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Award Number: DAMD17-01-1-0092

TITLE: Development of Gene Therapy with TRAIL for Prostate Cancer

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REPORT DATE: September 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-09-2003	Annual Summary	15 Aug 01 – 14 Aug 03

4. TITLE AND SUBTITLE
Development of Gene Therapy with TRAIL for Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-01-1-0092

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Young K. Song, Ph.D.
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E-Mail: None

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pittsburgh
Pittsburgh, PA 15260

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

See attached.

15. SUBJECT TERMS
TRAIL low pH caspase Bax Bid

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	13	19b. TELEPHONE NUMBER *(include area code)*

ABSTRACT

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL/Apo2L) is considered as one of the most promising cancer therapeutic agents due to its ability to selectively kill tumor cells. In this study, we investigated whether low extracellular pH affects TRAIL-induced apoptotic death. When human prostate carcinoma DU145 cells were treated with 200 ng/ml TRAIL for 4 h, the survival was ~10% at pH 6.3-6.6 and 61.3% at pH 7.4. The TRAIL-mediated activation of caspase, cytochrome *c* release, and PARP cleavage were promoted at low extracellular pH. Western blot analysis shows that the low extracellular pH-enhanced TRAIL cytotoxicity does not involve modulation of the levels of TRAIL receptors (DR4, DR5, and DcR2), FLIP, IAP and Bcl-2. Overexpression of Bcl-2 effectively prevented low extracellular pH augmented TRAIL cytotoxicity. Immunoprecipitation followed by western blot analysis shows that low extracellular pH enhances the association of trunacated Bid with Bax during treatment with TRAIL. Taken together, we propose that TRAIL-mediated cytotoxicity is greatly enhanced in low pH environments by facilitating interaction between Bax and truncated Bid and subsequently promoting the mitochondria-mediated apoptotic signal transduction.

Table of Contents

Introduction :

Early detection and new surgical and radiotherapy regimens have contributed to improved survival and quality of life for prostate cancer patients (Hanks et al., 1997; Keyser et al., 1997; Kupelian et al., 1997). However, approximately 25-60% of patients demonstrate an elevated level of a prostate specific antigen within 5 years following treatment, indicative of future recurrence. This translates to roughly 100,000 patients per year who face the possibility of recurrent prostate cancer following initial treatment (Vincini et al., 1997). Obviously, greater intervention will be required to significantly enhance primary local control of prostate cancer.

One approach to primary local control of prostate cancer is through the use of gene therapy techniques in which the cytotoxic gene products expressed by the transfected tumor cells specifically eliminate the neighboring tumor cells as well as themselves (Roth and Cristiano, 1997). This technique has attracted great attention as one of the strategies for treating cancer. For successful administration of this gene therapy, the therapeutic gene should be delivered specifically to tumor cells and produce gene products that act toxic only to tumor cells without killing normal cells.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a good candidate as a therapeutic gene for this therapy due to its toxicity to tumors. TRAIL is an apoptosis-inducing member of the tumor necrosis factor (TNF) gene family (Wiley et al., 1995; Pitti et al., 1997). It has been shown that TRAIL is nontoxic and can slow the growth and, in some cases, induce the regression of tumor cell xenografts (Walczak et al., 1999). In preclinical studies in mice and primates, it has been shown that the administration of TRAIL can induce apoptosis in human tumors without any cytotoxicity to normal organs or tissues (Walczak et al., 1999). Obviously, differential sensitivity between normal and tumor cells to TRAIL and the mechanism of TRAIL-induced apoptosis need to be further studied (Gura, 1997; Ashkenazi and Dixit, 1999; Keane et al., 1999).

