Potent Antitumor Activity of Oncolytic Adenovirus Expressing mda-7/IL-24 for Colorectal Cancer

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ABSTRACT

It has been demonstrated that interleukin 24 (IL-24, also called melanoma differentiation associated gene 7) exerts antitumor activity. In this study, we investigated whether oncolytic adenovirus-mediated gene transfer of IL-24 could induce strong antitumor activity. A tumor-selective replicating adenovirus expressing IL-24 (ZD55-IL-24) was constructed by insertion of an IL-24 expression cassette into the ZD55 vector, which is based on deletion of the adenoviral E1B 55-kDa gene. ZD55-IL-24 could express substantially more IL-24 than Ad-IL-24 because of replication of the vector. It has been shown that ZD55-IL-24 exerted a strong cytopathic effect and significant apoptosis in tumor cells with p53 dysfunction. Moreover, no cytotoxic and apoptotic effects could be seen in normal cells infected with ZD55-IL-24. Expression of IL-24 did not interfere with viral replication induced by oncolytic adenovirus. Activation of caspase 3 and caspase 9, and induction of bax gene expression, were involved in tumor cell apoptosis induced by ZD55-IL-24. Treatment of established tumors with ZD55-IL-24 showed much stronger antitumor activity than that induced by ONYX-015 or Ad-IL-24. These data indicated that oncolytic adenovirus expressing IL-24 could exert potential antitumor activity and offer a novel approach to cancer therapy.

OVERVIEW SUMMARY

In this study, we have successfully constructed a tumor-selective replicating adenoviral vector, ZD55, that can replicate and induce cytopathic effect in tumor cells with p53 dysfunction. To explore the maximal potency of this oncolytic vector, a therapeutic gene (IL-24) was incorporated into the vector system. Results showed that ZD55-IL-24 exhibited replication kinetics similar to those of ONYX-015. Importantly, the expression level of IL-24 increased with replication of the virus. The growth-suppressing and apoptosis-inducing effects on cancer cells infected with ZD55-IL-24 are more significant than with ONYX-015 or Ad-IL-24. Consequently, better antitumor efficacy was observed when ZD55-IL-24 was used in nude mice with subcutaneous human SW620 colon cancer. This is the first study to demonstrate that ZD55-IL-24, applied in a targeting gene-virotherapy strategy, has potential for gene therapy of human cancers.

INTRODUCTION

GENE THERAPY is the newest therapeutic strategy for treating human diseases and about 63% of gene therapy protocols have been used for cancer therapy. Great progress has been made in the field of adenoviral cancer gene therapy. For replication-defective adenoviruses, insufficient transduction has

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been the crucial obstacle (Bischoff et al., 1996). It is necessary to improve the efficiency of virus replication and cell killing while ensuring the specificity of adenoviral vectors for tumor cells. This goal can be attained by deletion of corresponding gene products that are not required for replication in tumor cells, but are needed in normal cells. The E1B 55-kDa gene-defective adenovirus ONYX-015 (CI-1042, or dl1520) was the first such adenovirus; it replicates selectively in p53-deficient tumor cells and kills cervical carcinoma, colon carcinoma, glioblastoma, and pancreatic adenocarcinoma cells with an efficiency comparable to that of wild-type adenovirus (Shinoura et al., 1999; Ries et al., 2000; Portella et al., 2002). It is currently undergoing phase II clinical trials for the treatment of squamous cell cancers of the head and neck (Kirm et al., 1998; Nemunaitis et al., 2000, 2001; Makower et al., 2003) and a phase I trial for primary carcinoma of the pancreas (Mulvihill et al., 2001). However, only a 15% remission rate could be obtained when using ONYX-015 alone (Khuri et al., 2000). A better result can be obtained by combining therapy with chemotherapeutic agents (Heise et al., 1997). Targeting gene-virotherapy strategies, in which tumor-targeting replicative adenovirus ZD55 (recombinant adenovirus with E1B 5-kDa gene deletion) is combined with therapeutic genes, is anticipated to increase antitumor efficacy. ZD55 is an E1B 55-kDa gene-deleted adenovirus that is similar to ONYX-015, but with the marked difference of a cloning site to insert foreign antitumor genes. Beyond the direct lytic function of the virus, additional cytotoxic effects induced by inserted therapeutic genes may be capable of eliminating tumor cells. Various antitumor genes such as suicide genes herpes simplex virus thymidine kinase (HSV-tk) and cytosine deaminase (CD), and apoptosis inducers hTRAIL (human tumor necrosis factor [TNF]-related apoptosis-inducing ligand) and SMAC (second mitochondria-derived activator of caspase), have been incorporated into oncolytic adenoviruses (Pei et al., 2004; Sova et al., 2004).

