Complete Elimination of Colorectal Tumor Xenograft by Combined Manganese Superoxide Dismutase with Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand Gene Virotherapy

Yanhong Zhang,1,6 Jinfu Gu,1 Lili Zhao,1 Lingfeng He,1 Wenbin Qian,3 Jinhui Wang,1 Yigang Wang,2 Qijun Qian,2,4 Cheng Qian,2,5 Jian Wu,6 and Xin Yuan Liu1,2

1Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences; 2Xinyuan Institute of Medicine and Biotechnology, School of Life Science, Zhejiang Sci-Tech University, Hangzhou, China; 3Department of Hematology, First Affiliated Hospital, Medical College, Zhejiang University; 4Laboratory of Gene and Virus Therapy, Eastern Hepatobiliary Surgery Institute, Second Military Medical University, Shanghai, China; 5Division of Hepatology and Gene Therapy, School of Medicine, Centro de Investigación Médica Aplicada, University of Navarra, Pamplona, Spain; and 6Department of Internal Medicine, Transplant Research Program, University of California-Davis Medical Center, Sacramento, California

Abstract
Manganese superoxide dismutase (MnSOD) is a latent tumor suppressor gene. To investigate the therapeutic effect of MnSOD and its mechanisms, a replication-competent recombinant adenovirus with E1B 55-kDa gene deletion (ZD55) was constructed, and human MnSOD and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) genes were inserted to form ZD55-MnSOD and ZD55-TRAIL. ZD55-MnSOD exhibited an inhibition in tumor cell growth ~1,000-fold greater than Ad-MnSOD. ZD55-TRAIL was shown to induce the MnSOD expression in SW620 cells. Accordingly, by the combined use of ZD55-MnSOD with ZD55-TRAIL (i.e., “dual gene virotherapy”), all established colorectal tumor xenografts were completely eliminated in nude mice. The evidence exists that the MnSOD overexpression led to a slower tumor cell growth both in vitro and in vivo as a result of apoptosis caused by MnSOD and TRAIL overexpression after adenoviral transduction. Our results showed that the production of hydrogen peroxide derived from MnSOD dismutation activated caspase-8, which might down-regulate Bel-2 expression and induce Bax translocation to mitochondria. Subsequently, Bax translocation enhanced the release of apoptosis-initiating factor and cytochrome c. Cytochrome c finally triggered apoptosis by activating caspase-9 and caspase-3 in apoptotic cascade. Bax-mediated apoptosis seems to be dependent on caspase-8 activation because the inhibition of caspase-8 prevented Bid processing and Bax translocation. In conclusion, our dual gene virotherapy completely eliminated colorectal tumor xenografts via enhanced apoptosis, and this novel strategy points toward a new direction of cancer treatment. (Cancer Res 2006; 66(8): 4291-8)

Introduction
Reactive oxygen species (ROS), such as superoxide anions (O₂⁻), hydroxyl radicals, and hydrogen peroxide (H₂O₂), are physiologically generated as a consequence of aerobic respiration and substrate oxidation in aerobic organisms. Recent studies have shown that ROS are important signaling molecules in various cellular responses. Intracellular ROS have been reported to be involved in tumor necrosis factor-α (TNF-α)–induced apoptosis (1). ROS are believed to participate in signaling cell transformation because ROS are mitogenic to different cells and capable of inducing tumor progression (2). For instance, H₂O₂ and O₂ induce the expression of the oncogenes c-fos, c-myc, and c-jun. When the level of H₂O₂ exceeds the capacity of the mitochondria and cells to detoxify it, the resulting oxidative stress will activate the mitochondrial permeability transition pore (3). This results in the release of cytochrome c, procaspase-2, procaspase-3, procaspase-9, apoptosis-initiating factor (AIF), and caspase-activated DNase. The released cytochrome c and the cytosolic factor Apaf1, in turn, activate the caspases, which degrade cytosolic proteins, whereas AIF and caspase-activated DNase translocate into the nucleus and degrade the chromatin (4).

Manganese superoxide dismutase (MnSOD) is located in the mitochondrial matrix, which converts O₂ into H₂O₂ and O₂. Thus, MnSOD is essential in protecting mitochondria against the damaging effects of O₂ and plays an important role in protection against oxidative stress. Data have shown that MnSOD activity is lower or undetectable in some human tumor cells that show the existence of different levels of other enzyme (5). Growing evidence supports the antitumor effects of MnSOD gene therapy in the models of colorectal, pancreatic, and human breast and prostate cancers (6–8). To our knowledge, there is no report available thus far in exploring the replication-competent recombinant adenovirus-mediated MnSOD gene therapy in colorectal cancer.

