Treatment of Malignant Melanoma by Downregulation of XIAP and Overexpression of TRAIL with a Conditionally Replicating Oncolytic Adenovirus

Xin-Qiu Li, Xian-Zhu Ke, Yu-Ming Wang*

Abstract

**Background and Aim:** Currently available systemic therapies for malignant melanoma produce low response rates in patients, and more effective treatment modalities are clearly needed. The tumor necrosis factor (TNF)-related apoptosis-inducing ligand has a significant impact on therapy for patients with X-linked inhibitor of apoptosis protein-downregulation malignant melanoma. The primary objective of this study was to assess its therapeutic potential. **Materials and Methods:** We employed a conditionally replicating oncolytic adenoviral vector, named CRAd5.TRAIL/siXIAP, with the characteristics of over-expression of the therapeutic gene TRAIL and downregulation of XIAP in one vector. B16F10-luc cells were employed to detect anti-tumor activity of CRAd5.TRAIL/siXIAP in vitro and in vivo. **Results:** CRAd5.TRAIL/siXIAP enhanced caspase-8 activation and caspase-3 maturation in B16F10 cells in vitro. Furthermore, it more effectively infected and killed melanoma cells in vitro and in vivo than other adenoviruses. **Conclusion:** Taken together, the combination of upregulation of TRAIL and downregulation of siXIAP with one oncolytic adenoviral vector holds promise for development of an effective therapy for melanomas and other common cancers.

**Keywords:** Malignant melanoma - TNF-related apoptosis-inducing ligand - conditionally replicating oncolytic adenovirus

Introduction

Malignant melanomas can spread to the blood and lymphatic systems of the body, which enables it to metastasize to other areas (Bellew et al., 2009). It is important to treat malignant melanoma as early as possible to prevent its spread beyond the top levels of skin. Treated early and effectively, it is possible to survive this form of skin cancer. When it was detected in the terminal cancer, traditional treatments such as surgery, radiotherapy, chemotherapy, alone or in various combinations, are not very effective. Therefore, innovative treatments are urgently needed. Cancer gene therapy has been evaluated as a novel and promising treatment modality (Verma and Somia, 1997; Ruiz et al., 1999; Mitry et al., 2000; Douglas, 2007).

Several vector systems, including adenovirus, retrovirus, herpes simplex virus, and cationic lipid vector systems, have been used for such treatments. Among them, adenovirus vectors have several advantages over other vectors; they can easily be grown to high titers and can efficiently transfer foreign genes into both dividing and nondividing cells (Volpers and Kochanek, 2004; Ghosh et al., 2006; Sharma et al., 2009). Conditionally replicating oncolytic adenovirus (CRAd) is a potential therapeutic adenovirus for cancer (Alemany, 2007; Short and Curiel, 2009), because it can selectively replicate in tumor cells and kill them, but not in normal cells. Among them, a mutant adenovirus named ONYX-015 which was engineered not to express the E1B55-kD viral protein was originally reported to preferentially target and destroy p53-dysfunctional tumor cells but not the normal cells (Bischoff et al., 1996; Khuri et al. 2000). In addition, the release of conditionally replicative adenovirus progeny by infected tumor cells provides a potential to amplify the oncolytic effect by lateral spread through solid tumors (Kirm et al., 2001; Jiang et al., 2003; Zhu et al., 2008).