A novel cancer growth-suppressing and apoptosis-inducing gene, mda-7 (melanoma differentiation-associated gene 7), was identified by subtraction hybridization using a human melanoma cell line (H-O-1) (Jiang et al., 1995). The mda-7 cDNA encodes an evolutionarily conserved protein of 206 amino acids with a predicted size of 23.8 kDa (Jiang et al., 1996). Because of structural homology to other members of the interleukin 10 (IL-10) family of cytokines, chromosomal localization, and cytokine-like properties, mda-7 has been redesignated IL-24 (Chiakna and Williams, 1996; Kotenko et al., 1997; Kotenko, 2002; Huang et al., 2001). Many studies have shown that enforced expression of mda-7/IL-24 suppresses cell growth and induces apoptosis in a variety of tumor types including melanomas, gliomas, and cancers of the breast, colon, lung, cervix, pancreas, and prostate (Su et al., 1998, 2001; Madireddi et al., 2000a,b; Mhashilkar et al., 2001; Ellerhorst et al., 2002; Lebedeva et al., 2002; Leath et al., 2004). In contrast, these investigations also demonstrated that elevated expression of mda-7/IL-24 in normal mammary epithelial cells has no cytotoxic effects (Su et al., 2003; Sauane et al., 2004). Studies clarified that mda-7/IL-24 induces growth suppression and apoptosis in diverse cancer cells including single or multiple genetic defects, including alterations in p53, p16/INK4a, and/or Rb (Huang et al., 2001; Lebedeva et al., 2002).

Replication-defective adenovirus has been used as a vector to carry mda-7/IL-24 for the therapy of cancer. It has been demonstrated that mda-7/IL-24-positive cells could secrete a soluble form of mda-7/IL-24 protein that can induce a profound bystander effect and selectively kill cancer cells (Sauane et al., 2003). In addition to its direct antitumor activity, mda-7/IL-24 also exerts antiangiogenic activity in vivo (Nishikawa et al., 2004). On the basis of its apparent antitumor cell activity in vitro and in vivo, Ad-IL-24 was evaluated in a phase I/II clinical trial in patients with advanced carcinoma. These studies demonstrated that a single intratumoral injection of Ad-IL-24 into patients was safe and that this gene could induce apoptosis in a large percentage of tumor volume.

Because mda-7/IL-24 is a novel and prospective gene for the therapy of multiple cancers, understanding the mechanism by which this gene induces apoptosis in cancer cells will be of immense value. Current studies indicate that the mechanism by which Ad-IL-24 induces apoptosis in multiple cancer cell types is complex, involving multiple signal transduction pathways and intracellular molecules (Sarkar et al., 2002a,b). These studies are beginning to shed light on the signaling cascades involved in mda-7/IL-24 induction of apoptosis (Pataer et al., 2002, 2003; Sarkar et al., 2002a,b). Experiments have also evaluated the effect of Bel-2 family proteins on Ad-IL-24-induced apoptosis of tumor cells (Reed et al., 1996; Cao et al., 2002; Lebedeva et al., 2003). Analyses of signaling pathways have revealed Ad-IL-24 regulation of inducible nitric oxide synthase (iNOS) and mitogen-activated protein kinase (MAPK) in melanoma (Ekmekcioglu et al., 2003) and Jun kinase, h-catenin, phosphatidylinositol 3-kinase (PI3K), and protein kinase R (PKR) in lung and breast tumor cells (Kawabe et al., 2002; Mhashilkar et al., 2003). Thus, different signaling pathways seem to be responsible for induction of apoptosis in different tumor types.