It is well known that severe architectural and functional abnormalities are commonly observed in the capillary network that develops during tumor growth (Vaupel et al., 1989). These abnormalities cause insufficient blood supply and development of a pathophysiological tumor microenvironment. Previous studies with the micropore chamber sampling procedure (Gullino et al., 1964) and tumor-isolated preparations (Gullino and Grantham, 1961) reveal differences in the constituents of serum (vascular compartment) compared to interstial fluid (interstitial compartment). Vascular and interstitial compartments are tow major compartments of the extracellular space of solid tumors. The tumor interstitial compartment is characterized by low oxygen tensions (hypoxia) (Vaupel et al., 1991), low glucose concentrations (Gullino, 1975), high lactate concentrations (Schwickert et al., 1995; Walenta et al., 1997) and low extracellular pH (Wike-Hooley et al., 1984). These characteristic features, which occur transiently or chronically, can markedly affect the therapeutic response Mueller-Klieser et al., 1989; Sartorelli, 1988). Recently, we demonstrated that low glucose augments the effect of TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), a potent anticancer agent, which induces apoptosis (Nam et al., 2002). This low glucose-induced augmentation is mediated through the ceramide-Akt-FLIP pathway (Nam et al., 2002). During this granting period, we investigated whether low extracellular pH alters TRAIL-induced cytotoxicity. We believe that understanding the role of low extracellular pH in TRAIL-mediated cytotoxicity will be important for predicting the effectiveness of this anticancer agent and developing new drug targets.

Revised STATEMENT OF WORK:

Task 1: Assessment of enhanced *in vitro* cytotoxicity of prostate tumor cells caused by TRAIL in low PH medium (1-6 months).

Task 2:: Assessment of enhanced *in vitro* cytotoxicity of prostate tumor cells caused by TRAIL in combination with amiloride, a substance capable of lowering the intracellular pH by being an inhibitor of the Na^+/H^+ antiporter (7-12 months).

Task 3: Examination of the tumoricidal effect of plasmid expressing TRAIL on human prostate tumor xenografts in mice (13-18 months):

a) Construction of hFlex/TRAIL recombinant plasmid (13-14 months),

b) Evaluation of the tumoricidal effect of TRAIL using hydrodynamics-based gene delivery protocol (15-16 months).

Task 4: Assessment of the tumoricidal effect of TRAIL gene therapy either alone or in combination with the intraperitoneal injection of amiloride in mice bearing the human prostate cancer xenograft by the tumor growth and animal survival assay (17-24 months).

Body :

We proposed to develop and evaluate novel strategies for enhancing gene therapy in human prostate cancer using a replication-competent adenoviral vector containing TRAIL and HSV-TK genes. As a first step in the preparation of a replication-competent adenoviral vector containing TRAIL and the Herpes simplex virus I thymidine kinase (HSV-TK) gene, we tried several times to make the replication-incompetent adenoviral vector containing the CMV promoter-driven TRAIL gene. This type of vector did not replicate in host cells, possibly because the cytotoxicity of TRAIL caused host cells' death prior to the production of progeny virus, even though the successful construction of the adenoviral shuttle vector containing the TRAIL gene was achieved.

Another difficulty for our research is that architectural and functional abnormalities in the tumor capillary network can markedly affect the therapeutic response (Mueller-Klieser et al., 1989; Sartorelli, 1988) and make it difficult to predict the effectiveness of new anticancer agents and develop new drug targets. Tumors are characterized by low oxygen tensions (hypoxia) (Vaupel et al., 1991), low glucose concentration (Gullino, 1975), high lactate concentration (Schwickert et al., 1995; Walenta et al., 1997), and low extracellular pH (Wike-Hooley et al., 1989). These characteristic features, which occur transiently or chronically, cause insufficient blood supply and produce pathophysiological conditions.

Task 1: Assessment of enhanced in vitro cytotoxicity of prostate tumor cells caused by TRAIL in low pH medium

Our initial studies were designed to investigate the effect of low extracellular pH on TRAIL-induced cytotoxicity of human prostate adenocarcinoma DU-145 cells. As DU-145 cells were treated with His-tagged TRAIL at various extracellular pH's (6.3–7.4), it was shown that no cytotoxicity was observed at low extracellular pH alone, while TRAIL-induced cytotoxicity was promoted at low extracellular pH (Fig. 1).