TNF-related apoptosis-inducing ligand (TRAIL) appears to be a promising candidate for cancer therapeutics because of its ability to preferentially induce apoptosis in malignant cells (Walczak and Krammer, 2000; Holoch and Griffith, 2009). The potential significance of TRAIL as an anti-cancer agent has been supported by studies in animal models showing selective toxicity to human tumor xenografts (Walczak et al., 1999). TRAIL induces apoptosis by recognizing and binding to its cognate receptors on cell surfaces. These receptors are known as death receptor 4 (DR4; TRAIL receptor 1; TRAILR1) and death receptor 5 (DR5; TRAIL receptor 2; TRAILR2). Binding initiates conformational changes in the receptors
and recruits an adaptor molecule (Fas-associated death domain) and initiator caspases (caspase-8 and -10) to form a death-inducing signaling complex. This process activates caspase-8 and -10, which can then directly activate effector caspases (caspase-3, -6, and -7) to cause apoptosis. Because TRAIL can induce apoptosis in cancer cells but has little effect on normal cells, it is considered a promising anticancer agent (Wang and El-Deiry, 2003; Hall and Clevet, 2007). TRAIL-based therapies, including recombinant human TRAIL and DR4/DR5-specific agonistic monoclonal antibodies, are currently undergoing phase I and II clinical trials (Huang and Sheikh, 2007).

However, the potential application of TRAIL in cancer therapy is limited as many cancer cells are found to be resistant to the cytotoxicity of TRAIL. The resistance may be due to low expression of proapoptotic molecules (death receptors or caspase-8) or high expression of antiapoptotic molecules (decay receptors, FLICE-like inhibitory protein (FLIP), inhibitor of apoptosis proteins (IAP), and Bcl-2) (Wang and El-Deiry, 2003). Thus, combination TRAIL with other agents has been a promising strategy to potentiate the cytotoxicity of TRAIL and its therapeutic applications (Huerta-Yepez et al., 2004; Rosato et al., 2004; von Haefen et al., 2004). Several phytochemicals, such as luteolin, also appear to be effective at overcoming TRAIL-resistance via degradation of XIAP (Shi et al., 2005), and 3, 3-diindolylmethane down-regulates cFLIP (Zhang et al., 2005). In addition, proteins of the Bcl-2 family, which are key regulators of apoptosis through the intrinsic mitochondrial pathway, are often deregulated in cancers and can be manipulated to achieve TRAIL sensitization (Gross et al., 1999; Suliman et al., 2001).

In this study, we employed a conditionally replicating oncolytic adenoviral vector named CRAd5.TRAIL/siXIAP with over-expression of the therapeutic gene TRAIL and downregulation of XIAP in one vector. CRAd5.TRAIL/siXIAP enhanced caspase-8 activation and caspase-3 maturation in B16F10 cells in vitro. Further, the synergistic effect was observed in inhibition of tumor cell proliferation and growth of tumor xenografts in nude mice. Our data showed that the antitumor effect of the combined use of CRAd5.TRAIL/siXIAP is more evident than each adenovirus used alone and suggest novel promising strategy for cancer gene therapy.

Materials and Methods

Cell lines and culture condition

HEK293 (from American Type Culture Collection, Manassas, VA), and B16-F10-luc (provided by Xenogen Corporation, Alameda, CA) cultured in 10% DMEM (high glucose) containing 10% newborn calf serum, 4mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin in a humidified 5% CO2/95% air atmosphere at 37 °C (Anderson et al., 2000).

Virus Construction

The human TRAIL gene was acquired by RT-PCR from HEK293 cells. The CMV-TRAIL and hU6-siXIAP (target sequence is 5’ GGA CAT CTA GTC ACT TGT AAA 3’) cassette was obtained by the common molecular method, named pcDNA-CMV-TRAIL-hU6-siXIAP. pShuttle and pAdenoX backbone were from Clontech, San Jose, CA (currently BD, Franklin Lakes, NJ) (Haviv, 2009). Digested pcDNA-CMV-TRAIL-hU6-siXIAP and pShuttle fragments were purified by gel extraction after agarose gel electrophoresis. To clone the CMV-TRAIL-hU6-siXIAP cassette from pcDNA-CMV-TRAIL-hU6-siXIAP into pShuttle, a ratio of 3:1 (insert: digested pShuttle) was optimal. Ligation of the I-CeuI/PI-SceI flanked pShuttle-CMV-TRAIL-hU6-siXIAP fragment (after digesting 1 μg of pShuttle-CMV-TRAIL-hU6-siXIAP for 3 hrs) into 750 ng of the pAdeno-X backbone was performed with T4 DNA ligase overnight at 16 °C. Cloning efficiency was enhanced by heat inactivation of the T4 DNA ligase and by digesting with SwaI that recognizes a unique site between the I-CeuI/PI-SceI sites of the Adeno-X backbone (but not within the I-CeuI/PI-SceI fragment from pShuttle-CMV-TRAIL-hU6-siXIAP), thereby preventing religation of the E1-deleted backbone. The recombinant CRAd5.TRAIL/siXIAP plasmid was used to transform 50 μl chemical-competent DH5α bacteria via 60 sec heat-shock and selection with ampicillin (100 μg/ml). The resulted recombinant adenovirus was named CRAd5.TRAIL, CRAd5.siXIAP and Ad5.eGFP, respectively. Particle titers of adenovirus preparations were determined using an absorbance at 260 nm and plaque assays in HEK 293 cells.

