Gene-Viral Cancer Therapy Using Dual-Regulated Oncolytic Adenovirus with Antiangiogenesis Gene for Increased Efficacy

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Abstract
Conditionally replicative adenovirus (CRAD) represents a promising approach for cancer therapy. Several CRADs controlled by the human telomerase reverse transcriptase promoter have been developed. However, because of their replicative capacity, the importance of cancer specificity for CRADs needs to be further emphasized. In this study, we have developed a novel dual-regulated CRAD, CNHK500-mE, which has its E1a and E1b gene controlled by the human telomerase reverse transcriptase promoter and the hypoxia response element, respectively. It also carries a mouse endostatin expression cassette controlled by the cytomegalovirus promoter. These properties allow for increased cancer cell targeting specificity and decreased adverse side effects. We showed that CNHK500-mE preferentially replicated in cancer cells. Compared with a replication-defective vector carrying the same endostatin expression cassette, CNHK500-mE–mediated transgene expression level was markedly increased via viral replication within cancer cells. In the nasopharyngeal tumor xenograft model, CNHK500-mE injection resulted in antitumor efficacy at day 7 after therapy. Three weeks later, it led to significant inhibition of xenograft tumor growth due to the combined effects of viral oncolytic therapy and antiangiogenesis gene therapy. Pathologic examination showed that most cancer cells were positive for adenoviral capsid protein and for apoptotic terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling in the CNHK500-mE–treated tumor tissues, and the microvessels in these tumor tissues were diminished in quantity and abnormal in morphology. These results suggest that, as a potential cancer therapeutic agent, the CNHK500-mE is endowed with higher specificity to cancer cells and low cytotoxicity to normal cells. (Mol Cancer Res 2008;6(4):568–75)

Introduction
The use of conditionally replicative adenovirus (CRAD) is a novel approach now being explored in the therapy of a variety of cancers (1). The replicative ability of CRAD is preserved in cancer cells but attenuated in normal cells, which allows for cancer cell–specific viral amplification and cell lysis (2). Large numbers of replicative virions are released from the lysed cancer cells, which are then capable of reinfecting adjacent cancer cells (3). Thus, when carrying a therapeutic gene, CRAD is expected to lead to a high level of therapeutic gene expression throughout the cancer mass. And so, as an antitumor therapeutic agent, it is superior to the replication-deficient adenovirus. Based on encouraging experimental results, several CRADs are now being evaluated in clinical trials (4-6).

Adenoviral replication can be restricted to just the cancer cells in a tumor by partial deletion of viral genes essential for adenoviral replication (3, 7-9) or by placing viral replicative genes under the control of cancer-specific promoters (10-14). Because activation and expression of telomerase is a common characteristic of most solid cancers, the promoter of human telomerase reverse transcriptase (hTERT) is used to control the adenoviral replicative genes in a series of CRADs. But the experimental results with these hTERT-regulated CRADs are inconsistent (15-17). We have previously reported the hTERT-promoter-regulated CRADs (18, 19) CNHK300, in which the Ela gene was controlled by the hTERT promoter, and its derivative CNHK300-mE, which in addition carries mouse endostatin gene. Both of these can selectively destroy telomerase-positive human cancer cells but do not significantly influence the growth of normal cells. After systemic delivery, they also superiorly suppressed xenograft tumor growth in vivo without detectable liver toxicity.