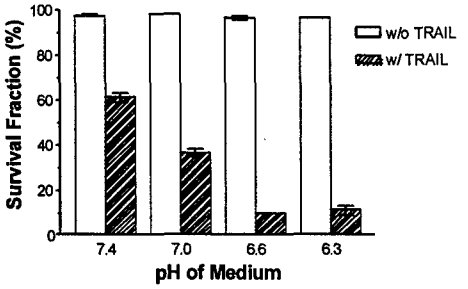

Figure 1. Low extracellular pH enhances TRAIL-induced cytotoxicity in human prostate adenocarcinoma DU-145 cells. Cells were treated for 4 h with 200 ng/ml His-tagged TRAIL or without TRAIL at various extracellular pH's (6.3–7.4). Cell survival was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean (SEM) from three separate experiments

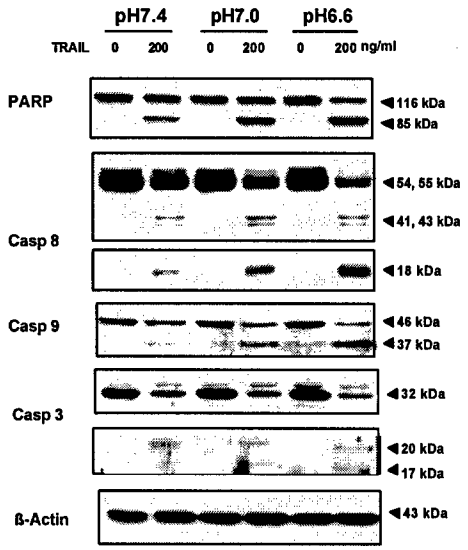

Figure 2. Low extracellular pH enhances TRAIL-induced apoptosis. DU-145 cells were treated with His-tagged TRAIL (200 ng/ml) or without TRAIL (0 ng/ml) for 4 h at various extracellular pH's (6.6–7.4). Poly (ADP-ribose) polymerase (PARP) cleavage and activation of caspases 8, 9, and 3 were determined by western blot analysis.

Additional studies were designed to determine whether the combination of TRAIL and low extracellular pH enhances poly (ADP-ribose) polymerase (PARP) cleavage, a hallmark feature of apoptosis (Fig. 2). Low extracellular pH alone failed to induce PARP cleavage. In contrast, TRAIL (200 ng/ml) alone caused PARP cleavage; PARP (116 kDa) was cleaved into characteristic 85 kDa fragments in the presence of TRAIL. PARP cleavage was markedly enhanced when TRAIL and low extracellular pH were combined; cleavage increased as the extracellular pH decreased (Fig. 2). We extended our studies to investigate whether low extracellular pH enhances TRAIL-induced cytotoxicity by increasing the activation of caspases. Using western blot analysis and enzyme activity assay, we found that low extracellular pH augmented the TRAIL-induced activation of caspases 8, 9, and 3 while low extracellular pH alone failed to induce the activation of these caspases (Fig.2 and data not shown). These results show that low extracellular pH significantly enhances TRAIL-induced apoptosis.

We further investigated whether low extracellular pH's affect the expression of TRAIL receptors (DR4, DR5, and DcR2) and apoptosis regulators (FLIP, cIAP-1, cIAP-2, and Bcl-2). Western blot analysis of DU-145 cells treated with 200 ng/ml His-tagged TRAIL for 4 h at various extracellular pH's reveals that the combined treatment did not significantly alter the levels of DR4, DR5, DcR2, FLIP, cIAP-1, or Bcl-2 (data not shown). Unlike cIAP-1, the level of cIAP-2 was decreased during treatment with TRAIL. However, the reduction of cIAP-2 during treatment with TRAIL was not promoted at low extracellular pH (data not shown). These data suggest that low extracellular pH enhances TRAIL-induced apoptosis by affecting the mitochondria-mediated apoptotic pathways.

We investigated the effect of low extracellular pH on the mitochondria-mediated apoptotic pathways, notably the cleavage of Bid, and the interaction between the truncated Bid and Bax. which induces cytochrome c release from the mitochondria into the cytosol, To determine the involvement of cytochrome c in the promotion of TRAIL-induced apoptosis at low extracellular pH, DU-145 cells were treated with 200 ng/ml His-tagged TRAIL at pH 7.4 or 6.8 for various times (1–4 h) after which TRAIL-induced cytochrome c release was determined. As shown in Fig. 3, TRAIL-induced cytochrome c release from mitochondria was increased at low extracellular pH.