Western blotting

A total number of 2 × 10^5 of B16F10-luc were plated into a 60 mm plate the day prior to adenovirus infection. 10 multiplicities of infection (MOI) of each recombinant adenovirus was used for infection. Cells were harvested in radioimmunoprecipitation assay (RIPA) buffer (1xphosphate buffered saline, 1% NP-40, 0,5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)) containing a protease inhibitor cocktail (Roche, St. Louis, MO) for 48 h after adenovirus infection. Proteins were quantified using Bradford methods and 25–60 mg protein per lane was analyzed on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Separated samples were transferred onto a polyvinylidene fluoride (PVDF) transfer membrane. Membranes were incubated for 3 h at room temperature in antibody dilution buffer (5% BSA, 0,3% Triton X-100 in 1xphosphate buffered saline) containing different dilutions of primary antibodies: anti-TRAIL (1:500); anti-XIAP (1:1,000) and anti-GAPDH (1:5,000). The anti-GAPDH was used as a loading control for western blot analysis. The membrane was then washed stringently in PBS-T (0.1% tween in 1xPBS buffer) three times followed by incubation for 1 h in 1:10,000 dilution of horseradish peroxidase–labeled antimouse or antirabbit secondary antibody. After washing three times in PBS-T, bands were visualized using an ECL Western blotting kit (Millipore, Piscataway, NJ).

MTT analysis

B16F10-luc and mouse embryonic fibroblast (MEF) cells were seeded in 96-well tissue culture plates (2×10^3 cells per well) and the next day treated with various adenoviruses. After exposure to adenovirus with 0.01, 0.1,
1, 5, 10 MOI for 48 h, 20 μL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma, St Louis, USA) solution (2 mg/ml) were added to each well, and the plates were incubated for additional 4 h at 37 °C. MTT solution in medium was aspirated off. DMSO (200 μL) was added to each well before absorbance at 570 nm was measured. A statistical analysis of the results was performed using the Analysis Tool Pack provided by Microsoft Excel.

Animal Studies

All mice received human care in compliance with the guidelines for the care and use of laboratory animals in research. Female 4-6 weeks old athymic nude mice were purchased from Shanghai Experimental Animal Center, China and were quarantined for 1 week under pathogen-free conditions. 2×10^6 B16-F10-luc cells were xenografted s.c. into the right flank of the mice under anesthesia (8 mice for each group). When tumor volumes reached approximately 50 mm³, the animals were assigned to four groups of Ad.eGFP, CRAd5.TRAIL, CRAd5.siXIAP and CRAd5.TRAIL/siXIAP to be treated with a total dose of 2×10^8 plaque-forming units (PFU). A dose of 5×10^5 PFU in 50 μL PBS was administered intratumorally every two days for four times. Tumor size was measured at intervals of two days. Tumor volume (mm³)=length×width²/2. At the end of the experiment, tumors were resected from the sacrificed mice for immunohistochemistry assay.

