Late Expression of Nitroreductase in an Oncolytic Adenovirus Sensitizes Colon Cancer Cells to the Prodrug CB1954

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ABSTRACT

We have constructed an oncolytic adenovirus expressing the Escherichia coli nitroreductase gene nfsB from an internal ribosome entry site (IRES) in the adenovirus L5 major late transcript. The virus (Tcf-NTR) has Tcf transcription factor-binding sites in the E1A, E1B, and E4 promoters, which restrict viral replication to cells that have activation of the Wnt signaling pathway. This virus was compared with an E1B-55K-deleted virus expressing nitroreductase (NTR) from a cytomegalovirus (CMV) promoter in the E1B-55K region [CRAd-NTR(PS1217H6)]. Both viruses express NTR in colorectal cancer cell lines and show increased cytopathic effect in the presence of the prodrug CB1954. Unlike the Tcf-NTR virus, the CMV-NTR virus expresses NTR in human lung fibroblasts and sensitizes these normal cells to CB1954. The in vivo activity of the viruses was tested in SW620 xenografts in nude mice by intravenous injection of $10^{11}$ particles of virus followed 1 week later by intraperitoneal injections of CB1954. The CMV-NTR virus produced minimal effects in this model. The median time to form 1000-mm$^3$ tumors in mice treated with the Tcf-NTR virus plus CB1954 was increased from 14 to 26 days ($p = 0.003$), but this was due mainly to the direct oncolytic effect of the virus. Combination therapy with $3 \times 10^{11}$ particles of Tcf-NTR virus (given intravenously) and the mammalian target of rapamycin (mTOR) inhibitor RAD001 (everolimus) (given orally) significantly improved survival (median, >50 days), and addition of CB1954 to this regimen further delayed tumor growth. These results show that the Tcf-NTR virus is more tumor selective and active than the CMV-NTR virus. At the level of transduction that can be achieved currently with oncolytic viruses given intravenously, drugs such as RAD001, which do not require activation by the virus, produce greater increases in efficacy than prodrugs such as CB1954.

OVERVIEW SUMMARY

Prodrug-activating enzymes are expressed from oncolytic viruses to permit conversion of harmless prodrugs into active chemotherapeutic agents in a tumor-specific manner. Diffusion of the active drug to surrounding cells leads to killing of uninfected tumor cells (a so-called bystander effect). This reduces the number of cells that must be directly infected with the virus to achieve a given oncolytic effect. Despite good efficacy in vitro, we show that the therapeutic gain in vivo is modest, probably because the foci of infection after intravenous injection of virus are too widely spaced for active drug to diffuse to all parts of the tumor. Improved delivery of oncolytic viruses to the tumor will thus be required before the full benefit of prodrug–enzyme systems can be realized in the context of systemic therapy.

INTRODUCTION

Many different types of replication-competent RNA and DNA virus are being developed as cancer therapeutics (Hawkins et al., 2002). Adenoviruses are the most widely used because their biology is well understood, they can be stably modified, and they can be produced to Good Manufacturing Practice (GMP) grade in large quantities. Despite showing good

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efficacy in tissue culture, oncolytic adenoviruses are barely able to cure human tumor xenografts in nude mice, especially on systemic administration of the virus. Expressing a toxic protein, such as tumor necrosis factor-α (TNF-α) (Marr et al., 1998), TNF-related apoptosis-inducing ligand (TRAIL) (Kagawa et al., 2001), or diphtheria toxin (Lipinski et al., 2004), can increase the efficacy of the virus in vivo but raises biosafety concerns. A more cautious approach is to express prodrug-activating enzymes such as thymidine kinase (Wildner et al., 1999), cytosine deaminase (Freytag et al., 1998), carboxyster-
ase (Studal et al., 2003), or nitroreductase (nfsB/NTR) (Searle et al., 2004), which are toxic only in the presence of the corresponding prodrug. One goal of therapy with these combinations is to kill tumor cells surrounding the infected cell, through a so-called bystander effect, to compensate for the low fraction of tumor cells that can be infected with currently available vectors. NTR converts CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide] into a highly toxic 4-hydroxylamino derivative that has a potent bystander effect (Anlezark et al., 1992; Djeha et al., 2000). NTR-expressing vectors are being developed for clinical use according to an incremental approach. The first virus, CTL102, expressed NTR from the cytomegalovirus (CMV) promoter in a nonreplicating adenovirus and has been tested by intratumoral injection in phase I trials in patients with colorectal metastases to the liver (Palmer et al., 2004). The CMV promoter is not tumor specific, so the next step was to develop vectors that were replication defective but used tissue- or tumor-specific promoters (Latham et al., 2000; Lipinski et al., 2001; Bilsland et al., 2003). All of these vectors have limited potential for clinical development because they are replication defective. Chen et al. (2004) described an E1B-55K-deleted replicating adenovirus expressing NTR from the CMV promoter. The viral backbone is similar to dl1520, which is safe but has shown poor efficacy in clinical trials (Reid et al., 2002). The logical next step would be to express NTR tumor selectively in a more active virus.

