J. Microbiol. Biotechnol. (2002), **12**(1), 89–95

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Adenovirus-Mediated Antisense Expression of Telomerase Template RNA Induces Apoptosis in Lung Cancer Cells

SONG, JOON SEOK[1], SANG BAE KIM[2], YOUNG HO LEE[2], KYU WAN LEE[2], HAK HUYN JUNG[3], MEE HYE KIM[4], KYUNG TAI KIM[5], ROBERT BROWN[6], AND YOUNG TAE KIM[1,2]*

[1]*Graduate School of Biotechnology,* [2]*Department of Obstetrics and Gynecology,* [3]*Department of Otolaryngology, Anam Hospital, Korea University, Seoul 136-705, Korea*
[4]*Sewha Pediatric Clinic, Seoul 139-050, Korea*
[5]*Department of Obstetrics and Gynecology, Hanyang University, Seoul 133-791, Korea*
[6]*CRC Dept. of Medical Oncology, University of Glasgow Beatson Labs, Garscube Estate, Switchback Road, Glasgow G61 1BD, U.K.*

Received: August 18, 2001
Accepted: January 3, 2002

Abstract Telomerase is a ribonucleoprotein complex, whose function is to add telomeric repeats $(TTAGGG)_n$ to chromosomal ends and is also known to play an important role in cellular immortalization. Telomerase is highly active in most tumor cells, yet not in normal cells. Therefore, it may have possible applications in cancer gene therapy. Telomerase consists of two essential components; a telomerase RNA template (hTR) and a catalytic subunit (hTERT). The current study attempted to inhibit the "open" part of the human telomerase RNA (hTR) with an antisense sequence-expressing adenovirus. It was found that the antisense telomerase adenovirus suppressed the telomerase activity, tumor cell growth, and survival *in vitro*. Furthermore, FACS analysis and TUNEL assay suggested that the reduced viability was mediated through the induction of apoptosis, indicating that this approach might be a useful method for suppressing cancer growth in targeted cancer gene therapy.

Key words: Telomerase, lung cancer cell, hTR, antisense sequence, adenovirus, targeted cancer gene therapy

Telomerase is highly active in both small and non-small-cell-lung cancers [10], colorectal and gastric cancers [5], hepatocellular carcinomas [24], hematologic malignancies [7], giant cell tumors of bone [27], prostate cancers [28], breast cancers [11], and ovarian carcinomas [6]. Telomerase is a ribonucleoprotein, which consists of several components. Among these, the functions of two components are already known: namely, the RNA component (hTR) and a telomerase catalytic subunit (hTERT). The hTR acts as a template for telomere synthesis [8]. Since telomerase activation and hTR expression are early signs in human cancer development, telomerase inhibitors are expected to function as novel anticancer therapeutics. Several approaches for inhibiting telomerase activity have already been reported; for example, inhibitors of retroviral reverse transcriptase [30], peptide nucleic acid [23], cisplatin [4], hammerhead ribozyme [15], hTR antisense RNA [2], and hTR gene deletion [3]. Recently, a synthetic antisense oligonucleotide against the "open" part of the hTR (2-5A-anti-hTR) was found to exhibit cytotoxic effects on malignant glioma cells [18] or prostate cancer cells [20]. As such, it has been hypothesized that the effect of the 2-5A-anti-hTR is mainly due to an active induction of caspase-dependent apoptosis, which is independent of the telomere length [17].

An expressed antisense sequence has various advantages over synthetic antisense oligomers. Synthetic antisense oligomers are unstable against nuclease, ineffective in the cells, and have a high toxicity [26], whereas an expressed antisense sequence can be easily delivered by a viral vector and has a low toxicity. Accordingly, it is essential to design a more effective antisense expressing vector system.

The current study attempted to inhibit the open part of human telomerase RNA (hTR) using an antisense sequence-expressing adenovirus, and found that the antisense telomerase adenovirus was effective in suppressing tumor cell growth and induced apoptosis, thus suggesting the usefulness of this method in suppressing tumor growth as a targeted cancer gene therapy.

Materials and Methods

Cell Culture

The 293 cells (a human cell line transformed by adenovirus 5 DNA) were grown in DMEM (GIBCO BRL, Grand

*Corresponding author
Phone: 82-2-920-5677; Fax: 82-2-926-5977;
E-mail: ytkim221@korea.ac.kr

Island, U.S.A.) supplemented with 10% FBS (HyClone, Logan, U.S.A.), penicillin (50 units/ml), and streptomycin (50 μg/ml) in the presence of 5% CO_2. The NCI-H358 cells (bronchoalveolar carcinoma) were grown in RPMI1640 (GIBCO BRL, Grand Island, U.S.A.) supplemented with 10% FBS (HyClone, Logan, U.S.A.), penicillin (50 units/ml), and streptomycin (50 μg/ml) in the presence of 5% CO_2. All cell lines were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.).