Immunohistochemistry assay

Tumor tissues were removed from treated mice, fixed in 4% paraformaldehyde at room temperature for 48 h and embedded with paraffin. Tissue sections from paraffin blocks were cut, dewaxed, and hydrated. Sections were treated with 3% H₂O₂ for 10 minutes to inactivate endogenous peroxidase, and incubated with the tissues with 20 μg/ml proteinase K for 1 h at 37 °C. Immunohistochemical staining was done with a monoclonal rat anti-mouse CD31 (Santa Cruz, USA) and a second antibody. All steps were performed according to the kit manual. Hematoxylin was used as counterstain. Antibody against the CD31 antigen on endothelial cells for the assessment of intratumoral microvessel density. The number of microvessels was evaluated using the weidner standard of scoring neovascular “hotspots” with the highest microvessel density.

TdT-mediated dUTP-biotin nick end-labeling assay

A terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) method was used for the detection of apoptotic cells. The TUNEL assay was performed according to the manufacturer’s procedures (Promega, USA). The number of apoptotic cells were calculated on ten randomly selected visual fields under a light microscope on 400x magnification. The apoptotic index was calculated by the formula: apoptotic index = (total number of apoptotic cells/total number of cells) × 100%.

Statistical analysis

Determinations of significant differences among groups were assessed by One-Way ANOVA analyses and student’s t test, results were expressed as mean±SD. P value<0.05 was considered significant.

Results

Recombinant adenovirus CRAd5.TRAIL/siXIAP

The expression cassette of CMV-TRAIL and hU6-siXIAP was inserted into modified pDNA3.1 (+) vector, and then the expression cassette of CMV-TRAIL and hU6-siXIAP was inserted into pAdshuttle vector. A pAdshuttle plasmid, constructed on the basis of the pShuttle of the AdEasy system, was employed to deletion of E1B55Kd of E1. However, instead of using this shuttle plasmid for HR, we further subcloned an E1B55Kddeleted, CMV-TRAIL and hU6-siXIAP construct from this shuttle plasmid. The modified E1 construct was then directly cloned into an E1/E3 deleted Ad backbone genome using in vitro ligation instead of HR. We inserted the expression cassette of CMV-TRAIL, hU6-siXIAP or CMV-eGFP into pAdshuttle vector, got pCRAd5.TRAIL, pCRAd5.siXIAP and Ad5.eGFP in the same way. All the vectors were then processed by transfecting PacI-linearized corresponding plasmid into HEK293 cells. The structures of pCRAd single expression and pCRAd dual expression were shown in Figure 1. To demonstrate that adenoviruses had the expected gene expression, virus-infected B16F10-luc cancer cells were examined for the expression of the target proteins by western blot assay (Figure 2).

