Antitumor Activity of an Oncolytic Adenovirus-Delivered Oncogene Small Interfering RNA

Yu-An Zhang,1 John Nemunaitis,2,4 Shirley K. Samuel,1 Patrick Chen,4 Yuqiao Shen,3 and Alex W. Tong1,2,4

1 The Mary Crowley Medical Research Center and the 2 Cancer Immunology Research Laboratory, Baylor-Sammons Cancer Center, 3Murex Pharmaceuticals, Dallas, Texas; and 4 Institute of Biomedical Studies, Baylor University, Waco, Texas

Abstract

Despite successes in animal models, cancer gene therapy with small interfering RNAs (siRNA) is hindered by the lack of an optimal delivery platform. We examined the applicability of the replication-competent, oncolytic adenovirus, ONYX-411, to deliver a mutant K-ras siRNA transgene to human cancer cells. Proof-of-principle studies showed an additive tumor growth–inhibitory response through siRNA-mediated K-ras knockdown and ONYX-411-mediated cancer cell lysis. A novel construct, termed Internavec (for interfering RNA vector), was generated by cloning a K-ras12-specific siRNA hairpin construct under the control of the human H1 promoter into the deleted E3b region of ONYX-411. Internavec acquired an increase in potency of ~10-fold in human cancer cells expressing the relevant K-ras12 mutation (H79, H441, and SW480), as defined by a reduction in the effective dose needed to achieve 50% growth inhibition (ED50). Internavec maintained attenuated in nonmalignant epithelial cells. Daily intratumoral injections of Internavec (five daily injections of 1 x 107 plaque-forming units) significantly reduced the growth of s.c. H79 pancreatic cancer xenografts in nu/nu mice by 85.5%, including complete growth suppression in three of five mice. Parental ONYX-411 or ONYX-411-siRNA19PT was markedly less effective (47.8% growth reduction, P = 0.03; and 44.1% growth reduction, P = 0.03, respectively). siRNA-mediated transgene expression contributed to cell cycle blockade, increased apoptosis, and marked down-regulation of Ras signaling–related gene expression (AKT2, GSK3β, E2F2, and MAP4K5). These findings indicate that Internavec can generate a two-pronged attack on tumor cells through oncogene knockdown and viral oncolysis, resulting in a significantly enhanced antitumor outcome. (Cancer Res 2006; 66(19): 9736-43)

Introduction

RNA interference (RNAi), originally referred to as “cosuppression” by Napoli and coworkers (1), describes the phenomenon of highly potent, sequence-specific, posttranscriptional gene silencing by double-stranded RNA (reviewed in refs. 2, 3). RNAi activity in mammalian cells was shown by Tuschil and coworkers, who introduced short, synthetic small interfering RNAs (siRNA) duplexes to attain sequence-specific RNA knockdown (4). Cleavage of the mRNA target involves the participation of a self-aggregating cytoplasmic protein complex called the “RNA interference silencing complex” (RISC) having an intrinsic, ATP-dependent helicase activity that unravels the duplex siRNA. Through Watson-Crick base pairing with the target mRNA sequence, the RISC-bound, antisense strand of the siRNA initiates ATP-independent cleavage by “Slicer,” a member of the Argonaute family of endonucleases (reviewed in refs. 2–5).

siRNA knockdown of the mutant K-ras oncogene, one of the most common oncogenic mutations in human cancers, has generated pronounced antitumor effects (6). When delivered as a nonreplicative retroviral transgene, siRNA19PT inhibited the relevant mutant K-ras12 allele and abrogated anchor-independent growth and tumorigenicity (6, 7). The collateral activity of K-ras knockdown has been ascribed to reduction in angiogenic potential. Fleming et al. (8) showed that K-ras knockdown increased the expression of thrombospondin-1, an endogenous inhibitor of angiogenesis, and decreased the production of vascular endothelial growth factor, a primary stimulant of angiogenesis. Antitumor activities were also attained in vivo through siRNA knockdown of other critical components for tumor cell growth, metastasis, angiogenesis, and chemoresistance (reviewed in refs. 2, 3). These findings suggest that siRNAs may serve as a novel and effective class of tumor therapeutics through posttranscriptional gene silencing.