TNF-related apoptosis-inducing ligand (TRAIL) is a member of recently identified TNF receptor superfamily and currently is under development as a potential therapeutic agent because it kills many types of tumor cells via an apoptotic cascade while spares normal cells (9, 10). Previous studies with adenovirus-mediated TRAIL gene therapy achieved notable therapeutic efficacy, and the function of TRAIL has been reviewed in detail elsewhere (11–13). However, up to date, there is no report available that combines replication-competent adenovirus-mediated dual gene (MnSOD and TRAIL) therapy of cancer. In this article, we report the antitumor effect of recombinant adenovirus with E1B 55-kDa gene deletion, ZD55-MnSOD and its combination with ZD55-TRAIL, and explore the possible mechanisms underlying the action of MnSOD and TRAIL in both colorectal tumor cell line and an animal model of colorectal tumor xenograft.

Note: Y. Zhang and J. Gu contributed equally to this work.

Requests for reprint: Xin Yuan Liu, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai 200031, China. Phone: 86-21-54921126; Fax: 86-21-54921126; E-mail: xyluo@sibs.ac.cn or Jian Wu, Department of Internal Medicine, Transplant Research Program, University of California-Davis Medical Center, Sacramento, CA 95817. Phone: 916-734-8071; Fax: 916-734-8097; E-mail: jdwu@ucdavis.edu.

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Materials and Methods

Cell lines and culture conditions. Human lung fibroblast cell line NHLF-1, human colorectal cancer cell line SW620, and human hepatoma cell line BEL7404 were purchased from Shanghai Cell Collection (Shanghai, China). Human breast cancer cell line Bcap-37 was purchased from the American Type Culture Collection (Rockville, MD). The HEK293 cell line was obtained from Microbix Biosystems, Inc. (Toronto, Ontario, Canada). The NHLF-1 and HEK293 cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS). The Bcap-37 cell line was grown in RPMI 1640 supplemented with 5% FCS (Life Technologies, Inc., Grand Island, NY), and other cell lines were cultured in DMEM supplemented with 5% heat-inactivated FBS at 37°C in 5% CO2.

Plasmid and virus construction. MnSOD cDNA was transcribed by reverse transcription-PCR from human fetal liver cells (LO2), and the MnSOD sequence from this cDNA was further amplified by PCR. The amplification was done using forward (5'-CGGAATTCTAGAAGCAGCAGCTCTCCCC-3') and reverse (5'-TGCTCTAGAGCATACGATGCTGTTATAC-3') primers. The MnSOD gene (excised by EcoRI/XhoI) from pBlueScript (+)-MnSOD was cloned into pCA13, which was excised by EcoRI/XhoI after cloning to construct pCA13-MnSOD. The pZD55-MnSOD was constructed by inserting the whole gene expression cassette cut from pCA13-MnSOD using BglII into the corresponding site of pZD55. All plasmid constructs were confirmed by restrictiv enzyme digestion, PCR, and DNA sequence. pZD55-TRAIL was kindly provided by Dr. Songbo Qiu (Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China).

Generation, purification, and titration of adenovirus. Ad-MnSOD, ZD55-MnSOD, and ZD55-TRAIL were generated by homologous recombination of pCA13-MnSOD, pZD55-MnSOD, and pZD55-TRAIL, with adenoviral packaging vector pBHG6 (Microbix Biosystems, Toronto, Ontario, Canada) in 293T cells. Appropriately identified plaques were purified by two subsequent passages through 293T cells. The presence of the transgenes in finally isolated viral stock was confirmed by PCR. Quantities of recombinant adenovirus were amplified by infecting 293T cells and purified by cesium chloride gradient ultracentrifugation. The preparation of recombinant adenovirus was titrated by a plaque assay using 293T cells.

Cytopathic assay. SW620, BEL7404, and Bcap-37 tumor cell lines as well as NHLF-1 cell line were grown to subconfluence and infected with adenoviruses at various multiplicity of infection (MOI). Seven days after infection, cells were exposed to 2% crystal violet in 20% methanol for 15 minutes, then washed with distilled water (dH2O), and documented as photographs.