Expression of a prodrug-activating enzyme early in the virus replication cycle risks killing the virus (Freytag et al., 1998; Rogulski et al., 2000). Late expression can circumvent this problem, at least for the infected cell itself, and can most conveniently be achieved by expressing the enzyme from the adenovirus major late promoter (MLP). This promoter drives expression of five groups of transcripts encoding mainly viral core and capsid proteins. Several strategies have been used to express transgenes from the MLP. Hermiston and colleagues replaced endogenous E3 genes with transgenes (Hawkins and Hermiston, 2001a,b; Hawkins et al., 2001). This is an efficient approach because no extra sequences are required outside the transgene, but it has the drawback that deletion of endogenous viral genes may worsen virus efficacy in vivo (Suzuki et al., 2002). Creation of entirely new splicing units within the major late transcript is feasible, but in our experience would require substantial optimization to achieve efficient expression (Fuerer and Iggo, 2004). Another possibility is to insert the transgene after an existing late gene and to reinitiate translation with an internal ribosome entry site (IRES). This approach has been successfully used to express p53 protein (Sauthoff et al., 2002) and cytosine deaminase (Fuerer and Iggo, 2004).

We have developed oncolytic adenoviruses with Tcf (T cell factor transcription factor)-binding sites in the early promoters (Brunori et al., 2001; Fuerer and Iggo, 2002). These viruses target cells with activation of the Wnt signaling pathway. This pathway is activated by mutations in the adenomatous polyposis coli (APC) and β-catenin genes, which are found occasionally in many cancers, and almost universally in colorectal cancers (Radtke and Clevers, 2005). Intravenous administration of the virus with Tcf sites in the E1A, E1B, and E4 promoters significantly slowed the growth of subcutaneous SW620 colon carcinoma xenografts, particularly when given in combination with RAD001 (everolimus), an orally active derivative of rapamycin (Homiczko et al., 2005). The bulk of the tumor was killed but a rim of viable cells remained in which oncolysis was balanced by tumor cell proliferation. In this situation, addition of a drug with a potent bystander effect might tip the balance in favor of oncolysis. To test this possibility, we have constructed a Wnt-targeting adenovirus that expresses NTR after the fiber gene in the L5 major late transcript. We compare this virus with CRAd-NTR(PS1217H6), the 55K-deleted virus described by Chen et al. (2004).

MATERIALS AND METHODS

Cell lines

SW480, SW620, Hct116, and HT29 cells were supplied by the American Type Culture Collection (ATCC, Manassas, VA). Human embryonic lung fibroblasts (HFs) were supplied by M. Nabholz (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland). Her911 cells (Fallaux et al., 1996) were supplied by P. Beard (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland). cR1 cells are C7 cells expressing Myc-tagged ΔN-β-catenin (Fuerer and Iggo, 2002). All cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin.

Adenovirus mutagenesis

The endogenous E4 enhancer was deleted from a luciferase reporter plasmid (pCV3; Volorio, 2001) carrying the Tcf-E4 region of the rCV2 virus (Fuerer and Iggo, 2002) by inverse polymerase chain reaction (iPCR) with primers GCGCTA-TATATACTCGCTCTGCAC (oCF90) and GCCACTAGC-AAAACA CCTGGGCGAGT (oCF91) to give pCF334. The left inverted terminal repeat (ITR) with four Tcf-binding sites was amplified from pCF25 (Fuerer and Iggo, 2002) with primers AAACAGTAGGAGATCAAAGGTTG (oCF102) and CGG- AATCATCGCCTTTATACATAAT (oCF103). The region downstream of the E4 TATA box was amplified from pCF361 with G76 and GCCAAGTCAGAG- (G76). The product was cut with SpeI and PacI and inserted into pCF334 to give pCF361. The new E4 promoter sequence was amplified from pCF361 with G76 and GCAAGTCAGAG-GG (oCF104). The region downstream of the E4 TATA box was amplified from pCF34 (Fuerer and Iggo, 2002) with primers AAACAGTAGGAGATCAAAGGTTG (oCF102) and CGG- AATCATCGCCTTTATACATAAT (oCF103). Both products were mixed and reamplified with G76 and oCF34. The final product was cut with EcoRI and PsiI and cloned into pCF34 to give pCF374. This plasmid carries the right end of the adenoviral genome with the new E4 promoter (an ITR with four Tcf-binding sites followed by the packaging signal and the E4 TATA box). The AvrII-EcoRI fragment of pCF374 including the new E4 promoter was then cloned into
a pUC19-derived plasmid carrying the right-end 4-kb HpaI fragment of Ad5, to give pJV9.

