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An oncolytic adenovirus enhances antiangiogenic and antitumoral effects of a replication-deficient adenovirus encoding endostatin by rescuing its selective replication in nasopharyngeal carcinoma cells

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ABSTRACT

A replication-deficient adenovirus (Ad) encoding secreted human endostatin (Ad-Endo) has been demonstrated to have promising antiangiogenic and antitumoral effects. The E1B55k-deleted Ad H101 can selectively lyse cancer cells. In this study, we explored the antitumor effects and cross-interactions of Ad-Endo and H101 on nasopharyngeal carcinoma (NPC). The results showed that H101 dramatically promoted endostatin expression by Ad-Endo via rescuing Ad-Endo replication in NPC cells, and the expressed endostatin proteins significantly inhibited the proliferation of human umbilical vein endothelial cells. E1A and E1B19k products are required for the rescuing of H101 to Ad-Endo replication in CNE-1 and CNE-2 cells, but not in C666-1 cells. On the other hand, Ad-Endo enhanced the cytotoxicity of H101 by enhancing Ad replication in NPC cells. The combination of H101 and Ad-Endo significantly inhibited CNE-2 xenografts growth through the increased endostatin expression and Ad replication. These findings indicate that the combination of Ad-Endo gene therapy and oncolytic Ad therapeutics could be promising in comprehensive treatment of NPC.

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1. Introduction

Oncolytic adenovirus (Ad) has been demonstrated to lyse selectively cancer cells but not normal cells [1]. E1B55k-deleted Ad *dl*1520 [2] and H101 [3] are the oncolytic Ad with the most extensive investigation, were firstly applied in clinical trials and H101 has been approved for the clinical application to treat squamous cell carcinoma of the head and neck in China.

The antiangiogenic therapy is another attractive strategy for cancer treatment. Endostatin was previously considered the most potent endogenous angiogenesis inhibitor [4] and was rapidly moved to clinical trials. However, the high instability and shorter half-life made it difficult for clinical application [5]. Promisingly, the antiangiogenic gene therapy can overcome these problems and is likely a potential new approach for the treatment of cancer.

An Ad vector encoding a secreted human endostatin (Ad-Endo) has been confirmed to inhibit tumor growth through antiangiogenesis [6]. The results of preclinical, phase I/II clinical trials suggested that the treatment of solid tumor with Ad-Endo is likely a safe and promising approach [7,8] (ClinicalTrials.gov identifier, NCT00634595). Even so, it is necessary to find a way for overcoming the problem of limited curative effect due to limited endostatin expression [7,9,10].

We presumed that the selective replication of oncolytic Ad would rescue Ad-Endo genome amplification and promote endostatin expression. In this study, we investigated the antitumor effects of the combined treatment of Ad-Endo and H101 on Nasopharyngeal carcinoma (NPC). The results indicate that Ad-Endo and H101 have a synergistic antitumor effect on NPC, which resulted from the promoted antiangiogenic effect of Ad-Endo by H101 and the enhanced oncolysis of H101 by Ad-Endo.

2. Materials and methods

2.1. Cells and plasmids

Human NPC CNE-1, CNE-2 cells contain a mutant p53 at codon 280, whereas C666-1 harbors a deletion at codon 249 of p53 and Epstein-Barr virus (EBV) DNA. Human umbilical vein endothelial cells (HUVEC) were cultured in Ham's F12 nutrient mixture

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supplemented with endothelial cell growth supplement (BD Biosciences) and 10% FBS. human embryonic kidney 293 cells and CNE-1, CNE-2 cells were cultured in DMEM containing 10% FBS (Invitrogen), and C666-1 cells were cultured in RPMI 1640 with 15% FBS. The plasmids pCD-E1A or pCD-E1B19k were constructed by inserting the relevant gene fragments, amplified with the corresponding primers (Table S1), into pcDNA3.1(+) vector. Plasmids were transfected into NPC cells using the Lipofectamine 2000 transfection reagent (Invitrogen).

2.2. Recombinant Ad, infection and titering

Recombinant Ad H101 [3,11] was purchased from Shanghai Sunway Biotech Co. Ltd., and Ad-Endo [6] was kindly supplied by Guangzhou Doublle Bioproduct Inc. NPC cells were infected with Ad in serum-free medium for 2 h, then were cultured in normal medium. The indicated time point post-infection corresponds to the one after the medium change. The Ad was titered by hexon immunoassay using Adeno-X rapid titer kit (BD Clontech™).

2.3. Quantitative real-time PCR

Ad-infected cells and tumor tissues were collected. The DNA was isolated using genomic DNA preparation kits (Axygen). The mRNA was isolated by TRIzol® reagent (Invitrogen) and reverse-transcribed into cDNA. The viral DNA or cellular cDNA were quantitatively measured by real-time PCR. The primers are listed in Table S1. The viral DNA copy number (DCN) is normalized to that of β-actin ($2^{-\Delta Ct}$), and mRNA level was presented as a relative value against GAPDH.

2.4. In vitro cytotoxicity assay

The cytotoxicity was assessed by CCK-8 (Dojindo). After treated for 72 h, cells were incubated with CCK-8 for 2–4 h, then measured OD_{450nm}.

