Effective Gene-Viral Therapy for Telomerase-Positive Cancers by Selective Replicative-Competent Adenovirus Combining with Endostatin Gene

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ABSTRACT

Gene-viral therapy, which uses replication-selective transgene-expressing viruses to manage tumors, can exploit the virtues of gene therapy and virotherapy and overcome the limitations of conventional gene therapy. Using a human telomerase reverse transcriptase-targeted replicative adenovirus as an antiangiogenic gene transfer vector to target new angiogenesis and making use of its unrestrained proliferation are completely new concepts in tumor management. CNHK300-mE is a selective replication transgene-expressing adenovirus constructed to carry mouse endostatin gene therapeutically. Infection with CNHK300-mE was associated with selective replication of the adeno virus and production of mouse endostatin in telomerase-positive cancer cells. Endostatin secreted from a human gastric cell line, SGC-7901, infected with CNHK300-mE was significantly higher than that infected with nonreplicative adenovirus Ad-mE in vitro (800 ± 94.7 ng/ml versus 132.9 ± 9.9 ng/ml) and in vivo (610 ± 42 ng/ml versus 126 ± 13 ng/ml). Embryonic chorioallantoic membrane assay showed that the mouse endostatin secreted by CNHK300-mE inhibited angiogenesis efficiently and also induced distortion of pre-existing vasculature. CNHK300-mE exhibited a superior suppression of xenografts in nude mice compared with CNHK300 and Ad-mE. In summary, we provided a more efficient gene-viral therapy strategy by combining oncolysis with antiangiogenesis.

INTRODUCTION

Conditionally replicative adenovirus (CRAD) is emerging as a promising new modality for the management of cancer. Replicating viral agents may lead to improved efficacy over nonreplicative adenoviruses in tumors because of their inherent ability to replicate, lyse infected cancer cells, and spread to surrounding cells (1–3). The key of CRADs lies in selective replication; these modified adenoviruses should specifically replicate in and destroy cancer cells while leaving the surrounding normal cells unaffected. Two major molecular strategies have been exploited to target CRADs selectively to tumor cells (4, 5). The first strategy involves the deletion of adenoviral genes that are necessary for virus replication in normal cells but not in tumor cells. The second strategy involves the use of tumor- or tissue-specific promoters, such as the α-fetoprotein promoter in hepatocellular cancer (6), the DF3/MUC1 antigen promoter in breast cancer (7) and the prostate-specific antigen promoter in prostate cancer (8). Although obvious antitumor effect of CRADs has been reported in many preclinical studies and in some clinical trials, there are still opportunities to achieve synergistic effect by combining virotherapy with gene therapy, chemotheraphy, or radiotherapy, making use of their different antitumor mechanisms. The management of human tumors with a replication-selective, transgene-expressing adenovirus is a natural extension of virus-mediated gene delivery, and this offers several potential advantages (9, 10). The oncolytic virus itself can specifically infect and replicate in tumor cells, inducing cell death and release of viral particles. The replicating viruses also serve as vectors for efficient transfection and expression of transgenes in the tumor cells in the process of viral replication. Gene-viral therapy, which uses these replication-selective, transgene-expressing viruses to manage tumors (11, 12), can exploit the virtues of gene therapy and virotherapy. Gene-viral therapy also can overcome the limitations of conventional gene therapy, such as poor gene transfection, low antitumor gene expression, and nontarget to tumor cells (13–15).

Solid tumors are characterized by unrestrained proliferation and new angiogenesis. To achieve unrestrained proliferation, tumors often are dependent on the maintenance of telomerase. Telomerase has been reported as the most common tumor molecular marker and is highly active in >85% human primary cancers but not in most normal somatic cells (16–18). Human telomerase reverse transcriptase (hTERT) is an essential catalytic subunit to maintain telomerase activity, and its expression is regulated at the transcription level (19). Studies making use of hTERT promoter to drive therapeutic genes, such as Bax, caspase-8, or suicide gene, to achieve selective expression of transgenes in telomerase-positive tumors have been reported (20–23). We thought that hTERT promoter could be used to regulate tumor-specific expression of genes necessary for viral replication, so that viral replication only occurs in telomerase-positive cancer cells. On the basis of this rationale, we constructed a replicative-competent adenovirus, named CNHK300, which used hTERT promoter to drive adenoviral E1A gene. Our previous studies have demonstrated that CNHK300 is superior to ONX-015, a conditional replicative adenovirus with E1B 55-kDa deleted (24) in terms of selective replication and oncolytic effects, with no sign of toxicity to liver cells even at a high dosage (25). Similar results also were reported from other research groups (26–29).