Another common characteristic of most solid cancers is the existence of low oxygen tension environment (20). Antiangiogenesis-based gene-virotherapy may result in cancer
cell necrosis and tumor angiogenesis inhibition, which further intensifies the hypoxia extent in cancer tissues. But there are contentions whether angiogenic inhibitors consistently increase tumor hypoxia. It has been shown that a significant decrease of tumor perfused vessels in endostatin-treated murine mammary carcinoma was observed in conjunction with an increase of tumor hypoxia (21). Local delivery of endostatin led to an induction of apoptosis, hypoxia, and large necrotic avascular areas within 77% of the treated BT4C glioma in rats (22), whereas it has been proved that the administration of recombinant endostatin had no effect on vessel spacing, tumor hypoxia, or tumor growth in highly vascularized MCa-35 murine mammary carcinomas, but in less vascularized MCa-4 murine mammary carcinomas. Although vessel spacings were also unchanged, tumor growth was inhibited and tumor hypoxia was significantly decreased (23). There was an emerging evidence supporting an alternative hypothesis that certain angiogenic agents can normalize tumor vasculature and alleviate hypoxia (24). Perhaps the different effect of angiogenic inhibitors on tumor hypoxia involves the xenograft model and administration schedule, and a feedback recruitment of new vasculature or not, which relies on the hypoxic response via a mechanism that is dependent or independent of hypoxia-inducible factor-1/vascular endothelial growth factor pathway, is a molecular determinant in cancer cells (25, 26). Hypoxia-inducible factor-1 is highly expressed in cancer cells, binds to the hypoxia response element (HRE), and up-regulates target gene expression for low oxygen adaptation (27). Therefore, HRE has been explored as an additional control for CRAD gene expression for low oxygen adaptation (27). Therefore, HRE has been explored as an additional control for CRAD gene expression for low oxygen adaptation (27). However, under hypoxic culture conditions (Fig. 2A), there was no significant difference in replicative capability in cancer cell lines between these CRADs ($P = 0.24$ for CNHK500-mE versus CNHK300; $P = 0.09$ for CNHK500 versus CNHK300), whereas there was a significant difference in normal cell lines ($P = 0.02$ for CNHK500-mE versus CNHK300; $P = 0.04$ for CNHK500 versus CNHK300). However, under hypoxic culture conditions (Fig. 2B), CNHK500-mE replicated more vigorously in most investigated cancer cell lines compared with CNHK300 (Fig. 2A), where there was no significant difference in replicative capability in cancer cell lines between these CRADs ($P = 0.03$), except in HepG2 and A549 cells. The replication potential of CNHK500-mE, as well as CNHK300 in A549 cells was decreased under hypoxia compared with normoxia. Compared with CNHK500, the replicative capacity of CNHK500-mE was slightly reduced in most cancer cells and normal cells. Of note, the replicative capability of CNHK300 was reduced in CNE-2, A549, and HeLa cells and developed a gene-viral therapeutic system CNHK500-mE by engineering an endostatin expression cassette into the CNHK500 vector (30). Compared with the previously generated CNHK300 and CNHK500 vector, the dual-regulated CNHK500-mE CRAD with antiangiogenesis gene exerts significantly stronger antitumor efficacy under hypoxic conditions by combining viral oncolytic therapy with antiangiogenesis gene therapy and thus provides a new strategy for human cancer therapy. The antitumor activity, viral replicative ability, and endostatin expression of the CNHK500-mE CRAD in telomerase-positive cancer cell lines and cancer xenografts have been studied.

Results
Selective Replication of CNHK500-mE in Cancer Cells

We have previously generated a CNHK300 adenovirus with $E_{1a}$ gene controlled by the $hTERT$ promoter and a CNHK500 adenovirus with $E_{1a}$ controlled by the $hTERT$ promoter and $E_{1b}$ controlled by HRE (Fig. 1; ref. 30). To generate the CNHK500-mE CRAD, we engineered a mouse endostatin gene expression cassette controlled by cytomegalovirus promoter into the CNHK500 vector. In vitro replicative assays showed that replication potential of the CNHK500-mE vector was about 100- to 1,000-fold higher in all cancer cell lines compared with normal cell lines. Under normoxic culture conditions (Fig. 2A), there was no significant difference in replicative capability in cancer cell lines between these CRADs ($P = 0.24$ for CNHK500-mE versus CNHK300; $P = 0.09$ for CNHK500 versus CNHK300), whereas there was a significant difference in normal cell lines ($P = 0.02$ for CNHK500-mE versus CNHK300; $P = 0.04$ for CNHK500 versus CNHK300). However, under hypoxic culture conditions (Fig. 2B), CNHK500-mE replicated more vigorously in most investigated cancer cell lines compared with CNHK300 ($P = 0.03$), except in HepG2 and A549 cells. The replication potential of CNHK500-mE, CNHK500, as well as CNHK300 in A549 cells was decreased under hypoxia compared with normoxia. Compared with CNHK500, the replicative capacity of CNHK500-mE was slightly reduced in most cancer cells and normal cells. Of note, the replicative capability of CNHK300 was reduced in CNE-2, A549, and HeLa cells and...
increased in HepG2 cells under hypoxia compared with normoxia.