Much like forerunning cancer-reactive antisense agents, a key barrier for siRNAs to attain clinical efficacy has been the lack of an optimal delivery platform (2, 5, 9). Earlier studies showed that stable transfection and expression of siRNA can be attained through delivery by nonreplicating viruses (10, 11), although this approach limits target cell penetration to the initial infectious event. The use of oncolytic virus platforms for siRNA transgene delivery is a natural extension from the success of nonreplicative viral delivery. Through early viral gene deletions and/or substitutions, oncolytic DNA viruses display restricted replicative activity in viral permissive cancer cells with inherent tumor suppressor gene defects (p53, pRB) or overexpressed transcription factors such as E2F-1 (12–14). siRNA delivery by an oncolytic virus is appealing from several fronts. First, tumor-selective infectivity implies that the viral delivery vehicle may be used to restrict transgene expression to the cancer microenvironment, hence, minimizing potential cytotoxicity to normal tissues (carrier-defined specificity). Second, transgene expression is extended through viral replication and re-infection of permissive cancer cells (15, 16). Furthermore, the viral oncolytic process is expected to augment antitumor outcomes by siRNA-mediated knockdown of the cancer genetic apparatus. Therapeutic doses (up to 1012 viral particles) of ONYX-015 and other oncolytic adenoviruses are well tolerated (reviewed in ref. 14), and have produced clinical efficacy at the locoregional level, particularly in advanced head and neck cancer (14, 17, 18).
To characterize the interaction of siRNA-mediated oncogene knockdown and viral oncolysis, we elected the use of siRNAs against the K-ras proto-oncogene (siRNA<sup>r</sup>ras), based on our prior success of inducing tumor growth inhibition by ribozyme-mediated K-ras knockdown (19). A number of siRNA<sup>r</sup>ras were designed against "stepped" nucleotide sequences at exon 1, codons 8 to 14, one of the three "hotspots" of ras oncogene mutations and a region previously shown to be susceptible to siRNA knockdown (6, 7). The prediction of a lack of "off-target" effects for irrelevant gene sequences was determined in silico by BLAST search of the National Center for Biotechnology Information (NCBI) GenBank database. This study examines the combined treatment outcome of mutant K-ras knockdown by a siRNA and oncolytic virus infection. Through coinfection studies and the subsequent generation of a novel viral-siRNA transgene construct, we showed that the siRNA-mediated K-ras knockdown acted in concert with viral oncolysis to achieve cell cycle arrest and increased apoptosis.

Materials and Methods

siRNAs and DNA oligonucleotides. K-ras siRNA gene target sequences were designed for targeting the K-ras exon 1 around commonly mutated codon 12. The designed sequences lacked homology with other known human genes as examined by BLAST search of the NCBI GenBank database. High-performance purity grade, customized siRNA<sup>r</sup>ras DNA duplexes were purchased from Qiagen (Valencia, CA). DNA oligonucleotides encoding for siRNA<sup>r</sup>ras<sup>−</sup> and control siRNA<sup>SP</sup> were synthesized by Integrated DNA Technologies (Coralville, IA). All other DNA oligos used were synthesized by Sigma-Genosys (The Woodlands, TX).

Cell lines. The human lines H441, A549, H522, H926 (all non–small cell lung cancers), H79 (pancreatic carcinoma), SW480 (colon carcinoma), and HEK293 (embryonic kidney) were obtained from American Type Culture Collection (Manassas, VA). All cell lines were cultured according to the vendor's instructions. Normal human mammary epithelial cells (HMEC) were obtained from Cambrex (Walkersville, MD).

Generation of Internaives. The DNA of hairpin siRNA<sup>r</sup>ras<sup>−</sup> sequence was cloned into the ONX-411 E3B region. Following restriction enzyme digestion, the hairpin siRNA template [forward strand: 5′-GCATTTTGCTATT-3′, reverse strand: 5′-AGGGTAT-3′] was ligated into the plasmid pIRE2-hs (Amion, Austin, TX). The H1 promoter–K-ras siRNA PCR product containing ClaI/SwaI restriction sites was generated by PCR, using the specific primers (forward primers: 5′-AGGCTAATCGATCA-3′, reverse primer: 5′-AGGGATCT-3′). The resulting hairpin construct was then transfected into permissive 293 cells (FuGENE 6 transfection reagent; Roche, Palo Alto, CA). The hairpin construct was then transfected into permissive 293 cells (FuGENE 6 transfection reagent; Roche, Palo Alto, CA). The cloned sequence was then cotransfected with the viral vector ONX-411 DNA (by EcoRI and BamHI) and the ONX-411 DNA (by EcoRI) was ligated to T4 DNA ligase, then transfected into permissive 293 cells (FuGENE 6 transfection reagent; Roche, Palo Alto, CA). The plasmid E2FGBV<sup>−</sup> was verified by PCR, using primers specific for the E3B region insertion sites (forward primer, 5′-CACCAACCAACCTCAGCTGCGGCCCAACTC-3′, reverse primer, 5′-AGGCCACATT-TAACATT-3′). Amplification products of the expected size were quantified by agarose gel electrophoresis (6, 7).