Angiogenesis is controlled by a balance between angiogenic stimulators and inhibitors, and this balance is perturbed in tumors by either overproduction of angiogenic inducers or lack of inhibitors (30). Distinct from other cancer gene therapies that target cancer cells, antiangiogenic gene therapy inhibits tumor growth by targeting endothelial cells. A number of antiangiogenic inhibitors have been tested in preclinical studies with obvious tumor repression observed (31–33). However, there has been no report using CRAD as an antiangiogenic gene delivery system, and we presented the first of such a model. Because the tumor xenografts in our experiments were established on nude mice and because tumor-supporting structure is murine in origin, we used mouse endostatin therapeutically in our experiments.

On the basis of CNHK300, we built a replication-selective, transgene-expressing adenovirus, designated as CNHK300-mE, which contained mouse endostatin gene-expressing cassette. The oncolysis
capability and endostatin expression mediated by this novel adenovirus were studied.

MATERIALS AND METHODS

**Cells and Cell Culture.** The following cells were purchased from the American Type Culture Collection (Manassas, VA): A549 (human lung cancer cell line), PANC-1 (human pancreatic cancer cell line), Hep3B and HepGII (human hepatocellular carcinoma cell line), HT29 (human colon cancer cell line), BJ (normal human fibroblast cell line), and MRC-5 (embryonic fibroblast cell line). Human embryonic kidney 293 cell line was obtained from Microbix Biosystems (Toronto, Canada). Human gastric cancer cell line SGC-7901 was obtained from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). SGC-7901, Hep3B, HepGII, HT29, and human embryonic kidney 293 cells were cultured in DMEM (Life Technologies, Rockville, MD). A549 and PANC-1 were cultured in RPMI 1640 medium. BJ and MRC-5 cell lines were cultured in Eagle’s MEM. All of the media were supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), 4 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and cultured under a 5% CO2 atmosphere at 37°C.

**Isolation and Culture of Primary Human Hepatocytes.** Surgical liver biopsies (30–50 g) were taken with informed consent from patients who underwent liver resection for hepatic angioma. These patients had no other known liver disease. Human hepatocytes were isolated using modified collagenase digestion technique as described by Liddle et al. (34). Liver tissues were cut into small pieces (~1 mm³) and washed three times in 4°C equilibrium liquid to remove the leftover blood, and then the liver pieces were digested for 30 min using RPMI 1640 medium containing 0.05% collagenase. The dissociated hepatocytes were placed into cold HBSS medium three times and centrifuged at 400 g for 40 s, 800 g for 50 s, and 1000 g for 60 s. Percoll-gradient centrifugation (1000–1200 rpm for 10 min) was required to get rid of the remaining blood thoroughly. Concentration and density of Percoll liquid were 55% (v/v) and 1.08 g/ml, respectively. Viability at plating was confirmed >90% after trypsin blue elimination, and hepatocytes were plated in RPMI 1640 medium supplemented with 100 units/ml penicillin G, 100 μg/ml streptomycin, and 15% heat-inactivated fetal bovine serum (Life Technologies). The hepatocytes (3 × 10⁶) were plated onto 60-mm culture plates previously coated with type I (rat tail) collagen. The cells were allowed to attach for 4 h at 37°C and 5% CO2, during which the media were replaced with serum-free ones with the supplements listed previously and changed every 24 h thereafter.

