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Conditionally Replicating Adenoviruses Expressing Short Hairpin RNAs Silence the Expression of a Target Gene in Cancer Cells

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Abstract

RNA interference (RNAi) is a posttranscriptional silencing mechanism triggered by double-stranded RNA that was recently shown to function in mammalian cells. Expression of cancer-associated genes was knocked down by expressing short hairpin RNAs (shRNAs) in cancer cells. By virtue of its excellent target specificity, RNAi may be used as a new therapeutic modality for cancer. The success of this approach will largely depend on efficient delivery of shRNAs to tumor cells. Tumor-selective replication competent viruses are especially suited to efficiently deliver anticancer genes to tumors. In addition, their intrinsic capacity to kill cancer cells makes these viruses promising anticancer agents per se. In this study, conditionally replicating adenoviruses were constructed encoding shRNAs targeted against firefly luciferase. These replicating viruses were shown to specifically silence the expression of the target gene in human cancer cells down to 30% relative to control virus. This finding offers the promise of using RNAi in the context of cancer gene therapy with oncolytic viruses.

Introduction

RNA interference (RNAi) is a conserved cellular surveillance system that recognizes double-stranded RNA and cleaves the double-stranded RNA into small interfering RNAs (siRNAs), which activate degradation of RNA species homologous to the double-stranded RNA (1). Introduction of synthetic siRNAs into mammalian cells leads to a sequence-specific degradation of the mRNA to which the siRNA is targeted (2). This method, exploiting RNAi, is now widely used to analyze the function of individual genes. In cancer research, RNAi is helpful in determining the functional role of genes associated with cancer initiation and progression and with response to treatment. Furthermore, there is the potential that siRNAs could be used as therapeutic anticancer agents. For example, it has been shown in vitro that siRNAs directed against Bcl-2 and c-Raf can induce apoptosis of human myeloid leukemia cells and chemosensitize these cells (3). RNAi-based genome-wide screens are currently being performed in mammalian cells (4) and are likely to identify additional siRNAs with selective anticancer effects. Reports that short hairpin RNAs (shRNAs) expressed from plasmids could also trigger RNAi (5–7) offer the promise of RNAi gene therapy using viral vectors. Indeed, nonreplicating adenoviral vectors expressing shRNAs could induce silencing of target genes in vitro and in vivo (8, 9). However, as with any gene therapy approach, efficient delivery of the shRNA to tumors remains a major obstacle. Replication-deficient viral vectors have thus far been used with limited success in cancer gene therapy, mainly due to low transduction efficiency of tumor cells and lack of penetration of viral vectors into the solid tumor mass. It is thus expected that delivery of shRNAs interfering with the expression of genes involved in tumor cell survival using nonreplicating vectors will meet with similar difficulties.

The limitations encountered with nonreplicating adenoviral vectors can be overcome by using conditionally replicating adenoviruses (CRAds), i.e., recombinant adenoviruses modified to selectively replicate in cancer cells (10). CRAds can be used in their own right as oncolytic agents because they lyse infected cancer cells to allow their progeny to spread to neighboring cells. Their ability to amplify and spread through a tumor mass also makes CRAds useful in cancer gene therapy as an efficient delivery vehicle for therapeutic genes, e.g., immunostimulatory genes or proapoptotic genes. Apart from causing a direct anticancer effect, the therapeutic transgene product expressed by a CRAd can also enhance the oncolytic potency of the CRAd. In this regard, we found recently (11) that expression of the tumor suppressor protein p53 expedited the lysis of CRAd-infected cancer cells, allowing a more rapid spread of the virus. CRAds thus represent a promising class of oncolytic agents, and their therapeutic efficacy can be further enhanced by expression of therapeutic genes (in particular, proapoptotic genes).

We hypothesize that the anticancer potency of CRAds might also be improved by inhibiting host factors that hinder adenovirus replication in cancer cells. Therefore, we set out to design a platform for CRAd-mediated RNAi. Here, we report that shRNAs expressed from the genome of a CRAd specific for cells with a dysfunctional pRb pathway can specifically silence a target gene in human cancer cells. This offers the promise of therapeutic delivery of shRNAs that silence cellular genes involved in cancer cell survival to augment the efficacy of anticancer therapy with CRAds.