Viral yield quantification. Cellular DNA from cell pellet or supernatants from virally infected cells was isolated (Qiagen DNA blood kit, Qiagen) and quantified by OD<sub>260</sub>/OD<sub>280</sub> measurements. Real-time PCR (TaqMan Universal PCR Master Mix, Applied Biosystems) was done using 0.1 μg of DNA and primer probes specific to the adenoaviral late exon gene (forward primer, 5′-TGGCCTTACGACGCTTCTCTC-3′, reverse primer, 5′-CGGTGTAGGTTGAGCAGTTTG-3′). FAM/BHQ labeled standard probe: 5′-CCA-CAACGCGCTCAGCCTGA; Biosearch Technologies, Novato, CA; ref. 16). DNA recovered from a predetermined copy number of wild-type Ad5 virus was used as internal reference control. Viral yield (viral particles/10<sup>5</sup> cells) at graded time points post-viral infection was normalized to the input dose that was determined at 4 hours post-infection.

Proliferative assay. The proliferative activity of siRNA–treated tumor cells was determined by bromodeoxyuridine (BrdUrd) incorporation. 1 × 10<sup>5</sup> H441 cells were transfected with siRNA (150 nmol/L; gene silencer siRNA transfection reagent; Gene Therapy Systems, San Diego, CA) for 24 hours, and cultured in 96-well plates. BrdUrd was then added according to the manufacturer's protocol (R&D Systems, Minneapolis, MN) after 48 hours of culture. Wells seeded with graded numbers (10<sup>3</sup> to 10<sup>5</sup>) of untreated cells were used to establish the linear relationship of cell number versus absorbance as a function of BrdUrd uptake (SpectraMax 340, Molecular Devices, Sunnyvale, CA). A minimum of three experiments was conducted to ensure the presence of more than 10% of a positive value for each treatment. Cell viability was determined by trypan blue exclusion analysis (19).

MTT assay. Virally infected target cells (1 × 10<sup>5</sup> cells/well) were treated with MTT (3-[4,5-dimethylthiazol-2-y]-2, 5-diphenyltetrazolium bromide; R&D Systems) as a measurement of mitochondrial metabolic activity according to the manufacturer's protocol. The colorimetric reaction was quantified by spectrophotometric determinations at 570 nm, with 690 nm as reference (SpectraMax 340, Molecular Devices).

Human tumor xenograft model in nu/nu mice. H79 cells (3 × 10<sup>6</sup> cells in 0.1 mL of PBS) were inoculated s.c. into the right flank of athymic nu/nu mice (5-6 weeks old; Harlan/Sprague, Indianapolis, IN). Treatment with viral constructs was initiated when tumor xenografts reached a size of >80 mm<sup>3</sup>. Each mouse received five daily intratumoral injections of Internaave, ONX-411, or ONX-411-siRNA<sup>r</sup>ras<sup>−</sup> at 1 × 10<sup>11</sup> or 1 × 10<sup>10</sup> plaque-forming units (pfu). Control mice received injections with PBS only. Xenograft sizes (width and length) were measured by a vernier caliper twice a week. Tumor volume (V) was calculated by the formula: V = (L × W)<sup>2</sup>/2 (20). Animals were euthanized when tumor xenograft exceeded 2 cm in diameter or at the end point of observations, whichever came first. Mean differences of tumor volume were analyzed statistically by the two-tailed, non-paired Student's t test. All procedures involving animal use were
approved by the Institutional Animal Care and Use Committee at Baylor University Medical Center.

Cell cycle and apoptotic analysis. Cells (1 × 10^6) were fixed in ethanol (80%; overnight), then stained with propidium iodide (40 μg/mL; Sigma, St. Louis, MO) and RNase A (100 μg/mL; Qiagen) at 37 °C for 30 minutes, then analyzed by flow cytometry immediately (FACSscan, BD Biosciences, San Jose, CA). DNA content distribution was analyzed by ModFit (Verity, Inc., Topsham, ME). Apoptotic cells were quantified by flow cytometry as a function of seven-aminomycin D (7-AAD, Invitrogen) incorporation (100 μmol/L; 20 minutes on ice, in PBS, 1% fetal bovine serum) following viral infection [5 MOI (multiplicities of infection)] or Fas ligand (0.4 μg/mL; Beckman Coulter, Fullerton, CA) as described previously (19).

cDNA microarray analysis. Total RNA was isolated with the RNeasy kit (Qiagen) at 36 hours post-viral infection (5 MOI). Gene expression profiles were determined by array hybridization reactions (21) with the Affymetrix Human Genome U133 Plus 2.0 oligonucleotide probe array (Affymetrix, Santa Clara, CA), according to standard protocols (Microarray Core Facility, UT Southwestern Medical Center, Dallas, TX). An absolute expression analysis was carried out by using Affymetrix MAS 5.0. Hybridization reactions with a minimum signal intensity of >50 were subjected to differential subtraction by the Gene Spring 7.2 software (Silicon Genetics, Santa Clara, CA) to identify transcripts that differed minimally by a magnitude of 2-fold, which were compared by hierarchical clustering analysis.