In this study, we constructed tumor-selective replicating adenoviral vector ZD55 (based on deletion of the adenoviral E1B 5-kDa gene) carrying mda-7/IL-24 and its antitumor effects were explored in vitro and in vivo. Our data showed that ZD55-IL-24 could allow for efficient tumor-specific viral replication and IL-24 expression, and induced the apoptosis of tumor cells effectively. These results indicate that ZD55-IL-24 can be used as a potential therapeutic for treatment of cancer cells.

**MATERIALS AND METHODS**

**Cell lines and culture conditions**

The human colorectal carcinoma cell lines SW620, HT-29, SW480, HCT116, HeLa (human cervical carcinoma), BEL7404 (human hepatocarcinoma), and NHLF (normal human lung fibroblasts) were purchased from the Shanghai Cell Collection (Shanghai, China). HEK293 (human embryonic kidney cells) was obtained from Microbix Biosystems (Toronto, ON, Canada). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO-BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO-BRL) at 37°C in a 95% air–5% CO₂ humidified incubator. Bcap37 (human breast carcinoma) was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 medium supplemented with 10% FBS.
Virus construction and production

The IL-24 expression cassette contains the IL-24 gene controlled by the human cytomegalovirus (CMV) promoter. The IL-24 expression cassette was cloned into adenovirus shuttle plasmid pCA13 to form pCA13-IL-24 and cut with BglII to clone into ZD55 to form pZD55-IL-24. Replication-defective adenovirus Ad-IL-24 and targeting oncolytic adenovirus ZD55-IL-24 were generated in HEK293 cells by homologous recombination between pCA13-IL-24 or pZD55-IL-24 and the adenovirus packaging plasmid pBHE3 (Microbix Biosystems), respectively. Plaques were picked and recombinant adenovirus was amplified in HEK293 cells and purified by cesium chloride gradient ultracentrifugation. Titers were determined by plaque assay on HEK293 cells.

Virus progeny assay

To determine virus progeny, tumor or normal cells were infected with ZD55-IL-24 or ONYX-015 at a multiplicity of infection (MOI) of 5. After 48 hr, medium and cells were collected, and virus was released by freeze–thawing for three cycles and centrifuged to collect the supernatant. Virus production was determined by standard plaque assay on 293 cells.

Cell viability assay

Cells were plated in 96-well plates and treated with various adenoviruses. At the indicated times, medium was removed and fresh medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/ml) was added to each well. Cells were incubated at 37°C for 4 hr and then an equal volume of solubilization solution (0.01 N HCl in 10% sodium dodecyl sulfate [SDS]) was added to each well and mixed thoroughly. Absorbance from the plates was read on a Bio-Rad (Hercules, CA) microplate reader at 655 and 595 nm.

Annexin V binding assay and DNA fragmentation

Cells were trypsinized and washed once with complete medium. Aliquots of cells (5 × 10^5) were resuspended in 500 μl of binding buffer and stained with fluorescein isothiocyanate (FITC)-labeled annexin V (BioVision, Palo Alto, CA) according to the manufacturer’s instructions. A fluorescence-activated cell-sorting (FACS) assay was performed immediately after staining. For the fragmentation assay, cells were seeded in 10-cm culture dishes (5 × 10^6 cells per dish) and DNA was extracted and assayed 3 days after infection of cells with ZD55-IL-24 at an MOI of 10.

Apoptotic cell staining

Cells seeded in chamber slides were treated with ZD55-IL-24 (MOI of 5) or phosphate-buffered saline (PBS). After 48 hr of treatment, cells were incubated with Hoechst 33342 (Molecular Probes, Eugene, OR) for 10 min, washed with PBS twice, and observed under a fluorescence microscope.

Western blot analysis

Cells were harvested from the plates and resuspended in lysis buffer. Protein concentrations were determined with the Bio-Rad protein assay system. Aliquots of cell extracts containing 30 μg of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on an 8–12% gel and transferred to nitrocellulose. The membrane was blocked for 30 min with blocking buffer (5% nonfat milk powder in TBS-T: 10 mM Tris-HCl [pH 8.0], 150 mM NaCl, and 0.05% Tween 20). The membrane was then incubated with primary and secondary antibodies and enhanced by chemiluminescence, using ECL Western blot detection reagents (Pierce Biotechnology, Rockford, IL) according to the manufacturer’s recommendation. Actin, caspase 3, caspase 9, E1A, E1B 55-kDa, poly(ADP-ribose) polymerase (PARP), and Bax antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). mda-7/IL-24 antibody was obtained from GenHunter (Nashville, TN).

