RAD001 (Everolimus) Improves the Efficacy of Replicating Adenoviruses that Target Colon Cancer

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Abstract

Selectively replicating adenoviruses have the potential to cure cancer but have shown little efficacy in clinical trials. We have tested the ability of the mTOR kinase inhibitor RAD001 (everolimus) to enhance the response of xenografts to an oncolytic adenovirus. The virus has Tcf sites inserted in the early viral promoters and replicates selectively in cells with activation of the Wnt signaling pathway. To enhance tumor cell infection, an integrin targeting peptide (CDCRGDCFC) was inserted into the fiber gene of the virus. RAD001 combines three useful properties: it inhibits tumor cell growth directly, blocks angiogenesis, and suppresses the immune response. RAD001 does not block viral protein expression, DNA replication, or cytopathic effect in tumor cells in vitro. After 6 weeks of daily RAD001 treatment, ongoing viral DNA replication could be detected in tumor xenografts, showing that RAD001 does not inhibit virus replication in vitro. I.v. injection of virus alone produced a small delay in xenograft growth, whereas combination therapy substantially prolonged the survival of the mice. We suggest that collapsing the tumor vasculature after the initial infection traps the virus and facilitates local spread within the tumor. Unlike conventional drugs, which require continued access to the tumor through the vascular system, oncolytic viruses are in principle less sensitive to late reductions in perfusion because they are produced locally within the tumor. (Cancer Res 2005; 65(15): 6882-90)

Introduction

Oncolytic viruses provide a means to target the causal oncogenic defects in tumors (1). Despite having great selectivity and efficacy in vitro, these viruses have shown only limited activity in preclinical xenograft models and in early stage clinical trials (2, 3). Many factors explain this low activity in vivo. The majority of i.v. administered virus is either sequestered on red cells or cleared by macrophages within minutes of injection (4, 5). This can be circumvented by depletion of Kupffer cells and coating the virus with hydrophilic polymers like hydroxypropyl methacrylamide (6), but delivery of the virus to the tumor in large amounts remains a major problem. Modification of the tropism of the virus by mutation of the fiber gene can reduce infection of nontumor cells, but it is difficult to achieve selectivity without loss of activity. The most successful strategies have instead sought to broaden the tropism to reduce the chance that tumor cells will be inefficiently infected because of poor expression of the natural receptors for human adenoviruses (7, 8). The CDCRGDCFC peptide was identified in a screen for homing of i.v.-injected phage to tumors (9). Tumor endothelial cells are known to express high levels of a receptor for RGD peptides, αvβ3 integrins, which explains the ability of RGD-modified phage to home to tumors (10). The RGD peptide binds to a wide range of integrins, some of which are highly expressed on tumors (11). Insertion of the CDCRGDCFC peptide into the HI loop of the fiber gene of adenovirus 5 (Ad5) has been shown to increase the efficiency of infection of many different cell types by recombinant adenoviruses (12).

To improve the efficacy of oncolytic adenoviruses, they are routinely combined with chemotherapeutic drugs (13) or with prodrugs that can be activated by enzymes expressed by the virus (14). The drugs normally used to treat colon cancer are unattractive for combination therapy because they can inhibit viral replication: irinotecan inhibits topoisomerase I, 5-fluorouracil blocks nucleotide synthesis, and oxaliplatin cross-links DNA. Newer drugs offer the possibility to inhibit tumor cell growth without killing the virus. RAD001 (4-hydroxyethyl-rapamycin, everolimus) is an orally available derivative of the immunosuppressant macrolide rapamycin (15). Like rapamycin, RAD001 inhibits mTOR kinase, a key component of growth factor and nutrient signaling pathways (16, 17). Phosphorylation and consequent inactivation of 4E-BP1 by mTOR allows eIF4E to recruits eIF4F to the mRNA cap. This leads to enhanced translation of mRNAs with highly structured 5′-untranslated regions, including genes that promote cell growth and oncogenic transformation (18). RAD001 inhibits the growth of many cancer cell lines in vitro (19) and can inhibit the growth of tumor xenografts in vivo (20). One of the mechanisms of antitumor activity in vivo seems to be inhibition of angiogenesis (21), resulting in activity against xenografts of cell lines that respond only weakly to the drug in vitro.