Results and Discussion

Selection of K-ras-reactive siRNA. siRNA<sup>K-ras</sup> that showed significant K-ras knockdown activity include oligonucleotides that target the consensus sequence upstream of codon 12 (siRNA<sub>K-ras<sup>-12</sup></sub>-<sup>-12</sup>), as well as codon 12 sequences that are unique to the K-ras<sup>-12</sup> gene (siRNA<sub>K-ras<sup>-12</sup></sub>-<sup>-12</sup>). Wild-type (K-ras<sup>-12</sup>; siRNA<sub>K-ras<sup>-12</sup></sub>-<sup>-12</sup>) and wild-type (K-ras<sup>-12</sup>; siRNA<sub>K-ras<sup>-12</sup></sub>-<sup>-12</sup>) genotypes (Fig. 1A). siRNA<sub>K-ras<sup>-12</sup></sub>-<sup>-12</sup>, siRNA<sub>K-ras<sup>-12</sup></sub>-<sup>-12</sup>, and siRNA<sub>K-ras<sup>-12</sup></sub>-<sup>-12</sup> reduced K-ras<sup>-12</sup> mRNA expression of the heterozygous H441 cells (K-ras<sup>-12</sup>; K-ras<sup>-12</sup>) by 49.2 ± 5.6%, 41.4 ± 17.8%, and 36.3 ± 14.6%, respectively, at 22 to 24 hours posttransfection (Fig. 1B). siRNA<sub>K-ras<sup>-12</sup></sub>-<sup>-12</sup>, with its consensus-binding capacity to both wild-type and mutant K-ras<sup>-12</sup> mRNA, is predisposed to a higher knockdown activity than siRNA<sub>K-ras<sup>-12</sup></sub>-<sup>-12</sup> or siRNA<sub>K-ras<sup>-12</sup></sub>-<sup>-12</sup>. However, an increased knockdown efficiency was not evident, probably due to closer proximity of the targeted site in reference to the K-ras exon 1 starting codon (3, 22). Based on evaluations with siRNA<sub>K-ras<sup>-12</sup></sub>-<sup>-12</sup>, K-ras<sup>-12</sup> knockdown was dose-dependent (50 nmol/L, 35.3 ± 9.9%; 100 nmol/L, 41.1%; 150 nmol/L, 67.9 ± 23.8%) and time-dependent, with optimal knockdown at 150 nmol/L at 20 to 24 hours, consistent with previous findings (7, 9, 10).

A pull-down assay based on binding with the RAS-binding domain of Raf-1 was used to determine the effect of siRNA treatment on active RAS protein expression. Control siRNA did not significantly affect K-ras<sup>-12</sup> mRNA or active RAS levels. siRNA<sub>K-ras<sup>-12</sup></sub>-<sup>-12</sup> reduced GTP-bound RAS by 42% (n = 2), as compared with reductions of 24% and <10%, respectively, by siRNA<sub>K-ras<sup>-12</sup></sub>-<sup>-12</sup> and siRNA<sub>K-ras<sup>-12</sup></sub>-<sup>-12</sup>. The pan-RAS antibody used in the pull-down analysis detects all active members of the RAS family, including H-RAS and N-RAS that are unaffected by K-ras<sup>-12</sup> siRNA treatment. This may explain the smaller reduction of total active RAS protein as compared with K-ras<sup>-12</sup> mRNA. Furthermore, the constitutively active ras mutant protein has been shown to predominate in cell lines with a heterozygous RAS phenotype, such as H441 (23). Thus, the wild-type-reactive siRNA<sub>K-ras<sup>-12</sup></sub>-<sup>-12</sup> can be expected to be less effective in reducing total active RAS, in spite of its capacity for wild-type K-ras<sup>-12</sup> mRNA knockdown.

Subsequent BrdUrd incorporation studies showed that the growth effect of siRNA treatment was proportionate to the extent of mutant K-ras<sup>-12</sup> knockdown. Significant growth reductions of 52% and 36% were attained by siRNA<sub>K-ras<sup>-12</sup></sub>-<sup>-12</sup> and siRNA<sub>K-ras<sup>-12</sup></sub>-<sup>-12</sup>, respectively (P = 0.05, n = 3). With a mean lipofection efficiency of 48%, our findings of a comparable level of target mRNA knockdown and growth reduction are indicative of the effectiveness of siRNA treatment.