Tumor xenograft in nude mice

All animals used in these experiments were maintained at institutional facilities in accordance with regulations and standards of the U.S. Department of Agriculture and the National Institutes of Health. Female BALB/c nude mice (4–5 weeks of age), obtained from the Animal Research Committee of the Institute of Biochemistry and Cell Biology (Shanghai, China), were used in all of the experiments. Aliquots of SW620 cells (2 × 10^6) were injected subcutaneously into the lower right flank of athymic female nude mice. When the tumors were 100–150 mm^3 in size, the animals were randomized into four treatment groups. Each group was treated with four consecutive daily intratumoral injections with PBS or with ZD55-IL-24, Ad-IL-24, and ONYX-015 (5 × 10^8 PFU/dose per day). At the end of the experiment, tumors were harvested for additional analyses as described below. Differences in tumor growth were tested for statistical significance.

Immunohistochemistry

De-paraffinized tumor sections were treated with mouse monoclonal anti-mda-7/IL-24 antibody (diluted 1:1000). After incubation with an anti-mouse secondary antibody, expression of mda-7/IL-24 in cells was detected with diaminobenzidine (DAB; Sigma, St. Louis, MO) by enhancement with an avidin–biotin reaction ABC kit (Vector Laboratories, Burlingame, CA). Tissue sections stained without primary antibody served as negative control. The slides were then counterstained with hematoxylin.

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. For this purpose, the In Situ cell apoptosis detection kit (Sino-American Biotechnology Company, Luoyang, China) was used. The staining was performed according to the manufacturer’s procedures. Tissue sections included in the kit were stained and served as positive controls. The TUNEL reaction preferentially labels DNA strand breaks generated during apoptosis, and allows discrimination of apoptosis from necrosis and primary DNA strand breaks induced by apoptosis agents.
Statistical analysis

The statistical significance of experimental results was calculated by analysis of variance (ANOVA) and Student t test. Differences among groups was regarded as significant if \( p < 0.05 \).

RESULTS

Characterization of oncolytic adenovirus expressing IL-24: ZD55-IL-24

ZD55 was constructed by deletion of E1B 55-kDa from Ad5 and ZD55-IL-24 was formed by inserting the IL-24 gene into ZD55. To characterize its function, SW620 colon cancer cells were infected with ZD55-IL-24, Ad-IL-24, ONYX-015, and wild-type Ad5, and lysates from 293 cells were used as a positive control. The expression of E1A and E1B 55-kDa proteins were examined. As shown in Fig. 1A, ZD55-IL-24 expressed E1A protein and failed to express E1B 55-kDa protein, which is similar to ONYX-015 (Fig. 1A, lanes 3 and 4). In contrast, wild-type adenovirus expressed both proteins (Fig. 1A, lane 6) and E1-deleted replication-defective Ad-IL-24 did not express either of them (Fig. 1A, lane 2).

To examine exogenous IL-24 expression, after infection with ZD55-IL-24 at an MOI of 5 for 48 hr, colorectal cancer cells (SW620, HT-29, and SW480) and normal human lung fibroblasts (NHLF) were harvested and subjected to Western blot. As shown in Fig. 1B, all three colorectal cell types infected with ZD55-IL-24 expressed much higher IL-24 protein compared with the control cell line. And NHLFs infected with ZD55-IL-24 only show less IL-24 expression.

The most important characteristic of oncolytic virus is its ability to selectively replicate in tumor cells. To determine whether IL-24 interferes with the tumor-selective replicative ability of ZD55 adenovirus vector, tumor cell lines (SW620, Bcap37, BEL7404, HeLa, HCT116, and HT-29) and normal cells (NHLF) were infected with ZD55-IL-24, Ad-IL-24, and ONYX-015 and the accumulation of viral particles in culture medium and cell lysate was determined by plaque assay on 293 cells. As shown in Fig. 2, ZD55-IL-24 replicated at levels comparable to ONYX-015; both showed much stronger specific replicative ability and produced more viruses in tumor cells than in normal cells. But Ad-IL-24 did not replicate in either nor-

![Figure 1A](image1.png)