**Construction of Plasmids and Recombinant Viruses.** The complete cDNA sequence of mouse endostatin gene was amplified by PCR from the plasmid pBluesmEndo (InvivoGen, San Diego, CA) by using the upstream primer 1 (5′-CCG CTG CTC AGT AGG GAT CTC CTG TCT TTA CCA AGT GGG AGC GCT CAT ACT CAT CAG GAC TTT CA-3′) and downstream primer 2 (5′-GGC GGA TTC TTA CTA TTT GGA GAA AGA GGT CA-3′). The PCR products were then cloned into a mammalian expression vector. A synthetic oligonucleotide coding M signal peptide was inserted upstream of the endostatin gene by PCR with primer 3 (5′-GGG GAA TTA ACC ATG GGG GAT CTG CTG CTA ACA GGG AGC AGG CTC AGT CAG AGT CTC GCT CTT GCA CTC-3′) and primer 2. The synthetic DNA sequence was released with EcoRI and BamHI (New England Biolabs, Beverly, MA) and ligated into plasmid pUC19 (Microbix Biosystems). After sequence confirmation, the endostatin cDNA plus M-signal peptide sequence was cloned into plasmid pCA13 (Microbix Biosystems) and generated pCA13-mEndo. Digestion of pCA13-mEndo with endonucleases BglII (New England Biolabs), a 1080-bp fragment containing cytomegalovirus promoter, mouse endostatin gene, and SV40 polyA was excised and inserted into BglII site of plasmid pCAG166-TP, which was constructed in our laboratory and contained hTERT promoter core sequence and endostatin expression cassette was released from the pCAG166-mEndo recombinant plasmid with NotI and XhoI (New England Biolabs) and cloned into pX20 (Ref. 25, constructed in our previous study) to generate pX20-mEndo.

To construct the recombinant adenoviruses, the plasmids pX20-mEndo and pCA13-mEndo were transfected respectively via Lipofectamine2000 (Life Technologies) into 293 cells together with pBHG35 (Microbix Biosystems), a plasmid-containing right arm of adenovirus type 5 with deletion of 188-1339-bp sequence. The viral plaques appeared 9–14 days after cotransfection and were sublimated three times. The recombinant adenoviruses, extracted using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA), were verified by PCR and named CNHK300-mE and Ad-mE, respectively.

The viruses were purified by cesium chloride density purification and propagated in 293 cells. After 72 h, the detached cells were harvested by centrifugation at 1,000 × g for 5 min at 4°C, resuspended in 10 ml cold PBS (free Ca²⁺ and Mg²⁺), and then lysed with three cycles of freeze and thaw. The lysate was collected by centrifugation at 1,500 × g for 10 min at 4°C, and the supernatant was placed on a gradient prepared with equal parts of CsCl in PBS and then centrifuged at 15,000 × g for 2 h at 4°C. The virus band was removed and placed in a preformed CsCl gradient by ultracentrifugation for 18 h and dialyzed into 10 mm Tris-HCl (pH 7.4) containing 10 mM MgCl₂ and 10% glycerol. Titers of the purified adenoviruses were determined by plaque assays of the tissue culture infectious dose 50 method and shown as plaque-forming unit per milliliter (pfu/ml). All of the viral preparations were free of endotoxin.

**In Vitro Viral Replication Assay.** Monolayer cells, including logarithmically growing cancer cells A549, SGC-7901, PANC-1, Hep3B, HepGII, BJ, MRC-5, and primary human hepatocytes (10⁶ cells/well), and contact-inhibition normal cells BJ, MRC-5, and primary human hepatocytes (10⁶ cells/well) were cultured in six-well dishes overnight and infected with CNHK300-mE or CNHK300 at a multiplicity of infection (MOI) of 5.0 pfu/cell. Virus inocula were removed after 2 h. The cells then were washed twice with PBS and incubated at 37°C for 0, 12, 24, 48, or 96 h. Lysates of cells were prepared with three cycles of freeze and thaw. Serial dilutions of the lysates were tiered on human embryonic kidney 293 cells with the tissue culture infectious dose 50 method, normalized with that at the beginning of infection, and reported as multiples. The wild-type adenovirus 5 and ONYX-015 (a gift from A. J. Beri, University of California-Los Angeles, Los Angeles, CA) also were used as controls.