The fiber-encephalomyocarditis virus (EMCV) IRES sequence is identical to that in the vCF125/AIC4 virus (Fuerer and Iggo, 2004), with replacement of the yeast cytosine deaminase (yCD) gene by NTR. The starting plasmid (pCF315) has the fiber-IRES cassette of pCF328 (Fuerer and Iggo, 2004) in pUC19. Escherichia coli NTR was amplified by PCR with primers TCTCT/H11005 1475/H11003 Tris-/H11003/H11003/H11003 mice were purchased from CB1954, respectively.

Nunclon were expanded on SW480 and Her911 cells, respectively, using forming unit (PFU) titers were measured on Her911 cells.

Iggo, 2002) and the IRES-NTR region of vpCF22 was sequenced with primers oCF137 (GGTCTGGCCACAACTATA- CATTA) and oCF138 (GGTGGGCTATACACTGGAAT). The PCR product was cut with PstI and SpeI and inserted into pUC19 to give pCF287. The F124N mutation was inserted into pCF287 by iPCR using the primers AAACTT/H9004 and SpeI and inserted downstream of the IRES in pCF315 cut with BgRII and SpeI to give pCF419. The F124N mutation was inserted into pCF287 by iPCR using the primers AAACTT/SalI and SpeI and inserted into the same sites in pJV9 to create pJV12. This plasmid carries the right end of the adenoviral genome, starting from the fiber, and includes the IRES-NTRF124N sequence and the new E4 promoter. pCF358 carries the right 1748 nucleotides of the vCF22 virus, including the Tcf-E1A and Tcf-E1B promoters (C.F., unpublished data; and Fuerer and Iggo, 2002) cut with PacI and SalI and inserted into pCF1 (Fuerer and Iggo, 2002) cut with PacI to create pCF456, a gap repair vector containing both viral ends separated by a single SalI site. Similarly, pCF358 and pCF374 PacI–SalI fragments were inserted into pCF1 to create pCF396. Plasmids carrying the full Tcf and Tcf-NTR viral genomes were created by gap repair (Gagnebin et al., 1999) of pCF396 with vKH1 (Homicsko et al., 2005) and of pCF456 with vpCF12 (Fuerer and Iggo, 2004) to give pCF16 and pCF22, respectively. The pCF16 genome has Tcf-binding sites in the E1A and E1B promoters, as well as the new Tcf-E4 promoter described above. The pCF22 construct carries the same mutations plus the IRES-NTRF124N cassette. The E4 region was sequenced with the primer IR215 (Fuerer and Iggo, 2002) and the IRES-NTR region of pCF22 was sequenced with primers oCF137 (GGTCTGGCCACAACTATCATTA) and oCF138 (GGTGGGCTATACACTGGAAT). vpCF16 and vpCF22 were cut with PacI and converted into the viruses vCF167 and vCF226, using ER1 cells for the initial transfection and SW480 cells for amplification and plaque purification. After expansion in SW480 cells, the cell extracts were brought to 10% glycerol and stored at −70°C. Particle counts were based on the optical density at 260 nm (OD260) of virus in 0.1% sodium dodecyl sulfate, using the following formula: 1 OD260 = 1012 particles/ml.

Western blotting

Cells were infected with 10 PFU/cell. Two hours later, medium was replaced by complete medium. Where indicated, cytosine arabinoside (ara-C, 20 μg/ml; Sigma, St. Louis, MO) was added to the medium. Cells were harvested at various times in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer. E1A, DBP (DNA-binding protein), fiber, and NTR were detected with M58 (BD Biosciences Pharmingen, San Diego, CA), B6 (Reich et al., 1983), 4D2 (RDI Division of Fitzgerald Industries, Flanders, NJ), and 4F11G10 (Bilsland et al., 2003) antibodies, respectively.

Cytopathic effect assays

Cells in 6-well plates were infected with 10-fold dilutions of virus. Two hours after infection, medium was replaced with complete medium containing CB1954 (Sigma). Fresh medium including CB1954 was added 4 days postinfection. After 6 to 9 days, the cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) and stained with crystal violet. SW480 cells, Hct116 cells, HT29 cells, and HLFs were treated with 200, 140, 20, and 150 μM CB1954, respectively.