Oligonucleotide Hybridization

For the design of the antisense oligonucleotide, an "open" part of the telomerase RNA (hTR) structure, between residues 94 and 76 of the telomerase template sequence as determined by Seiji Kondo *et al.* [18], was selected. This part of the sequence was named as "antisense OA (open arm)".

Each pair of oligonucleotides was synthesized as sense (5'-GATCCCGCGCGGGGAGCAAAAGCACG A-3') and antisense (5'-AGCTTCGTGCTTTTGCTCCCCGCGCGG-3'), heated at 90°C for 10 min, and annealed at 55°C for 4 h in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The resulting fragments included restriction endonuclease sites of *Bam*HI and *Hin*dIII at the 5' end and 3' end, respectively.

Construction of Recombinant Antisense hTR OA Adenovirus (Ad-OA)

The resulting fragment was cloned into *Bam*HI/*Hin*dIII digested pΔACMV-PA (adenovirus shuttle plasmid; a generous gift from Dr. Dae Sik Kim, Samsung Medical Center, Seoul, Korea). Direct dideoxynucleotide sequencing ensured a correct sequence and direction. The antisense telomerase shuttle plasmid was then cotransfected with pJM17 (Microbix, Canada) into the 293 cells, using FuGENE™6 (Roche, Germany) according to the manufacturer's protocol. This constructed adenovirus vector was named as Ad-OA. The constructed Ad-OA was then purified from the lysates of infected 293 cells by two rounds of CsCl gradient centrifugation [1], and the titers of the virus stock were then determined by a plaque dilution assay [9]. The recombinant virus was stored at −80°C until use. The negative control vector (Ad5.CMV-Null; Ad-CMV) was purchased from Quantum-Appligene (Quantum-Appligene, U.S.A.). Ad-CMV is an empty vector, which contains no coding sequences between the CMV promoter and PA (polyadenylation) site. The infection efficiency was determined by the method previously described [25].

RCV Assay by PCR

The replication defective adenovirus was confirmed by PCR amplification. The presence of a replication competent virus (RCV) in the virus stock was examined, based on the assumption that the RCV contains the E1 region of an adenovirus type 5 in its genome, using E1A primers, 5'-AGCTGATCGAAGAGGTACTG-3' (624–643; 1.74 mu) and 5'-GAGTCACAGCTATCCGTAC-3' (1376–1358; 3.83 mu), and a pair of E1B primers, 5'-GGTTACATCTGACCTCA-TGGAG-3' (1698–1719; 4.71 mu) and 5'-CAGTACCTC-AATCTGTATCTTC-3' (3516–3495; 9.76 mu), which gave rise to 752 bp and 1,818 bp DNA segments, respectively. A control PCR using non-E1 (Ad) primers, 5'-TCGTTTC-TCAGCAGCTGTTG-3' (3955–3974; 11.01 mu) and 5'-CATCTGAACTCAAAGCGTGG-3' (4815–4796; 13.4 mu), generated an 861 bp segment regardless of the presence of the E1 region. The reactions were carried out at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 1 min for 30 cycles.

Dot Blotting

The adenoviral lysates from the individual plaques were screened for the antisense telomerase sequence using a dot blot analysis with a sense oligonucleotide primer. The synthesis and detection of Dig-labeled DNA probes were carried out using a DIG DNA Labeling and Detection Kit in accordance with the manufacturer's instructions (Boehringer-Mannheim, Germany). The viral DNA samples were applied to a positively charged Nylon membrane (Boehringer-Mannheim, Germany) as described in the manufacturer's protocol.

RT-PCR

The total RNA was isolated from the NCI-H358 cells using an RNeasy Mini kit (QIAGEN, Germany) according to the manufacturer's protocol and treated with DNase I to avoid any genomic DNA contamination. The cDNA was then synthesized from 1 μg of RNA using a SuperScript II reverse-transcriptase assay (GIBCO BRL, Germany) with the Oligo$(dT)_{15}$ primer (Promega, U.S.A.). For amplification, 2 μl aliquots of the cDNA were subjected to 30 cycles of PCR (at 94°C for 1 min, 55°C for 1 min, and 72°C for 90 sec) with an hTR specific primer set (5'-TTTGTCTAACC-CTAACTGAGAAGG-3' as forward and 5'-TGTGAGCC-GAGTCCTGGGTGCACG-3' as backward) or expressing antisense specific primer (the sense primer for antisense hTR as forward and 5'-CCACAACTAGAATGCAGTG-3' as backward from vector sequence upstream of SV40 poly-A site), which gave rise to 400 bp and 120 bp sequences, respectively. The efficiency of the cDNA synthesis from each sample was estimated using PCR with GAPDH-specific primers (5'-TTCGTCATGGGTGTGAACCA-3' as forward and 5'-TGGCAGGTTTTTCTAGACGG-3' as backward) producing 350 bp DNA segment.