Western blot analysis was done to measure E1a and E1b expression in virus-infected cancer and normal cell lines. The E1a expression was positive in both CNHK500-mE–infected (Fig. 3A) and CNHK500–infected (Fig. 3B) cancer cell lines, whereas it was negative in both infected normal cells. CNHK500 expressed E1b 55kDa at low levels in A549 cancer cells under normoxia and at higher levels under hypoxia. In contrast, the wild serotype 5 adenovirus (WAd5) and CNHK300 expressed E1b 55kDa at the same levels under both hypoxic and normoxic conditions (Fig. 3C). CNHK500-mE also expressed significantly more E1b 55kDa in cancer cells under hypoxia than under normoxia. In normal cells, E1b 55kDa expression was not detected under normoxia, although slight induction of E1b 55kDa expression was observed under hypoxia, albeit significantly less than seen for the cancer cell lines (Fig. 3D).

In vitro and In vivo Endostatin Expression Mediated by CNHK500-mE

CNHK500-mE expressed mouse endostatin with high efficiency in cancer cells compared with the replication-deficient adenovirus, Ad-mE. ELISA showed that CNHK500-mE–generated endostatin concentrations varied from 110.68 ± 47.36 ng/mL to 560.27 ± 111.13 ng/mL at day 7 after infection of the various cancer cell lines, and these were between 11.4- and 32.4-fold higher than those from the Ad-mE vector-infected cultures (Fig. 4A). Conversely, CNHK500-mE produced endostatin at much lower levels in normal cells compared with cancer cells \( (P = 0.04) \), which was comparable with the Ad-mE–mediated endostatin production in cancer cells \( (P = 0.29) \). In SGC-7901 cells, CNHK500-mE expressed comparable levels of endostatin as CNHK300-mE under normoxia \( (P = 0.81) \) but significantly more endostatin than CNHK300-mE under hypoxia \( (P = 0.03) \;\text{Fig. 4B}.\)

In CNE-2 xenograft models, the endostatin concentrations in mouse sera were 80.16 ± 30.77 ng/mL at day 3 and reached a peak value of 402.42 ± 55.95 ng/mL at day 7 after treatment with CNHK500-mE, whereas the corresponding endostatin concentrations were 39.30 ± 8.34 ng/mL at day 3 and 114.19 ± 36.71 ng/mL at day 7 in the Ad-mE–treated group \( (P < 0.01\text{ for day 7 data}) \). At day 14 after treatment, the endostatin expression concentration declined to 315.06 ± 50.59 ng/mL and 86.43 ± 10.96 ng/mL in the CNHK500-mE and the Ad-mE groups, respectively (Fig. 4C).

Antitumor Efficacy of CNHK500-mE in Xenograft Nasopharyngeal Tumor Model

After showing the high continuous transgene expression, we further investigated the antitumor efficacy of the

FIGURE 3. Conditional expression of viral E1a and E1b 55kDa in cancer and normal cell lines. Cell lines were infected with various viruses at a multiplicity of infection of 1, and the cell lysates containing 20 μg total protein were examined by Western blot. A, E1a was expressed by CNHK500-mE in cancer cell lines but not in normal cells. B, Under hypoxic conditions, CNHK500 and WAd5 viruses expressed E1a at the same levels in CNE-2 and A549 cancer cells but not in MRC-5 control normal cells. C, E1b 55kDa was expressed at similar levels in A549 cancer cells when infected with WAd5 or CNHK300, but CNHK500 expressed more E1b 55kDa under hypoxia compared with normoxia. CNHK500-mE expressed significantly more E1b 55kDa in cancer cells under hypoxia compared with normoxia, and no or weak expression of E1b 55kDa was detected in MRC-5 cells infected with CNHK500-mE under normoxia and hypoxia, respectively.

