Overexpression of Soluble TRAIL Induces Apoptosis in Human Lung Adenocarcinoma and Inhibits Growth of Tumor Xenografts in Nude Mice

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Abstract
Recombinant adeno-associated virus 2/5 (rAAV2/5), a hybrid rAAV-2 with AAV-5 capsid, seems to be a very efficient delivery vector for the transduction of the lung adenocarcinoma cell line A549. Infection of the A549 cell line with a rAAV2/5 vector encoding the extracellular domain of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, amino acids 114-281) resulted in secretion of soluble TRAIL (sTRAIL) and induction of apoptosis in these cells. rAAV2/5-sTRAIL mediated delivery and stable expression of sTRAIL resulted in the presence of the trimeric form of sTRAIL in sera of nude mice that were implanted with s.c. or orthotopic A549 tumors. The rAAV2/5-sTRAIL transduction of the tumors resulted in a statistically significant reduction in tumor growth and prolonged survival of the tumor-bearing animals. Primary cell culture, histologic examination of the tumors, and serum analyses showed the absence of detectable TRAIL-induced toxicity in normal tissues including the liver. The successful inhibition of lung cancer growth and the absence of detectable toxicity suggest a putative role for rAAV2/5-sTRAIL114-281 in the therapy of lung cancer.

Introduction
Lung carcinoma is the leading cause of malignancy-related mortality (1) with little change in survival rates over the past two decades (2). Non–small cell lung cancer now accounts for about two-thirds of lung cancers and metastasize as well as resistance to chemotherapy and radiation. Attempts to overcome resistance through increased dosage results in unacceptable toxicity and bystander damage to normal tissues.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo-2L) is a typical member of the tumor necrosis factor family, inducing apoptosis by activating death receptors (4). A number of studies have shown that both the membrane-bound and the soluble extracellular domain of TRAIL can induce apoptosis in a wide variety of tumor cell lines without affecting most normal cells (4–8). Repeated administration of recombinant and biologically active soluble TRAIL (sTRAIL) can induce tumor cell apoptosis, suppress tumor progression, and improve survival in tumor-bearing mice (5–7). Furthermore, combination treatment with TRAIL and chemotherapy or ionizing radiation can substantially enhance the cytotoxic effects of these agents, even in otherwise resistant tumors (8). However, the application of sTRAIL in cancer therapy is limited by its short half-life in vivo (9). In addition, the transfer and expression of adenovirus encoded TRAIL, or TRAIL/green fluorescent fusion protein (GFP/TRAIL), can induce apoptosis and apoptotic bystander effects in several human cancer cells in vitro (10) and in the in vivo xenograft models of human glioblastoma, breast (11), prostate (12), and liver carcinoma (13). However, the use of adenovirus causes hepatotoxicity through the innate and cell-mediated immune responses. In contrast, adeno-associated virus (AAV)-based vectors are less pathogenic but efficient vehicles for gene delivery. A recent report showed that a single i.v. administration of AAV resulted in extensive transduction of the cardiac and skeletal muscles of adult animals, thus demonstrating its potential in a range of clinical applications of gene therapy (14).

In this study, we used a recombinant AAV 2/5 (rAAV2/5) vector, expressing TRAIL114-281, to examine the effect of expression of this soluble form of TRAIL in A549 cells both in vitro and in two in vivo xenograft models of A549 lung carcinoma. The results indicate that rAAV2/5-sTRAIL may provide an effective form of therapy for lung cancer.

Materials and Methods

Animals. Surgical procedures and care of animals, approved by the Ethics Committee of the University of Hong Kong, were done according to institutional guidelines. Male 5- to 6-week-old BALBc nude mice were housed at a constant temperature and supplied with laboratory chow and water ad libitum on a 12-hour dark/light cycle.

Construction of the rAAV-sTRAIL and rAAV-eGFP Vectors. The sTRAIL cDNA insert encoding TRAIL amino acids 114 to 281 was amplified from TRAIL cDNA as previously reported (15). The cytomegalovirus enhancer/chicken β-actin promoter, cDNA, and poly(A) sequences were inserted into the inverted terminal repeats using appropriate restriction enzymes. To boost transgene expression, a woodchuck hepatitis virus posttranscriptional regulatory element was inserted into both constructs immediately after the expression cassette (16). DNA sequence analysis confirmed the integrity of the cDNA constructs and the vector.