**FIG. 1.** Characterization of ZD55-IL-24 and mda-7/IL-24 expression in colorectal cell lines. (A) Western blot assay of E1A and E1B 55-kDa gene expression. Lysates from 293 cells were used as a positive control (lane 1). SW620 cells were infected with Ad-IL-24 (lane 2), ZD55-IL-24 (lane 3), or ONYX-015 (lane 4), mock infected (lane 5), or infected with wild-type Ad (lane 6) at an MOI of 10. At 24 hr, lysates from uninfected and infected cells were subjected to Western blot assay with anti-Ad E1A antibody and anti-Ad E1B 55-kDa antibody. (B) IL-24 expression. Colorectal cancer cells and normal cell were infected with ZD55-IL-24 or PBS. Twenty-four hours after infection, expression of mda-7/IL-24 was analyzed.
mal cells or tumor cells because of the absence of adenoviral E1A proteins. These results confirmed that ZD55-IL-24 could selectively replicate in many tumor cell lines and that IL-24 did not affect the replication properties of the virus.

Inhibition of human colorectal cancer cell proliferation by ZD55-IL-24

To detect the replicative capacity of ZD55-IL-24 in cells, human colorectal cancer cells (SW620 and HT-29) and normal human lung fibroblasts (NHLFs) were infected with ZD55-IL-24, Ad-EGFP, ZD55-EGFP, ONYX-015, and Ad-IL-24 at various MOIs. The two tumor cell lines lack functional p53 whereas NHLFs have wild-type p53. Cells were stained with crystal violet 7 days later. Significant cytopathic capacity was observed in both tumor cell lines infected with ZD55-IL-24, as compared with cells infected with ZD55-EGFP or Ad-IL-24 (Fig. 3). Moreover, the cytopathic capacity of ZD55-EGFP was comparable to that of ONYX-015 and was about 100 times greater than that of Ad-EGFP. The cytotoxicity of Ad-IL-24 is slight less than that of ONX-015, but significantly different from that of Ad-EGFP because of IL-24 expression. For all these viruses, significant suppression of cell proliferation was not observed in NHLFs. These results suggest that ZD55-IL-24 can selectively replicate in p53-dysfunctional tumor cells and inhibit the growth of tumor cells more effectively than ONX-015, ZD55-EGFP, and Ad-IL-24.

To further evaluate the kinetics of cytotoxicity induced by ZD55-IL-24, a panel of tumor cells (SW620 and HT-29) and normal cells (NHLF) were plated in 96-well plates and infected with Ad-IL-24, ZD55-EGFP, and ZD55-IL-24. As shown in Fig. 4, the cytotoxic effect of ZD55-IL-24 on the cancer cell lines was more apparent than the effect of Ad-IL-24 or ZD55-EGFP. This cytopathic effect was time dependent. ZD55-IL-24, Ad-IL-24, and ZD55-EGFP had little or no cytopathic effect on normal cells. Similar results were obtained from other tumor cell lines and normal cell lines (data not shown).

ZD55-IL-24 selectively induces apoptosis in colorectal cancer cells

Previous studies demonstrate that Ad-IL-24 induces apoptosis in a wide range of human cancers, but not in normal cells (Lebedeva et al., 2002). On the basis of these observations, experiments were performed to determine whether ZD55-IL-24 can selectively induce apoptosis of colorectal cancer cells. The colorectal SW620 cell line was analyzed for apoptosis by annexin V staining in conjunction with FACS analysis. Colon cancer cells infected with ZD55-IL-24 showed the highest percentage of cell apoptosis ($p < 0.01$; Fig. 5A). Whereas, less percentage of cell apoptosis was observed in the colon tumor cells infected with Ad-IL-24 or ONX-015 (Fig. 5A). At the same time, nucleosomal DNA degradation (DNA ladder) assays were also done to verify the selective induction of the apoptosis capacity of ZD55-IL-24 in colorectal cancer cells. DNA ladders were apparent only in colorectal cancer cells, not in normal human lung fibroblasts (NHLF) (Fig. 5B).
To confirm these results further, tumor cells (SW620, HT-29, and HCT116) and normal cells (NHLF) were analyzed for apoptotic changes by Hoechst 33258 staining after treatment with ZD55-IL-24 for 48 hr. Tumor cells but not normal cells treated with ZD55-IL-24 showed obvious apoptosis and no changes were observed in any of the cells treated with PBS (Fig. 5C).