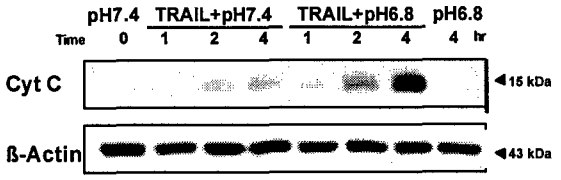

Figure 3. Low extracellular pH facilitates TRAIL-induced cytochrome c release in DU-145 cells. Cells were incubated in the presence of His-tagged TRAIL (200 ng/ml) for various times (1–4 h) at pH 7.4 or 6.8. Cytochrome c (15 kDa) release into cytosol was determined by immunoblotting for cytochrome c in the cytosolic fraction. Untreated control cells at pH 7.4 and cells incubated for 4 h at pH 6.8 in the absence of TRAIL were used as control groups.

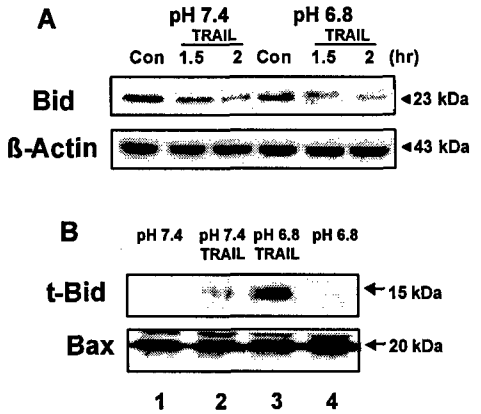

Figure 4. Effect of low extracellular pH on the level of Bid during treatment with TRAIL (A) or association of truncated Bid (t-Bid) with Bax (B). DU-145 cells were treated with 200 ng/ml His-tagged TRAIL for 1.5–2 h (A) or 10 min (B) at pH 7.4 or 6.8. (Panel A) The level of Bid were determined by western blot analysis. (Panel B) Lysates were immunoprecipitated with anti-Bax antibody and then immunoblotted with anti-Bid antibody or anti-Bax antibody.

In order to determine the cause of low extracellular pH-mediated cytochrome c release from mitochondria, we investigated the upstream events, that is, the cleavage of Bid by caspase 8. It has been shown that the 15-kDa form of truncated Bid binds to Bax and triggers a change in the conformation of Bax. As a result, Bax oligomerizes and inserts into the outer mitochondrial membrane, which results in cytochrome c release from the mitochondria (Eskes et al., 2000; Korsmeyer et al., 2000; Luo et al., 1998). We hypothesized that the binding affinity of truncated Bid with Bax can be affected by cytosolic pH. To examine this possibility, we first examined whether low extracellular pH enhances Bid cleavage during treatment with TRAIL. DU-145 cells were treated with His-tagged TRAIL at pH 6.8 or 7.4. Fig. 4A shows that TRAIL reduced the level of Bid. This is probably due to the cleavage of Bid. The reduction of Bid level increased as the treatment time increased. However, TRAIL-induced reduction of Bid was not significantly promoted at low extracellular pH at less than 2 h. Next, we investigated whether low extracellular pH enhances interaction between truncated Bid and Bax. Immunoprecipitation followed by western blot analysis shows that Bax associated with truncated Bid and the interaction between them was increased at low extracellular pH (Fig. 4B). These results suggest that an increase in cytochrome c release is mediated by promoting association of truncated Bid with Bax at low pH. These results also indicate that low pH accelerates the kinetics of caspase activation by promoting association of truncated Bid with Bax.

Task 2: Assessment of enhanced in vitro cytotoxicity of prostate tumor cells caused by TRAIL in combination with amiloride, a substance capable of lowering the intracellular pH by being an inhibitor of the Na^+/H^+ antiporter

Next, we investigated whether amiloride, an inhibitor of the Na^+/H^+ antiporter capable of lowering the intracellular pH, can potentiate TRAIL-induced apoptotic death. LNCaP cells were treated with TRAIL in the presence or absence of amiloride. Little or no cytotoxicity was observed with TRAIL (1-200 ng/ml) alone (Fig. 5A) or amiloride (0.1-1 mM) alone (Fig. 5B). In contrast, TRAIL in combination with amiloride significantly induced cytotoxicity (Fig. 5C). TdT-mediated dUTP Nick end labeling (TUNEL) assay showed that apoptotic death occurred during combined treatment with TRAIL and amiloride (Fig. 5D). Similar results were observed by DAPI staining (Fig. 5E). DAPI staining of cells treated with TRAIL in combination with amiloride showed the presence of many cells with condensed nuclei, a morphological change that is associated with apoptosis.