Mathematical modeling shows that the balance between the rate of tumor cell growth and virus spread is a critical determinant of the outcome of oncolytic virus infection (22). Whether it acts directly on tumor cells or indirectly by blocking tumor perfusion, the resulting delay in tumor growth induced by RAD001 should allow the virus to spread further within the tumor before the tumor cells can grow away from the foci of infection or the immune system halts the infection. The initial dosing regimens of RAD001 in cancer patients were chosen to avoid immunosuppression (23), but for combination therapy with virus, transient immunosuppression to extend the period of viral replication would be an advantage.

Wnt pathway activation by mutations in the APC or β-catenin genes is seen in many different types of tumor, but is particularly common in colon cancer. We have previously developed oncolytic adenoviruses that replicate selectively in cells with constitutive activation of the Wnt signaling pathway (24, 25). These viruses have binding sites for the Tcf signaling factor in the early promoters. We describe here Tcf-regulated adenoviruses that have the peptide CDCRGDCFC inserted into the HI loop of the fiber gene to enhance...
infection of tumor cells. We show that RAD001 does not inhibit the growth of these viruses in vitro or in vivo, and that combination therapy with RAD001 plus virus is much more effective in a xenograft model than virus or RAD001 alone.

Materials and Methods

Chemicals. RAD001 (everolimus) was supplied by Novartis (Basel, Switzerland) as dry powder for in vitro use and microemulsion for oral use. The powder was dissolved in ethanol and stored as 10 mmol/L stock solution at −20°C. The microemulsion (2% RAD001) and placebo emulsion were aliquoted and stored at −20°C.

Cell lines. The colon cancer cell lines SW620, HT29, and Hct116 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). LS174T and SW480 cells were provided by Dr. B. Sordat (ISREC, Epalinges, Switzerland). HeLa cells were provided by Dr. D. Lane (Department of Surgery, University of Dundee, Scotland). cR2 cells are derivatives of HEK293 cells that express a stable mutant of β-catenin, the viral polymerase and viral preterminal protein (27, 28). All cells were cultured in DMEM (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum. LS174T and SW480 cells were provided by Dr. B. Sordat (ISREC, Epalinges, Switzerland). HeLa cells were provided by Dr. D. Lane (Department of Surgery, University of Dundee, Scotland). cR2 cells are derivatives of HEK293 cells that express a stable mutant of β-catenin, the viral polymerase and viral preterminal protein (27, 28). All cells were cultured in DMEM (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum and 1% penicillin/streptomycin. RAD001 was used at final concentration of 3.6 μmol/L in vitro.

Viruses. The E1B-Tcf integrating vector, pBDD-241, was described by Brunori et al. (25). The KpnI/XbaI Ad5 fragment (nucleotides 30,470 to 33,598) containing the fiber region was cloned from Ad5 genomic DNA (ATCC VR5) into pUC19 to give pCFl9. The CdcRGRDCF motif was inserted into the HI loop of the fiber by inverse PCR from pCFl9 using primers GGAGACTGTTTCTGGCAGGGATCAGTATCTATGTGC (oKH11) and GCGGCGTACAAATGTGTCATCGTGCTTCT (oKH12) to create pKH67. The Coxsackie-adenovirus receptor (CAR) binding site was mutated in the knob of the fiber by inverse PCR from pKH67 using primers GGAGACTGTTTCTGGCAGGGATCAGTATCTATGTGC (oKH11) and ATTTAGACTACATGTAGGA-GATGGAGCTGG (oKH10) to create pKH68. The amino acid sequence of the fiber gene of vKH6 was deleted to create vKH3. To avoid contaminating the fiber sequences of vKH6, the fiber gene of vKH6 was deleted to create vKH3. To avoid contaminating the fiber sequences of vKH6, the fiber gene of vKH6 was deleted to create vKH3.