Materials and Methods

Cell Lines. Human non-small cell lung carcinoma A549 cells, breast carcinoma MCF-7 cells, cervical carcinoma HeLa cells, and human embryonic kidney 293 cells were obtained from the American Type Culture Collection (Manassas, VA). Osteosarcoma SaOs-2 cells were a kind gift of Dr. F. van Valen (Westfalsiche Wilhelms-Universitat, Munster, Germany). Cells were maintained in DMEM:Ham’s F-12 supplemented with 10% FCS, 50 IU/ml1 penicillin, and 50 μg/ml1 streptomycin (Life Technologies, Inc., Paisley, United Kingdom).

Construction of Plasmids. Reporter plasmid pHAR-FF-RL contains the firefly luciferase and the Renilla luciferase genes under the bidirectional human aldehyde reductase (hAR) promoter (12, 13). To construct pHAR-FF-RL, the hAR promoter (nucleotides –124 to +29 relative to the transcription start site, GenBank accession number AF112482) was obtained by PCR using subcloned genomic DNA as template and primers 5′-CCAGAAAGAGCTCG-
CAACGTCGATCTGATA-3' and 5'-GTTGTGAGAGCTCCTGGGGCACAAT-5'GAGC-3', creating flanking SacI sites (underlined). The PCR product was inserted in the SacI site of pGL3-basic (Promega, Madison, WI) with the 3'-end of the promoter toward the firefly luciferase gene, creating phAR-FF. Next, the Renilla luciferase cDNA was obtained by PCR using pRL-TK (Promega) as template and primers 5'-ACACAGTGTCGCCCTGGTGATT-GGAATCG-3' and 5'-CAGAAACCTGGTCGCATTATCT-3', creating flanking KpnI sites (underlined) and inserted into the KpnI site of phAR-FF in an orientation opposite to that of the firefly luciferase gene, such that the 5'-end of the hAR promoter faces the 5' side of the Renilla luciferase gene. phAR-FF* was similar to phAR-FF but contains silent mutations in the target recognition site of firefly luciferase changing the recognition sequence into 5'-ACCAGTGTCGCCCTGGTGAT-3' and 5'-CGATTCCTACCTGACGCGCAACCTGGT-3' (mutations are underlined). The mutations were first introduced in pGL2 (Promega), where the target recognition site is fortuitously flanked by EcoRV and ClaI restriction sites, allowing the use of a small linker to introduce these mutations. For this purpose, the oligonucleotides 5'-ACCAGGTTCGCCCTGGTGAT-3' and 5'-CGATTCCTACCTGACGCGCAACCTGGT-3' were annealed and treated with kinase. This linker was ligated into pGL2 digested with EcoRV and ClaI, replacing the unmodified target sequence. From this vector, the 765-bp SpHl-SgrI fragment containing firefly sequence with the mutated target site was ligated into phAR-FF-RL, replacing the corresponding SpHl-SgrI region.

pSHAG-1 and pSHAG-Ff1 (Ref. 5; generously provided by Dr. G. J. Hannon; Cold Spring Harbor Laboratory, NY) contain a U6 promoter-driven expression cassette that can be transported into destination plasmid vectors using the Gateway system (Invitrogen, Carlsbad, CA). pSHAG-Ff1 encodes a shRNA homologous to nucleotides 1340–1368 of the coding sequence of the firefly luciferase gene; pSHAG-1 is the negative control without shRNA sequence.

The adenoviral shuttle vector pEndK/SpeI was made by first digesting pTG3602 (14) with KpnI and religating the vector fragment comprising adenovirus serotype 5 (Ad5) map units 0–7 and 93–100. Next, a unique SpeI site was introduced by changing Ad5 nucleotide 35813 from A to T by site-directed mutagenesis. pEndK/SpeI was made compatible with the Gateway system by ligating the Gateway destination cassette (Invitrogen) as a blunt fragment into the SpeI site (filled in with Klenow) of pEndK/SpeI. Plasmids were selected that contained the Gateway destination cassette with the coding sequence of the ccdB gene on the adenovirus R or L strand and designated pEndK/DEST-R and pEndK/DEST-L, respectively. The shRNA expression cassette of pSHAG-Ff1 was transported to these pEndK/DEST vectors via a Gateway in vitro recombination reaction according to the manufacturer's protocol, generating pEndK/DEST-Ff1-R and pEndK/DEST-Ff1-L containing the U6 promoter and firefly luciferase shRNA in the right-hand and leftward orientation of the adenoviral genome, respectively.