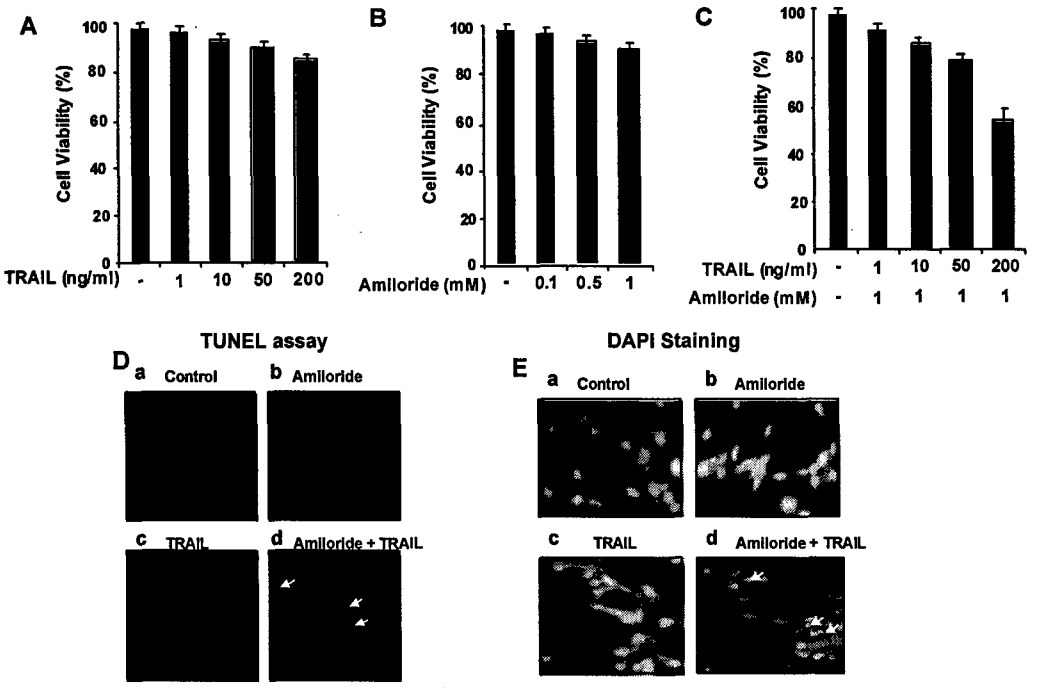

Figure 5. Effect of amiloride on TRAIL-induced cytotoxicity in human prostate adenocarcinoma LNCaP cells. (**A**) Cells were treated for 4 h with various concentrations of TRAIL (1-200 ng/ml). (**B**) Cells were treated with various concentrations of amiloride (0.1-1 mM). (**C**) Cells were treated with various concentrations of TRAIL (1-200 ng/ml) in the presence of 1 mM amiloride. Cell survival was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean (SEM) from three separate experiments. (**D**) Cells were treated for 4 h with TRAIL (200 ng/ml) in the presence or absence of 1 mM amiloride. After treatment, apoptosis was detected by the TUNEL assay. Apoptotic cells are indicated by arrows. (a) untreated control; (b) amiloride; (c) TRAIL; (d) amiloride + TRAIL. (**E**) Cells were treated for 4 h with TRAIL (200 ng/ml) in the presence or absence of 1 mM amiloride. After treatment, cells were stained with DAPI (1 μg/ml), and morphological features were analyzed with a fluorescence microscope.