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Results

The structure of the viruses used in this study is shown in Fig. 1A. The parental virus, vKH1, has Tcf sites inserted into the inverted terminal repeats and EIB promoter, resulting in Tcf-dependent expression of E1A, EIB and, to a lesser extent, E4 (24). The peptide CdcRGRDCF was inserted into the HI loop of the fiber gene to confer on vKH6 the ability to infect cells either through binding to the normal receptor (CAR) or through binding to integrins, particularly αvβ3 and αvβ5. The CAR binding site in the fiber gene of vKH6 was deleted to create vKH3. To avoid selection for escape from the Tcf-based regulation of the early promoters, the viruses were produced in SW480 cells, which have constitutively high Tcf activity because of a mutation in the APC gene (35). The particle to plaque-forming unit (pfu) ratio of the viruses is shown in Fig. 1B. The microemulsion viral genome was labeled using the DIG DNA labeling kit (Roche). Tumor sections were fixed with 4% formaldehyde in PBS and stained with crystal violet.

Quantitative PCR assay. Cells were infected in vitro with one viral particle per cell in DMEM. The medium was replaced after 4 hours and cells were harvested after 48 hours. DNA was purified from tissue culture cells and mouse tissues using a DNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Quantitative PCR was done on a Light Cycler (Roche, Basel, Switzerland) using FastStart SYBR Green (Roche), 500 nmol/L primers (Invitrogen), and 10 ng template DNA. Results were expressed relative to cell number assuming 6 pg DNA/cell. The primers used for quantitative PCR were hexon forward primer, CTTCTGATTGCCCCAGTGG and hexon reverse primer, GGCTCTGAGTAGCTCCAGGG.

Animal experiments. Four-week-old female NMBR nu/nu mice were purchased from Elevage Janvier (Le Genest St Isle, France). SC. SW620 flank xenografts were made by injecting 10^6 cells. Mice (five per group) were injected with virus when tumors reached 80 to 150 mm^3 in size. A total of 1 × 10^9 particles were injected into the tail vein per day, given as four shots of 2.5 × 10^10 particles at four hourly intervals. Two regimens were tested: virus injection on a single day at the start of the experiment (total dose per mouse 1 × 10^10 particles) or virus injection at weekly intervals for 3 weeks (total dose 3 × 10^11 particles). RAD001 microemulsion was diluted in water and 5 mg/kg/d was administered by daily gavage starting the day after virus injection. For mice receiving weekly injections of virus, RAD001 was omitted the day before and on the day of the injection; RAD001 administration continued daily thereafter to the end of the experiment. Tumor size was measured every 2 days. Tumor volume was calculated according to the formula, volume = length × width^2 × 0.5. Differences in growth curves were calculated by two-tailed Student’s t test. Differences in survival curves were calculated by log-rank test in R (33).

ELISA. Three weeks after i.v. injection of 10^9 viral particles of adenovirus (vKH1), 200 μL of blood was taken from each mouse. The serum titer of antibodies against adenoviral proteins was measured by ELISA using ELISA plates (Nunc) coated with Ad5 as described (34).

Fluorescence in situ hybridization. Viral genomic DNA was labeled using the DIG DNA labeling kit (Roche). Tumor sections were fixed in 4% PFA, digested for 30 minutes with proteinase-K, acetylated with TEA for 10 minutes, and blocked with hybridization buffer without probe for 2 hours. Labeled probe was added and slides were hybridized overnight at 50°C. After washing in SSC, slides were incubated overnight with anti-DIG horseradish peroxidase at 4°C and label was detected with TSA Cy3 reagent kit (Perkin-Elmer, Boston, MA). 4,6-Diamidino-2-phenylindole (DAPI) was used to counterstain nuclei.

Results

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Combination Therapy with Oncolytic Virus and RAD001

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misleading criteria, particle counts rather than pfu were used to calculate the viral titer for the experiments described below.