Cell viability assay. Cells were plated on 96-well plates at a density of 5 x 103 per well. When cells were grown to subconfluence, they were infected with adenoviruses at 10 MOI. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 20 μg/mL) was added to lyse the cells. Absorbance was read at 595 nm with an A595 mean value of untransduced cells and A595 mean value of transduced cells calculated as (length/C2 × 100%)

Gel assay for MnSOD activity. Cells were harvested with ice-cold PBS (0.05 mol/L; pH 7.8). After sonication and centrifugation, MnSOD activity was analyzed in the supernatant. Protein of each sample (30 μg) was loaded and fractionated on a 12% native polyacrylamide gel. Sodium cyanide (5 mmol/L) was used to inhibit CuZnSOD. The gel was stained according to the method of Beauchamp et al. (14), and achrromatic bands against dark blue background represented MnSOD activity.

Animal experiments. All procedures with experimental animals were done according to the institutional and NIH guidelines for the ethical use of animals. Male BALB/c nude mice at 4 to 5 weeks obtained from the Animal Research Committee of the Institute of Biochemistry and Cell Biology (Shanghai, China) were used in all of the experiments. Mice were inoculated s.c. with SW620 cells (2 x 106). After 10 days, when most of the tumor volume reached 100 to 150 mm3 in size, inoculated mice were randomly divided into six groups (eight mice per group). Intratumoral injection of different adenoviruses (5 x 105 daily) or PBS was done once every other day for a total of four times. After the beginning of the injection, tumor size was measured with a Vernier caliper every 3 days. The tumor volume (mm3) was calculated as (length x width2)/2.

Analysis of ROS generation. Intracellular ROS generation was assessed by the conversion of 2',7'-dichlorofluorescein (DCF) diacetate (DCFH-DA; Molecular Probes, Eugene, OR) to a fluorescent product, DCF, which was determined by flow cytometry. Cells were infected either with different adenoviruses or without adenovirus at 10 MOI for 48 hours. During the last 30 minutes of incubation, 10 μmol/L DCFH-DA was applied to cells. After quickly detached from the plate, cells were immediately subjected to flow cytometry. DCF-positive cells were counted as an indicator of H2O2 generation.

Cell cycle analysis. To analyze the DNA distribution, cells were harvested by trypsinization followed by washing with PBS and then fixed in 70% ethanol. The cell pellets were incubated at 37°C with 1 μg/mL RNase A. After 30 minutes, cells were washed with PBS and then stained with 500 μL (25 μg/mL) propidium iodide (PI) for 1 hour. Cellular DNA distribution was analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Bedford, MA). Cell cycle profile was analyzed using CellQuest software.

Subcellular protein fractionation. Cells with different treatments were washed with ice-cold PBS and then scraped from plates in 100 μL ice-cold lysing buffer [20 mmol/L HEPES (pH 7.4), 10 mmol/L NaCl, 1.5 mmol/L MgCl2, 20% glycerol, 0.1% Triton X-100, 1 mmol/L DTT, and protease inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN)]. The cells were spun at 1,000 rpm for 1 minute at 4°C, and the supernatant was centrifuged at 10,000 rpm for 30 minutes at 4°C to obtain mitochondrial fraction. The protein of the cytoplasmic fraction was used for Western blot analysis of Bcl-2, cytochrome c, AIF, caspase-2, caspase-3, caspase-9, poly(ADP-ribose) polymerase (PARP), and β-actin. For mitochondrial protein isolation, cells were harvested in MSH buffer [210 mmol/L mannitol, 70 mmol/L sucrose, 5 mmol/L HEPES, 1 mmol/L EDTA (pH 7.4), and protease inhibitor mixture]. After homogenizing, samples were centrifuged at 500 x g for 5 minutes at 4°C to eliminate nuclei and unbroken cells. The resulting supernatant was centrifuged at 10,000 x g for 30 minutes at 4°C to obtain mitochondrial fraction for Western blot analysis of Bax and tBid. Protein concentration was measured with a kit from Bio-Rad (Hercules, CA).

Western blot analysis. Proteins samples were diluted with SDS-PAGE loading buffer, boiled for 3 minutes before loading on a 15% SDS-PAGE gel, and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA). The membranes were blocked with 5% nonfat dry milk and then incubated with corresponding primary antibodies. After incubation with peroxidase-conjugated secondary antibodies, immunodetection was done using an enhanced chemiluminescence detection kit (Santa Cruz Biotechnology, Santa Cruz, CA). All primary antibodies were purchased from Santa Cruz Biotechnology, except the human anti-MnSOD (Stressgen Biotechnologies, San Diego, CA) and tBid (R&D Systems, Minneapolis, MN) primary antibodies.