**ELISA Determination of Mouse Endostatin Expression In Vivo.** SGC-7901 cells were seeded in 24-well plates at a density of 5 × 10⁶ cells/well and cultured for 24 h, followed by infection with CNHK300-mE and Ad-mE at an MOI of 0.1. On days 3, 5, 7, and 10 postinfection, the supernatants of cell cultures were collected and assayed for mouse endostatin gene expression levels using the ChemiKine Mouse Endostatin EIA Kit (Chemicon International, Teme culpa, CA) according to the manufacturer’s instructions.

**Western Blot Determination of Mouse Endostatin and E1A Expression.** For mouse endostatin expression determination, the supernatants of 10⁶ SGC-7901 cells were collected on day 3 postinfection at an MOI of 1. Thirty μl unconcentrated samples were mixed with the same volume of SDS loading buffer, separated by SDS-PAGE on 15% (w/v) polyacrylamide gels. Proteins were electrophoretically blotted onto PROTRAN nitrocellulose transmembrane (Schleicher & Schuell, Dassel, Germany) and blocked at room temperature with 5% nonfat milk in Tris-buffered saline [10 mm Tris (pH 7.5), 0.9% NaCl] containing 0.1% Tween-20 for 1 h. Blots then were incubated overnight at 4°C in blocking solution with rat-antimouse endostatin monoclonal antibody (Oncogene Research Products, Cambridge, MA). Thereafter, membranes were extensively washed in Tris-buffered saline/Tween-20 and incubated with horseradish peroxidase-conjugated antirat IgG (1:1000) in blocking solution for 1 h. Bands were detected by Lumiglo chemiluminescence detection kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and visualized with Kodak Biomax film (Rochester, NY). For E1A gene expression, the cultured cells were lysed in cell lysis buffer (M-PEI Mamalian Protein Extraction Reagent; Pierce, Rockford, IL), incubated on ice for 10 min, and then centrifuged at 1,500 × g for 10 min at 4°C. The supernatants were collected, and other procedures were performed in a similar way as described previously.

**CAM Assay.** Fertilized chicken eggs were incubated at 37°C with 70% humidity for 8 days. An artificial air sac was created over a region containing small vessels in the chicken choriorallantoic membrane (CAM) as described. A small window was cut in the shell over the artificial air sac. The CAM was treated with 100 μl conditioned medium collected from 1 × 10⁶ SGC-7901 cells infected with CNHK300, CNHK300-mE, or Ad-mE, respectively, at an MOI of 1 on day 7. One hundred μl sterile saline were used as negative control, and 20 ng mouse endostatin were used as positive control. After
incubated for 3 days, CAMs were fixed in methanol/acetone, observed under a stereoscope for the formation of avascular zones, and photographed using a digital camera.

**Animal Experiments.** A total of $1 \times 10^7$ logarithmically growing SGC7901 cells, a human gastric cancer cell line established and commonly used in China and possessing high activity of telomerase (35), were injected s.c. into the right flank of BALB/c nude mice (Institute of Animal Center, Chinese Academy of Sciences, Shanghai, China) aged 6–8 weeks. Typically 10–14 days after inoculation, when the s.c. nodules grew and reached 7–9 mm in diameter, the mice were randomly assigned to three treatment groups (CNHK300, CNHK300-mE, and Ad-mE) and one control group (treated with virus preservation buffer). There were 11 mice in each group. Pre-established tumors then were injected with 100 μl control buffer or $2 \times 10^6$ pfu viruses in the same medium, respectively. The injections were repeated every other day for five times with a total dosage to $1 \times 10^6$ pfu. Tumor growth was monitored by periodic measurements with calipers, and tumor volume was calculated by the following formula: $(\text{maximal length} \times \text{perpendicular width})^2 / 2$. All of the procedures were performed in accordance with the institution guidelines and approved by the Committee on the Use and Care on Animals. All of the animals were sacrificed 28 days after treatment, and the tumors were resected and weighed. The tumor reduction rate was calculated using the formula: $(V_{\text{control group endpoint}} - V_{\text{control group initial point}}) / V_{\text{control group initial point}} \times 100\%$.

Mouse serum samples were obtained by retro-orbital puncture and assayed for endostatin gene expression on days 1, 3, and 10 after intratumoral injection with the recombinant adenoviruses.