ZD55-IL-24 induces caspase cascade activation and results in upregulation of proapoptotic proteins in colorectal cancer cells

To determine the underlying mechanism by which ZD55-IL-24 induces apoptosis in tumor cells, the activation of caspases was studied in SW620 tumor cells by Western blot analysis. Significant overexpression of IL-24 is time dependent after infection with ZD55-IL-24. Cleaved caspase 3 increased and pro-caspases 3 and 9 decreased in a time-dependent manner, beginning at 12 hr and peaking 48 hr after infection (Fig. 6A). To further confirm the involvement of caspase in ZD55-IL-24-induced apoptosis, we examined the specific cleavage of poly(ADP-ribose) polymerase (PARP) by caspase during apoptosis. PARP was cleaved, indicating that infection with ZD55-IL-24 resulted in activation of the caspase cascade. Similar caspase activation results were also observed in HT-29 and SW480 cells (data not shown). These results showed that mda-7/IL-24 transferred by tumor-selective replicating adenovirus ZD55 could induce apoptosis via the caspase cascade in colorectal cancer cells.

Previous studies in multiple tumor models indicate that expression of mda-7 induces changes in the levels of proapoptotic proteins. Moreover, this change, which occurs in cancer cells but not in normal cells, may be a defining element in mda-7/IL-24 induction of apoptosis. Therefore proapoptotic protein expression was also determined in human colorectal cancer cells infected with ONYX-015, Ad-IL-24, and ZD55-IL-24 for 48 hr. Cells were fixed and stained with mda-7/IL-24 antibody. The expression of mda-7/IL-24 in colorectal cancer cells infected with ZD55-IL-24 was much higher than that in cells infected with Ad-IL-24 (Fig. 6B). No mda-7/IL-24 expression was found in uninfected cells or cells infected with ONYX-015 or PBS. Analysis of Bax protein confirmed that Bax protein expression was increased in SW620 carcinoma cells by ZD55-IL-24 infection, whereas this effect was not apparent in cells infected with control vector ONYX-015 (Fig. 6B). Moreover, mitochondrial cytochrome c was released much more by cells

FIG. 3. Tumor-selective cytopathic effect of ZD55-IL-24. Tumor cells (SW620 and HT-29) and normal cells (NHLF) were seeded at a density of $1 \times 10^5$ cells and infected with ZD55-IL-24, ONYX-015, Ad-EGFP, ZD55-EGFP, and Ad-IL-24 at the indicated MOIs. Seven days later, cells were stained with crystal violet.
FIG. 4. ZD55-IL-24 inhibits the growth of colorectal cells. Tumor cells and normal cells were infected with ZD55-IL-24, Ad-IL-24, and ZD55-EGFP at an MOI of 10. On days 1, 2, and 3 postinfection, cells were stained with MTT as described in Materials and Methods. Results represent means ± SD (error bars) of triplicate experiments and are expressed as a percentage of untreated control cells.
infected with ZD55-IL-24 than by cells infected with Ad-IL-24. These results suggest that the mitochondrial pathway is primarily involved in the ZD55-IL-24-induced apoptosis of colorectal cancer cells.

**Antitumor efficacy of ZD55-IL-24 in nude mice**

Our *in vitro* data clearly demonstrated that ZD55-IL-24 could specifically induce tumor cell apoptosis via activation of caspase activation and release of cytochrome c. To determine whether ZD55-IL-24 could induce strong antitumor activity *in vivo*, we established SW620 subcutaneous tumors in nude mice. When the tumors reached 100–150 mm³, they were treated with ZD55-IL-24, Ad-IL-24, ONYX-015, or saline by intratumoral injection. In the control group receiving saline, tumors grew progressively during the course of the experiment (8 weeks). On the other hand, animals treated with ZD55-IL-24 exhibited statistically significant suppression of tumor development (*p* < 0.01; Fig. 7). The antitumor efficacy of ZD55-IL-24 was much higher than that of Ad-IL-24 or ONYX-015.