Recombinant Adenoviruses. The Ad5-derived CRAd Ad5-Δ24E3 carrying a 24-bp deletion in the prb-binding CR2 domain in E1A and containing the wild-type E3 region has been described previously (15). Ad5-Δ24E3-derivative CRAds expressing shRNA were generated by homologous recombination in Escherichia coli BJ5183 between Ad5-Δ24E3 viral DNA and KpnI-digested pEndK/Ff1-F and pEndK/Ff1-L to form plasmids pAdΔ24E3-Ff1-R and pAdΔ24E3-Ff1-L, respectively. These plasmids were digested with PacI to release the full-length adenoviral DNA from the plasmid backbone and transfected into human 293 cells. Ad5-Δ24E3-Ff1-R and Ad5-Δ24E3-Ff1-L CRAds were harvested and further propagated on A549 cells. The E1Δ24 deletion and the U6-Ff1 insertion and orientation were confirmed by PCR on the final products, and functional plaque-forming unit titers were determined by limiting-dilution plaque titration on 293 cells according to standard techniques.

Gene Silencing Assays. A549 cells at 50–70% confluence in 24-well plates were transfected with 50 ng of phAR-FF-RL with or without pSHAG-1 or pSHAG-Ff1 at the amount indicated and irrelevant plasmid pBlueScript SK− carrier DNA (Stratagene, La Jolla, CA) to obtain a total of 300 ng of DNA using LipofectAMINE Plus (Invitrogen) according to the manufacturer’s protocol. Infections with CRAds were performed at a multiplicity of infection of 500 plaque-forming units/cell for 2 h at 37°C, followed immediately by transfection. Activities of firefly and Renilla luciferase were determined using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol.

Results

Silencing of Luciferase Expression in Human Cancer Cells. To accurately quantify silencing efficiency in transient transfection assays, we used the firefly luciferase gene as RNAi target. To adequately compensate for experimental variation, we used the reporter plasmid phAR-FF-RL that expresses firefly and Renilla luciferase genes from the bidirectional hAR promoter (Fig. 1A), thus allowing normalization of firefly luciferase silencing relative to Renilla luciferase expression. As silencing construct, we chose the shRNA expression cassette from pSHAG-Ff1 (5), consisting of the RNA polymerase III U6 promoter and a hairpin RNA with a stem of 29 nucleotides targeting the firefly luciferase gene (Fig. 1B). A549 lung carcinoma cells were transfected with phAR-FF-RL and various amounts of pSHAG-FF1. One day later, firefly and Renilla luciferase activities were measured. As can be seen in Fig. 2A, pSHAG-Ff1 suppressed firefly luciferase expression in a dose-dependent manner. Maximum silencing obtained was at approximately 25% of expression in the absence of pSHAG-Ff1. Hence, the mammalian RNAi pathway appeared intact in A549 cells, and Ff1 shRNA silenced firefly luciferase expression in these cells.
Adenoviral Replication Does Not Interfere with RNAi in Cancer Cells. For the use of CRAds as delivery vehicle for shRNA, it is a prerequisite that adenovirus replication does not interfere with RNAi. Therefore, we investigated whether adenoviral replication influenced shRNA-induced silencing. To this end, A549 cells were infected with the CRAd Ad5-Δ24E3 or mock infected, followed by cotransfection of pSHAG-1 or pSHAG-Ff1 together with phAR-FF-RL. Efficient infection and replication of Ad5-Δ24E3 were confirmed by the observation of cytopathic effects in the majority of cells in control cultures 2 days after infection (data not shown). Thirty h after infection, firefly and Renilla luciferase expressions were measured. As shown in Fig. 2B, there was no significant difference in silencing in the presence or absence of CRAd infection. This experiment thus demonstrated that adenoviral replication per se does not inhibit shRNA-mediated gene silencing in cancer cells.