Animal experiments

Four-week-old male NMRI nu/nu mice were purchased from Harlan (Horst, The Netherlands). Subcutaneous SW620 flank xenografts were made by injecting 106 cells. Mice (six per group) were injected with virus when tumors reached 80–150 mm3 in size (day 0 of the experiment). A total of 1011 particles was injected into the tail vein, given as four doses. The first injection of 1010 particles was followed by three injections of 3 × 1010 particles at four hr intervals. Mice in groups receiving RAD001 received additional injections of 1011 particles on days 1 and 2 (total dose, 3 × 1011 particles). CB1954 (Sigma) was first diluted in dimethyl sulfoxide (DMSO) to a concentration of 100 mg/ml. The DMSO solution was then diluted 1:200 in PBS, pH 7.4, to yield a final concentration of 0.5 mg/ml. CB1954 was administered to mice by intraperitoneal injection at 25 mg/kg per day on days 7–11 and, when possible, on days 20–23, 30–33, and 40–43. Mice in control groups received intraperitoneal injections of 0.5% DMSO in PBS on these days. RAD001 (everolimus) was supplied by Novartis (Basel, Switzerland) as a microemulsion for oral use. The microemulsion (2% RAD001) was aliquoted and stored at −20°C. Before administration, the microemulsion was diluted in water (final volume, 100 μl) and 5 mg/kg per day was administered by gavage, starting on day 6 after virus injection. RAD001 administration continued daily to the end of the experiment. Tumor size was measured every 2 days. Tumor volume was calculated according to the following formula: volume = (length × width2 × 3.14)/6. Quantitative PCR and fluorescence in situ hybridization (FISH) were performed as described by Homcsko et al. (2005).
in the L5 transcript after the fiber gene. The CMV-NTR virus ([CRAd-NTR(PS1217H6)]) is similar to dl1520, but with a CMV-NTR expression cassette replacing the E1B-55K gene. The NTR gene in the Tcf-NTR virus contains a point mutation that lowers the IC50 for CB1954/H11011 5-fold in a bacterial toxicity assay (Grove et al., 2003).

**NTR is expressed with late kinetics**

NTR expression was tested by Western blotting in three colon cancer cell lines: SW480 cells, which are highly permissive for Tcf-regulated viruses, and Hct116 and HT29 cells, which are less permissive for these viruses because of lower Tcf promoter activity (Fuerer and Iggo, 2002). The parental Tcf-regulated virus was used as a negative control. NTR expression by the Tcf-NTR virus was detectable at 48 hr in all three cell lines (Fig. 2A, left panel). We previously showed that the yeast cytosine deaminase (yCD) gene in an analogous L5-IRES construct was expressed with late kinetics (Fuerer and Iggo, 2004). To check the kinetics of NTR expression, cells were treated with ara-C, a drug that blocks DNA replication and expression of late proteins. DBP expression and fiber protein expression were used as negative and positive controls, respectively, for the ara-C treatment, because they are expressed from early and late promoters. Treatment with ara-C did not affect expression of DBP, but completely blocked expression of the fiber and NTR proteins (Fig. 2A, right panel). This indicates that NTR is expressed as a late gene, as expected from its location in the L5 transcript.

**Selectivity of NTR expression**

The Tcf-NTR virus is based on a virus that replicates selectively in cells with activation of the Wnt signaling pathway (Fuerer and Iggo, 2002). The CMV-NTR virus is similar to dl1520, which derives most of its tumor specificity from a defect in late viral RNA export in normal cells (O’Shea et al., 2004). Because NTR is expressed from a CMV promoter in the CMV-NTR virus, it should show similar expression in normal and tumor cells. To test whether the Tcf-NTR virus is more selective for tumor cells, E1A, DBP, and NTR expression by the Tcf-NTR and CMV-NTR viruses was compared in SW480 colon cancer cells and normal human lung fibroblasts (HLFs) (Fig. 2B). The CMV-NTR virus gave higher NTR expression in SW480 cells. This is reasonable, given the strength of the CMV promoter and the limited efficiency of IRES-mediated gene ex-

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**FIG. 1.** Schematic diagram showing the structure of the two NTR-expressing viruses used in this study. The clone names are vCF226 for the Tcf-NTR virus and CRAd-NTR(PS1217H6) for the CMV-NTR virus. Both viruses are replication competent. The Tcf virus derives its tumor specificity from the insertion of Tcf sites in the viral promoters. The CMV virus derives its tumor specificity from deletion of the E1B-55K gene.