Pseudotyping. Different pseudotypes of rAAV were generated by standard production and purification protocols (17). The rAAV titer was quantified by real-time PCR analysis.

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Transgene Expression In vitro. HEK293 or A549 cells were cultured in the complete medium before the addition of rAAV at a dose of 10^5 particles per cell in culture medium containing 2% fetal bovine serum for 8 hours, followed by incubation in complete medium containing 10% fetal bovine serum. The transduction efficiency with rAAV-eGFP was analyzed by fluorescence microscopy. The culture media of the transduced cells were collected and concentrated by vacuum desiccation for analysis of the secreted TRAIL.

Apoptosis and Cell Death. Cells, fixed for 20 minutes in 4% paraformaldehyde in PBS, were stained with Hoechst 33258 at 1 μg/mL in PBS for 15 minutes. Apoptosis was visualized with fluorescence microscopy and cytotoxicity was quantified by 3-(4, 5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium assay (Sigma, St. Louis, MO).

S.c. Tumor Xenografts and Assessment of Growth. A549 cells (5 × 10^6) were administered in the s.c. tissue of the left dorsal flank of 7-week-old male nude mice. Tumor growth was monitored regularly and volume (V) was calculated using the formula V = 1/2 × length × width^2. When tumor size reached ~50 mm^3 (about 10 days post-inoculation), animals were randomized into three groups and vectors were administered by intra-tumoral injection. Groups 1 and 2 received 3 × 10^11 particles rAAV2/5-sTRAIL or rAAV2/5-eGFP, respectively; and group 3 mice received PBS as control. Experiments were terminated when tumors reached 500 to 600 mm^3 in the control groups.

Orthotopic Lung Cancer Model and Assessment of Growth. Orthotopic implantation of A549 cells was done as described by Yamaira et al. (18). The left chests of anesthetized mice were incised and 20 μl aliquots of the A549 cell suspensions (5 × 10^7/mL), containing 1 mg/mL of Matrigel (BD Biosciences, Bedford, MA), were injected into the lung parenchyma through the intercostal space (~3 mm depth). One week later, animals were randomized into three groups receiving intratracheal injections of 30 μL PBS or 5 × 10^11 vector particles. Mice were sacrificed 45 days after tumor cell implantation. Animals were euthanized if they developed gross ascites, palpable tumors >2 cm, dehydrated, or weight loss >20% of initial body weight.

Analysis of Gene Expression and Microvessel Density. The primary antibodies, TRAIL, CD31, caspase-3, and caspase-8 were from Santa Cruz Biotechnology (Santa Cruz, CA), and CD34 were obtained from PharMingen (San Diego, CA). Western blot analysis, histology, immunohistochemical staining, ELISA, and terminal nucleotidyl transferase (TdT)–mediated nick end labeling assays were carried out as previously described (19). Microvessel density was assessed by the hotspot method (20).

Statistical Analysis. Log-rank tests were done for survival data. Student’s t test was used to evaluate statistical significance. P values were considered to be statistically significant when <0.05.

Results

To investigate the transduction efficiency of different rAV serotypes, rAAV2/1, rAAV2/2, rAAV2/5, rAAV2/6, and rAAV2/8 vectors expressing eGFP were used to infect the A549 human lung cancer cell line at a multiplicity of infection (MOI) of 5 × 10^5. Flow cytometric analysis showed transduction rates to be 32.6 ± 3.7% for rAAV2/1, 50.8 ± 7.4% for rAAV2/2, 70.4 ± 6.7% for rAAV2/5, 55.5 ± 3.5% for rAAV2/6, and 18.6 ± 7.8% for rAAV2/8 (see Fig. L1).