Effect of siRNA<sup>K-ras<sup>-12</sup></sub> knockdown on ONYX-411-infected tumor cells. Coinfection studies were carried out with ONYX-411, a late generation, conditional replicative, oncolytic adenovirus, whose antitumor potency was 10-fold to 100-fold higher than ONYX-015 (16, 24). A549 human lung cancer cells were used as the target, in view of their permissiveness to adenoviral infection and moderate resistance to viral oncolysis at low MOI (25). To simulate peak siRNA transgene expression at the “late” phase of viral replication, siRNA<sub>K-ras<sup>-12</sup></sub>-<sup>-12</sup>, which targets the consensus K-ras exon 1 sequence upstream of codon 12, was introduced by lipofection at 24 hours post-viral infection. The highest level of growth-inhibitory response by siRNA<sub>K-ras<sup>-12</sup></sub>-<sup>-12</sup> alone was observed at 48 hours posttreatment, when viable tumor cells were reduced by 35%. By comparison, low doses of ONYX-411 (5 MOI) reduced viable tumor cells by 49% to 63% at 72 to 120 hours post-helipoxing infection (Fig. 1C). Cotreatment with siRNA<sub>K-ras<sup>-12</sup></sub>-<sup>-12</sup> and ONYX-411 displayed an additive antitumor effect, resulting in a >90% reduction of viable tumor cells at 120 hours post-helipoxing infection (Fig. 1C). These findings indicate that the antitumor effect was augmented and prolonged through siRNA-mediated K-ras knockdown and viral oncolysis.
RAS knockdown did not abort viral replication. To consider the effect of RAS knockdown on viral replication, quantitative PCR analysis was carried out on ONYX-411 + siriRNAras−2−treated A549 cells, using primers specific to the adenovirus late hexon gene. Viral yield did not differ significantly from cells infected with parental ONYX-411 (Fig. 1D), indicating that viral “late phase” coexpression of siriRNAras (at 24 hours post-viral infection) did not adversely affect viral replication. A limitation of this model is an incomplete overlap between siRNA-transfected and viral-infected cells (efficiency of ≈50% for either treatment). Nonetheless, the observed additive cytotoxic effect supports the premise that late phase RAS knockdown enhances viral oncolysis without adversely altering viral replication.

Generation of Internavec. Based on these favorable findings, we have cloned a siRNA that specifically targets the common K-ras oncogene mutant (K-ras12) into the E3B region of ONYX-411. siriRNAras−4, chosen for its highly effective tumor growth–inhibitory activity during screening (Fig. 1B), was integrated in a short hairpin configuration encompassing a PolIII (H1) promoter upstream of siRNA sense and antisense encoding sequences that are separated by a 3 to 9 nucleotide intervening loop (Fig. 2A). The PolIII promoter causes termination after the second uridine and produces a transcript that mimics the natural siRNA configuration after Dicer processing. A similarly configured siriRNA transgene for the reporter gene luciferase was recently shown to be highly effective when delivered from the E4 region of the replication-competent adenovirus, Ad324E3 (26). We elected the E3B region as the cloning site, in view of prior findings that peak expression of transgenes inserted in this region coincided with the viral “major late transcription unit” following initiation of viral DNA replication (16, 24). Hence, nonmalignant cell types that are nonpermissive for viral replication may have a correspondingly attenuated effect from E3 transgene-mediated effects (24, 25).

Cytotoxic activity of Internavec in human cancer lines. The molecular integrity of siriRNAras−4 in the ONYX-411 siRNA construct, named Internavec, was confirmed by PCR and bidirectional DNA sequence. Functional evaluations were carried out with the homozygous K-ras12 mutant Capan-1 (H79) pancreatic cancer cells. Internavec treatment, but not the parental ONYX-411 virus, significantly reduced K-ras mRNA expression at 48 hours posttreatment (63% reduction as compared with untreated control; P < 0.02; Student’s t test). Viable H79 cells were reduced by 48%, 61%, and 75% on days 1, 3, and 5 post-Internavec treatment according to trypan blue exclusion analyses, as compared with 16%, 31%, and 53%, respectively, in ONYX-411-treated cultures (Fig. 2B).

MTT analysis confirmed that cellular activity was reduced by 88% in Internavec-treated H79 cells (5 MOI) at day 5, as compared with a 39% reduction by parental ONYX-411 or the control construct carrying the siriRNA GFP (Fig. 2C). Comparative analysis indicates that the effective dose for inhibiting cancer cell growth by 50% (ED50) was reduced by ~10-fold through incorporation of the siriRNAras−4 transgene (MOI of 1.03 as compared with an MOI of 11.6 by parental ONYX-411 and MOI of 14.7 by ONYX-411-siriRNA GFP; Fig. 2C). K-ras mutations in human cancer are localized in a limited number of “hotspots” (codons 12, 13, or 61) that differ from the wild-type by a single nucleotide substitution (19). The enhanced antitumor activity of Internavec was evident in human cancer lines expressing the relevant, homozygous K-ras12 mutation (SW480 colon carcinoma), as well as in H441 lung cancer cells that have a heterozygous K-ras12/wt phenotype and were resistant to ONYX-411 oncolysis (Fig. 2D). By contrast, incorporation of siriRNAras did not markedly improve antitumor activity in cancer lines lacking the relevant K-ras mutation (H522, H596, both with wild-type K-ras12/g12 phenotypes; Fig. 2D). These findings showed that Internavec retains the specific knockdown activity of K-ras12, which translates into an additive antitumor response with ONYX-411 oncolytic activity.