To verify that the therapeutic effect was due to mda-7/IL-24 overexpression, further evidence was demonstrated by immunohistochemical analysis. Strong mda-7/IL-24 expression was observed in tumor cells treated with ZD55-IL-24 (Fig. 8D). Tumors treated with Ad-IL-24 displayed less mda-7/IL-24 expression. In contrast, no mda-7/IL-24 expression was observed in tumors received saline or ONYX-015. Moreover, to understand the mechanism of tumor inhibition mediated by ZD55-IL-24, tumor sections were further analyzed for apoptosis by TUNEL staining. As shown in Fig. 8, tumors from mice treated with ZD55-IL-24 demonstrated extensive apoptosis (Fig. 8H), whereas treatment with Ad-IL-24 or ONYX-015 resulted in less tumor cell apoptosis (Fig. 8F and G).

**DISCUSSION**

Adenovirus-based vectors have become gene delivery vehicles that are widely used for cancer gene therapies. A major limitation of cancer gene therapy is the inadequacy of replication-deficient Ad vectors to efficiently infect tumors (Dobbelstein, 2004). To solve this problem, replicating vectors such as ONYX-015 have been used to enhance infection efficiency (Morley *et al*., 2004). Although these viruses replicate selectively in tumor cells, their efficacy is limited if they are used alone. The significant antitumor activity of ONYX-015 was evident only when the viral treatment was combined with chemotherapy or radiation therapy (Georger *et al*., 2003). To avoid the toxicity caused by systemic use of chemotherapeutic agents, a therapeutic transgene is incorporated into a replicating Ad vector to enhance the potency of replication-competent vectors. This therapeutic method is called the gene-virotherapy strategy. We have successfully constructed tumor-selective replicating

**FIG. 5.** ZD55-IL-24 selectively induced apoptosis in colorectal cancer cells. (A) Annexin V binding assay. SW620 cells were harvested 48 hr after infection at an MOI of 5 with various vectors (ZD55-IL-24, ONYX-015, and Ad-IL-24) or PBS. Infected cells were stained with FITC-labeled annexin V and immediately analyzed by flow cytometry. The percentage of apoptotic cells was calculated with CellQuest software. Colon cancer cells infected with ZD55-IL-24 showed a higher percentage of cell apoptosis than did cells infected with ONYX-015 and Ad-IL-24 (*p* < 0.01). Colon cancer cells treated with ONYX-015 or Ad-IL-24 showed significant apoptosis compared with cells treated with PBS. (B) ZD55-IL-24 induced nucleosomal DNA degradation in colorectal cancer cells. The indicated cell types were infected with ZD55-IL-24 and analyzed for nucleosomal DNA degradation 3 days after infection. (C) Colorectal cancer cells (SW620, HT-29, and HCT116) and normal cells were stained with Hoechst 33258 after treatment with ZD55-IL-24 and PBS. ZD55-IL-24 induced apoptosis in cancer cells but not in normal cells. Arrows indicate apoptotic cells. Original magnification, ×400 for all cell lines.
FIG. 5C.
adenoviral vector ZD55, which can replicate and induce cytopathic effect in tumor cells with p53 dysfunction. To increase the potency of this oncolytic vector, the new therapeutic gene IL-24 was incorporated into this vector system. The most important characteristic of ZD55-IL-24 is its selective replication similar to ONYX-015, and our experiments also demonstrated that the expression of IL-24 had no effect on its replication in tumor cells.

Data have demonstrated that mda-7/IL-24 inhibits the proliferation and induces apoptosis of most cancer cells (Mhashilkar et al., 2001). Moreover, because elevated expression of mda-7/IL-24 in normal cells does not elicit deleterious effects, the mechanism by which mda-7/IL-24 differentially inhibits growth and induces apoptosis in cancer cells versus normal cells remains to be determined. Our data showed that more exogenous IL-24 protein expression was detected in cancer cell lines compared with normal cells after infection with ZD55-IL-24. Correlating with these results is the observation that ZD55-IL-24 has the ability to selectively replicate, suppress cell proliferation, and induce apoptosis in tumor cells, but not in normal cells. These results are similar to findings previously reported for adenovirus-mediated IL-24 (Lebedev et al., 2002). However, one major difference distinguishing our results from others is the significant tumor-suppressive activity of ZD55-IL-24.