2.5. Animal models and in vivo antitumor activity

BALB/c-nu/nu mice (5–6 weeks old) were obtained from Guangdong Medical Laboratory Animal Center (License No. SCXK (Yue) 2008-0002), and fed under specific pathogen-free conditions according to protocols approved by the Sun Yat-sen University Institutional Animal Care and Use Committee. Pieces (about Ø1.5 mm) of CNE-2 tumor were subcutaneously transplanted into the flanks to construct xenograft model.

To assess Ad DCN dynamic change and endostatin expression, mice were injected intratumorally with Ad-Endo or H101 (alone or together) when xenografts reached Ø7–8 mm. Tumor tissues and heparin-anticoagulant blood were sampled After treatment, DNAs from tumor tissues were analyzed for DCN, blood plasma and tumor tissue homogenate were measured for endostatin concentration.

To analyze the antitumor effects, mice carrying CNE-2 xenografts were randomly assigned to five groups when the xenografts reached Ø5–6 mm. The mice were treated as described in (Fig. 4E). Body weight and tumor size were measured every 5 days, and the tumor volumes were calculated according to the formula $V = 0.52 \times L \times W^2$ (L, length; W, width) [6]. The xenografts were weighed at the end point of the experiments.

2.6. Statistical analysis

All *in vitro* experiments were repeated at least three times, and the animal experiments were repeated over two times. The data were analyzed with ANOVA. $p < 0.05$ indicates statistically significant. The combined effect was assessed with the Q value using Zheng-Jun Jin's method [12]: $Q = E_{AB}/[E_A + E_B(1 - E_A)]$ (E_A, E_B and E_{AB} indicate the effects of A, B and combination). And the effect of the combination effect can be classified as antagonistic ($Q < 0.85$), additive ($0.85 < Q < 1.15$), or synergistic ($Q > 1.15$).

3. Results and discussion

Ad-Endo has been showed that can inhibit tumor growth through antiangiogenic effects in our previous study [6]. E1B55kD-deficient Ad, such as H101 or Onyx-015, has been confirmed that can selectively lyse cancer cells with abnormal p53 pathway [1,13]. In addition, late viral RNA export, the cell cycle status of host cells, viral infectivity, and the expression of heat shock proteins may also determine the tumor selectivity of E1B55kD-deficient Ad [13,14]. In previous studies, E1B55k-deleted oncolytic Ad was demonstrated to replicate selectively in and destroy NPC cells [13]. In this paper, we try to investigate the antitumor effects of combination treatment with H101 and Ad-Endo on NPC.

3.1. H101 enhanced the antiangiogenic effect of Ad-Endo by promoting endostatin expression in vitro

Firstly, we assessed the influence of H101 on endostatin expression by Ad-Endo in NPC cells. The results showed that H101 dramatically promoted endostatin expression in Ad-Endo-infected NPC cells (Fig. 1). The endostatin concentrations in cultural supernatants from NPC cells infected with Ad-Endo plus H101 were much higher than those infected with Ad-Endo alone (Fig. 1A–C). Then we infected CNE-2 cells with 10 MOIs of Ad-Endo in combination with H101 at increasing doses, and analyzed endostatin concentrations at 48 h post-infection. The results showed that endostatin amounts increased along with the increases of H101 doses, presenting a dose-dependent manner (Fig. 1D).

Next, we tested the anti-proliferation effect on HUVEC of endostatin in these cultural supernatants after removing viral particles by ultra-filtering, and found that all of supernatants from Ad-Endo-infected NPC cells significantly inhibited HUVEC growth, compared with those from mock-infected NPC cells. Moreover, the supernatants from Ad-Endo+H101-infected cells showed stronger inhibitory effect on HUVEC proliferation than those from Ad-Endo-infected cells (Fig. 1F).

In summary, oncolytic Ad H101 likely enhances the antiangiogenic effect of Ad-Endo by promoting endostatin expression in NPC cells *in vitro*.

3.2. H101 rescued Ad-Endo replication in NPC cells by supplying E1A and E1B19k proteins

Since H101 can replicate in NPC cells, we presumed that H101 rescued the replication of Ad-Endo by supplying some Ad early proteins, then promoted endostatin expression. To test this hypothesis, we detected Ad-Endo DCN in NPC cells infected with Ad-Endo alone or in combination with H101. The results showed that Ad-Endo DCN in CNE-1 or CNE-2 cells infected with Ad-Endo+H101 increased by over 100-fold after infection, whereas the DCN decreased gradually in cells infected with Ad-Endo alone (Fig. 2A and B). These data indicated that H101 rescued the Ad-Endo replication in CNE-1 and CNE-2 cell. Moreover, the rescue effects enhanced along with the increases of H101 doses in CNE-2 cells (Fig. 2D).

Surprisingly, different from in CNE-1 and CNE-2 cells, Ad-Endo DCN increased in C666-1 cells infected with Ad-Endo alone, though the DCN increased more in Ad-Endo+H101-infected C666-1 cells than in Ad-Endo-infected C666-1 cells (Fig. 2C). These data gave

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