CNHK500-mE vector in vivo. In CNE-2 xenograft tumor models, the antitumor effect was evident at day 7 after therapy in the CNHK500-mE–treated group but at day 15 in the CNHK500-treated, CNHK300-treated, and Ad-mE–treated groups. Three weeks later, all virus-injected groups, except for the Ad-mE group, revealed pronounced antitumor efficacy when compared with the control group, with the tumor sizes of 723.49 ± 181.32, 1,563.46 ± 400.73, 1,273.47 ± 444.81, and 2,228.85 ± 394.45 mm³ in CNHK500-mE, CNHK500, CNHK300, and control groups, respectively (P < 0.01). The effect of CNHK500-mE was the best out of all groups studied (Fig. 5).

Pathologic Examination

Having shown the significant tumor inhibition effects, we further studied the pathologic changes in tumors injected with the CNHK500-mE vector and compared the results with other vectors. Mice were sacrificed after the observation period as shown in Fig. 5. Tumors were collected and examined pathologically by H&E staining and immunohistochemistry. The H&E staining showed many large necrotic regions in tumors from each group, especially in the CNHK500-mE–treated group. But in the control group, cancer cells grew unhindered with only small focal areas of necrosis. Around the necrotic areas, most cancer cells were positive for adenoviral capsid protein hexon in tumor tissues of CNHK500-mE–treated, CNHK500–treated, and CNHK300–treated groups (Fig. 6A) and also positive for the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL; Fig. 6B). In regions away from necrosis area, the cancer cells positive for hexon distributed sporadically, but no cell was positive for hexon in the control group. The percentage positive indices of TUNEL labeling in the CNHK500-mE, CNHK500, and CNHK300 groups were 44.67 ± 9.61, 36.00 ± 10.58, and 32.33 ± 8.74, respectively, whereas there were a few cancer cells positive for TUNEL labeling with the positive index of 5.23 ± 2.36 (Fig. 6C). There was a significant difference in TUNEL labeling positive index between the control group and any replicative adenovirus-treated group (P < 0.01 for CNHK500-mE group; P = 0.01 for CNHK500 and CNHK300 groups).

Quantitative analysis of blood vessels was also done by CD31 immunohistochemistry. Compared with the control group, the microvessels in tumor tissues of the CNHK500-mE group were irreparably damaged or occlusive in morphology, and decreased in quantity (Fig. 6D and E). The microvessel density was 7.67 ± 2.08, 32.33 ± 5.13, 22.33 ± 12.34, and 42.00 ± 13.08 in the CNHK500-mE, CNHK500, Ad-mE, and control groups, respectively. There was a significant difference between them (P = 0.01 for CNHK500-mE versus control group; P < 0.01 for CNHK500-mE versus CNHK500). Compared with the control group, the microvessel densities in the CNHK500 and Ad-mE groups were slightly decreased (P = 0.15 for CNHK500; P = 0.07 for Ad-mE). There were some microvessels growing into the cancer mass from the peripheral matrix in the Ad-mE–treated group (Fig. 6F), and the cancer cells near the peripheral matrix grew well with obvious nuclear atypia.

Discussion

Telomerase is activated in most types of human cancers and serves as a promising target for CRAD-mediated cancer gene therapy (15, 31-33). Besides the unrestrained proliferation due to the activation of telomerase, hypoxia is another outstanding characteristic of most solid cancers. Hypoxia has been considered to be a major factor that leads to resistance to cancer radiotherapy and chemotherapy (34). Therefore, tumor hypoxia can be exploited as a target in developing cancer therapy. Hypoxia-inducible factor-1 triggers a transcriptional signaling pathway that responds to hypoxia by binding to the HRE enhancer in target genes. These findings lead to the use...
of HRE to drive therapeutic gene expression in solid tumors (35-37). Recently, we and others have provided compelling evidence that hypoxia-dependent CRADs can selectively lyse tumor cells under hypoxia (29,30,38).