Previous reports suggested that Ad-IL-24 was able to suppress cell growth and induce apoptosis in a series of cancer cell lines through a variety of apoptotic signaling pathways (Chada et al., 2004). Our studies also demonstrated that ZD55-IL-24 has a broad spectrum of effects, which can induce the apoptosis of many kinds of cancers. Most tumor cells could be killed efficiently by ZD55-IL-24. This effect was due to the apoptosis mediated by IL-24 and cell death induced by viral replication. However, ZD55 vector with reporter gene EGFP (ZD55-EGFP) had less antitumor activity in vitro. This partial tumor response was consistent with our previous data (Pei et al., 2004). ZD55-IL-24 may become a new therapeutic agent for clinical cancer gene therapy, with the potential to extend the repertoire of antitumor gene therapy agents for the treatment of cancer.

To further understand the mechanisms by which ZD55-IL-24 suppressed cell growth and induced apoptosis, the down-
stream caspase cascade was analyzed. Our results found that overexpression of mda-7/IL-24 induced apoptosis and activated the caspase cascade, including cleavage of caspase 9 and caspase 3. Activation of the caspase cascade in colorectal cancer cells infected with ZD55-IL-24 was time dependent. This observation is consistent with the specificity of selectively replicating adenovirus ZD55. Like other reports, the upregulation of Bax was detected through mda-7/IL-24 expression, resulting in the release of cytochrome c from the mitochondria and activation of the downstream caspase cascade.

The anticancer activity of mda-7/IL-24 was demonstrated both in vitro and ex vivo in breast cancer cells and lung tumor xenografts (Su et al., 1998). Our research demonstrated that the potent antitumor activity of ZD55-IL-24 is more evident than that of Ad-IL-24 in colorectal cancer xenograft tumors. Moreover, this strong antitumor activity by ZD55-IL-24 is probably due to dramatic induction of tumor cell apoptosis in treated tumors. In addition to its direct apoptotic effect on tumor cells, IL-24 has antiangiogenic activity and immune stimulatory activity. The antiangiogenic effect of IL-24 has been demonstrated in tumor models in nude mice (Ramesh et al., 2003). IL-24 also has immune stimulatory activity. It activates IL-6, tumor necrosis factor α (TNF-α), and interferon α production and has been shown to significantly downregulate transforming growth factor β (TGF-β) (a potent immune suppressor of anticancer activity) (Saeki et al., 2002). In our study, treatment of colon tumor cells with ZD55-IL-24 in vivo is more efficient than in vitro. Inhibition of tumor angiogenesis and immune stimulation may also be important for the in vivo efficiency of the IL-24 system.

In conclusion, we constructed an armed oncolytic adenovirus system named ZD55-gene, which has both the lytic ability of oncolytic adenovirus and the capacity to deliver therapeutic factors. This is the first report of using the IL-24 gene, mediated by targeting vector ZD55, for cancer therapy. Ectopic expression of mda-7/IL-24 by means of this adenoviral delivery approach induces growth suppression and apoptosis in human colorectal cancer cell lines in vitro and inhibits human colorectal tumor growth in vivo in nude mice. Consequently, these findings should provide important insight into the role of this cancer growth suppressor gene in tumor development and progression. This study also verified that mda-7/IL-24 mediated by
FIG. 8. mda-7/IL-24 expression and induction of apoptosis after treatment with ZD55-IL-24 in vivo. Tumor sections were excised and analyzed for mda-7/IL-24 expression by immunohistochemistry (A–D) and for induction of apoptosis by TUNEL staining (E–H). In (B) and (D) arrows denote cells expressing IL-24. Expression of mda-7/IL-24 was higher in tumors treated with ZD55-IL-24 (D) than in tumors receiving ONYX-015 (C), Ad-IL-24 (B), or saline (A). For the TUNEL test, tumors treated with ONYX-015 (F), Ad-IL-24 (G), and ZD55-IL-24 (H) stained positive for cells undergoing apoptotic cell death. Tumors injected with PBS served as control (E). In (G) and (H) arrows denote cells undergoing apoptosis. Original magnification, ×400.
selectively replicative adenovirus ZD55 can attack tumors at multiple levels and represents a potentially exciting new treatment paradigm for human cancer.

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