**FIG. 2.** Western blots for NTR and viral proteins. (A) Colon cancer cells infected with Tcf-regulated adenoviruses in the presence or absence of ara-C to block progression to the late phase of the viral cycle. Tcf, parental virus with Tcf sites in the E1A, E1B, and E4 promoters (vCF167); Tcf-NTR, the NTR-expressing derivative. (B) Infection of tumor cells (SW480) and normal cells (HLFs) with the Tcf-NTR and CMV-NTR viruses. (C) Infection of HLFs with the NTR viruses, wild-type Ad5, and a virus expressing yeast cytosine deaminase (yCD), using the same IRES in L5 as is used for the Tcf-NTR virus. SW480 cells were infected at an MOI of 10 PFU/cell; HLFs were infected at 100 PFU/cell.
pression. The Tcf-NTR virus expressed E1A and DBP earlier and to a higher level in SW480 cells. This reflects the strong Tcf activity in these cells. The poor viral protein expression by the CMV-NTR virus cannot be explained by a p53 restriction, because SW480 cells are mutant for p53. As expected, the Tcf-NTR virus did not express viral proteins or NTR in HLFs. In contrast, the CMV-NTR virus expressed E1A, DBP, and NTR normally in HLFs (Fig. 2B). We thus conclude that the Tcf-NTR virus is more tumor specific than the CMV-NTR virus.

Leakiness of E2 expression in Tcf-regulated viruses

We have previously shown that expression of yCD in the L5 transcript reduces the stringency of regulation of E2 and E4 expression in a Tcf-E1A/Tcf-E4 virus (Fuerer and Iggo, 2004). A possible explanation is that the yCD sequence contains enhancer elements that trans-activate the E4 promoter and, through E4orf6/7, the E2 promoter. The original Tcf-E4 promoter had three Tcf sites in the ITR, followed by the packaging signal and the normal E4 enhancer. To increase the stringency of E4 regulation, the normal E4 enhancer was deleted and an extra Tcf site was added in the Tcf-NTR virus (this means the virus has symmetrical ITRs containing four Tcf sites). Luciferase assays showed that the new E4 promoter is inducible by activation of the Wnt signaling pathway, for example, by transfection of a stable mutant of β-catenin (data not shown). To determine whether the Tcf-NTR virus is more specific than the Tcf-yCD virus, HLFs were infected with wild-type Ad5 and the Tcf-regulated viruses. As shown previously, DBP was weakly expressed 24 hr after infection with the Tcf-yCD virus (Fig. 2C). No DBP expression was seen with the Tcf-NTR virus. This indicates that the new virus is more stringently regulated than its predecessor. The mechanistic basis for the improved regulation (different transgene versus promoter modification) was not addressed further in this study.

Cytopathic effect in vitro

Cytopathic effect (CPE) assays were used to test the ability of CB1954 to sensitize cells to the Tcf-NTR and CMV-NTR viruses in vitro. In the absence of CB1954, Tcf-NTR was more active in SW480 cells, CMV-NTR was more active in HT29 cells, and both viruses were equally active in Hct116 cells (Fig. 3). Because the cells differ substantially in their sensitivity to CB1954 alone, a titration was performed to determine the IC_{50} of the prodrug in each cell line, and ~70% of this concentration was used for the CPE assays. Addition of CB1954 increased the CPE of both viruses in all three colon cancer cell lines. The CPE increased ~100-fold in the cell lines that were least sensitive to virus alone, and ~10-fold when the virus alone showed more activity (Fig. 3). In normal fibroblasts, the Tcf-NTR virus did not produce any CPE at a multiplicity of infection (MOI) of 300 PFU/cell, with or without CB1954, consistent with the lack of NTR or viral protein expression on Western blots (Fig. 2). The CMV-NTR virus was not cytopathic alone, but showed CPE equivalent to that seen in tumor cells in the presence of CB1954. This is expected, given the strong NTR expression seen on Western blots (Fig. 2). We conclude that expression of NTR sensitizes cells to CB1954 and that the expression of nitroreductase by the CMV-NTR virus is not selective for tumor cells.

FIG. 3. CPE assays in the presence or absence of CB1954. Colon cancer cells and normal fibroblasts were infected with log dilutions of virus at the indicated MOI (PFU per cell), and then CB1954 was added 2 hr after infection. The concentration of CB1954 was adjusted to ~70% of the IC_{50} for each cell line: SW480, 200 μM; Hct116, 140 μM; HT29, 20 μM; HLF, 150 μM. The cells were harvested at 6–9 days.

