and/or ethnic origin data will report aggregate statistical findings only and will not identify individuals. If you decline to provide this information, it will in no way affect consideration of your application. Your cooperation will be appreciated.

<table>
<thead>
<tr>
<th>ETHNICITY</th>
<th>PII Redacted</th>
</tr>
</thead>
</table>

1. Do you consider yourself to be Hispanic or Latino? (See definition below.) Select one.

- **Hispanic or Latino.** A person of Mexican, Puerto Rican, Cuban, South or Central American, or other Spanish culture or origin, regardless of race. The term, “Spanish origin,” can be used in addition to “Hispanic or Latino.”

- [ ] Hispanic or Latino

- [x] Not Hispanic or Latino

<table>
<thead>
<tr>
<th>RACE</th>
<th>SEX/GENDER</th>
</tr>
</thead>
</table>

2. What race do you consider yourself to be? Select one or more of the following.

- [ ] **American Indian or Alaska Native.** A person having origins in any of the original peoples of North, Central, or South America, and who maintains tribal affiliation or community attachment.

- [x] **Asian.** A person having origins in any of the original peoples of the Far East, Southeast Asia, or the Indian subcontinent, including, for example, Cambodia, China, India, Japan, Korea, Malaysia, Pakistan, the Philippine Islands, Thailand, and Vietnam. (Note: Individuals from the Philippine Islands have been recorded as Pacific Islanders in previous data collection strategies.)

- [ ] **Black or African American.** A person having origins in any of the black racial groups of Africa. Terms such as “Haitian” or “Negro” can be used in addition to “Black” or African American.

- [ ] **Native Hawaiian or Other Pacific Islander.** A person having origins in any of the original peoples of Hawaii, Guam, Samoa, or other Pacific Islands.

- [ ] **White.** A person having origins in any of the original peoples of Europe, the Middle East, or North Africa.

- [ ] Check here if you do not wish to provide some or all of the above information.