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Therapeutic Efficacy of Adenoviral-Mediated *p53* Gene Transfer Is Synergistically Enhanced by Combined Use of Antisense Oligodeoxynucleotide Targeting *Clusterin* Gene in a Human Bladder Cancer Model[1]

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Abstract

To establish a more effective therapeutic strategy against advanced bladder cancer, we investigated the effects of combined treatment with antisense (AS) oligodeoxynucleotide (ODN) targeting the anti-apoptotic gene *clusterin* and adenoviral-mediated *p53* gene transfer (Ad5CMV-p53) using the human bladder cancer KoTCC-1 model. Clusterin expression in KoTCC-1 cells was highly upregulated by Ad5CMV-p53 treatment; however, AS clusterin ODN treatment further suppressed clusterin expression in KoTCC-1 cells after Ad5CMV-p53 treatment. AS clusterin ODN treatment synergistically enhanced the cytotoxic effect of Ad5CMV-p53, and DNA fragmentation characteristic of apoptosis was observed only after combined treatment with AS clusterin ODN and Ad5CMV-p53, but not after treatment with either agent alone. Administration of AS clusterin ODN and Ad5CMV-p53 into nude mice resulted in a significant inhibition of KoTCC-1 tumor growth as well as lymph node metastases compared to administration of either agent alone. Furthermore, combined treatment with AS clusterin ODN, Ad5CMV-p53, and cisplatin completely eradicated KoTCC-1 tumors and lymph node metastases in 60% and 100% of mice, respectively. These findings suggest that combined treatment with AS clusterin ODN and Ad5CMV-p53 could be a novel strategy to inhibit bladder cancer progression, and that further additional use of a chemotherapeutic agent may substantially enhance the efficacy of this combined regimen.

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Introduction

To date, cisplatin-based combination chemotherapy has been the mainstay of treatment for patients with advanced bladder cancer, and it is at least palliatively effective. However, the efficacy of cisplatin-based combination chemotherapy is limited due to *de novo* drug resistance, or the development of cellular-resistant phenotype during treatment; therefore, there has been no study demonstrating the significant prognostic benefit of this therapy for patients with advanced disease [1,2]. Accordingly, novel therapeutic strategies targeting molecular mechanisms mediating resistance to conventional treatments must be developed to achieve a significant impact on the survival of advanced bladder cancer patients.

Gene transfer techniques may provide novel therapeutic strategies for a variety of malignancies. Among them, replication-deficient adenoviral vectors, which are capable of highly efficient infection in target cells, have been regarded as one of the most attractive tools [3]. To date, a number of studies have demonstrated marked growth-inhibitory effects of adenoviral-mediated gene therapy of wild-type p53 on various types of tumor growth both *in vitro* and *in vivo*, including bladder cancer, through the induction of apoptotic cell death; based on promising findings of these preclinical studies, several clinical trials of *p53* gene therapy have been carried out [4–6]. Recent studies, however, reported that overexpression of antiapoptotic genes inhibits the therapeutic efficacy of adenoviral-mediated *p53* gene transfer (Ad5CMV-p53) [7,8], suggesting that it would be necessary to consider the additional treatment using another type of adequate agent if adenovirus p53 vectors are used for patients expressing high levels of antiapoptotic genes.

Clusterin, also known as testosterone-repressed prostate message-2, sulfated glycoprotein-2, or apolipoprotein J, was first isolated from ram rete testes fluid, and has been shown to be implicated in several pathophysiological processes,

Abbreviations: AS, antisense; MM, mismatch; ODN, oligodeoxynucleotide

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including tissue remodeling, reproduction, lipid transport, and apoptotic cell death [9]. Because clusterin expression is highly upregulated in various benign and malignant tissues undergoing apoptosis, clusterin was initially regarded as a marker for cell death [10,11]. Recent studies, however, reported conflicting findings that clusterin acts as a cell survival gene through the inhibition of apoptosis induced by a wide variety of stimuli [12–16]. Our previous studies demonstrated that overexpression of clusterin is closely associated with disease progression and recurrence in patients with bladder cancer [17], and that antisense (AS) oligodeoxynucleotide (ODN) therapy targeting *clusterin* gene enhances the efficacies of cytotoxic chemotherapy, resulting in an inhibition of the growth and metastasis of bladder cancer cells [18,19]. However, because numerous genes mediate tumor progression, inhibition of a single target gene with AS technology is likely insufficient to completely suppress tumor progression. Although there have not been any reports demonstrating complete eradication of established tumors *in vivo* using AS ODN alone, combined use of AS ODN with another ODN targeting a different gene or an other type of therapeutic modality, such as chemotherapeutic agents and radiotherapy, has been shown to induce more potent antineoplastic effects in some tumor model systems [14–16,20].