Telomerase Activity Assay (TRAP assay)

The telomerase activity was examined by a telomeric repeat amplification protocol (TRAP) assay with 2,000 cells, using a Telosay Kit in accordance with the manufacturers instructions (Intron, Korea). This method was based on an

original method described by Kim *et al.* [16]. The PCR products were electrophoresed in 10% nondenaturing PAGE in TBE buffer and stained with the Silver Staining System (Promega, U.S.A.).

Growth Rate Assay

The cytotoxic effect of the antisense adenovirus on the NCI-H358 cells was determined using a trypan blue dye exclusion assay. Cells were seeded in 60-mm dishes at a density of 5×10^4 cells/dish for 24 h before viral infection. The exponentially growing cells were then infected with the Ad-CMV or Ad-OA adenovirus at a multiplicity of infection (MOI) of 100. The culture medium was also used for mock infection. Triplicate dishes for each treatment were counted every two days for 6 days after the infection.

Flow Cytometry

The apoptotic cells were detected by an Annexin V-FITC Kit (Clontech, U.S.A.) according to the manufacturer's instructions. Briefly, the Ad-CMV and Ad-OA infected cells were rinsed with 1× Binding buffer and then resuspended in 200 μl of a 1× Binding buffer. After adding 5 μl of Annexin V-FITC (20 μg/ml) and 10 μl of propidium iodide (PI; 50 μg/ml), the mixture was incubated at room temperature for 10 min in dark. The cells were then analyzed by a FACSCalibur flow cytometer (Becton Dickinson, CA, U.S.A.) using the CELLQuest software (Becton Dickinson, San Jose, CA, U.S.A.).

TUNEL Assay

To further determine whether treatment with Ad-OA induced apoptosis, the NCI-H358 cells were stained using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) technique with an ApopTag® Plus Peroxidase In Situ Apoptosis Detection Kit (INTERGEN, U.S.A.).

RESULTS

Design of Antisense Oligonucleotide

For obtaining a successful antisense sequence to get the maximum homologous binding, it is essential to find the most open part of the RNA molecule. Seiji Kondo *et al.* [18] analyzed the telomerase RNA (hTR) structure using the MFOLD program, and found that the most open part of the hTR was between residues 94 and 76 of the telomerase template sequence. Therefore, we selected an antisense sequence against the predicted loop comprising nucleotides 94–76.

Generation of Recombinant Adenovirus

The adenoviral shuttle plasmid containing the antisense hTR OA (pΔACMV-OA) is shown in Fig. 1. The presence

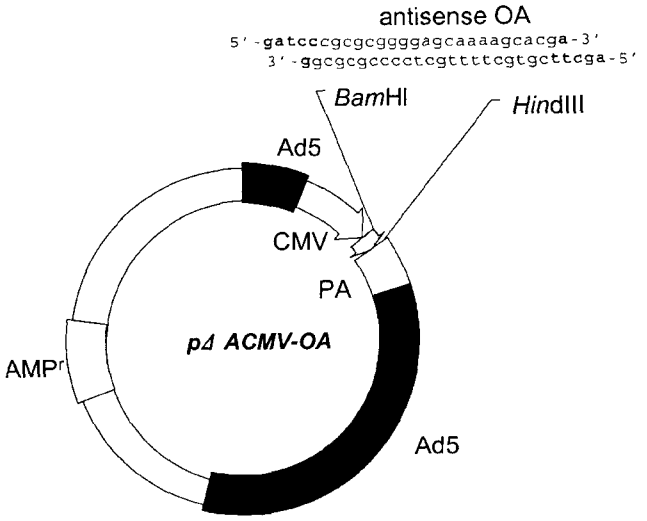

Fig. 1. Adenoviral shuttle plasmid containing the antisense hTR OA (pΔACMV-OA).
The hTR antisense sequence is shown. Each oligomer, which has a *Bam*HI and *Hin*dIII site at the 5'-end and 3'-end, respectively, is in boldface.

of the recombinant adenovirus in the viral stock was confirmed by a Dot blot analysis of the viral DNA. Only the viral DNA from the Ad-OA infected cells showed positive signals (Fig. 2). The absence of replication competent adenovirus (RCV) in the viral stock was confirmed by PCR amplification of the viral DNA (Fig. 3). Both 0.7-kb E1A (Lane 5) and 1.8-kb E1B (Lane 6) fragments were amplified from the 293 genomic DNA, yet not from the viral DNA (Lanes 2 and 3). A 0.8-kb non-E1 region (Lane 1) was amplified from the viral DNA, yet not from the 293 genomic DNA (Lane 4). These results confirmed that there was no RCV in the viral stock.