CRAds Expressing shRNA Efficiently Silence a Target Gene in Cancer Cells. Next, we investigated whether shRNAs can be delivered to and functionally expressed in cancer cells by a CRAd. We chose the CRAd Ad5-Δ24E3 (15), for which replication is restricted to cancer cells with a disrupted Rb pathway (16), as backbone for this endeavor. The U6-F1 shRNA expression cassette of pSHAG-Ff1 was inserted in the genome of Ad5-Δ24E3 at the extreme 3′-end, directly preceding the rightward ITR, in either the rightward or leftward orientation, generating Ad5-Δ24E3.Ff1-R and Ad5-Δ24E3.Ff1-L, respectively (Fig. 1C). A549 cells were infected with these silencing CRAds or with Ad5-Δ24E3 as control, followed by transfection with the reporter plasmid phAR-FF-RL. Thirty h after infection, firefly and Renilla luciferase activities were determined. Ad5-Δ24E3.Ff1-R and Ad5-Δ24E3.Ff1-1 each suppressed the normalized firefly luciferase expression to approximately 50% of the expression level observed after infection with the parental CRAd Ad5-Δ24E3 (Fig. 3A). Similar results were obtained in three other cancer cell lines (Fig. 3B). This demonstrated that shRNAs expressed from CRAds are able to suppress the expression of a target gene.

Efficiency of Silencing Increases During Viral Replication. Because the shRNAs are encoded by a replicating virus, it was assumed likely that as a consequence of virus genome replication, their expression would increase in time. Pilot experiments using an Ad5Δ24 CRAd encoding a marker protein revealed that in A549, an exponential increase in transgene expression started at 20 h after infection and reached a plateau at 32 h (data not shown). Assuming that the expression of the shRNAs driven by the U6 promoter follows a similar expression profile, we anticipated that the silencing effect induced by the shRNAs would increase during the replicative cycle. To test this, we performed a time course experiment measuring the silencing by Ad5-Δ24E3.Ff1-R at 12, 24, 36, and 48 h after infection. This experiment showed that Ad5-Δ24E3.Ff1-R progressively suppressed firefly luciferase expression in A549 cells over the first 2 days after infection (Fig. 3C). At 48 h after infection, when cytopathic effects became apparent, Ad5-Δ24E3.U6-Ff1-R had silenced firefly luciferase down to approximately 30% of the Ad5-Δ24E3 control. This CRAd-induced silencing was substantial but somewhat less efficient than silencing induced by the pSHAG-Ff1 plasmid, which silenced firefly luciferase down to approximately 10% at 48 h after transfection (Fig. 3C).

CRAd-shRNA-Induced Silencing Is Sequence Specific. Because the firefly luciferase expression values were normalized using Renilla luciferase expression as an internal control, and effects of shRNA-expressing CRAds were compared with expression after infection with the Ad5-Δ24E3 parental virus, it is unlikely that the observed suppression of firefly luciferase expression was due to nonspecific effects caused by viral replication. However, to formally exclude this possibility, we investigated silencing of a mutant firefly luciferase. To this end, we introduced six silent point mutations in the shRNA target sequence in the firefly luciferase gene encoded by phAR-FF-RL. As expected, these mutations did not influence the activity of the firefly luciferase protein but abolished Ff1 shRNA-mediated silencing, as was confirmed by cotransfection of the mutated reporter plasmid (phAR-FF*-RL) with pSHAG-Ff1 (data not shown). A549 cells were infected with Ad5-Δ24E3, Ad5-Δ24E3.U6-Ff1-R, or Ad5-Δ24E3.U6-Ff1-L, followed by transfection with the reporter plasmid phAR-FF-RL or the mutated reporter plasmid phAR-FF*-RL. Fig. 4 shows that both silencing CRAds suppressed firefly luciferase expression 30 h after infection to approximately 50% (as before) but did not change expression of mutant firefly luciferase. This confirmed that the observed silencing of firefly luciferase expression was dependent on the shRNA target sequence and strongly suggests that the silencing is brought about via the mammalian RNAi pathway.