Efficacy of NTR-expressing viruses in vivo without CB1954

Xenograft experiments were performed to test the efficacy of the Tcf-NTR virus in vivo. SW620 cells were derived because we demonstrated that a systemically administered Tcf-regulated oncolytic virus showed significant efficacy in this model when combined with RAD001, a rapamycin derivative (Homicsko et al., 2005). SW620 cells were derived from a metastasis of the tumor that gave rise to SW480 cells, and show similar high Tcf activity. Xenografts were allowed to grow to a size of ~100 mm³ and then virus was injected into the tail vein in four aliquots at four hr intervals (10^{10} particles and then three injections of 3 × 10^{10} particles, for a total of 10^{11} particles). This fractionated dosing schedule was used to reduce Kupffer cell-mediated hypotension and to prolong the half-life of the virus in the circulation (Tao et al., 2001; Schiedner et al., 2003).

The growth rate of tumors treated with the CMV-NTR virus alone was slowed on days 2–4 (Fig. 4A), but was similar to that of the control group at later time points. There was a 2-day increase in median survival (defined as tumor size below 1000 mm³), which was not statistically significant (Fig. 4B). The growth rate of tumors in the group treated with Tcf-NTR alone was slower than in the control and CMV-NTR-treated groups, and on day 14 (the last complete time point in the control group) tumors in the Tcf-NTR group were substantially smaller than in the control group (T_{cf}/C ratio, 34%; p = 0.005) and CMV-NTR group (T_{cf}/T_{CMV} ratio, 64%; p = 0.01). The median survival was almost doubled compared with the control group (26 versus 14 days; p = 0.003) and with the CMV-NTR-treated group (26 versus 16 days; p = 0.004).
CB1954 on days 20–23. There was a small and insignificant effect of CB1954 on the growth of Tcf-NTR-treated tumors (T_{Tcf}/T_{Tcf+CB}, ratio, 75% on day 30; p = NS; median survival, 30 days in both groups) (Fig. 4).

**Efficacy of NTR-expressing viruses plus CB1954 and RAD001 in vivo**

We have previously shown that the mammalian target of rapamycin (mTOR) inhibitor RAD001 significantly prolongs the survival of mice treated with Tcf-regulated adenoviruses (Homiczko et al., 2005). Combination therapy with virus and RAD001 leads to the formation of tumors with a necrotic core, caused by the antivascular effect of RAD001, surrounded by a rim of viable tumor cells. Virus can be detected in the rim 6 weeks after virus injection despite continuous RAD001 therapy. In this situation, in which ongoing virus replication balances tumor cell replication, a drug with a substantial bystander effect could potentially tip the balance in favor of oncolysis. To determine whether the addition of CB1954 to our most effective existing regimen would result in tumor clearance, SW620 xenografts were treated intravenously with 10^{11} particles of Tcf-NTR virus on days 0, 1, and 2 (total dose, 3 × 10^{11} particles) followed by daily gavage with RAD001 starting on day 5 and cycles of CB1954 on days 7–10, 20–23, 30–33, and 40–43. The experiment was performed only with the Tcf-NTR virus because the constitutive early expression of NTR by the CMV-NTR virus was mildly toxic to the cells we used to produce the virus, which made it difficult to produce the virus in large amounts. RAD001 was started 2 days before CB1954 to exploit the possibility of a transient normalization of the vasculature that may improve tumor perfusion a few days after starting antiangiogenic therapy (Winkler et al., 2004). Unlike CB1954, RAD001 had no significant effect on the mean body weight of the mice (data not shown). Consistent with our previous data, the growth of tumors in the group receiving the combined Tcf-NTR virus and RAD001 therapy was substantially slower than in the control group (Fig. 5A). Triple therapy was more effective than any other treatment, although the difference in tumor size (Fig. 5A) and median survival (Fig. 5B) attributable to CB1954 therapy did not reach statistical significance, and no cures were seen.