Antisense OA Expression and hTR Elimination

To confirm the production of the antisense OA and the elimination of the hTR, the RT-PCR method was performed

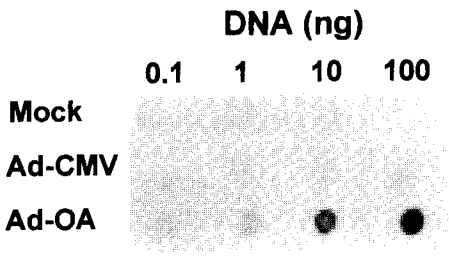

Fig. 2. Presence of recombinant adenovirus in viral stock, confirmed by dot blot analysis of viral DNA.
After infecting the 293 cells, the viral DNA was isolated by proteinase K treatment. Each aliquot was blotted onto a nylon membrane with concentration of 0.1–100 ng and hybridized with a Dig-labeled sense primer (Material and Methods). The image was developed on X-ray film. Mock, mock infected; Ad-CMV, viral DNA from Ad-CMV infected cells; Ad-OA, viral DNA from Ad-OA infected cells.

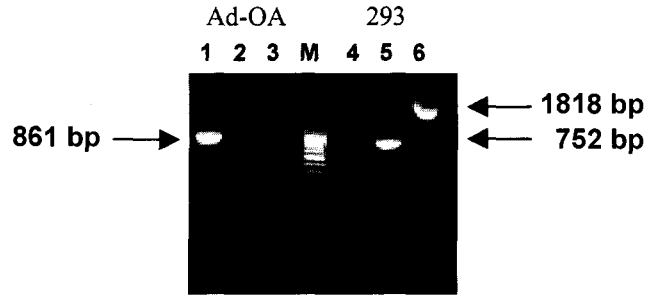

Fig. 3. Absence of replication competent adenovirus (RCV) in the vial stock, confirmed by PCR amplification of viral DNA.
Lanes 1–3 : non-E1, E1A, and E1B fragment from Ad-OA viral DNA. M, 100 bp marker. Lanes 4–6: non-E1, E1A, and E1B fragment from 293 genomic DNA.

in the NCI-H358 cells, 6 days after infection with the mock, Ad-CMV, and Ad-OA (Fig. 4). The antisense OA-specific product was detected only in the Ad-OA infected cells (Lane 6), but not in the mock and Ad-CMV infected cells (Lanes 4 and 5). The NCI-H358 cells infected with Ad-OA had no detectable hTR (Lane 9), however, the hTR was present in the mock and Ad-CMV infected cells (Lanes 7 and 8). These results showed that the hTR could be selectively eliminated by Ad-OA.

Cytotoxic Effects of Antisense Telomerase Adenovirus (Ad-OA)

An 80% efficiency of viral infection was shown at a MOI of 100 for the NCI-H358 cells, as determined by the Ad5-CMV-LacZ (Quantum-Appligene, U.S.A.; Data not shown). Next, to estimate the cytotoxic effect of Ad-OA, the NCI-H358 cells were infected with Ad-OA at a MOI of 100. Two days after the infection, the morphology of the minority of the Ad-OA infected cells had been changed into apoptotic cells. After 6 days, the majority of the cells

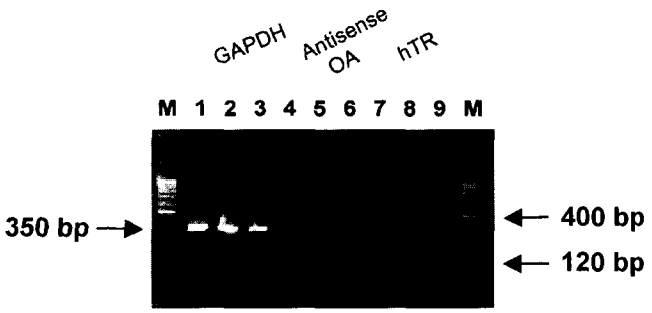

Fig. 4. Antisense OA expression and hTR elimination in Ad-OA infected NCI-H358 cells.
Six days after the Ad-OA infection, the total RNA was isolated and used for RT-PCR in the presence GAPDH, the antisense OA, and hTR specific primers. The PCR products were visualized after 1.5% agarose gel electrophoresis and ethidium bromide staining. M, 100 bp marker. Lanes 1, 4, 7: mock infected. Lanes 2, 5, 8: Ad-CMV infected. Lanes 3, 6, 9 :Ad-OA infected.

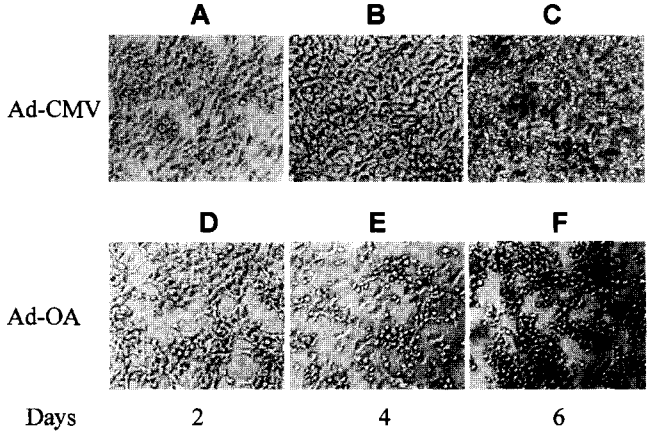

Fig. 5. Cytotoxic effect of antisense adenovirus on NCI-H358 (magnification 100×).
A, B, C: Ad-CMV-infected NCI-H358. D, E, F: Ad-OA-infected NCI-H358.