Supression of cancer cell growth in vitro

In this study, to evaluate the cytotoxicity of the dual-expression of oncolytic adenoviruses in different cells, B16F10-luc and MEF were infected with CRAd5. TRAIL, CRAd5.siXIAP and CRAd5.TRAIL/siXIAP or replication-deficiency adenovirus Ad5.eGFP at MOIs of 0.01, 0.1, 1, 5 and 10 for 48 h. Cell viability was measured. A statistical analysis of the results was performed using the Analysis Tool Pack provided by Microsoft Excel.
An average of three independent experiments are shown ± s.d. Results were expressed as percentage of untreated control. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cytotoxicity of Oncolytic Adenoviral Vectors in Tumor Cells in Vitro. B16F10-luc cells were infected with Ad5.eGFP (lane 1), CRAd5.TRAIL (lane 2), CRAd5.siXIAP (lane 3) and CRAd5.TRAIL/siXIAP (lane 4) for 48 h and lysates underwent western blotting with anti-TRAIL, anti-XIAP and anti-GAPDH antibodies. Western Blot Assay of Target Gene Expression. The in vivo antitumor efficacy of the viruses was determined by MTT assay. The data showed that the virus could induce cytotoxicity. The cytotoxic effect of the CRAd5.TRAIL/siXIAP adenovirus in B16F10-luc cells were more apparent than that of CRAd5.TRAIL, CRAd5.siXIAP and Ad5.eGFP (Figure 3). In contrast, the replicative capacity was much reduced in normal MEF cells. These data indicated that over-expression of TRAIL gene and downregulation of XIAP did not affect the selective replicative ability of oncolytic adenoviruses. To determine the mechanism responsible for the synergistic inhibitory effects of the viruses CRAd5.TRAIL/siXIAP, activation of caspases were studied (Figure 4). The analysis of infected B16F10-luc cancer cells demonstrated the activation of the caspase cascade, including cleavage of caspase-3, caspase-8 and poly(ADP-ribose) polymerase (PARP) 48 h after infection with different oncolytic adenoviruses. The cleaved forms of caspase-8 and PARP increased and the uncleaved form of caspase-3 decreased, which indicated the activation of caspase cascade after treatment with CRAd5.TRAIL or CRAd5.TRAIL/siXIAP. The most obvious effect of caspase activation was observed in the cells treated with CRAd5.TRAIL/siXIAP. These results suggest that the combined use of TRAIL and siXIAP can induce the caspase cascade activation in a synergistic manner.

Antitumor efficacy of CRAd5.TRAIL/siXIAP in nude mice

The in vivo antitumor efficacy of the viruses was examined by intratumoral administration in B16F10-Luc xenograft tumors in nude mice. The tumor growth curves of each treatment group are shown in Figure 5. The tumors grew rapidly and reached 2,000 mm³ within 24 days in the Ad5.eGFP group. However, in the CRAd5.TRAIL and CRAd5.TRAIL/siXIAP groups, tumor was growing slowly. This was especially notable in the CRAd5.TRAIL/siXIAP group (155.7±45.67) in which the tumor volume of 8 mice was about 1/10 of the control group (1504.3±551.13) 14 days after adenoviruses injection. These results indicate that CRAd5.TRAIL/siXIAP has strong anti-melanoma activity in vivo.

Pathologic analysis of tumor samples

24 days after tumor cells injection, we sacrificed the mice and analyzed sections of the B16F10-luc tumors by immunohistochemistry. We measured the microvessels density (MVD) and apoptosis of tumor samples. Blood vessels were visualized using an antibody against CD31, a platelet/endothelial cell adhesion molecule. Immunohistochemical staining detection of anti-CD31, the morphological observation of vascularity of the tumor tissue sections (black arrow, Figure 6A) and microvesdels density analysis (Figure 6B) demonstrated that a significantly low microvessel density (8.40±2.12) (P<0.01) in CRAd5.TRAIL/siXIAP group, as compared with other groups, group of expressing TRAIL (23.50±2.22), group of TRAIL (23.50±2.22), group of siXIAP (26.50±2.55), and Ad5.eGFP (32.90±3.17).

To understand the mechanism underlying the antitumor effect of the CRAd5, we analyzed the apoptosis induced in different adenoviruses treated tumors by TUNEL staining. The results shown that the group of CRAd5.TRAIL/siXIAP has highest apoptotic index (25.95±2.35)%. The average percentages of TUNEL-positive cells from 10 random visual fields in the Ad5.eGFP, CRAd5. TRAIL and CRAd5.siXIAP groups were (2.03±0.59)%,
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Figure 6. Immunohistochemical Staining with Anti-CD31 antibodies to Assess the Microvessels Density (MVD). (A) Morphological observation of vascularity of the tumor tissue sections after incubated with anti-CD31 (black arrows for blood vessels); (B) analysis of tumor MVD, the MVD was calculated via immunohistochemical staining of CD31. Groups of CRAd5.TRAIL/siXIAP significantly blocking angiogenesis. Each bar represents the mean ± SD. **P<0.01. (n=10 in each group)

Figure 7. TUNEL Staining for the Detection of Apoptosis Cells. Tumors were harvested 24 days after the injection of different adenoviruses. Sections were stained with TUNEL to reveal the apoptotic activity in the tissue. TUNEL-positive cells were quantified and presented as mean ± SD. **P<0.01 (n=10 in each group)

(12.85±2.37)% and (7.08±1.58)%, respectively (Figure 7). These results indicate that TRAIL and siXIAP induced apoptosis in melanoma xenografts in nude mice.