**Histopathologic and Immunohistochemical Study.** The transplanted tumors, resected from nude mice, were fixed in 10% formalin, paraffin embedded, and then cut into 4-μm-thick sections. All of the sections were baked, deparaffinized, and heated in citrate buffer (pH 6.0) in a microwave oven. After inactivation by exposure to 1.5% H$_2$O$_2$/methanol for 10 min to block the endogenous peroxidase, the sections were incubated with blocking serum (goat serum) at room temperature for 30 min. Immunohistochemistry was carried out using the UltraSensitive Streptavidin-Peroxidase Kit (Maxim Pharmaceuticals, San Diego, CA) and primary antibody, including antiadenoviral hexon protein (Biodesign International, Saco, ME) and anti-mouse CD3 (PharMingen, San Diego, CA). Microvessel areas were quantified by manual counting of hotspots in sections, as described by Weidner et al. (36).

**Statistical Analysis.** Mean tumor volume and microvessel density (MVD) were compared at a given time point using unpaired, two-tailed $t$ test; $P < 0.05$ was considered significant.

**RESULTS**

**Construction of Adenovirus CNHK300-mE.** A novel conditionally replicative adenovirus CNHK300-mE was constructed, in which the $E1A$ gene was placed under the control of hTERT promoter plus three extra E-boxes, and the mouse endostatin-expressing cassette was inserted between adenoaviral package signal and hTERT promoter (Fig. 1). Adenoviral DNA was extracted, and recombination was confirmed by PCR amplification without contamination by wild-type adenoviruses.

**Selective Replication of CNHK300-mE in Vitro.** The selective replication of CNHK300 in telomerase-positive tumor cells had been demonstrated in our previous study (25). The selective replication of the new recombinant adenovirus CNHK300-mE was re-evaluated using telomerase-positive cancer cell lines SGC-7901, A549, PANC-1, Hep3B, and HepG2 and telomerase-negative normal cell lines MRC-5, BJ, and primary human hepatocytes. For all of the tumor cell lines tested, the selective replication of CNHK300-mE increased by 30,776–252,000-fold 4 days after infection, which was comparable with or only slightly weaker than that of CNHK300 (Fig. 2A). CNHK300-mE replicated as efficiently as CNHK300 and wild-type adenovirus 5 and more efficiently than ONYX-015 in telomerase-positive cancer cells (Fig. 2B). In telomerase-negative normal cells MRC-5, BJ, and primary human hepatocytes, CNHK300-mE basically did not replicate, whereas wild-type adenovirus 5 and ONYX-015 replicated continuously (Fig. 2B). These findings are consistent with our previous study. All of the cancer cells infected with CNHK300-mE or wild-type adenovirus 5 were positive for $E1A$ expression. $E1A$ expression was detected in wild-type adenovirus 5-treated normal cell groups but not in CNHK300-mE-treated normal cell groups (data not shown).

**Expression of Mouse Endostatin Produced by CNHK300-mE in Vitro.** To verify that the mouse endostatin expressed by CNHK300-mE could be secreted efficiently into the media, the conditioned media from $5 \times 10^4$ SGC-7901 cells infected with CNHK300-mE or Ad-mE at an MOI of 0.1 were collected and analyzed for the presence of endostatin by ELISA. For CNHK300-mE, the level of mouse endostatin increased by 270-fold and reached 800 ± 94.7 ng/ml on day 10 postinfection (Fig. 3A). For Ad-mE, the expression of endostatin also increased with time but was substantially lower than that of CNHK300-mE. On day 10 postinfection, endostatin reached only 132.9 ± 9.9 ng/ml in the Ad-mE treatment group. Western blot analysis revealed a clear band of $M_r$ 20,000 in the conditioned media after SGC-7901 cells were infected with CNHK300-mE or Ad-mE at an MOI of 1 on day 3, suggesting that the protein in the media was mouse endostatin (Fig. 3B).