Additional studies were designed to examine whether the combination of amiloride and TRAIL treatment in LNCaP cells enhances poly (ADP-ribose) polymerase (PARP) cleavage, the hallmark feature of apoptosis, and also whether the combination of amiloride and TRAIL treatment activates caspases. Previous studies show that PARP (116 kDa) is cleaved yielding a characteristic 85 kDa fragment in the presence of TRAIL alone in human prostate adenocarcinoma DU-145 cells (Lee et al., 2004). Figures 6A and 6B show that the cleavage of PARP was not observed by treatment with amiloride (0.1-1 mM) alone or TRAIL (1-1000 ng/ml) alone in LNCaP cells. Interestingly, PARP was cleaved by combined treatment with TRAIL (50-1000 ng/ml) and amiloride (0.5-1 mM). Similar results were observed with HMA, an analogue of amiloride (Fig.

6C). Figure 6D demonstrates that TRAIL in combination with amiloride, but not TRAIL alone, activated caspases. Amiloride alone did not activate caspases (data not shown). However, western blot analysis shows that procaspase-8 (54/55 kDa) was cleaved to the intermediate forms (41 and 43 kDa) and active form (18 kDa) by treatment with TRAIL in the presence of amiloride. The combined treatment of TRAIL and amiloride also resulted in caspase-9 activation (Fig. 6D). TRAIL in combination with amiloride induced proteolytic processing of procaspase-9 (48 kDa) into its active form (37 kDa). The combined treatment with TRAIL and amiloride also induced caspase-3 activation. Western blot analysis shows that procaspase-3 (32 kDa), the precursor form of caspase-3, was cleaved to active form (17 kDa) in the presence of TRAIL and amiloride.

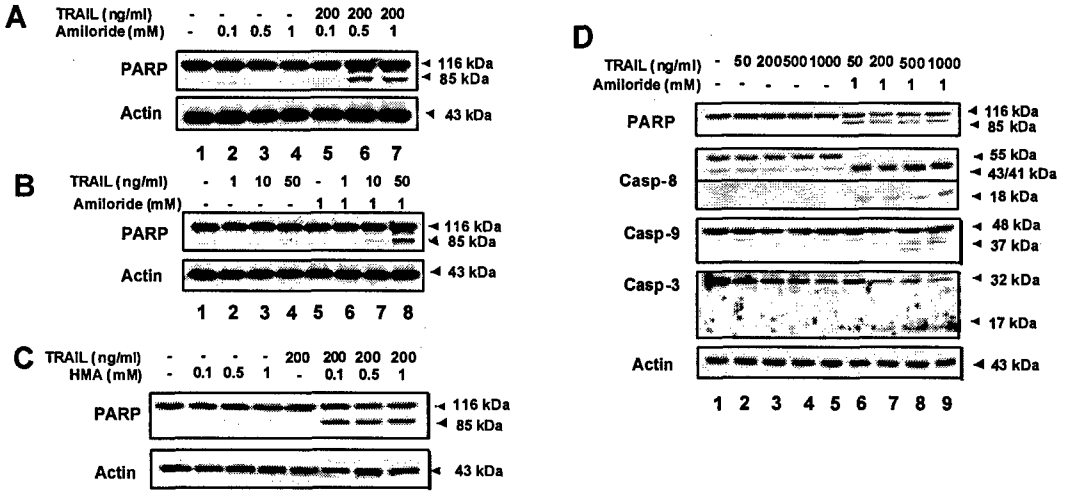

Figure 6. Effect of amiloride or HMA on TRAIL-induced proteolytic cleavage of PARP and activation of caspases in LNCaP cells. Cells were treated for 4 h with various concentrations of amiloride (0.1-1 mM) in the presence or absence of 200 ng/ml TRAIL (**A**), various concentrations of TRAIL (1-50 ng/ml) in the presence or absence of 1 mM amiloride (**B**), various concentrations of HMA (0.1-1 mM) in the presence or absence of 200 ng/ml TRAIL (**C**), or various concentrations of TRAIL (50-1000 ng/ml) in the presence or absence of 1 mM amiloride (**D**) and then harvested. Cell lysates were subjected to immunoblotting for PARP, caspase-8, caspase-9, or caspase-3. Antibody against caspase-8 detects inactive form (55 kDa), cleaved intermediates (41, 43 kDa), and active form (18 kDa). Anti-caspase-9 antibody detects both inactive form (48 kDa) and cleaved intermediate (37 kDa). Anti-caspase-3 antibody detects inactive form (32 kDa), and cleaved active form (17 kDa). Immunoblots of PARP show the 116 kDa PARP and the 85 kDa apoptosis-related cleavage fragments. Actin was used to confirm the amount of proteins loaded in each lane.