Immunohistochemical analysis of human MnSOD. Tumor tissues were fixed in 4% formaldehyde, dehydrated with gradient ethanol, and embedded in paraffin. Tissue sections were then dewaxed and rehydrated according to a standard protocol. The sections were washed with PBS, treated with 3% H2O2, and then blocked with the blocking solution. This was followed by incubation with anti-human MnSOD primary antibody (1:2000 dilution) overnight. After being washed with PBS, tumor sections were incubated in PBS containing biotinylated rabbit anti-human secondary antibody and followed by incubation in the presence of avidin-biotin complex method reagent (Biogenex Laboratories, San Ramon, CA), and color was developed with 3,3′-diaminobenzidine (DAB). All sections were examined with bright-field microscopy.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. An in situ apoptosis detection kit (Sino-American
Biotechnology Co., Luoyang, China) was used to detect apoptotic cells in tumor tissue sections. Briefly, after being incubated with proteinase K (Merck Co., Darmstadt, Germany) and rinsed with dH2O, tumor sections were dewaxed with dimethylbenzene and rehydrated with gradient ethanol twice, each for 5 minutes. Endogenous peroxidase was blocked with 3% H2O2, and sections were incubated with equilibration buffer and terminal deoxynucleotidyl transferase (TdT) enzyme. Finally, the sections were incubated with antidigoxigenin-peroxidase conjugate. Peroxidase activity in each tissue section was shown by the application of DAB (peroxidase substrate kit, Sino-American Biotechnology). Sections were counterstained with hematoxylin.

Statistical analysis. The statistical significance of the difference between various groups in the same experiments was determined by ANOVA and Newman-Keuls test for multiple comparisons. In vivo survival curves were estimated with the Kaplan-Meier method by the log-rank test for pair-wise survival analysis. Statistical significance was assumed when \( P < 0.05 \). All experiments were repeated at least twice to confirm the reproducibility. All data were displayed as mean ± SD.

Results

Tumor-selective cytopathic effect of different adenoviruses. The replication-competent ZD55 was constructed for this study. The human MnSOD gene was inserted into pZD55 to form pZD55-MnSOD, and ZD55-MnSOD was produced by homologous recombination with pBHGE3. To investigate the tumor-specific cytopathic effects, tumor cells BEL7404, SW620, and Bcap-37, which are all lack of functional p53, as well as NHLF-1 cell, which has functional p53, were transduced with different adenoviruses. Cells were treated with MTT at indicated time points after transduction. Data were summarized from three experiments and expressed as histogram to reflect the cell viability after different adenoviral transductions. Transduced with Ad-MnSOD (a), ZD55-MnSOD (b), ZD55-TRAIL (c), the combination of ZD55-MnSOD with ZD55-TRAIL (d), and ONYX-015 (e).

Figure 1. A, selective cytopathy of different adenoviruses. NHLF-1 cell line and tumor cell lines SW620, BEL7404, and Bcap-37 were transduced either with different adenoviruses or without adenovirus at the indicated MOI. Seven days later, cells were stained with crystal violet. The remaining color formation was documented by photographs to reflect the survival cells. B, tumor cell viability at different time points after different adenoviral transductions. Cells seeded in 96-well plate were transduced with adenoviruses. Cells without adenoviral transduction were used as control. Cells were treated with MTT at indicated time points after transduction. Data were summarized from three experiments and expressed as histogram to reflect the cell viability after different adenoviral transductions. Transduced with Ad-MnSOD (a), ZD55-MnSOD (b), ZD55-TRAIL (c), the combination of ZD55-MnSOD with ZD55-TRAIL (d), and ONYX-015 (e).
1,000- to 10,000-fold titers compared with ZD55-MnSOD or ZD55-TRAIL alone (Fig. 1A). As expected, ZD55-MnSOD and ZD55-TRAIL or their combination barely caused any cell death in NHLF-1 cells. The cytopathic effects of various adenoviruses were further confirmed by MTT test as shown in Fig. 1B. The viability of different tumor cells significantly decreased after transduction with the combination of ZD55-MnSOD and ZD55-TRAIL at 10 MOI. However, the viability of NHLF-1 cells remained unchanged.