Western blotting for the E1A, DBP, and fiber proteins was done to test whether the new Tcf viruses are able to infect colon cancer cells efficiently and retain selectivity. We tested three colon cancer cell lines with strong Tcf activity, SW480, SW620, and LS174T, and two colon cancer cell lines with weak Tcf activity, HT29 and Hct116 (35). A cervical cancer cell line without activation of the Wnt pathway, HeLa, was used as a negative control. Viral protein expression by the Tcf viruses was reduced or absent in HeLa cells, confirming our previous results with Tcf-regulated viruses and showing that fiber modification does not compromise promoter selectivity (Fig. 1B). The RGD viruses were at least as good as vKH1 in all of the colon cancer cell lines, and gave substantially higher viral protein expression in LS174T, indicating that integrin binding plays an important role in infection of LS174T cells.

To determine whether addition of the RGD peptide changes the in vitro efficacy of the viruses, cytopathic effect assays were done (Fig. 1C). At low multiplicities of infection, virus must undergo multiple rounds of productive infection to kill all the cells. Thus, the assay measures the ability of the virus both to infect cells and to replicate. Insertion of the RGD peptide into the fiber increased the efficacy of the Tcf-regulated viruses on colon cells by a factor of 10 to 100. vKH3, in which the CAR-binding site in the fiber has been deleted, showed intermediate cytopathic effect in LS174T and Hct116, indicating that both CAR and integrins are used by vKH6 to infect these cells. The efficacy of vKH6 was comparable with, or greater than, that of wild-type Ad5 in all of the colon cancer cell lines tested except HT29, which has the lowest Tcf activity. vKH1 and vKH3 were 10,000-fold less cytopathic than wild-type virus on HeLa cells. vKH6 was 10-fold more cytopathic than the other Tcf-regulated viruses on HeLa cells, showing that integrins and CAR both contribute to HeLa infection. The perfect control for vKH6 would be a virus with wild-type promoters and RGD insertion in the fiber, but this virus was not constructed for biosafety reasons. The increase in cytopathic effect of vKH6 on HeLa cells probably reflects a large increase in the effective multiplicity of infection (number of viral genomes entering the cell).
caused by the RGD modification, rather than any change in the Tcf regulation of the viral promoters. We conclude that the viruses with modified fiber genes retain selectivity for colon cancer cells and show similar or greater cytopathic effect than the parental virus. This is consistent with previous reports on the behavior of RGD-modified adenoviruses (36).

To determine whether addition of the RGD peptide alters the pattern of Tcf virus infection in vivo, the virus was injected into the tail vein of nude mice with s.c. SW620 xenografts. The virus was injected in four aliquots at 4 hourly intervals. This schedule was used to prolong the circulation time of the virus in the blood and hence the probability of infection (37). The first dose is rapidly cleared by Kupffer cells, which are themselves eliminated in the process. Fluorescence in situ hybridization (FISH) to viral DNA was used to identify sites of virus replication; single viral genomes were below the detection limit of the assay. Nine days after injection, vKH1 produced compact islands of infected cells, whereas vKH6 produced a more diffuse pattern, suggesting that vKH6 may spread better through tumor tissue (Fig. 2A). To determine whether the spectrum of organs infected was altered by insertion of the RGD peptide, which can bind to multiple different integrins, the biodistribution was tested (Fig. 2B). Virus was injected into the tail vein as described above and organs were harvested for quantitative PCR 24 hours later. Compared to other organs, there was less viral DNA than expected in liver after injection of the virus with native tropism (vKH1). This may be related to the fractionated dosing schedule, which leads to clearance of Kupffer cells. The amount of viral DNA was 10 to 100 times higher in the tumors infected with the RGD viruses, consistent with the in vitro data showing that SW620 is more susceptible to infection with vKH6 than vKH1. The amount of viral DNA was similar for the viruses with native or modified tropism in all mouse organs except liver, where the amount was increased 50-fold for vKH3 and vKH6. Wild-type adenovirus is lethal after i.v. injection of $10^{10}$ particles (Fig. 2C). This is caused by virus replication in the liver and fulminant hepatocyte necrosis (38). None of the mice treated with the Tcf viruses developed fatal liver necrosis after injection of 10 times the lethal dose of wild-type virus (Fig. 2C). Consistent with the increased liver infection seen by quantitation of viral DNA, there was an inflammatory infiltrate in the region around the central veins of the mice 3 days after receiving vKH6 (Fig. 2C, arrows). The inflammatory infiltrates resolved completely and were undetectable 20 days after the last virus injection (data not shown). The lack of overt signs of illness in the mice receiving the Tcf viruses indicates that the increased ability of the RGD viruses to infect the liver is more than offset by the reduction in toxicity caused by Tcf regulation of the early promoters.