Discussion

Because cancer cells are genetically and phenotypically complex and frequently harbor multiple abnormalities (El-Serag and Rudolph, 2007), it is reasonable that single agent such as oncolytic adenovirus alone is not sufficient for complete eradication of malignancies. Using oncolytic adenovirus with therapeutic genes has become an important issue in the treatment of cancers (Hermiston and Kuhn, 2002). The clinical results of using ONXY-015 as a therapeutic agent that a significant antitumor activity was only seen when viral treatment was combined with chemotherapy (Heise et al., 1997; Opyrchal et al., 2009). In order to improve the efficacy of ONXY-015 while avoiding the toxicity caused by systemic using chemotherapeutic agents, we presumed that the combination of ONXY-015 with therapeutic gene into the vector might be a good choice. Among different therapeutic genes, TRAIL appears to be a relatively safe and promising agent for cancer-specific therapy. TRAIL induces apoptosis in a wide range of cell lines and shows substantial antitumor activity in rodent xenograft models, such as colon, breast, multiple myeloma, glioma, and prostate cancers (Wang, 2010; Mahalingam et al., 2011). Systemic administration of TRAIL exhibited little or no toxicity in mice or nonhuman primates (Wang, 2010). Given the effectiveness of rhTRAIL in preclinical oncology studies, it is currently in clinical trials.

Despite aggressive therapies, resistance to current treatment protocols has been a major obstacle in clinical oncology. Most anticancer agents act through induction of apoptosis in target cells and defects in apoptosis programs may confer resistance (Debatin, 1997; Kaufmann and Earnshaw, 2000). X-linked inhibitor of apoptosis protein (XIAP) is associated with tumor genesis, growth, progression and metastasis, and acts by blocking caspase-mediated apoptosis (Holcik and Korneluk, 2001; Hunter et al., 2007). Knocking out XIAP with siRNA or antisense oligonucleotides restores chemosensitivity to a variety of malignant cell lines (Holcik et al., 2000; McManus et al., 2004). Finally, knocking out XIAP is not toxic to normal cells, as evidenced by a lack of significant pathology in the XIAP knockout mouse (Harlin et al., 2001). Hence, in this study, we used siXIAP to downregulate XIAP, thereby inducing apoptosis in melanoma cells.

Previous studies show that the combined use of different antitumor genes had significant antitumor effect. The combination of Ad-IL-2 and Ad-IL-12 (Addison, et al., 1998), Ad-Bax and Ad-TRAIL (Huang et al., 2002), D55-IL-24 and ZD55-TRAIL (an adenovirus with E1B55kDa deleted) has been studied (Zhao et al., 2006). All the above experiments have got good antitumor effect, but experimental adenovirus vectors in all studies can express only one target gene. So the adenovirus status affect the therapeutic effect in the cancer cells. In this study, we employed a conditionally replicating oncolytic adenoviral vector, named CRAd5.TRAIL/siXIAP, with the characteristics of over-expression of the therapeutic gene TRAIL and downregulation of XIAP in one vector. CRAd5.TRAIL/siXIAP enhanced caspase-8 activation and caspase-3 maturation in B16F10 cells in vitro. Further, CRAd5.TRAIL/siXIAP more effectively infected and killed melanoma cells in vitro and in vivo than other adenoviruses. Our study points the way for development of novel strategies for cancer gene therapy that involve the combined delivery of two aspects of the genes that can synergistically induce apoptosis in cancer cells.

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