This study investigated whether apoptotic cell death induced by Ad5CMV-p53 is synergistically enhanced by AS clusterin ODN treatment, and determined whether combined treatment with Ad5CMV-p53 and AS clusterin ODN inhibits tumor growth and metastasis in the human bladder KoTCC-1 tumor model.

Materials and Methods

Tumor Cell Line

The human bladder cancer cell line KoTCC-1 was established in our laboratory [8] and was maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal calf serum.

Chemotherapeutic Agent

Cisplatin, a generous gift from Nippon Kayaku Co. (Tokyo, Japan), was dissolved in PBS at 1 mg/ml and diluted in medium before each experiment.

Adenovirus Vectors

Replication-deficient recombinant adenovirus vectors [8] were used in this study (i.e., Ad5CMV-p53 expresses the wild-type human p53 under the control of the human cytomegalovirus promoter, and Ad5CMV-Luc–encoding *Luciferase* gene was used as a control vector). Adenoviruses were propagated in human embryonic kidney 293 cells, which contain E1 and are thus highly permissive of the replication of the E1 replication-deficient adenovirus. It was stored as previously reported, and the viral infection titers were determined by plaque assays [8].

AS Clusterin ODN

Phosphorothioate ODNs used in this study were generously supplied by Dr. Brett P. Monia (Isis Pharmaceuticals, Carlsbad, CA). The sequence of AS clusterin ODN corresponding to the human clusterin translation initiation site was 5′-CAGCAGCAGAGTCTTCAT-3′. Two-base clusterin mismatch (MM) ODN (5′-CAGCAGCAGAGTATTTAT-CAT-3′) was used as the control. ODNs were diluted in 10 mM Tris (pH 7.4) and 1 mM ethylenediaminetetraacetic acid (EDTA) and kept at −20°C.

Treatment of Cells with ODN

KoTCC-1 cells were treated with various concentrations of ODN after a preincubation for 20 minutes with 4 μg/ml lipofectin (Life Technologies, Inc.) in serum-free OPTI-MEM (Life Technologies, Inc.). Four hours later, the medium containing ODN and lipofectin was replaced with the standard culture medium described above.

Northern Blot Analysis

Total RNA was isolated from cultured KoTCC-1 cells by the acid–guanidium thiocyanate–phenol–chloroform method. Electrophoresis, hybridization, and washing conditions were carried out as previously reported [14]. Human clusterin and GAPDH cDNA probes were generated by reverse transcription polymerase chain reaction (RT-PCR) from total RNA of human kidney using primers 5′-AAG-GAAATTCAAAATGCTGTCAA-3′ (sense) and 5′-ACAGA-CAAGATCTCCCGGCACTT-3′ (antisense) for clusterin, and 5′-TGCTTTTAACTCTGGTAAAGT-3′ (sense) and 5′-ATATTTGGCAGGTTTTTCTAGA-3′ (antisense) for GAPDH. The density of bands for clusterin was normalized against that of GAPDH by densitometric analysis.

Real-Time RT-PCR

Total RNA was isolated as described above, and 1 μg of each total RNA was reverse-transcribed using an Oligo dT and Superscript preamplification system (Life Technologies, Inc.). To examine the expression levels of clusterin, real-time quantitative PCR was performed using Sequence Detector (ABI PRISM 7700; PE Applied Biosystems, Foster City, CA). Selected sequences of primers and probes are as follows: 5′-GAGCAGCTGAACGAGCAGTTT-3′ (sense), 5′-CTTCGCCTTGCGTGAGGT-3′ (antisense), and 5′-ACTGGGTGTCCCGGCT-3′ (probe) for clusterin; and 5′-GAAGGTGAAGGTCGGAGTC-3′ (sense), GAPDH 5′-GAAGATGGTGATGGGATTTC-3′ (antisense), and 5′-CAAGCTTCCCGTTCTCAGCC-3′ (prove) for GAPDH. The TaqMan probes consisted of an ODN with a 5′ FAM reporter dye and a 3′ TAMRA quencher dye. Each cDNA was analyzed by quantitative PCR in a 50-μl volume using Master Mix (PE Applied Biosystems). The condition of thermal cycling was 50 cycles of amplification consisting of 15 seconds at 95°C and 1 minute at 60°C. Real-time quantitation was performed using TaqMan assay according to the manufacturer's instruction. After the generation of a real-time amplification plot based on the normalized fluorescence signal, the threshold cycle (C_T) was determined. The C_T was then used for kinetic