**Virus distribution in tumor at late stages of therapy**

To determine whether CB1954 adversely affected replication of the Tcf-NTR virus in vivo, quantitative PCR and FISH were used to measure the viral content and distribution after treatment. Tumors were harvested once most of the tumors in the group exceeded 1000 mm^3 or, in the surviving mice, on day 50, the predetermined study end point. The amount of viral DNA in tumors in the minus-RAD001 group was slightly lower after CB1954 treatment, a difference that was not statistically significant, and unaffected by CB1954 in the plus-RAD001 group (Fig. 6). Thus, although there may have been a small effect at early time points, CB1954 was clearly unable to eliminate the virus, even after four courses of treatment. Because the treated tumors continued to grow slowly despite persistence of the virus, the distribution of virus within the tumors was examined by FISH. Untreated SW620 tumors contained cylinders of viable tumor cells around blood vessels, which coalesced to form confluent sheets of tumor cells in some regions (Fig. 7A; hema-
toxylin and eosin (H&E) stain). Low-power views of tumors treated with the Tcf-NTR virus alone (Fig. 7B) or Tcf-NTR virus plus CB1954 (Fig. 7C) showed that the tumors were more heterogeneous after viral treatment, with nodules of viable tumor cells scattered within large areas of necrosis, which presumably resulted in part from virus oncolysis. There was no conspicuous histological difference between tumors treated with Tcf-NTR alone or with Tcf-NTR and CB1954. Essentially the same pattern was seen in tumors treated with Tcf-NTR and RAD001: virus could be found in the rim of viable cells and at the border between necrotic and viable regions (Fig. 7D-I/II), particularly where few viable cells remained, but was absent from a substantial fraction of the rim (Fig. 7D-III) and from the larger nodules of viable cells (Fig. 7D-IV). We conclude that neither CB1954 nor RAD001 can prevent ongoing replication of the Tcf-NTR virus in vivo, even after multiple cycles of treatment. The progressive growth of tumors receiving triple therapy (Fig. 5) is explained by the growth of regions devoid of virus. In most cases, we observed that regions containing abundant virus were surrounded by necrotic material or connective tissue, which probably acted as a barrier to the effective spread of the virus into the proliferating areas of the tumor.

**DISCUSSION**

The main conclusion from this study is that the CB1954–NTR prodrug system can substantially increase the CPE of an oncolytic adenovirus in vitro, but this translates poorly into increased efficacy in a xenograft model in vivo. We previously observed that relapse of SW620 xenografts is caused by overgrowth of tumor nodules devoid of virus (Homicsko et al., 2005), which is a consequence of inefficient delivery of the virus to the tumor after intravenous injection. This patchy distribution of virus is the most likely explanation for the limited response to combination therapy with virus plus CB1954. Alternative explanations could relate to the specific design of the viruses or inhibition of viral replication by the active drug. The potential design issues concern, for the CMV-NTR virus, the low efficacy of the parental vector, and for the Tcf-NTR virus, the low efficiency of IRES-mediated gene expression. The use of an E1B-55K-deleted virus to express NTR from the CMV promoter was a logical extension of previous work by the Birmingham group (Latham et al., 2000; Weedon et al., 2000; Djeha et al., 2004).
FIG. 7. Tumor morphology and distribution of virus within tumors. (A–D) Low-power views (H&E stain) showing the overall appearance of tumors (scale bars, 1 mm). (A) negative control, day 14; (B) Tcf-NTR alone, day 32; (C) Tcf-NTR plus CB1954, day 32; (D) Tcf-NTR plus CB1954 plus RAD001, day 50. (B-I to D-IV) Enlargements of the circled regions in the upper panels. H&E and FISH of adjacent sections are shown (scale bar, 100 μm). Blue, DAPI-stained nuclei; red, FISH for viral DNA.
et al., 2001; Chen et al., 2004; Palmer et al., 2004; Searle et al., 2004). The CMV promoter guarantees high expression in a wide range of cell types, and the dl1520 vector backbone has proven safe in clinical trials involving hundreds of patients (Reid et al., 2002). Against this impressive record of safety must be set the fact that the 55K deletion attenuates the virus in a manner that is not fully complemented in most tumor cells, resulting in low efficacy. The main tumor restriction of dl1520 intervenes after viral DNA replication, resulting in amplification of the viral DNA in normal cells. Because the CMV promoter is active in normal cells, there is no barrier to high NTR expression in normal cells. Our results confirm these two points. In the absence of CB1954, the CMV-NTR virus was no better than control treatment of the SW620 xenografts. Addition of CB1954 produced a 2-day increase in survival, which meets a formal test of significance, but examination of the curves shows that the difference occurred before starting CB1954; thereafter, the growth rate was similar in the virus, virus plus CB1954, and control groups. No mice in any group died of side effects of CB1954 therapy, but it is nevertheless a source of concern that the CMV-NTR virus was toxic to normal fibroblasts in the presence of CB1954 in vitro. The lack of overt liver toxicity in the CMV-NTR group may be related to the drug schedule: CB1954 was started only on day 7, at which stage the liver has eliminated most of the injected virus and is in a phase of regeneration. Without significant new virus production by the tumor, chronic re-infection and CB1954 activation in liver cells is unlikely to occur. The low specificity of NTR expression by the CMV-NTR virus may not be a major issue for intratumoral virus therapy, but is clearly undesirable for systemic therapy.