**Induction of MnSOD synthesis by ZD55-TRAIL transduction.** In addition to the increased expression of MnSOD in ZD55-MnSOD-transduced SW620 cells, enhanced MnSOD expression was also seen in ZD55-TRAIL-transduced cells tested by Western blot, whereas ONXY-015, which was used as a control viral vector, did not cause significant change in MnSOD protein expression compared with cells without transduction (Fig. 2A). To confirm this unexpected phenomenon, MnSOD activities were assayed by native gel electrophoresis. As shown in Fig. 2B, MnSOD activity was undetectable in untransduced SW620 cells nor in cells transduced with ONXY-015, whereas MnSOD activity in ZD55-TRAIL-transduced cells was almost as high as that in Ad-MnSOD-transduced cells. As expected, the MnSOD activity was much higher in SW620 cells transduced with ZD55-MnSOD or its combination with ZD55-TRAIL, which was consistent with Western blot analysis as shown in Fig. 2A. These results showed that the overexpression of MnSOD/TRAIL may account for the inhibition of tumor cell growth.

To further verify the enhanced MnSOD expression in ZD55-TRAIL-transduced cells, a H2O2-sensitive probe DCFH-DA was used to detect H2O2 generation. Figure 2C shows that there was a significant increase in the percentage of DCF-positive tumor cells infected with ZD55-MnSOD (P < 0.05) and its combination with ZD55-TRAIL (P < 0.01) compared with untransduced cells and that there was a marginal increase in the percentage of DCF-positive cells infected with ONXY-015 (P > 0.05) compared with control. It is also notable that there were almost the same DCF-positive cells after infection with ZD55-TRAIL as that of Ad-MnSOD-transduced cells. It is well known that O2- can be dismuted to H2O2 and O2 by MnSOD expression. Thus, these results indirectly indicate that MnSOD expression could be induced after ZD55-TRAIL transduction in SW620 cells and that the enhanced MnSOD expression led to the accumulation of H2O2 as evidenced by the elevated percentage of DCF-positive cells.

**Antitumor efficacy of ZD55-MnSOD and ZD55-TRAIL in vivo.** A model of SW620 human colorectal tumor xenograft was established in nude mice to investigate the antitumor efficacy of different recombinant adenoviruses in vivo. Tumor growth curves were plotted to compare the difference of their antitumor efficacy during a 13-week observation. As shown in Fig. 3A, there was a rapid decrease in mean tumor volume in animals receiving intratumoral injections of ZD55-MnSOD, ZD55-TRAIL, and their combination compared with those receiving injections of PBS, Ad-MnSOD, or ONXY-015 alone. Tumor growth was significantly inhibited after intratumoral injection of ZD55-MnSOD combined with ZD55-TRAIL, and the inhibition was more profound than those receiving injection of ZD55-MnSOD or ZD55-TRAIL alone. Moreover, intratumoral injection of ZD55-MnSOD or its combination with ZD55-TRAIL resulted in an improved survival rate compared with PBS, Ad-MnSOD, or ONXY-015 groups (Fig. 3B). Only one mouse died 63 days after inoculation with ZD55-MnSOD, whereas all established colorectal tumor xenografts were completely eliminated, and all nude mice survived until euthanasia on day 91 after receiving a combined injection of ZD55-MnSOD with ZD55-TRAIL.

**Immunohistochemistry of MnSOD and in situ detection of apoptosis.** To further verify the possibility that the antitumor effect of ZD55-MnSOD was due to the MnSOD overexpression in tumor xenografts, the presence of human MnSOD was examined by immunohistochemical staining using anti-human MnSOD antibody. It is evident that there was a strong expression of MnSOD in all xenografts with the injection of ZD55-MnSOD and that the highest expression of MnSOD was observed in tumor sections that received combined injections of ZD55-MnSOD with ZD55-TRAIL. MnSOD staining was also seen in tumor tissue receiving ZD55-TRAIL injection alone (Fig. 4A). These findings are consistent with the level of MnSOD detected by Western blot analysis in vitro and suggest that the antitumor effect of ZD55-MnSOD on colorectal tumor may be a consequence of MnSOD overexpression.

To reveal the possible mechanisms underlying MnSOD-induced tumor growth inhibition, a TdT-mediated dUTP nick end labeling technique was used.
Bax translocation induced the release of AIF and cytochrome c. Western blot analysis showed an increased expression of tBid and Bax in mitochondria, whereas Bcl-2 expression was decreased after infection with ZD55-MnSOD, ZD55-TRAIL, and their combination (Fig. 5A). At the same time, the expression of cytochrome c and AIF was found in the cytoplasm with the increased level of Bax/tBid in tumor cells transduced with different adenoviruses, whereas the levels of AIF and cytochrome c were barely detectable with the low-level Bax/tBid in SW620 cells without adeno viral transduction. These results indicate that the translocation of Bax occurred in cells transduced with ZD55-MnSOD, ZD55-TRAIL, and their combination and that the release of the cytochrome c and AIF from the mitochondria may be a consequence of the overexpression of MnSOD or TRAIL.