CRADs can be exploited as gene therapeutic vectors by insertion of transgene expression cassettes (39). Using the cancer-selective replication properties of CRADs, the therapeutic transgenes are transferred to cancer cells, which leads to the production of transgenes at therapeutic concentrations in the tumor region. A selection of genes can be used in cancer gene-viral therapy, such as tumor suppressor genes, cytokine genes, prodrug-activating enzyme genes, and antiangiogenesis genes (40). The understanding of tumor angiogenesis has led to the development of a new generation of CRADs that target tumor vascularization. Chen et al. (41) have constructed a replication-deficient adenovirus vector, Av3mEndo, which encodes a secretory form of mouse endostatin. The secreted endostatin from Av3mEndo-transduced cells was shown to potently inhibit endothelial cell migration in vitro. A single i.v. administration of Av3mEndo in mice could result in prolonged and elevated levels of circulating endostatin, which resulted in partial inhibition of vascular endothelial growth factor–induced angiogenesis and complete prevention of tumor growth in 25% of mice (41). We have also shown a profound antitumor efficacy of endostatin in xenograft models of human cancers (19,42).

By controlling the expression of Ela gene with hTERT promoter and Elb gene with HRE, the CNHK500-mE vector achieved the desired cancer cell targeting specificity under hypoxia conditions, whereas the normal cells, negative for telomerase activity, were spared. Compared with CNHK300 with Ela controlled by the hTERT promoter, the replicative capacity of CNHK500 and CNHK500-mE was markedly

![FIGURE 4. CNHK500-mE-mediated high levels of endostatin expression in cancer cell culture and xenograft tumor model. A, CNHK500-mE expressed more endostatin than Ad-mE in cancer cell supernatants at day 7 after infection and expressed as much endostatin as Ad-mE in normal cells under normoxia. B, CNHK500-mE expressed more endostatin than CNHK300-mE under hypoxia but expressed the same levels of endostatin under normoxia in SGC-7901 cells at day 7 after infection. C, Tumor-bearing mice were given five intratumoral injections of viruses, one injection every other day with 2 × 10^8 plaque-forming units/dose/mouse. At days 0, 3, 7, 10, and 14 after injection, mice serum samples were collected and measured for the expression of mouse endostatin by ELISA. CNHK500-mE expressed more endostatin than Ad-mE in mouse sera in CNE-2 xenograft models, reaching a peak value at day 7 after treatment and then declining subsequently.](image-url)

![FIGURE 5. Antitumor efficacy of the CRADs in CNE-2 tumor xenografts. Mice were given five intratumoral injections to introduce viruses into CNE-2 xenografts, one injection every other day with 2 × 10^8 plaque-forming units/dose/mouse. The potent antitumor effect was shown in all virus-treated groups. CNHK500-mE showed the best antitumor growth effect. The antitumor effect of CNHK300, CNHK500, and CNHK500-mE was stronger than that of Ad-mE.](image-url)
stronger in cancer cells and significantly weaker in normal cells, suggesting that these dual-regulated CRADs can target malignancies with a higher specificity. Concomitantly, CNHK500-mE efficiently expressed more endostatin than Ad-mE in cancer cell lines and in xenograft cancer models, which could overcome the disadvantages of low gene transfer rate and poor gene expression in replication-deficient adenovirus-mediated gene therapy. The replicative capability of CNHK500-mE reached the same extent as that of CNHK300 in cultured cancer cells under normoxia conditions. However, under hypoxia, the hypoxia-inducible factor-1 expression in cancer cells led to HRE-dependent E1b expression, which additionally enhanced the replication and oncolysis effect of CNHK500-mE. This is of advantage, as new viral particles are capable of reinfection and may initiate a cascade of cell death.

Compared with CNHK500, CNHK300, and Ad-mE, the improved antitumor efficacy of CNHK500-mE in human nasopharyngeal cancer CNE-2 xenograft models originated from (a) the selective viral replication and oncolysis of cancer cells and (b) the antiangiogenesis effect due to enhanced endostatin gene expression, suggesting that the combination of gene therapy and virotherapy can enhance the potential of cancer treatment. Pathologic examination confirmed that the replicative progeny virions of CNHK500-mE expressed the capsid protein hexon only in xenograft cancer cells and that the endostatin expression reduced the numbers and disrupted the structure of microvessels in tumor tissues. The combined effect from both of these two antitumor factors led to cancer cell apoptosis and cancer tissue necrosis, thus inhibiting xenograft tumor growth.