Induction of A549 Tumor Cell Apoptosis by rAAV2/5-sTRAIL. To determine the apoptosis-inducing activity of rAAV2/5-sTRAIL, A549 cells were infected with rAAV2/5-sTRAIL or rAAV2/5-eGFP, stained with Hoechst 33258, and microscopically examined for evidence of apoptosis (Fig. 1B). Condensed nuclei, a characteristic of apoptosis, were present in substantially greater numbers in the rAAV2/5-sTRAIL, but not the rAAV2/5-eGFP–infected cells. Similarly, analysis of toxicity by the 3-(4, 5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium assay showed the induction of toxicity in the rAAV2/5-sTRAIL, but not the rAAV2/5-eGFP–infected cells. There was also no evidence of toxicity in the transduced HEK293 cells (Fig. 1C). To further test apoptosis, A549 cells infected with rAAV2/5-sTRAIL (MOI = 5 × 10^5) were subjected to Western blot analysis, revealing the presence of activated caspase-3 and decreased levels of caspase-8 precursor protein (Fig. 1D).

Next we tested whether the encoded TRAIL could be secreted into the medium. ELISA showed a peak value of 282.9 ± 69.7 ng/mL at 48 hours in the culture media of rAAV2/5-sTRAIL–transduced HEK293 cells. In contrast, the maximal sTRAIL concentration was only 97.6 ± 41.9 ng/mL in the similarly transduced A549 cells, and this level was reached 24 hours post-infection. The lower maximal expression level and the absence of further increases are likely to be due to the toxic effects of TRAIL on A549 cells. To test the potential toxicity of rAAV2/5-sTRAIL on normal cells, primary human hepatocytes were infected with rAAV2/5-eGFP or rAAV2/5-sTRAIL.
There was no apparent evidence of rAAV2/5-sTRAIL–induced toxicity in these cells, even when the vector dose was increased 4-fold to an MOI of $2 \times 10^5$ and the duration extended to 5 days (Fig. 2). Together, these results show that sTRAIL is secreted from rAAV2/5-sTRAIL–infected cells and that it can induce apoptosis in the human lung cancer A549 cells, but not in the normal human hepatocytes.

**Suppression of S.c. Tumor Growth.** The s.c. model was employed (see Materials and Methods) to analyze the therapeutic potential of rAAV2/5-sTRAIL. The monitoring of tumor growth for ~7 weeks revealed substantial growth reduction in the rAAV2/5-sTRAIL–injected tumors (Fig. 3A), but not in the rAAV2/5-eGFP or PBS control groups. Histologic examinations confirmed smaller tumors in the rAAV2/5-sTRAIL group, with large areas of cell death and substantially reduced numbers of scattered tumor cells (Fig. 3B). Evidence to support tumor cell apoptosis was also provided by terminal nucleotidyl transferase (TdT)–mediated nick end labeling assays demonstrating significantly higher levels of apoptotic cell death in the sTRAIL–transduced tumors on day 45 (Fig. 4A). Immunohistochemical studies confirmed TRAIL expression 45 days after intratumoral injection of rAAV2/5-sTRAIL, but not in the PBS-injected tumors (Fig. 4B). Moreover, there were significant reductions in the microvessel density in the rAAV2/5-sTRAIL–treated animals compared with the two control groups ($P < 0.05$; Fig. 4C and D).

**Suppression of Orthotopic Lung Tumor Growth Following Intratracheal Administration of rAAV Viruses.** Gross macroscopic examination of the chest cavity showed the presence of tumor nodules on both the left and right lobes of the lung of orthotopic model (Materials and Methods), particularly in the control groups (Fig. 5A), with heart metastases in ~30% of the PBS and 20% of the rAAV2/5-eGFP–treated animals (data not shown). Microscopic examination of H&E-stained lung tissue sections confirmed extensive metastatic disease in the control groups, but substantially reduced tumor presence in the lung tissue of the rAAV2/5-sTRAIL–transduced animals (Fig. 5B).
Intratracheal administration of rAAV2/5-sTRAIL suppresses orthotopic A549 tumors in nude mice. Tumor cells (1 × 10^6) were implanted into the lung parenchyma through the intercostal space. One week later, animals were randomized into three groups and subjected to intratracheal administration of 5 × 10^11 particles of rAAV2/5-sTRAIL, rAAV2/5-eGFP or the carrier PBS (n = 7). Mice were sacrificed on day 45. A substantial reduction in the number of tumor nodules was visible in the lungs of the rAAV2/5-sTRAIL–transduced animals, but not in those injected with rAAV2/5-eGFP or the carrier PBS (A and C). B, histologic examination of H&E-stained lung tissue sections (×100 magnification). C, columns, the number of tumor nodules in the lungs; bars, SD (n = 5). D, immunochemical detection of sTRAIL protein in the lung tissue was examined 38 days after rAAV2/5-sTRAIL transduction (brown, ×800 magnification). E, Western blot analysis of serum proteins 38 days after the intratracheal transduction with rAAV2/5-sTRAIL. The trimeric form of sTRAIL presents in the serum of the rAAV2/5-sTRAIL group (lanes 2-4), but not in the rAAV2/5-eGFP group (lane 1).