Major late promoter activation occurs only after viral DNA replication, which is dependent on activation of the early promoters by the Tcf transcription factor in our Tcf-regulated viruses. By expressing NTR from an IRES in the L5 major late transcript, we can restrict NTR expression to cells with activation of the Wnt signaling pathway. Constitutive activation of this pathway in the adult is restricted mainly to tumor cells, particularly colorectal cancer cells. Consistent with this, we saw NTR expression by the Tcf-NTR virus in colon cancer cells but not in normal fibroblasts. Capsid proteins represent a substantial fraction of all new proteins synthesized by the cell late in infection, but it is unlikely that NTR is expressed in similar amounts, because the level of expression of the second open reading frame in IRES vectors rarely exceeds 50% of the level of the first, and is frequently much lower (Mizuguchi et al., 2000). The low level of NTR expression achieved was sufficient to sensitize cells to CB1954 in vitro, but it is possible that a more efficient expression strategy is required to produce equally strong responses in vivo.

The lack of a major effect of CB1954 in vivo could reflect a flaw in the overall strategy or a failing of the CB954–NTR combination, rather than a weakness in the specific design of the vectors. The obvious question is whether a prodrug system that is toxic to the virus can sensibly be incorporated into an oncolytic virus (Freytag et al., 1998; Rogulski et al., 2000). Early treatment with CB1954 definitely blocks replication of the CMV-NTR virus (Chen et al., 2004). Late expression of NTR in the Tcf-NTR virus is designed to minimize inhibition of virus replication by the drug. This expectation is based partly on our previous observation that viruses expressing cytosine deaminase from the major late promoter could replicate in the presence of the prodrug 5-fluorocytosine (Fuerer and Iggo, 2004). In practice, CB1954 did not clear virus from tumors in vivo, even after four cycles of treatment (Fig. 6). A truly effective bystander effect might be expected to overwhelm the virus after the initial rounds of infection. Indeed, the body uses interferon to fight virus infections in exactly this way. We conclude that the bystander effect probably does not play a major role in the response to the current vectors, and that substantial increases in efficacy may be achievable with more potent vectors before toxicity of the activated prodrug limits viral replication in vivo.

Consistent with our previous results, combination therapy with RAD001 and the Tcf-NTR virus substantially delayed tumor growth. This was probably due mainly to an effect of RAD001 on the tumor vasculature. Because RAD001 is not cytotoxic, it must be given regularly to suppress tumor growth. We postulated that the addition of CB1954 would allow the virus to kill the residual viable tumor cells that persist in the tumor rim during treatment with oncolytic virus and RAD001. Addition of CB1954 did slow the growth of tumors receiving Tcf-NTR virus plus RAD001, but the virus plus RAD001 combination was already so effective that much larger numbers of mice would be required to achieve high statistical significance. Examination of the Tcf-NTR virus-treated tumors by H&E staining showed that large regions of the core and rim of the tumor were replaced by necrotic or scar tissue. This effect was even more pronounced in the tumors treated with virus and RAD001. FISH confirmed that virus replication was occurring in limited parts of the rim and at the borders between necrotic and viable regions, even at late times after infection. The general approach thus appears sound, but poor local spread of virus from sites of replication to remote masses of viable cells within the tumor prevents elimination of the tumor. At the level of transduction that can currently be achieved after intravenous therapy, the distance over which the bystander effect must act is unrealistically large. Several approaches are being actively pursued to improve virus delivery and spread. Kupffer cell depletion by fractionated virus injection increases the half-life of the virus in the blood (Tao et al., 2001; Denby et al., 2004), and thus the availability of virus to infect the tumor, but we have not formally shown that it increases the amount of virus taken up by the tumor cells. Alternative strategies to improve virus delivery involve modification of the fiber gene (reviewed by Mizuguchi and Hayakawa, 2004) and physically coating the virus with hydrophilic polymers (Green et al., 2004). Improved viral spread within the tumor can be achieved by improving lysis of infected cells (Doronin et al., 2003) and by injection of proteases into the tumor (Kuriyama et al., 2001). Given the limited success of the Tcf-NTR virus–CB1954 treatment, we predict that techniques to improve delivery and spread of virus will be required before the full benefit of prodrug therapy can be realized.

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LAT E EXPRESSION OF NTR IN AN ONCOLYTIC ADENOV IRUS


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