Although we operated multipoint intratumoral injections of viral agents, the xenograft cancer cells positive for hexon and TUNEL labeling were seen to be predominantly distributed around the necrotic foci. Several hypotheses could explain this phenomenon: (a) the existence of barriers, which could be the mouse-originated extracellular matrix and the tumor-supporting cells, concentrates the viruses mainly in the injection sites (43); (b) cancer cell necrosis is caused by the oncolysis and the endostatin expression and thus there is a high probability that hexon- and TUNEL-positive cells are enriched in perinecrotic regions; (c) the necrosis of cancer tissues diminishes the quantity of microvessels in the necrotic and perinecrotic regions and then increases the hypoxia extent and regulates the HRE-controlled virus replication in the perinecrotic regions; and (d) cancer cell necrosis makes the viruses lose the base for replication in the necrotic regions and limits the viruses to spread across the necrotic regions. We also found that these viruses inhibited the growth of xenografts for only a certain period, with the tumor growth recovering slowly in some cases. Quantitative analysis of blood vessels in these tumor tissues showed that there were some microvessels growing into the cancer nests from peripheral matrix in adenovirus-treated groups. This phenomenon was especially obvious in the Ad-mE–treated group. The impaired viral spread and the recovered microvessel growth were possibly the main reasons for tumor regrowth. These findings suggest that it is necessary to reintject the gene-viral therapeutic agents at multiple sites in tumors. Future studies addressing adenoviral spread in cancer tissues may improve the antitumor efficacy of CRADs.

In summary, the dual-regulated CRAD CNHK500-mE provides a novel strategy for cancer gene therapy. It holds advantages of higher specificity to cancer cells under hypoxia and lower cytotoxicity to normal cells. As a gene delivery vector, CNHK500-mE can mediate high levels of therapeutic gene expression in cancer cells. Therefore, the combination of oncolytic viral therapy and antitumor gene therapy improves the antitumor effect of CRADs in cancer therapy.

**Materials and Methods**

**Adenoviruses and Cell Lines Used in This Study**

The adenovirus plasmid pSG500, in which the *E1a* promoter was replaced by *hTERT* promoter (−212 bp to +46 bp) and the *E1b* promoter was replaced by HRE (five copies of...
TCCACAGTGCATACGTTGGGCTCACAAGGTCTCTCT, with 60 bp additive nucleotides), was constructed by overlap PCR method from the plasmid pXC1 (Microbix Biosystems, Inc.) as described previously (30). The HRE was synthesized according to the sequence derived from the hypoxia-inducible factor–regulated gene [e.g., the vascular endothelial growth factor (VEGF)] from 1,377 to 1,411 bp in the promoter region (27, 44). The expression cassette of mouse endostatin was released from pCA13-mE by using BglI (19) and inserted into pSG500 to generate pSG500-mE. The plasmids pSG500, pSG500-mE, and pCA13-mE were individually transfected into HEK293 cells using Effectene Transfection Reagent (Qiagen) together with the adenovirus packaging plasmid pBGHE3 (Microbix Biosystems). After homologously recombining in HEK293 cells, we obtained two sets of novel dual-regulated tumor-selective CRADs, namely CNHK500 and CNHK500-mE, and one set of replication-deficient adenovirus, named Ad-mE.

Human hepatocyte cancer cell lines Hep3B and HepG2, human pancreatic cancer cell line PANC-1, human lung cancer cell line A549, human breast cancer cell line MDA-MB-231, human nasopharyngeal cancer cell line CNE-2, human cervical cancer cell line HeLa, and human normal fibroblast cell lines MRC-5 and BJ were purchased from the American Type Culture Collection. Human embryonic kidney cell line HEK293 was obtained from Microbix Biosystems. Human gastric cancer cell line SGC-7901 was obtained from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in medium recommended by the providers. The cancer cell lines were all shown positive for hTERT expression by reverse transcription-PCR with the sense primer 5′-CGGAAGATGTGCTGGAGCA-3′ and the antisense primer 5′-GGATGAAGCGGAGTCTGGA-3′, but the normal cell lines were negative for hTERT (data not shown). To simulate hypoxic conditions of in vivo tumor mass, the cultured cells were exposed to 0.1% partial oxygen tension for 16 h.