became rounded up and detached from the culture dishes to undergo cell death (Fig. 5). The cytotoxic effect of Ad-OA was determined using a trypan blue dye exclusion assay (Fig. 6), and it was found that about 60% of the Ad-OA infected cells were inhibited by Ad-OA. The viability of the cells infected with Ad-CMV was 10–20% less than that of the mock infected cells, whereas the change was insignificant compared to the viability of the Ad-OA infected cell.

Effect of Ad-OA on the Telomerase Activity

The telomerase activity in the Ad-OA-infected cells was measured after 6 days, and the relative activity was

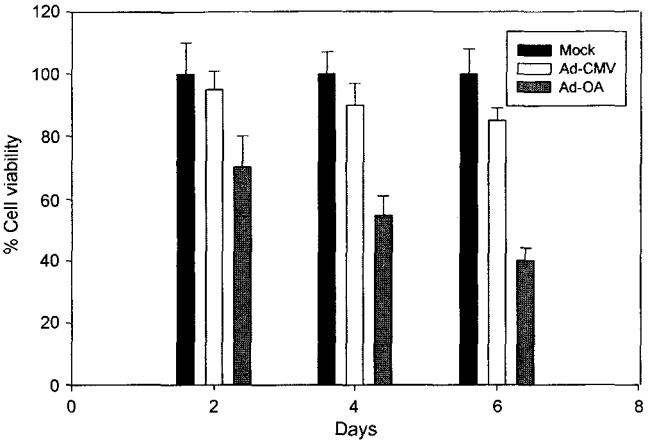

Fig. 6. Growth inhibition of Ad-OA infected cells determined by cell count assay.
The NCI-H358 cells were seeded at 5×10^4 cells/60-mm dish, 24 h prior to infection, with Ad-CMV or Ad-OA at a MOI of 100. At the indicated time points, triplicate dishes of each treatment were counted during 6 days after infection relative to the mock-infected cells. The cell viability was determined by trypan blue exclusion.

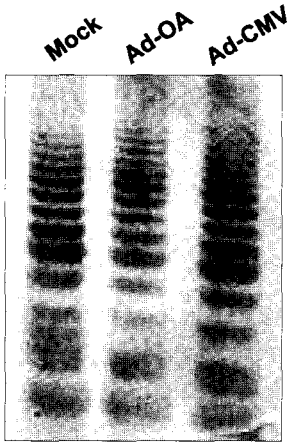

Fig. 7. Telomerase activity in Ad-OA-infected NCI-H358 cells.
After 6 days, the telomerase activity was assayed by a telomeric repeat amplification protocol (TRAP) assay using 2,000 cells. The relative telomerase activity was calculated by Density Analysis Software (Total Lab., Phoretix, U.S.A.) based on data from the mock-infected cells.

calculated using Density Analysis Software (Total Lab., Phoretix, U.S.A.) based on data from the mock-infected cells. The level of telomerase activity in the Ad-OA-infected cells decreased about 40% compared to that in the mock-infected cells (Fig. 7).

Ad-OA Induced Apoptosis of NCI-H358 Cells

The apoptotic cells in the Ad-OA-infected cells were analyzed by FACSCalibur flow cytometery using PI staining and FITC-conjugated Annexin V (Fig. 8). After inducing apoptosis, phosphatidylserine (PS) redistributes to the outer layer of the membrane, and becomes exposed to the extracellular environment [22], therefore, the exposed PS

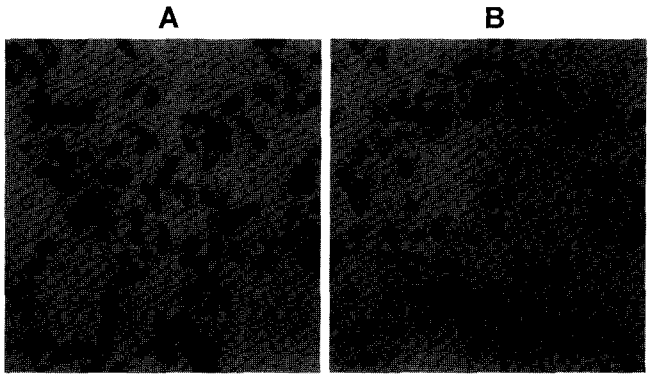

Fig. 9. Comparison of TUNEL assay of NCI-H358 cells infected with Ad-CMV (A) and Ad-OA (B).
The nuclei of the apoptotic cells were darkly stained, indicating DNA fragmentation. The cells were also counterstained with methyl green (magnification 200×).