To test whether the increased ability of vKH6 to infect SW620 cells in vitro leads to increased efficacy in vivo, xenograft growth was analyzed after i.v. injection of virus. Two regimens were compared: injection on a single day (total $10^{11}$ particles injected) and injection on 3 days at weekly intervals (total $3 \times 10^{11}$ particles injected). In both cases, the virus was fractionated on the day of injection to circumvent Kupffer cell clearance. Three courses of injection at weekly intervals (Fig. 3A, open symbols) were more effective than injection on a single day (closed symbols). The RGD-modified virus, vKH6 (triangles), was more effective than vKH1 (circles). Even in the best case, however, most of the tumors eventually relapsed. Quantitative PCR showed that there was a large amount of viral DNA present in the tumors at the time of relapse (data not shown). To understand why the virus had failed to control tumor growth, tumors were examined for viral DNA by FISH at different time points. Three days after infection, virus was found in isolated clusters of cells, corresponding to the cells infected with the injected virus and its progeny after one cycle of replication. At later
time points, the infected regions expanded to form confluent areas of infection in some parts of the tumor (Fig. 3B). Relapse can be explained by uncontrolled growth of tumor regions that are devoid of virus. This is consistent with theoretical models showing that tumor cell spread generally outpaces virus spread (22).

RAD001 is an mTOR inhibitor that inhibits tumor cell growth directly, blocks angiogenesis, and suppresses the immune response. To determine whether RAD001 interferes with translation of adenoviral proteins, a Western blot was done in the presence or absence of RAD001. There was no evidence of inhibition of viral protein expression (Fig. 4A). Quantitation of viral DNA 48 hours after infection showed only a small effect of RAD001 (in most cell lines, there was a slight increase in replication in the presence of the drug; Fig. 4B). In vitro efficacy was tested in cytopathic effect and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays (Fig. 4C; data not shown). Both showed either no effect or a 5- to 10-fold increase in efficacy in the presence of the drug. To test whether RAD001 inhibits replication in vivo, an SW620 xenograft was infected by the i.v. route as above, and the amount and distribution of viral DNA in the tumor were examined after prolonged treatment of the mice with RAD001. Quantitative PCR showed that a large amount of viral DNA was present in tumors even after treatment with RAD001 for 6 weeks (Fig. 5A). Widespread areas of the tumor were also positive by FISH for viral DNA after RAD001 treatment for 6 weeks (Fig. 5B). Some of the FISH signal comes from cell debris in areas of necrosis, but the strongest signals are from the nuclei of living cells that are undergoing productive viral infection. We conclude that RAD001 does not inhibit virus growth in tumor cells in vitro or in vivo.

Figure 3. Xenograft infection with virus alone. A, growth curves of s.c. SW620 xenografts in nude mice (five mice per group). Viral treatment started when tumors reached ~100 mm². Circles, vKH1. Triangles, vKH6. Closed symbols, 10¹¹ particles of virus i.v. on day 0. Open symbols, 10¹¹ particles of virus i.v. on days 0, 7, and 14. ×, control (buffer injection). B, FISH for viral DNA in tumors 9 days after i.v. injection of 10¹¹ particles of virus. The whole tumor is shown to demonstrate the patchy distribution of virus. Red, viral DNA (FISH); blue, cell nuclei (DAPI). Bar, 500 µm. The red fluorescence at the tumor margin (arrow) is caused by autofluorescence of erythrocytes not hybridization to viral DNA.