Limited cytotoxicity of Internavec to nonmalignant cells. ONYX-411 carries an E1A deletion and E2F-1 conditional promoters in the E1 and E4 regions, hence, limiting replication to cells with a defective retinoblastoma tumor suppressor protein (pRb) pathway and E2F-1 overexpression (16, 24). We examined the cytotoxic effects of Internavec as compared with ONYX-411 on nonmalignant, human epithelial HMEC cells, in order to determine the effect of siriRNA arming on the conditional replicative activity of ONYX-411. Both ONYX-411 (Fig. 3D) and Internavec (Fig. 3C) produced low levels of toxicity (loss of viability in 18% and 4% of total cells, respectively), as compared with cytotoxic effects that were observed in >90% of HMEC cells following wild-type, d309 viral infection (Fig. 3B). The differential cytotoxic outcome is likely attributed to the minimum viral load (>10^3 vp per cell) needed to
achieve a cytopathic effect (27). Although Internavec and ONYX-411 showed comparable replicative activities as dl309 in H79 cancer cells (Fig. 4A), their viral replicative activity was attenuated by 750-fold in HMEC cells (Fig. 4B). Accordingly, the viral load for Internavec and ONYX-411 was ~100 virions/cell, as compared with 10^4 virions/cell in HMEC-infected cultures after 72 hours. These findings indicate that integration of siRNA ras did not produce added cytotoxicity in nonmalignant HMEC cells. Compared with H79 cells, HMEC displayed an increased susceptibility to wild-type adenoviral infection, as evidenced by markedly increased viral yield (Fig. 4) and a higher frequency (~2-fold) of E1A-expressing cells following dl309 infection. The expression of early adenoviral genes such as E1A and E4 immediately after cell entry functions to override the fundamental control of the host cell cycle, forcing progression into the cell cycle, thereby facilitating wild-type viral replication (13, 28). The finding of Internavec (and ONYX-411) replication in HMEC cells with an intact pRb pathway, albeit at a markedly lower level as compared with dl309, likely reflects viral replication in the growth fraction of these nonmalignant cells that releases E2F-1 through physiologic phosphorylation of retinoblastoma after entry into the cell cycle. Nonetheless, the limited proliferative activity of normal cells in vivo is expected to foster minimal viral replication and cytotoxicity in nonmalignant tissues (13, 14, 16–18), because E1 and E4 expression of Internavec is under the control of an E2F-1 promoter (16).

Antitumor activity in vivo. The in vivo antitumor efficacy of Internavec was examined with a s.c. H79 xenograft model in athymic nu/nu mice (Fig. 5). Internavec at 1 × 10^8 pfu completely inhibited tumor growth in three of five treated mice for up to 32 days. The mean reduction in tumor size (mean of 85.5%, n = 5) by Internavec differed significantly from that in animals treated with ONYX-411 (47.8% growth reduction, P = 0.03), or ONYX-411-siRNA GFP (44.1%, P = 0.03; Fig. 5). These findings showed that the combined activities of siRNA ras knockdown and viral oncolysis produced markedly enhanced antitumor outcomes in vivo.

Growth-regulatory activity of Internavec. Currently, the precise mechanism by which a conditional replicative adenovirus effects tumor cell kill is unclear. The self-limiting disease course of wild-type adenoviral infections in immunocompromised patients is indicative of limited virulence (29). Host cells with viral protein expression are commonly seen post-infection, indicative of an aborted viral replicative process and/or a latent infection state (13, 28, 29). Coupled with the common defects in apoptotic and IFN-induction pathways in cancer cells, the aborted oncolytic process is an explanation for incomplete clinical responses seen in oncolytic virotherapy trials (14, 18). The incorporation of a regulatory siRNA transgene that can disrupt cell survival mechanisms, such as through K-ras knockdown, could conceivably tilt the cellular balance towards cancer cell death.