Discussion

RNAi holds promise in the treatment of cancer by virtue of its exceptional target specificity. Currently, synthetic lethal high-throughput screenings are being performed to evaluate shRNAs for their ability to kill engineered tumorigenic cells but not their isogenic normal cell counterparts (4, 17). Thus, identification of selective anticancer shRNAs is foreseen. However, to be effective in cancer treatment, it is essential that these therapeutic shRNAs are delivered to most, if not all, cells of a tumor in vivo. Nonviral delivery or delivery by nonreplicating viruses is efficient in vitro, but transduction...
of tumor tissue in vivo is generally poor. In contrast, tumor-selective replicating viruses possess intrinsic property to spread through a tumor mass, thus amplifying the input dose and extending the reach and duration of treatment. These viruses may therefore be more appropriate delivery agents for anticancer shRNAs.

Apart from this, clinical trials with CRAds have shown that their oncolytic efficacy per se clearly requires improved potency (18–20). In this regard, expression of therapeutic transgenes (e.g., tumor necrosis factor (TNF-α) and p53) has already drastically augmented the oncolytic potency of replicating adenoviruses in preclinical investigations (11, 21, 22). In particular, restoration of p53 function in cancer cells augmented CRAd efficacy by accelerating cell death and virus progeny release (11). We propose that the potency of oncolytic viruses could be similarly improved by expression of synthetic lethal shRNAs. Instead of expressing proapoptotic transgenes, endogenous antiapoptotic genes could be silenced. Antiapoptotic proteins, such as Bcl-2, Mdm2, and members of the inhibitor of apoptosis (IAP) family of proteins, are often overexpressed in cancer cells. We speculate that such proteins may compromise the efficacy by which a CRAd lyses a cancer cell at the end of its replication cycle. This assumption is supported by the observation that an adenovirus containing a deletion of the adenoviral E1B-19K gene, which encodes a functional homolog of Bcl-2, showed enhanced oncolytic potency (23).

In this study, we show as a proof of principle that shRNAs expressed from a CRAd specifically silence expression of a target gene in cancer cells. We used firefly luciferase as target and Renilla luciferase as internal control to conveniently and accurately measure the silencing caused by shRNA-expressing CRAds during the course of replication in human cancer cell lines. The expression inhibition was evident during later stages of infection, reaching a maximum of approximately 70% silencing at the time when cytopathic effects became apparent. This silencing profile could be important when synthetic lethal shRNAs targeting endogenous genes are used because it has been shown that premature apoptosis induction may compromise effective virus production (24).

As expected for genuine RNAi, gene silencing by CRAd-encoded shRNA was highly sequence specific. Silencing efficiency, however, was only partial, ranging from 40% to 70% expression reduction, depending on the cancer cell type and the time after infection. There are no standardized criteria to judge the biological relevance of silencing efficiency. Anything between approximately 50% and 100% reduction of mRNA concentration, protein expression, or enzyme...
activity is considered as successful knockdown (25). Moreover, the level of silencing depends to a great extent on which region of the mRNA is targeted (26). For the particular shRNA used in this study, the maximal observed silencing efficiency in the context of CRAds was not much less than the silencing induced by a plasmid encoding the same shRNA under direction of the same RNA polymerase III promoter. Therefore, we expect that when a synthetic lethal phenotype is observed in cancer cells after transfection of plasmid-encoded shRNA, the same phenotype will become apparent when the same shRNA is delivered by a CRAd. This should allow direct translation of the results from plasmid-based screens to application in the CRAd context.

In a direct side-by-side comparison, plasmid-induced silencing was somewhat more efficient than CRAd-induced silencing (see Fig. 3C). This may be due to suboptimal transcription from the U6 promoter in the replicating adenoviral genome. Alternatively, the formation during adenoval viral replication of large amounts of adenovirus virus-associated RNAs, which structurally resemble shRNAs and microRNAs, may reduce silencing efficiency by competing for cellular factors involved in RNAi. In this respect, it was shown recently that adenovirus-associated RNAs, shRNAs, and pre-microRNAs all use Exportin-5 for their export out of the nucleus (27, 28).

In conclusion, we have demonstrated that CRAd-delivered shRNA can induce proper gene silencing in human cancer cells. This offers the prospect of improving the efficacy of these oncolytic viruses by interfering specifically with antiapoptotic functions in cancer cells.

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References
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