Figure 4. Effect of RAD001 on vKH6 virus infection in vitro. A, Western blot for E1A and DBP protein expression 24 and 48 hours after infection. B, viral DNA replication was analyzed by quantitative PCR for viral DNA 48 hours after infection. Cells were infected at a moi of 1. RAD001 was added 4 hours after infection. C, cytopathic effect assay in the presence (+) or absence (−) of RAD001. Cells were infected with 10-fold dilutions of virus starting at a moi of 1,000 and stained with crystal violet on day 6.

In addition to its effect on mTOR in the tumor, RAD001 is known to suppress the immune response by inhibiting mTOR in lymphocytes. The induction of a neutralizing antibody response is thought to limit the efficacy of oncolytic adenoviruses in vivo (39). To confirm that RAD001 can prevent induction of a primary antibody response against adenovirus, immunocompetent mice were treated with RAD001 and then immunized with adenovirus. As expected, RAD001 was able completely to block the primary antibody response to virus (Fig. 5C).
The efficacy of combination therapy in vivo was tested by giving i.v. virus followed by daily oral RAD001. The virus was given according to the same schedules as in Fig. 3A. RAD001 was withheld the day before and on the day of virus injection, on the grounds that antivascular effects of the drug might limit access of the virus to the tumor. Mice treated with placebo (gavage with the vehicle used for the RAD001) all had to be sacrificed because of uncontrolled tumor growth by day 20 (Fig. 6A). This reflects the rapid growth of SW620 xenografts once the tumors reach ~100 mm³. The start of the experiment was delayed until the tumors reached 80 to 150 mm³ to ensure that they had developed a vascular tree that could be called on to deliver the virus after i.v. injection. Consistent with the known low toxicity of RAD001, there was no weight loss in RAD001-treated mice after 20 days of treatment. RAD001 alone was more effective than virus alone in slowing growth of the tumors, but by 40 days most mice still had to be sacrificed because of progressive tumor growth (Fig. 6B). Tumors treated with the combination showed markedly better responses: Only one mouse in each vKH1 plus RAD001 group had to be sacrificed, and no mice in either vKH6 plus RAD001 group had to be sacrificed before the end of the experiment 40 days after the first virus injection (Fig. 6B). The difference between the survival of the control and virus + RAD001 groups was significant in every case (P < 0.01). The smallest difference in survival was between that of the RAD001 alone and virus + RAD001 groups: It achieved significance (P < 0.05) for the 1×vKH1, 1×vKH6, and 3×vKH6 groups but not the 3×vKH1 group. The failure of the 3×vKH1 + RAD001 versus RAD001 alone survival difference to reach significance is explained by the small number of animals. Analysis of the growth curves (Fig. 6A) showed that at the time the first mouse was sacrificed in the RAD001 alone group (day 28), there was a significant difference in tumor size in the 3×vKH1 + RAD001 versus RAD001 alone groups (P < 0.05). The mechanism for the interaction between oncolytic virus and RAD001 is unlikely to be related to the immunosuppressant effect of the drug because the mice were immunodeficient to allow xenografting. It could be partially explained by a direct inhibitory effect on the tumor cells, given that the drug slightly increased the cytopathic effect of the virus in vitro (Fig. 4E). Histologic examination of the tumors showed that RAD001-treated tumors had the typical appearance of poorly vascularized tumors, with a rim of viable cells and a large central necrotic core, in contrast with the untreated tumors, which contained uniform sheets of viable cells with only patchy necrosis (Fig. 5D). It is likely that this antivascular effect contributed importantly to the interaction between virus and drug, substantially delaying the growth of the tumor while still allowing the virus to spread within it.