Task 3: Examination of the tumoricidal effect of plasmid expressing TRAIL on human prostate tumor xenografts in mice

In vivo studies were performed to investigate the effects of TRAIL on tumor growth. A recombinant plasmid, hFlex/TRAIL, was constructed, encoding the soluble form of the human Flt3L gene (hFlex) at the 5' end and the human TRAIL gene at the 3' end (Fig. 7). This design allows the TRAIL gene product to be secreted into the body circulation. An isoleucine zipper was added to the N-terminus of TRAIL. It is well known that addition of the zipper greatly enhances the trimerization of the fusion protein and dramatically increases its anti-tumor activity. The study consisted of 10 male athymic mice (nu/nu) mice randomized as to control and experimental status. Human prostate adenocarcinoma DU-145 tumors were established by subcutaneously injecting 2 x 10^6 cells into the dorsal surface of the mice. Tumors were measured 2-3 times per week and treatment was initiated when the tumors reached a mean volume of 100 mm^3. Mice were intravenously injected

with saline or the hFlex/TRAIL recombinant plasmids (10 μg). The hydrodynamic-based gene delivery protocol (Liu et al., 1999) was employed to deliver the recombinant DNA plasmids (10 μg) every week. Although this experiment is still in progress, Figure 8 demonstrates that tumor growth is being significantly delayed by hFlex/TRAIL plasmids administration.

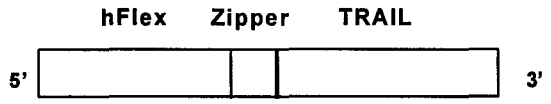

Figure 7. Schematic map of a recombinant DNA construct (phFlex/TRAIL) for TRAIL production.

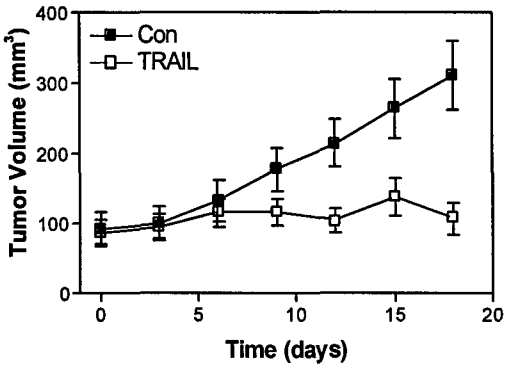

Figure 8. Effect of phFlex/TRAIL on growth of DU-145 cell xenograft tumors in nude mice.

Key research accomplishments

1) Low extracellular pH enhances TRAIL-induced cytotoxicity in human prostate carcinoma DU-145 cell line.
2) Low extracellular pH enhances TRAIL-induced apoptosis by increasing the mitochondria-mediated caspase signal transduction pathway (interaction between truncated Bid and Bax, and cytochrome c release).
3) Amiloride, an inhibitor of the Na^+/H^+ antiporter capable of lowering the intracellular pH, potentiates TRAIL-induced apoptotic death.
4) The growth of xenograft prostate tumor is delayed by hFlex/TRAIL plasmids administration.

Reportable Outcomes

Lee, Y. J., Song, J. J., Kim, J. H., Kim, H. R. C., and Song, Y. K. (2004) Low extracellular pH augments TRAIL-induced apoptotic death through the mitochondria-mediated caspase signal transduction pathway. Exp. Cell Res., 293:129-143.

Conclusions

We observed that low extracellular pH promotes tumor cell killing by TRAIL. We also observed that amiloride, an inhibitor of the Na^+/H^+ antiporter capable of lowering the intracellular pH, potentiates TRAIL-induced apoptotic death. These results suggest that acidification of the intracellular pH by blocking the Na^+/H^+ antiporter enhances TRAIL cytotoxicity. Therefore, it can be useful to combine TRAIL gene therapy with compounds that cause the acidification of tumor microenvironments, such as amiloride, for cancer gene therapy.

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