can easily be detected with Annexin V, a 35.8-kDa protein that has a strong affinity for PS. In the experiment shown in Fig. 8, the Annexin V binding was coupled with propidium iodide staining to quantify the early stage apoptotic events (i.e., Annexin V^+/propidium $iodide^-$). The dual parameter analysis using PI and Annexin V showed that about 50% of the Ad-OA treated cancer cells had undergone apoptosis (Fig. 8). Namely, the NCI-H358 cells infected with Ad-OA for 6 days exhibited 55.91% Annexin V^+/propidium $iodide^-$ apoptotic events and less than 1.14% Annexin V^+/propidium $iodide^+$ events that represented dead cells. To confirm the apoptotic events by Ad-OA, the NCI-H358 cells infected with Ad-OA were also analyzed by a TUNEL assay. As shown in Fig. 9, a large number of darkly stained nuclei, indicating apoptosis, were detected. Consequently, all the experiments clearly showed that the hTR was eliminated in the NCI-H358 cells infected with Ad-OA, which then underwent cell death by apoptosis.

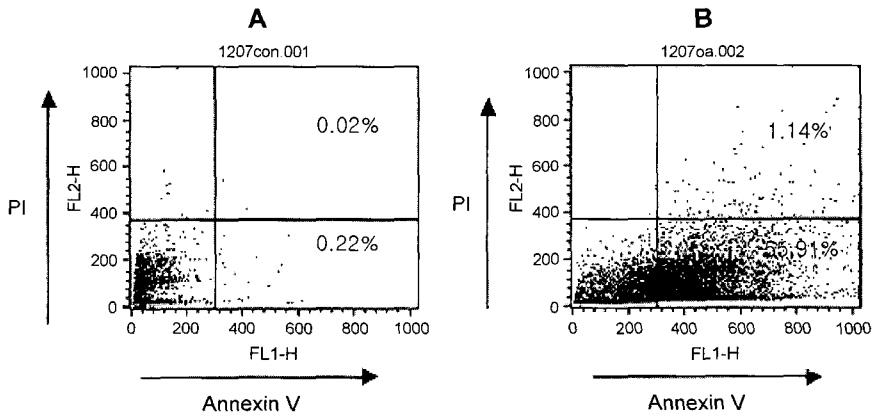

Fig. 8. Flow cytometry analysis of Ad-OA-treated apoptotic NCI-H358 cells stained with AnnexinV-FITC and propidium iodide (PI).
The NCI-H358 cells were infected with Ad-CMV (A) or Ad-OA (B) at a MOI of 100. After 6 days, the cells were stained with AnnexinV-FITC/ PI and analyzed.

DISCUSSION

Gene therapy using an antisense sequence would appear to be an attractive approach for the treatment of viral infections, cancers, and genetic disorders [13]. One of the major advantages of this approach is that it inhibits the expression of the disease-associated gene in a sequence-specific manner. Ever since the first attempt to inhibit gene expression by an antisense sequence [32], there have been numerous studies on the structure of an antisense sequence [14] or tumor vaccine [31] to modulate gene expression.

There are two kinds of antisense sequences; synthetic antisense oligomers and expressed antisense sequence. For drug development, synthetic antisense oligomers have been more commonly used due to the ease of dose control and advantages in clinical trials. However, synthetic antisense oligomers also have certain disadvantages, including instability against nuclease, toxicity, and ineffective infusion into cells [26]. On the other hand, an expressed antisense sequence has a few advantages over synthetic antisense oligomers; for example, it can easily be delivered by a viral vector and has a low toxicity. There have already been several approaches that use expressed antisense sequences, including inhibition of human telomerase by a retrovirus expressing telomeric antisense RNA [2], suppression of established human prostate cancer by antisense *c-myc* retroviral vector [29], and antitumor effects of adenovirus expressing antisense insulin-like growth factor on human lung cancer cell lines [21].

Although telomerase activity is undetectable in most human somatic tissues, it is generally present in highly replicative tissues and in most human cancers. Therefore, its exclusive expression in most tumor cells makes it a very strong candidate for targeted cancer gene therapy. As a result, there have been several attempts to inhibit telomerase activity; for example, inhibitors of retroviral reverse transcriptase, peptide nucleic acid, hammerhead ribozyme, hTR antisense RNA and hTR gene deletion.

Adenoviral vectors have many advantages over other gene therapy vectors, including high transduction efficiency, extremely high viral titers, and infectivity in both replicating and differentiated cells. Also, since they lack integration, they do not cause any mutagenic effects due to random integration into the host genome.