Discussion
The major current limitation to the development of oncolytic viruses is the low efficacy of the viruses in vivo. This is the first report showing systemic efficacy of Tcf-regulated adenoviruses in mice. Virus plus RAD001 significantly slowed tumor growth and prolonged the survival of the treated animals. Combination therapy with RAD001 and RGD-modified viruses gave the best results. Although the ideal targeting ligand would lead to selective infection of cells in the tumor, no viruses selectively infecting tumor cells have yet shown high efficacy in vivo (7). The main concern is to preserve activity of the virus on tumor cells. The two approaches most widely used are to target heparan sulfate proteoglycans with poly-lysine or to target integrins with RGD.
Neither change impairs the ability of the virus to infect cells through CAR. A virus using all three routes of infection has been produced and shows promise in ovarian cancer (8). A potential drawback of this approach is that the viruses are not tumor specific, and side effects caused by infection of normal cells are potentially increased. Insertion of the RGD peptide into the Tcf-regulated viruses substantially enhanced its ability to infect tumor cells, and increased the amount of viral DNA that could be recovered from tumor and liver at 24 hours. The normal tissues where the Wnt pathway is likely to be active are stem cells in the skin, hematopoietic system and intestine, and neurons in the several regions of the brain, including the subventricular zone, cortex, and hippocampus (40–42). There was a small increase in the amount of viral DNA detectable in skin with vKH6, albeit to a very low level, and no change in the amount of viral DNA in brain or intestine. The liver was the major site of toxicity. The efficiency of infection depends on the relative expression of receptors on the cells and ligands on the viruses. It is likely that insertion of the RGD peptide will allow for more specific targeting of the virus to tumors.

![Figure 6. Effect of RAD001 on tumor response in vivo. A, growth curves of s.c. SW620 xenografts in NMRI nu/nu mice. ▲, vKH6 and daily RAD001; ●, vKH1 and daily RAD001; ■, daily RAD001 alone; ×, control. Virus injections are indicated by arrows. 1× virus injection: mice received $10^{11}$ particles of the virus i.v. on day 0; 3× virus injection: mice received $10^{11}$ particles of the virus i.v. on days 0, 7, and 14. The control group received placebo (RAD001 vehicle) by daily gavage. B, Kaplan-Meier curves showing the fraction of mice with SW620 xenografts <1,000 mm$^3$ [the corresponding growth curves are shown in Figs. 3A and (A)]. Each group contained five mice. Virus injections are indicated by arrows. V+R, virus plus RAD001; R, RAD001 alone; V, virus alone; C, control.](image-url)
peptide alters the distribution of virus within the liver to take advantage of integrin expression by hepatocytes (43). Liver infection resulted in the formation of inflammatory infiltrates at early time points. In immunocompetent mice, the acute response is normally followed by T-cell infiltration and acquired immunity. In the immunodeficient mice used here, the inflammatory infiltrates completely resolved by 20 days. Inflammatory infiltrates around the central veins in the liver were accompanied by viral replication in occasional hepatocytes, which could be detected by FISH. Compared with infection by wild-type virus, which produced fulminant liver necrosis after injection of $10^{10}$ particles, the RGD viruses were very well tolerated by the mice after injection of 10 times more virus. This must reflect the decrease in expression of viral proteins caused by Tcf regulation of the early promoters. We have not constructed Ad5 with wild-type promoters and RGD in the fiber, but one might reasonably expect such a virus to produce liver necrosis at lower doses than the wild-type virus. Compared to such a virus, our Tcf-regulated viruses would probably be >100-fold less toxic to the liver. The other major toxicity that has been reported for systemically administered adenoviral vectors in mice is acute hypotension (44). About one third of the mice in the vKH6 group showed signs consistent with hypotension after the first injection of virus, but no mice died as a result. There were no overt clinical changes after subsequent injections, consistent with reports that the initial hypotension is caused by activation of Kupffer cells, which are then eliminated (44).