was consistent with the macroscopically visible number of lung tumor nodules on day 45: 61 ± 12 and 58 ± 8 in the PBS and rAAV2/5-eGFP groups, compared with 20 ± 12 in the rAAV2/5-sTRAIL treated group (P < 0.01 for either the PBS or rAAV2/5-eGFP groups; Fig. 5C). By day 60, the number of lung tumor nodules had increased further in the TRAIL-treated groups. However, this was still significantly lower than the number of nodules in the PBS (P = 0.017) or the rAAV2/5-eGFP–treated groups (P = 0.015).

The expression of TRAIL in lung tissue sections isolated from the rAAV2/5-sTRAIL–transduced group was confirmed by immunohistochemistry. The highest levels of TRAIL expression were in the airway epithelial cells (Fig. 5D) with biologically active trimeric form of TRAIL in the sera of the rAAV2/5-sTRAIL–transduced animals (Fig. 5E), corresponding to a circulating serum level of 114 ± 29 ng/mL at 38 days after the intratracheal rAAV2/5-sTRAIL administration. The statistically significant reduction in the microvessel density in the rAAV2/5-sTRAIL–treated mice, measured on day 45 after tumor cell implantation (P < 0.05; Fig. 6A), was no longer significant by day 60.

Discussion

Adenovirus-based vectors are among the most efficient vehicles for the transfer and expression of therapeutic genes (11–13). However, immunologic responses to adenovirus vectors result in
the immune-mediated clearance of the transduced cells, hence reduced level and duration of expression of the encoded gene products, as well as limited scope for repeat administration of these vectors (21). The reduced immunogenicity of AAV-based vectors provides distinct advantages with respect to each of these limitations. Nevertheless, despite some success with AAV2-based vectors in the lung (22), the luminal introduction of AAV has had largely disappointing results (23). Vectors based on AAV5 or AAV6 have been shown to enter from the apical side and more efficiently transduce airway epithelial cells (24). Capsid proteins from AAV5 and AAV6 have also been shown to bind to sialic acid (24), a component of the putative specific receptor present on the apical surface of airway epithelial cells. Consistent with these observations, the rAAV2/5 vector generated in the present studies has provided an efficient vehicle for the delivery and expression of TRAIL in both mouse airway epithelia and in the A549 human lung adenocarcinoma cells. As a consequence, high levels of TRAIL protein were readily detectable both at the site of vector administration and in the serum of these animals for at least 38 days after a single intratracheal administration of the vector. The serum levels detected for TRAIL (114 ± 29 ng/mL) were similar to the levels detected 2 hours after a single i.v. injection of 10 mg/kg recombinant TRAIL protein in nude mice (9). A tet- or rapamycin-dependent regulatory system has been developed for AAV-mediated delivery and inducible expression of the encoded genes (25). The regulated expression of TRAIL is likely to be an important requirement for the clinical application of TRAIL-based gene therapy, as it would reduce the risk of toxicity due to long-term expression.