**Inhibition of Angiogenesis in the CAM.** The biological activity of endostatin secreted in the SGC-7901 cultures by CNHK300-mE or Ad-mE was tested in a CAM assay. Obvious inhibition of new embryonic blood vessel development and formation of avascular zones were observed in the group treated with CNHK300-mE. Within the avascular zones, a large number of newly formed vessels were regressed (Fig. 4F). Inhibition of new blood vessels also was seen in the endostatin or Ad-mE treatment groups, although not as strong as that in the CNHK300-mE treatment group, and there was no apparent formation of avascular zones (Fig. 4, B and C). Interestingly, the CNHK300-mE and CNHK300 treatment groups showed distortion of
of the CNHK300-mE treatment group was 800.50 ± 130.83 mm³, significantly greater reduction in tumor volume was observed in all of the treatment groups (68.25%, 56.23%, and 43.93%, respectively).

On day 28, all of the mice were sacrificed, and tumors were resected and weighed. Compared with the CNHK300 and Ad-mE treatment groups, significantly greater reduction in tumor volume was observed in the CNHK300-mE treatment group. The mean tumor size of the CNHK300-mE treatment group was 800.50 ± 115.63 mm³, much smaller than that of the CNHK300 treatment group (1226.65 ± 205.44 mm³; P = 0.002) and the Ad-mE treatment group (1402.83 ± 210.14 mm³; P < 0.0001). There were no significant differences in tumor size and weight between the Ad-mE treatment group and the CNHK300 treatment group (P > 0.05).

The serum level of endostatin in the CNHK300-mE treatment group increased nearly 10-fold and reached 610 ± 42 ng/ml on day 10 after treatment, which in theory is sufficient to inhibit tumor metastasis. For the Ad-mE treatment group, the serum level of endostatin was 126 ± 13 ng/ml on day 10, which was significantly lower than that of the CNHK300-mE treatment group. Endostatin also was detected in the control group, which suggests that tumor xenografts secreted endostatin into the circulation. The endostatin level of mice treated with CNHK300 was nearly equal to that of the control group (data not shown).

All of the tumor samples were examined histologically using H&E staining and immunohistochemical staining for hexon or CD31. In the control group, the cancer cells grew luxuriantly with small foci of necrosis. In the groups treated with replicating viruses, many wide areas of necrosis were observed, and hexon was detected in the cytoplasm of tumor cells (data not shown). These results indicated that CNHK300-mE and CNHK300 selectively replicated in and lysed tumor cells. In all of the treatment groups, the reduction of MVD was observed. Mean MVD in the CNHK300-mE treatment group was 11.33 ± 2.52, much less than that of the control group (87.67 ± 6.11; P = 0.0029) and the other two treatment groups (26 ± 2.65 for Ad-mE, P = 0.0357 and 48 ± 5.57 for CNHK-300, P = 0.0139; Fig. 6).

**DISCUSSION**

The concept of angiogenesis-dependent tumor growth was first introduced by Folkman et al. (30, 37). Angiogenesis is a complex process that includes activation, proliferation, and migration of endothelial cells (30, 37, 38). In tumorigenesis, this process also involves disruption of the vascular basement membrane, formation of vascular tubes and networks, and linkage to pre-existing vascular networks. Folkman also demonstrated that suppression of tumor angiogenesis leads to tumor starvation and tumor regression (30). Thus, inhibition of these angiogenic processes may provide a powerful strategy to suppress tumor growth. Antiangiogenesis therapy targets endothelial cells of tumor vasculature rather than tumor cells. A theoretical advantage of this approach is that endothelial cells are not transformed and are unlikely to acquire drug resistance through mutations (37). Furthermore, treatment targeted on endothelial cells is applicable to all of the solid tumors, irrespective of the origin and histologic types of tumors.