Many classic chemotherapeutic drugs have been tested in combination with oncolytic viruses (13). With the exception of taxanes, which act on microtubules, most of these drugs are expected to inhibit viral DNA replication. RAD001 has several attractive properties for combination therapy with oncolytic viruses. It has no known inhibitory effect on viral DNA replication, it inhibits tumor cell growth directly, it blocks tumor angiogenesis, and it specifically inhibits the immune response. A major advantage of RAD001 is its low toxicity compared with standard chemotherapeutic agents. RAD001 inhibits translation of cellular mRNAs with highly structured 5'-untranslated regions by blocking translation of capped cellular mRNAs by competing for Mnk1 binding to eIF4G (46). The 100 K protein contains an RNA-binding domain that binds to the tripartite leader sequence in late mRNAs. This allows selective translation of late viral RNAs by ribosome shunting, a process that is independent of scanning by eIF4A (47). We speculate that this process is largely resistant to RAD001.

RAD001 is an orally active derivative of rapamycin, a well-characterized immunosuppressant that is used clinically in organ transplant recipients. Therapy with oncolytic adenoviruses induces a strong neutralizing antibody response in all patients (48). This is a particular concern for treatment schedules involving multiple cycles of virus injection. Most viruses already manipulate the immune system in some way, but total suppression of the immune response by a virally encoded gene would raise obvious biosafety concerns. Transient immune suppression by a drug avoids this problem. Several approaches have been tested to suppress the immune response, including the use of anti-CD40 antibodies (49). RAD001 is more suitable for combination with viral therapy than cyclosporin, because cyclosporin stimulates the formation of tumor blood vessels (21). We have shown that RAD001 can suppress the primary immune response to adenovirus in mice (Fig. 5C), but most patients already have anti-Ad5 antibodies following Ad5 infection in childhood. This means that what is required is the ability to suppress a secondary immune response, which is a much more difficult task. One way to investigate this problem would be to use immunocompetent mouse tumor models, some of which may be permissive for our Tcf-regulated viruses (50).

We have shown that combination therapy with oncolytic viruses and RAD001 is more effective than treatment with either agent alone. Mathematical modeling suggests that seeding of virus to multiple sites in the tumor is essential to achieve rapid spread of the virus throughout the tumor mass, which is a prerequisite for cure of the disease (22). The main requirement is to deliver large amounts of virus to as many regions of the tumor as possible at the start of treatment. Tumor blood vessels are disorganized and leaky, which should favor escape of the virus from the circulation, but this is counterbalanced by high interstitial fluid pressure within the tumor and a thickened basement membrane, which are known to reduce delivery of macromolecules to the tumor (51). Antiangiogenic therapy, for example, with antibodies against vascular endothelial growth factor (VEGF, bevacizumab) or VEGFR2 (CD101), transiently normalizes the vasculature and lowers interstitial fluid pressure (52, 53). This so-called “normalization window” is associated with pericyte recruitment and thinning of the basement membrane. Adenovirus is large (90 nm) compared with normal drugs, but smaller than the pores in tumor blood vessels (>200 nm; ref. 54). The ideal solution would be to normalize the vessels to thin the basement membrane and then transiently increase vessel permeability at the time of virus injection. TNFα is known to increase the permeability of tumor blood vessels, but in preliminary experiments we observed increased hepatotoxicity after low-dose TNFα therapy. Systemic therapy with VEGF is unattractive because it increases permeability indiscriminately (55). Rapamycin and RAD001 block VEGF induction (21). We tried to exploit this fact in our weekly dosing schedule by withdrawing RAD001 the day before giving the virus. This schedule might result in normalization of the vasculature during RAD001 treatment, followed by induction of vessel leakiness by a burst of VEGF secretion around the time of virus injection. Because the precise timing of the change in perfusion upon RAD001 treatment is unclear, it is possible that other schedules would give even better results. There are clearly many possible levels of interaction between antiangiogenic therapy and oncolytic viruses. The important conclusion from our study is that oncolytic viruses are particularly well suited to combination with antiangiogenic therapy.

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1 K. Homicsko and R. Iggo, unpublished data.

2 H.A. Lane, personal communication.
References

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