Caspase-8 mediated Bax translocation via H2O2 accumulation. It is reported that Bax and Bid required caspase-8 as an upstream regulator (15) and that Bax translocation to mitochondria is dependent on caspase-8 activation (16). In the present study, it was found that caspase-8 was activated as a result of MnSOD/TRAIL overexpression. With a high level of intracellular H2O2 dismuted from MnSOD, the activation of caspase-8 was elevated (Figs. 2C and 5A), which may promote Bax/tBid translocation as shown by the increased mitochondrial level of Bax/tBid. This finding suggested that the activated caspase-8 resulted in the cleavage of Bid, which, in turn, enhanced Bax translocation. To determine whether Bax/tBid translocation resulted from caspase-8 activation, a pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) was used. Figure 5C shows that Bax translocation was significantly blocked after treatment with Z-VAD-fmk, whereas no change of Bax was found in tumor cells without Z-VAD-fmk treatment. In contrast, the expression of Bcl-2 significantly increased after the use of Z-VAD-fmk. Taken together, we postulated that the activated caspase-8 resulted in the increased mitochondrial level of H2O2, which led to the increased MnSOD protein levels but also elevated MnSOD activity and the release of H2O2 from the mitochondria, thereby activating caspase-8. This is consistent with recent observation that H2O2 enhances caspase-8 activation, which cleaves Bid to its active truncated form, BID, in human leukemia HL60 cells (17). However, how H2O2 activates caspase-8 is not immediately apparent from our data. Thus, further study is needed to validate these explanations. Therefore, at this moment, we cannot absolutely rule out the possibility that Bax may be induced by H2O2 through other pathways (18).

Apoptosis cascade. To clarify the possible pathways leading to apoptosis, a specific family of broadly conserved proteases, named caspase (cysteine-dependent aspartate protease), which are the major effectors responsible for key pathways and cascades of apoptosis, has been investigated. A notable cleavage of caspase-9 and caspase-3 was observed after different adenoviral transductions. However, neither procaspase-9 nor procaspase-3 was cleaved in tumor cells without any adenoviral transduction. PARP, a target protein of caspase-3, was cleaved into 85-kDa protein by activated caspase-3 after adenoviral transduction as shown in Fig. 4B. a). A flow cytometric analysis was done to further determine the apoptotic cells after adenoviral transduction. As shown in Fig. 4C, MnSOD overexpression induced a distinct sub-G1 peak, which represents the population of apoptotic cells. The proportion of cells in sub-G1 phase was increased by MnSOD and TRAIL overexpression. Percentage of apoptotic cells infected with ZD55-MnSOD (49.2 ± 10.4%) and its combination with ZD55-TRAIL (77.2 ± 13.3%) was much higher (P < 0.01) than that in SW620 cells (3.3 ± 0.13%) without transduction. ZD55-TRAIL infection led 35.7 ± 6.9% cells to be apoptotic, which was higher than that in ONX-015 (24.2 ± 1.4%) or Ad-MnSOD (15.8 ± 1.4%; Fig. 4D). These results further documented that the combination of ZD55-MnSOD with ZD55-TRAIL transduction induced remarkable apoptosis in SW620 cells, which coincided with the antitumor effect in vivo (Fig. 3A).

Figure 3. A, antitumor effect of different adeno viral injections on SW620 tumor xenograft. Male BALB/c nude mice were inoculated s.c. with SW620 cells (2 × 106). Tumor size was recorded, and tumor volume was calculated. Arrow, starting of adenoviral injection. Points, mean (n = 8); bars, SD. Significant suppression of tumor growth was observed after the treatment with Ad-MnSOD, ZD55-MnSOD, ZD55-TRAIL, and their combinations at 8 weeks after intratumoral injection of adenoviruses, NS, P > 0.05; *, P < 0.05; **, P < 0.01. B, Kaplan-Meier survival curves of animals after the same treatments as above. Pair-wise log-rank test was used to analyze survival rates in different groups. Statistical significance: a, P < 0.05; b, P < 0.001, compared with PBS; c, P < 0.01; d, P < 0.001, compared with Ad-MnSOD; e, P < 0.05; f, P < 0.01, compared with ONX-015; g, P < 0.05, compared with ZD55-TRAIL; h, P < 0.05, compared with ZD55-MnSOD.