The present study attempted to inhibit the "open" part of the human telomerase RNA (hTR) using an antisense sequence-expressing adenovirus. After infection, the morphology of the majority of the Ad-OA-infected cells changed into apoptotic cells, with rounded-up shapes and detachment from the culture dishes, and undergoing cell death (Fig. 5). The growth of Ad-OA-infected cells was significantly suppressed (Fig. 6). Although the telomerase activity of the Ad-OA-infected cells was about 40% lower than that of the mock-infected cells, the effect was not significant (Fig. 7). Kondo *et al.* reported that it took about 1 month to induce 50% apoptosis in U251-MG cells by telomere shortening following telomerase inhibition [19]; however, after treatment with the 2-5A-anti-hTR to activate caspase function, the apoptosis was induced in 70% of U251-MG cells in only 4 days [18]. Accordingly, they suggested that the effect of the 2-5A-anti-hTR (antisense OA) was due to active induction of caspase-dependent apoptosis, which was independent of the telomere length [17]. Therefore, it is possible that the cytotoxic effects of Ad-OA are mainly due to caspase-dependent apoptosis. Telomerase mainly consists of hTR and hTERT, and the expression of hTERT is closely associated with telomerase activity [12]. Consequently, for the treatment of tumors with a long telomere, antisense OA would appear to be more attractive, whereas for higher telomerase activity, hTERT inhibition would be better [20]. Using an FACSCalibur flow cytometer, the apoptotic cells within the Ad-OA infected cells were analyzed, and it was found that about 50% of the Ad-OA treated cancer cells had undergone apoptosis (Fig. 8). Also, TUNEL assay confirmed that the cytotoxic effect of Ad-OA was mainly due to apoptotic events. In conclusion, the antisense telomerase adenovirus suppressed tumor cell growth and induced apoptosis; therefore, it may be a useful in suppressing tumor growth in targeted cancer gene therapy.

Acknowledgment

This study was supported by a grant from the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (#01-PJ1-PG1-01CH05-0001).

REFERENCES

1. Becker, T. C., R. J. Noel, and W. S. Coats, *et al.* 1994. Use of recombinant adenovirus for metabolic engineering of mammalian cells. *Methods Cell Biol.* **43:** 161–189.
2. Bisoffi, M., A. E. Chakerian, M. L. Fore, J. E. Bryant, J. P. Hernandez, R. K. Moyzis, and J. K. Griffith. 1998. Inhibition of human telomerase by a retrovirus expressing telomeric antisense RNA. *Eur. J. Cancer* **14:** 1242–1249.
3. Blasco, M. A., H. W. Lee, and M. P. Hande, *et al.* 1997. Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell* **91:** 25–34.
4. Burger, A. A., J. A. Double, and D. R. Newll. 1997. Inhibition of telomerase activity by cisplatin in human testicular cancer cells. *Eur. J. Cancer* **33:** 638–644.
5. Chadeneau, C., K. Hay, H. W. Hirte, S. Gallinger, and S. Bacchetti. 1995. Telomerase activity associated with acquisition of malignancy in human colorectal cancer. *Cancer Res.* **55:** 2533–2536.