Internavec-treated cultures displayed comparable, albeit moderately elevated viral replicative activity as ONYX-411 treatment in H79 cancer cells (Fig. 4A), suggesting that the facilitation of viral replication may not be the primary contribution for enhanced tumor cell kill. Cell cycle analyses were carried out to characterize the treatment effect on the growth fraction (S + G2-M) distribution. The percentage of cycling cells were relatively constant (54-49%) in untreated cultures over 96 hours, whereas virus-treated cultures exhibited a progressively increasing growth fraction. As shown in a representative study (Fig. 6A), S + G2-M cycling cells constituted

Figure 3. Cytotoxicity to human nonmalignant cells. The viral cytopathic effect of HMEC cells was examined by light microscopy at day 5 post-infection with Internavec, ONYX-411, or wild-type dl309 (0.1 MOI). The proportion of live cells, as defined by cells that lacked the cytopathic features of rounding and increased granularity, was quantified over two high-power fields (magnification, ×200).

Figure 4. Viral replicative activity in viral permissive and nonpermissive human cells. Viral DNA replication was determined by real-time PCR quantification of DNA from viral-infected (0.1 MOI) H79 (A), or HMEC cells at quiescent state (B). Relative viral yield (viral particles/1 × 10^5 cells) at different time points was normalized to the input dose determined at 4 hours post-initial infection.
81% of dl309-treated cells, 73% of Internavec-treated cells, and 58% of ONYX-411-treated cells at 96 hours post-infection. These findings were consistent with previous reports (28, 29) of $E1A$ and $E4$ early adenoviral genes overriding the fundamental control of the host cell cycle, forcing progression into the cell cycle. Furthermore, Internavec treatment, but not ONYX-411, produced significantly elevated accumulation of cells in $S + G_2-M$ phases as compared with untreated cultures at 72 hours ($68 \pm 8.4\%$ versus $52 \pm 0.4\%$, $n = 3$; $P = 0.028$) and 96 hours ($71 \pm 7.2\%$ versus $49 \pm 3.8\%$, $n = 3$; $P = 0.01$; mean ± SD). Internavec seemed to be more effective in driving cells into growth phase at the level that approached that of dl309 (Fig. 6A), with increases in both $S$ phase (48 ± 11.4%) and $G_2-M$ (23 ± 4.9% at 96 hours) compartments, as compared with 43 ± 5.2% and 18 ± 8.0%, respectively, in ONYX-411-treated cells. Cell cycle blockage by Internavec was accompanied by modestly increased apoptotic activity (18 ± 3.5% as compared 8.2 ± 3.3% by ONYX-411; $P < 0.001$), as determined by 7-AAD staining and flow cytometric analysis (Fig. 6B).

An activated K-RAS has been reported to modulate cycling cells through a wide spectrum of cell cycle regulators (30), but in particular, facilitates transition from $G_2-M$ into the next cycle (31). Our findings with Internavec were consistent with cell cycle blockage events that resulted from early viral gene expression as well as $K-ras$ knockdown, culminating in $S$ and $G_2-M$ phase arrests. Previously reported antiapoptotic activities of adenoviral $E1B$ and $E3$ genes (13, 14) may account for the observed low levels of apoptosis. Hence, enhanced tumor cell kill by Internavec may engage alternative pathways of cell death induction, such as autophagy, the process of programmed cell death that involves the degradation of cytoplasmic structures preceding nuclear collapse (32), which has been shown to be suppressed by oncogenic RAS activity (33).

**Gene array analysis of Internavec treatment outcome.** To define the transcriptional effect of Internavec treatment, gene expression array analysis was carried out with Affymetrix Human Genome U133 Plus 2.0 oligonucleotide probe array and total RNA from untreated, or ONYX-411, ONYX-411-siRNA<sub>GFP</sub>, and Internavec-infected H79 cells. The expression profile of the Internavec-treated culture was compared with ONYX-411 and ONYX-411-siRNA<sub>GFP</sub> cultures following normalization against the corresponding hybridization signals from the untreated culture. Differential subtraction of the gene profile signature of ONYX-411-treated cultures from Internavec-treated cells yielded 198 genes that were uniquely down-regulated by Internavec (>2-fold). Using Affymetrix and David 2.0 software, 41 genes were assigned to cell cycle, cell-cell interaction and signal transduction, RNA and protein metabolism, and stress and immune response (data not shown). In particular, four genes which were associated with the RAS signaling network in the modulation of cell cycle and proliferation were down-regulated, i.e., protein kinase $\beta$ (AKT2).
glycogen synthase kinase-3 β (GSK3β), the transcription factor E2F2, and the mitogen-activated protein kinases, MAP4K5 (Fig. 6C). AKT2 is the only member of the Akt/PKB family implicated in several types of human malignancies, and is known to be collaterally activated by RAS (34). AKT2 knockdown has been shown to reduce viable cell numbers, and suppresses tumor clonogenicity, cell migration, and invasion. The down-regulation of AKT2 also increases apoptosis and necrosis (34). These AKT2-dependent outcomes may contribute to the antitumor outcome of Internavec treatment.