In vitro Viral Replication and Transgene Expression Study

To investigate the cancer cell–specific replication of CNHK500-mE in comparison with our previous generated adenovirus CNHK300 and CNHK500, various cancer cell lines and normal fibroblast cell lines were seeded in six-well plates at 5 × 10^5 cells per well and cultured until cancer cells were in log phase and normal cells at contact inhibition stage. Subsequently, the cultured cells were infected with the adenoviruses at a multiplicity of infection of 5. At 48 h after infection, the cells were harvested and lysed by three cycles of freeze and thaw, and the cell lysates were titered on 293 cells according to the tissue culture infectious dose 50 (TCID50) method (45). The titer data at 48 h were normalized with those at the beginning of infection. The expression of E1a and E1b 55KDa in cell lysates was examined using Western blot as described in earlier report (18).

To study the cancer-specific production of endostatin, cells were seeded in 24-well plates at a density of 5 × 10^4 per well. Following 1-d culture, cells were infected with CNHK500-mE, CNHK300-mE, or Ad-mE at a multiplicity of infection of 1. At day 7 after infection, the cell culture supernatants were collected. The ELISA was done to detect the mouse endostatin expression using ChemiKine Mouse Endostatin EIA kit (Chemicon) as described previously (19).

Xenograft Tumor Models for in vivo Evaluation of Antitumor Effect

CNE-2 cancer cells were s.c. injected into the right flanks of athymic BALB/c (nu/nu) mice at 2 × 10^6 per mouse. Seven days later, when CNE-2 tumor xenografts were established and reached ~100 mm^3 in volume, mice were randomly divided into the following groups: CNHK300, CNHK500, CNHK500-mE, Ad-mE, and the control groups, with 10 mice in each of the groups for studying the gene-viral therapeutic efficacy. In the CNHK500-mE, Ad-mE, and the control groups, additional 15 mice per group were simultaneously treated for detecting systemic mouse endostatin expression. Mice were given five intratumoral injections for 9 d, one injection every other day, with a total dosage of 10^9 plaque-forming unit viruses per mouse in the virus-treated groups and with 100 µL viral preservation solution [10 mmol/L Tris-HCl (pH 8.0), 2 mmol/L MgCl2, 4% sucrose] per mouse per time in the control group. Tumor size was measured and estimated using the formula a × b^2 × 0.5, where a and b are the maximal and minimal diameters, respectively. The mice were killed humanely when their average volume of xenografts was >2,000 mm^3 at a regular measurement.

At days 0, 3, 7, 10, and 14 after injection, three mice from the CNHK500-mE, Ad-mE, and control groups were sacrificed at each time point to collect serum samples by tail vein incision. The expression level of mouse endostatin gene in mouse sera was measured using the ChemiKine Mouse Endostatin EIA kit. All other mice were sacrificed after the observation period by cervical dislocation. Tumors were excised, fixed in 10% formaldehyde for 6 h, paraffin embedded, and serially sliced into 5-µm-thick consecutive sections for H&E staining and immunohistochemical examination. The expression of adenoviral capsid protein hexon was visualized using mouse anti-adenoviral hexon antibody (Biodesign International). Quantitative analysis of blood vessels in tumor tissues was done by CD31 immunohistochemistry using rat anti-mouse CD31 monoclonal antibody (BD Biosciences Pharmingen). The microvessel density was calculated within five random high-power fields under microscope and shown as mean ± SD (46).

To show apoptotic cell death of tumor cells, the TUNEL assay was done on paraffin-embedded sections using an In situ Cell Death Detection kit (Roche Diagnostics) according to the manufacturer’s instructions. The positive indices were counted from five randomly selected high-power fields and expressed as the percentage of total cells counted. This study was approved by the local ethical committee, and we followed the criterion in animal experiments established by American Veterinary Medical Association to dispose animals scientifically and humanely (47).

Statistical Analysis

All in vitro experiments were done thrice, and the in vitro and in vivo experimental data were presented as the mean ± SD and analyzed by Student’s t test for statistical significances.

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