6. Counter, C. M., H. W. Irte, S. Bacchtti, and C. B. Harley. 1994. Telomerase activity in human ovarian carcinoma. *Proc. Natl. Acad. Sci. USA* **91:** 2900–2904.
7. Counter, C. M., J. Guota, C. B. Harley, B. Leber, and S. Bacchetti. 1995. Telomerase activity in normal leukocytes and in hematologic malignancies. *Blood* **85:** 2315–2320.
8. Feng, J., W. D. Funk, S. S. Wang, A. A. A. Weinric, C. P. Ciu, R. R. Adams, E. Chang, R. C. Allsopp, J. Yu, S. Le, M. D. West, C. B. Harley, W. H. Andrew, C. W. Greider, and B. Villeponteau. 1995. The RNA component of human telomerase. *Science* **269:** 1236–1241.
9. Graham, F. L. and A. J. Van Der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52:** 456–467.
10. Hiyama, K., E. Hiyama, S. Ishioka, M. Yamakido, K. Inai, F. G. Gazddar, M. A. Piatyszek, and J. W. Shay. 1995. Telomerase activity in small-cell and non-small-cell lung cancer. *J. Natl. Cancer Inst.* **87:** 895–901.
11. Hiyama, E., L. Gollahon, T. Kataoka, K. Kuroi, T. Yokoyama, A. F. Gazdar, K. Hiyama, M. A. Piatyszek, and J. W. Shay. 1996. Telomerase activity in human breast tumors. *J. Natl. Cancer Inst.* **88:** 116–122.
12. Ito, H., S. Kyo, T. Kanaya, M. Takakura, M. Inoue, and M. Namiki. 1998. Expression of human telomerase subunits and correlation with telomerase activity in urothelial cancer. *Clin. Cancer Res.* **4:** 1603–1608.
13. J. Thomas A. 1997. pp. 1–49, *Gene Therapy, Advances in Pharmacology.* Vol. 40, Academic Press, CA, U.S.A.
14. Jung, K. E., Y. Mirim, L. Kwangjun, L. Hong, J. Jihyun, K. Bonjung, S. J. Lak, S. Donghoon, L. Chulhoon, C. Youlhee, and L. Yoongho. 2000. Structural study of antisense dimers, modified adenosine-thymidine phosporothioate. *J. Microbiol. Biotechnol.* **10:** 889–892.
15. Kanazawa, Y., K. Ohkawa, and K. Ueda. 1996. Hammerhead ribozyme-mediated inhibition of telomerase activity in extracts of human hepatocellular carcinoma cells. *Biochem. Biophys. Res. Commun.* **225:** 570–576.
16. Kim, N. W., M. A. Piatyszek, K. R. Prowse, C. B. Harley, M. D. West, P. L. C. Ho, G. M. Coviello, W. E. Wright, S. L. Weinric, and J. W. Shay. 1994. Specific association of human telomerase activity with immortal cells and cancer. *Science* **266:** 2011–2015.
17. Komata, T., Y. Kondo, S. Koga, S. C. Ko, L. W. Chung, and S. Kondo. 2000. Combination therapy of malignant glioma cells with 2-5A-antisense telomerase RNA and recombinant adenovirus p53. *Gene Ther.* **7:** 2071–2079.
18. Kondo, S., Y. Kondo, G. Li, R. H. Silverman, and J. K. Cowell. 1998. Targeted therapy of human malignant glioma in a mouse model by 2-5A antisense directed against telomerase RNA. *Oncogene* **16 :** 3323–3330.
19. Kondo, S. *et al.* 1998. Antisense telomerase treatment: Induction of two distinct pathways, apoptosis and differentiation. *FASEB J.* **12:** 801–811.
20. Kondo, Y., S. Koga, T. Komata, and S. Kondo. 2000. Treatment of prostate cancer *in vitro* and *in vivo* with 2-5A-anti-telomerase RNA component. *Oncogene* **19:** 2205–2211.
21. Lee, C. T., S. Wu, D. Gabrilovich, H. Chen, S. Nadaf-Rahrov, I. F. Ciernik, and D. P. Carbone. 1996. Antitumor effects of an adenovirus expressing antisense insulin-like growth factor I receptor on human lung cancer cell lines. *Cancer Res.* **56:** 3038–3041.
22. Martin, S. J., C. P. Reutelingsperger, A. J. McGahon, J. A. Rader, R. C. van Schie, D. M. LaFace, and D. R. Green. 1995. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: Inhibition by overexpression of Bcl-2 and Abl. *J. Exp. Med.* **182(5):** 1545–1556.
23. Norton, J. C., M. A. Piaryszek, W. E. Wright, J. W. Shay, and D. R. Corey. 1996. Inhibition of human telomerase activity by peptide nucleic acids. *Nature Biotechnol.* **14:** 615–619.
24. Sahara, H., T. Nakanishi, M. Kitamoto, R. Nakashio, J. W. Shay, E. Tahara, G. Kajiyama, and T. Ide. 1995. Telomerase activity in human liver tissue: Comparison between chronic liver disease and hepatocellular carcinoma. *Cancer Res.* **55:** 2734–2736.
25. Sanes, J. R., J. L. R. Rubenstein, and J. F. Nicholas. 1986. Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. *EMBO J.* **5:** 3133–3142.
26. Scanlon, K. J., Y. Ohta, H. Ishida, H. Kijima, T. Ohkawa, A. Kaminski, J. Tsai, G. Horng, and M. Kashani-Sabet. 1995. Oligonucleotide-mediated modulation of mammalian gene expression. *FASEB J.* **9:** 1288–1296.
27. Schwartz, H. S., S. F. Juliao, M. F. Sciadini, L. K. Miller, and M. G. Butler. 1995. Telomerase activity and oncogenesis in giant cell tumor of bone. *Cancer* **75:** 1094–1099.
28. Sommerfeld, H. J., A. K. Meeker, M. A. Piatyszek, G. S. Bova, and D. S. Coffey. 1996. Telomerase activity: A prevalent marker of malignant human prostate tissue. *Cancer Res.* **56:** 218–222.
29. Steiner, M. S., C. T. Anthony, Y. Lu, and J. T. Holt. 1998. Antisense c-myc retroviral vector suppresses established human prostate cancer. *Hum. Gene Ther.* **9:** 747–755.
30. Strahl, C. and E. H. Blackburn. 1996. Effects of reverse transcriptase inhibitors on telomere length and telomerase activity in two immortalized human cell lines. *Mol. Cell Biol.* **16:** 53–65.
31. Yoo, Y. C., Y. P. Seung, B. K. Kyung, and A. Ichiro. 2000. MDP-Lys (L18), a synthetic muramyl dipeptide derivative, enhances antitumor activity of an inactivated tumor vaccine. *J. Microbiol. Biotech.* **10:** 399–404.
32. Zamecnik, P. and M. L. Stephenson. 1978. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc. Natl. Acad. Sci. USA* **75:** 280–284.