Endostatin is a novel potent inhibitor of angiogenesis with little toxicity, immunogenicity, and resistance (39). Because inhibition of angiogenesis by endostatin just induces dormancy without tumor cell killing, there is a need for combined use with other agents that kill tumor cells directly. Another problem inherent to endostatin is its short half-life and rapid clearance from blood. To achieve significant tumor regression, endostatin protein needs to be administered continuously. However, large-scale production of endostatin is difficult, and the attendant cost of continuous use is high. Antiangiogenesis using gene therapy is an attractive solution, but this method suffers from toxicity, immunogenicity, and resistance (39). Because inhibition of angiogenesis by endostatin just induces dormancy without tumor cell killing, there is a need for combined use with other agents that kill tumor cells directly. Another problem inherent to endostatin is its short half-life and rapid clearance from blood. To achieve significant tumor regression, endostatin protein needs to be administered continuously. However, large-scale production of endostatin is difficult, and the attendant cost of continuous use is high. Antiangiogenesis using gene therapy is an attractive solution, but this method suffers from toxicity, immunogenicity, and resistance (39).
cornea and tumor growth. However, the antitumor effect produced by this endostatin gene therapy was less dramatic than that produced by direct injection of endostatin protein. This difference possibly could be explained by the fact that the blood level of expressed endostatin was not high enough to induce tumor dormancy. Although other studies on endostatin gene therapy using plasmids, liposome, or cationic liposome as vectors have demonstrated inhibition of tumor growth by endostatin in vivo (40–42), there is no effective system for cancer gene therapy using endostatin gene at present. Viral vectors are efficient transgene-carrying systems, and some studies showed antitumor ability in vitro and in vivo by using replication-deficient adenovirus, adenovirus-associated virus, or retrovirus (43–47). Joseph et al. (48) recently reported the use of a recombinant adenovirus Adk3-HAS, in which the human angiostatin kringle 1–3 was directly fused to human serum albumin HAS, to treat human NB IGR-N835 tumor models in different experiment situations. There was no delay in tumor growth observed in animals treated with Adk3-HAS as compared

![Fig. 3. Detection of mouse endostatin expression in vitro. A, mouse endostatin in supernatants of SGC-7901 cells infected with CNHK300-mE, Ad-mE, and the control at days 3, 5, 7, and 10 postinfection. The levels of mouse endostatin in both treatment groups were increased with time. B, mouse endostatin expression in supernatants of SGC-7901-infected cells by Western blot analysis. a, positive control by 20 ng mouse endostatin protein. b, SGC-7901 cells infected with CNHK300-mE. c, SGC-7901 cells infected with CNHK-300. d, SGC-7901 cells infected with Ad-mE. e, negative control.](image)

![Fig. 4. Inhibition of angiogenesis in vitro by chorioallantoic membrane assay. A, angiogenesis in the sterile saline-treated group. B, slight inhibition of new vessels in the 20 ng endostatin-treated group. C, slight inhibition of new vessels in the Ad-mE-treated group. D, distortion of pre-existing vasculature in the CNHK300-treated group (black arrow). E, distortion of pre-existing vasculature and deadwood-like transformation of main vessels in the CNHK300-mE-treated group (black arrow). F, avascular zones in the CNHK300-mE-treated group (open arrows).](image)
with those treated with the empty virus AdCO1. A possible explanation for this observation was that the serum level of K3-HAS was not able to counteract the effect of high level of vascular endothelial growth factor released by the IGR-N835 tumors (48). To our knowledge, there is no report of using selective replication adenovirus as a vector to carry antiangiogenic gene. Our current approach takes advantage of virotherapy and gene therapy to achieve synergistic antitumor effect.

In the gene-viral therapy systems, selective replicating adenoviruses draw extensive attention because of their dual mechanisms as efficient gene carriers and as effective oncolytic agents (9, 10). In our previous study, we constructed a replication-competent adenovirus CNHK300, in which hTERT promoter with three extra E-boxes downstream of the promoter core sequence was introduced and used to drive E1A gene. Our previous experiments showed that the three extra E-boxes downstream of hTERT promoter can inhibit hTERT promoter activity in telomerase-negative cells, and CNHK300 had significant antitumor efficacy in vitro and in vivo (25).

On the basis of CNHK300, we constructed a novel replication-selective, transgene-expressing adenovirus CNHK300-mE, which contains mouse endostatin gene as therapeutic agent. CNHK300-mE, although containing a 1080-bp transgene-expressing cassette, demonstrated unimpaired selective replication in telomerase-positive tumor cells, whereas its replication was greatly attenuated in normal tissue cells. In telomerase-positive cancer cell lines, CNHK300-mE multiplied 30,776–252,000-fold 4 days after infection; the replication was similar to wild-type adenovirus 5 and was stronger than ONYX-015. We consistently observed neither viral replication nor E1A expression in CNHK300-mE-infected telomerase-negative normal cells. These findings indicate that adenovirus CNHK300-mE possessed the characteristic of selective replication.