(TUNEL) assay was done to prove whether the action of ZD55-MnSOD, ZD55-TRAIL, and their combination was due to their proapoptotic effect. As shown in Fig. 4B, injections of ZD55-MnSOD, ZD55-TRAIL, and their combination caused profound cell death in tumor mass via apoptosis (Fig. 4B, b-d), whereas no apoptosis was found in tumor tissue receiving PBS injection (Fig. 4B, a).
3–4-fold higher than the controls. Our findings suggest that MnSOD, a new type of tumor suppressor gene, can inhibit tumor cell growth by inducing apoptosis in tumor cells. We showed here that the antitumor effect of ZD55-MnSOD was much more potent (∼1,000-fold) than that seen in Ad-MnSOD-transduced cells both in vivo and in vitro. Moreover, a combined injection of ZD55-MnSOD with ZD55-TRAIL in SW620 tumor xenograft resulted in a profound inhibition in tumor growth and completely eliminated all tumor masses in nude mice. These findings imply the great clinical application of ZD55-MnSOD and its combination with ZD55-TRAIL in addition to currently available chemotherapy or radiotherapy.

The function of TRAIL in induction of apoptosis had been studied in detail by others (19, 20). We showed in this study that ZD55-TRAIL transduction induced the expression of MnSOD in colorectal tumor cells (Fig. 2A–C). However, the mechanism of the induction of MnSOD by TRAIL was not clear and needs to be investigated in the future studies.

It is known that a moderate increase of H2O2 inhibits cell proliferation and contributes to apoptosis (21). Our results showed that MnSOD/TRAIL overexpression caused an accumulation of H2O2 in SW620 cells. We investigated the effect of H2O2 on cell cycle arrest and apoptosis in tumor cells with MnSOD overexpression and confirmed the enhanced production of H2O2 after MnSOD/TRAIL overexpression as indicated by DCF, which is a specific fluorescent probe for detection of intracellular H2O2 level (22). It is suggested that MnSOD/TRAIL overexpression enhanced the production of H2O2, which, at least in part, contributes to decreased tumor cell growth by prolonged cell cycle transition time from G1 to S phases.

The Bcl-2 family of proteins includes members that either promote (Bax and BID) or inhibit (Bcl-2 and Bcl-xL) apoptosis. All of them are responsible for signal transduction between cytosol and mitochondria in many pathways. The overexpression of MnSOD/TRAIL resulted in the accumulation of H2O2, which may elicit the cleavage of caspase-8 (Fig. 5B). Bid could be cleaved to tBid by caspase-8, and tBid promoted the Bax translocation from cytoplasm to mitochondria (23). This translocation was confirmed in the experiment with the presence of Z-VAD-fmk. The inhibition of caspase-8 by Z-VAD-fmk blocked the cleavage of Bid and the Bax

![Figure 4.](image)

**Figure 4.** A, representative micrographs of immunohistochemical staining for MnSOD in tumor tissue. Tumor sections were fixed, dewaxed, and incubated with primary antibodies against human MnSOD. Representative images of at least three experiments. Colorectal tumor in nude mice injected with PBS (a), ZD55-MnSOD (b), ZD55-TRAIL (c), and the combination ZD55-MnSOD with ZD55-TRAIL (d). B, detection of apoptotic cells in tumor tissue by TUNEL assay. Tumor sections were processed as described in Materials and Methods. Intratumoral injection of PBS (a), ZD55-MnSOD (b), ZD55-TRAIL (c), and the combination of ZD55-MnSOD with ZD55-TRAIL (d). C, representative record of flow cytometric analysis of apoptotic cells. Cells were trypsinized and fixed with 70% ethanol. Nuclei were counterstained with PI and analyzed for the DNA content by a flow cytometer. a, without adenoviral transduction; b, transduced with ZD55-MnSOD; c, transduced with ZD55-TRAIL; d, transduced with the combination of ZD55-MnSOD with ZD55-TRAIL. D, percentage of apoptotic cells with different treatments with the same experimental protocol in (C). *, P < 0.05; **, P < 0.01.
translocation. It is reported that cleaved caspase-2 is also necessary for efficient Bid cleavage (24), but we did not detect the cleaved caspase-2 in the present study. Thus, with respect to the initiation of apoptosis via caspase-8 activation, we speculated that H₂O₂-activated caspase-8 cleaves Bid to its active truncated form, tBid, which collaborates with Bax by translocation from the cytosol to the mitochondria. This action markedly enhanced the ability of Bax to induce the release of cytochrome c from mitochondria (23, 25), whereas cytochrome c elicited the activation of caspases and induced apoptosis (26). Therefore, the translocation of Bax accelerated apoptotic death in response to death signals.