The major concern with the application of TRAIL in the treatment of tumors in vivo is its controversial role in hepatic cell death (26–28). Interestingly, TRAIL studies showing hepatotoxicity are all either with the full-length membrane-bound form of the protein (28) or, if soluble, in combination with exogenous sequence tags (27). A histidine-tagged TRAIL has been shown to have an altered protein conformation, reduced stability, decreased solubility, and hepatotoxicity (29). However, the same protein without the histidine tag was able to trimerize adequately, giving it biological activity and neoplastic cell toxicity, with little or no evidence of toxicity to primary human hepatocytes in vitro (29). These observations are consistent with the absence of toxicity in the present study. Even the addition of 1 µg/mL recombinant sTRAIL prepared in our laboratory did not induce apoptosis in freshly isolated human hepatocytes in culture (data not shown). It would therefore seem that sTRAIL lacks the hepatotoxicity that is associated with other forms of TRAIL, but has the ability to induce apoptosis in a variety of tumor cell lines, including the A549 lung adenocarcinomas. The hepatotoxicity of TRAIL may be affected not only by the nature of TRAIL, but also by the physiologic condition of the hepatocytes and other interacting factors, such as innate and adaptive immune responses to the vector. Therefore, the adenovirus-induced up-regulation of TRAIL receptor DR5 may be an important contributory factor in the reported hepatotoxicity of adenovirus-encoded TRAIL (30).

Although TRAIL has been shown to induce apoptosis in various cancer cells, there are discrepancies in the published data on the sensitivity of HEK293 cells to TRAIL. Some studies have found HEK293 cells to be sensitive to TRAIL toxicity (31), whereas others suggest the resistance of these cells to TRAIL-induced apoptosis (32). In the present study, we could detect no apoptosis in the rAAV2/5-sTRAIL–transduced HEK293 cells for about 4 days. One possible explanation is that the effect of sTRAIL is likely to be dose-and/or conformation-dependent for different cell lines. Cell line differences in receptor status and effector pathways may also account for different responses to TRAIL. The present study shows that the in vivo transduction of A549 tumors with rAAV2/5-sTRAIL resulted in decreased presence of endothelial cell markers CD31 and CD34. Regulation of the angiogenic process involves a delicate balance of factors to promote and inhibit neovascularization. Tumors seem to be able to disrupt this balance, resulting in neoangiogenesis and development of a microenvironment favoring tumor growth and metastasis (33). Li et al. (34) have shown that isolated endothelial cells from human umbilical veins or human dermal microvessels, expressing DR4 and DR5, are sensitive to TRAIL-induced apoptosis. However, several other studies have shown the resistance of primary human endothelial cells to TRAIL-induced apoptosis, and even greater survival and proliferation of these cells by TRAIL-induced activation of the Akt and extracellular signal-regulated kinase pathways (35). Furthermore, adenovirus-mediated overexpression of MDA-7 seems to result in significant inhibition of growth in s.c. lung cancer tumors with decreased expression of CD31 and up-regulation of TRAIL (36). We observed reduced vasculature in s.c. tumors and in the initial but not in the later vascularization of orthotopic tumors, even though tumor growth was still significantly inhibited. The mechanism responsible for the observed effects of TRAIL on the proliferation of vascular endothelial cells remains to be elucidated.

In summary, the present studies provide the first report of rAAV-mediated delivery and expression of sTRAIL114-281, demonstrating that intratumoral or intratracheal injection of rAAV2/5-sTRAIL result in the presence of the trimeric form of sTRAIL in sera, a statistically significant reduction in the rate of tumor growth, and the prolonged survival of tumor-bearing mice. The rAAV2/5-sTRAIL administration did not cause any detectable toxicity either in the primary human hepatocytes in culture, or to any of the examined organs of the transduced mice in vivo, suggesting that AAV2/5-mediated sTRAIL gene therapy may provide a feasible and effective form of treatment for lung cancer.

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In the article on TRAIL gene therapy for lung cancer in the March 1, 2005, issue of Cancer Research (1), Dr. Dexian Zheng should have been listed as a co-corresponding author: Dexian Zheng, National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & Peking Union Medical College, 5 Dong Dan San Tiao, Beijing 100005, China. Phone: 8610-6529-6409; Fax: 8610-6510-5102; E-mail: zhengdx@pumc.edu.cn.

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