Using ELISA and Western blot analysis, mouse endostatin was detected in the conditioned media after SGC-7901 cells infected with CNHK300-mE or Ad-mE. The biological activity of endostatin produced by CNHK300-mE also was confirmed by CAM assay, and obvious inhibition of angiogenesis was observed. The level of endostatin achieved 800 ± 94.7 ng/ml on day 10 after infection with CNHK300-mE, which is much higher than the data reported by Feldman et al. (43). In vivo, CNHK300-mE was injected intratumorally to achieve sustained expression of endostatin. The serum level increased greatly and reached the level of 610 ± 42 ng/ml on day 10 after treatment, which is in theory sufficient to inhibit tumor metastasis. In our control group treated with adenovirus preserved buffer, the serum endostatin level was 61.04 ± 8.98 ng/ml on day 10 after treatment, which indicated the base level of endostatin in mice bearing tumors. These findings proved that endostatin expression could be maintained at a therapeutic level in mice circulation after intratumoral administration of CNHK300-mE for a relatively long time.

In the gastric cancer xenograft model, significant tumor growth inhibition was demonstrated in all of the treatment groups when compared with the control group, which was treated with adenovirus preserved buffer. There was no significant difference in tumor volume between groups treated with Ad-mE and with CNHK300 during the entire course (P > 0.05). From day 14 after treatment, the tumor volume in the mice treated with CNHK300-mE was suppressed more than that in the mice treated with CNHK300. The mice were killed 4 weeks after treatment, and the tumors were harvested and measured. When compared with the control group, the tumor volume was reduced by 75.44% and 51.70%, respectively, in the CNHK300-mE and CNHK300 treatment groups, and CNHK300-mE showed more tumor repression than CNHK300 (P = 0.0001). Tumor reduction also was observed in the Ad-mE treatment group compared with the control group (P = 0.0001), although not as greatly as in the CNHK300-mE treatment group. These results indicated that all of the treatments (including CNHK300-mE, CNHK300, and Ad-mE) inhibited tumor growth, and CNHK300-mE is the most efficient agent among them. Gene-viral therapy is superior to simple gene therapy and virotherapy.

Immunohistochemistry revealed that hexon was expressed in tumor cell cytoplasm of the groups treated with CNHK300-mE and CNHK300 but not of the groups treated with Ad-mE or adenovirus preservation buffer, which indicated efficient replication of CNHK300-mE and CNHK300 in SGC-7901, a human gastric cancer cell line. Significant reduction of MVD was observed in all of the treatment groups compared with the control group, and the CNHK300-mE treatment group demonstrated more reduction of MVD on SGC-7901 xenografts compared with the other two treatment groups. These results indicated that SGC-7901 cells infected with CNHK300-mE can efficiently express and secrete endostatin into the circulation with biological activity. Interestingly, reduction of MVD also was observed in the CNHK300 treatment group; it may be explained by the toxicity of replicating viruses to endothelial cells.
Chen et al. (49) also reported similar results in prostate cancer animal models.

Immune response is a major obstacle for repetitive administration of adenoviruses as vector in gene therapy. The route of viral administration will be a critical determining factor. Intratumoral injection may elicit fewer neutralizing antibodies compared with systemic administration. Other means to reduce neutralizing antibody formation and/or titers, such as anti-CD20 antibody therapy with rituxan and plasma pheresis, also may be helpful to reduce the immune response and allow repeated adenovirus use (51).

In summary, the tumor-specific replication-competent adenovirus driven by hTERT promoter as an antiangiogenic gene delivery vector takes the advantages of viral therapy and gene therapy. CNHK300-mE can selectively replicate in and lyse telomerase-positive cancer cells, and the containing mouse endostatin gene can express and amplify to hundreds of times to inhibit angiogenesis efficiently. Gene-viral therapy is a promising strategy to combat tumor. Further increase in antitumor ability relies on construction of more efficient viral vectors and use of more potent angiogenesis inhibitors or combined therapy with multiple genes. Our findings may provide new insights into cancer management with gene-viral therapy, a completely new concept in the battle against human cancers.

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REFERENCES


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