The unique down-regulation of AKT2, GSK3β, and E2F2 by Internavec was confirmed by real-time RT-PCR analyses with predefined primer probe sets. E2F2 mRNA levels were reduced by 53% as compared with untreated culture (n = 2). AKT2 and GSK3β were reduced by 25% and 22%, respectively, whereas ONXY-411 or ONXY-411-siRNA GFP garnered minimal effects on all three mRNAs (<5%). Quantitative differences in the absolute level of reduction of AKT2 and GSK3β by microarray and quantitative RT-PCR determinations may be related to discrepancies of assay dynamic range of the two methods, efficiency of reverse transcription, and/or sequences and concentrations of primer and probe molecules employed (35). The effect of Internavec treatment on protein translation is currently being investigated by proteomic analysis.

Recently, Andersson and coworkers (36) found that expression of the adenoviral genes, virus-associated (VA) RNAI and VA RNAII, led to the suppression of RNAI activity, possibly through competitively interfering with Dicer or RISC activity. The validity of this phenomenon needs to be further confirmed because the studies were done only in HEK293 cells with integrated viral DNA and corresponding helper activities for viral gene expression and replication. RNAI suppression was evident only when high concentrations (up to 10^5 copies/cell) of VA RNA were present at a very late phase of the replicative cycle (36). By comparison, our study showed maximal siRNA-additive effects at the earlier time frame of 48 to 72 hours post-infection. Other siRNAs have been integrated into the E3 region of nonreplicative adenoviral constructs with a wild-type VA RNAI and RNAI configuration. Their RNAi activity was also unaffected by the wild-type VA RNAI and RNAI phenotype of the vector backbone.5 Hence, further studies are needed with viral permissive cancer lines to better define the interaction of VA RNA expression and RNAI in human cancer cells.

In comparative studies whereby ribozyme and siRNAs were directed against a single hybridization accessible region of epidermal growth factor receptor, the siRNA construct was significantly more potent in inhibiting cancer cell growth (37). Other siRNAs have shown prolonged in vitro and in vivo knockdown activity when compared with ribozyme (3) or DNA enzyme (38). These findings infer theoretic advantages for the use of siRNA in gene knockdown applications, although this premise needs to be evaluated for individual target genes. Our previously conducted studies involved the delivery of K-ras ribozymes by a nonreplicative adenovirus (19). Hence, a direct comparison of relative efficacy with siRNA is not immediately feasible. Lipofection studies are planned to address this issue, using siRNA<sup>nuc</sup>- and a hammerhead Rbz sequence targeting the same K-ras region.

To the best of our knowledge, this is the first report that documents the use of an oncolytic viral-transgene platform to attain siRNA-mediated posttranscriptional gene silencing that results in tumor cell death. Our findings indicate that siRNA-mediated K-ras knockdown manifested as enhanced cell cycle blockage via multiple molecular pathway perturbations, and can achieve an additive antitumor response in combination with viral oncolysis. The Ras family of oncogenes is mutated in ~30% of all human cancers, and represents the most frequently mutated oncogenes (39). K-ras codon 12 point mutations were found in up to 80% of colorectal, 90% of pancreatic, and 40% of non–small cell lung cancers (39–41), of which 29% to 37% were of the GTT genotype (42) and susceptible to siRNA<sup>nuc</sup> knockdown. Hence, Internavec may be directly applicable against these tumor subsets. Our proof-of-principle study also suggests that the oncolytic virus may be used to deliver siRNAs directed against other oncogene sequences that critically contribute to cancer cell growth. The strategy of incorporating multiple, tandem siRNAs with distinct knockdown specificities could conceivably address the issues of tumor phenotypic heterogeneity and the development of resistance to siRNA knockdown from single base pair mutations (18).

References


Acknowledgments

Received 5/3/2006; revised 7/11/2006; accepted 7/26/2006.

Grant support: Baylor-Sammons Cancer Center, Mary Crowley Medical Research Center, and the PHS grant CA108039.

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The authors thank Dr. Joseph Newman (Baylor University Medical Center) for his assistance in the performance of flow cytometry analyses, Dr. Donald Rao (Murex Pharmaceuticals) for his technical input regarding DNA sequencing of shRNA transgenes, Dr. Lynda Bennett (Baylor Institute for Immunology Research) for her valuable insights on microarray data analysis, Dr. Leonard Post (ONYX Pharmaceuticals) for providing the ONXY-411 virus, Dr. Erica Gomes (Baylor University), Ila Oxendine and Viviana Manguel (Murex Pharmaceuticals) for their technical assistance, and Dr. Frank McCormick (University of California, San Francisco Comprehensive Cancer Center) for discussions of the manuscript.

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Antitumor Activity of an Oncolytic Adenovirus-Delivered Oncogene Small Interfering RNA


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