Findings in the present study also showed that the elevated release of cytochrome c occurred with the translocation of Bax in SW620 cells transduced with ZD55-MnSOD or its combination with ZD55-TRAIL (Fig. 5A) and that the elevated cytochrome c in cytosol triggered the activation of caspase-9, which, in turn, activated caspase-3 (Fig. 5B) and eventually initiated and executed the apoptotic cascade as indicated by Western blot analysis and TUNEL assay (Fig. 4B). Thus, overexpression of MnSOD may finally result in enhanced apoptosis and shrinkage of the colorectal tumor xenografts transduced with ZD55-MnSOD, ZD55-TRAIL, or their combination. This complicated pathway is illustrated in Fig. 6.

In addition to the elevated release of cytochrome c, an increase in release of cytosolic AIF was also detected after adenoviral transduction (Fig. 5A). AIF is a proapoptotic flavoprotein, which is a key regulator of cell death. AIF is essential for normal mammalian development and participates in pathologic apoptosis. In the nuclei, it stimulates peripheral chromatin condensation and large-scale 50-kb DNA fragmentation (27). The enhanced apoptotic staining with Hoechst 33258 in AIF-positive cells implied its role in nuclear DNA fragmentation and may be attributed to AIF release in this study (data not shown).

In conclusion, we showed that the MnSOD overexpression by adenoviral transduction with ZD55-MnSOD, ZD55-TRAIL, and their combination inhibited tumor cell growth both in vitro and in vivo. The inhibitory ability of replication-competent ZD55-MnSOD is much more potent (1,000-fold) than replication-deficient Ad-MnSOD. We also showed that the accumulation of H₂O₂ resulted from MnSOD overexpression and led to the activation of caspase-8, which triggered an apoptotic cascade via Bax translocation. Another significant finding is that TRAIL overexpression induced MnSOD synthesis in adenovirus-transduced cells, which may partially account for the enhanced proapoptotic effect in colorectal tumor xenografts. A combined intratumoral injection of ZD55-MnSOD with ZD55-TRAIL completely eradicated all of the colorectal tumor xenografts and remarkably improved animal survival, which implied for the first time that this novel strategy of "dual gene virotherapy" approach may have great clinical potential in cancer therapy.

Figure 5. A. Western blot analysis of Bax, tBid, Bcl-2, cytochrome c, and AIF. After treatment with different adenoviruses for 48 hours, cytosolic or mitochondrial proteins extracted from SW620 cells were quantified and loaded. After SDS-PAGE, proteins were transferred to nitrocellulose membranes, and different primary antibodies were applied to analyze the expression of Bax, tBid, Bcl-2, cytochrome c (cyto C), AIF, and β-actin. B. roles of cytochrome c release in the cleavage of caspase-9, caspase-3, and PARP. After transduction with different adenoviruses, total cell extracts were loaded and Western blot analysis was done. Cleaved and uncleaved caspases and PARP. C. inhibition of Bax translocation by a pan-caspase inhibitor, Z-VAD-fmk (20 μmol/L) was added 1 hour before ZD55-MnSOD transduction. Samples from total mitochondrial fraction and cytosolic fraction were loaded. Primary antibodies to Bax, tBid, and Bcl-2 were used to detect the expression of Bax, tBid, and Bcl-2.

Figure 6. Possible mechanisms of MnSOD-induced apoptosis in colorectal tumor cells. Apoptosis is initiated by production of H₂O₂ resulted from MnSOD overexpression. The accumulation of H₂O₂ results in the activation of caspase-8, which leads to the translocation of truncated Bid and Bax from the cytosol to the mitochondria. The translocation of Bax may induce the release of cytochrome c, which, in turn, activates caspase-9 and initiates onset of apoptosis. Bax also induces the release of AIF, which may lead to the DNA fragmentation, and triggers the apoptotic cascade in nucleus directly. However, this pathway is inhibited by Z-VAD-fmk via enhancing the expression of Bcl-2. In addition, H₂O₂ may also induce the translocation of Bax from the cytosol to the mitochondria by an unknown pathway, which subsequently triggers the release of cytochrome c and AIF, and thus accelerates the apoptosis.
References