In Vivo Antitumor Activity of ONYX-015 Is Influenced by p53 Status and Is Augmented by Radiotherapy

Kenneth R. Rogulski, Svend O. Freytag, Kang Zhang, Jeff D. Gilbert, Dell L. Paielli, Jae Ho Kim, Carla C. Heise, and David H. Kirn


Abstract

The E1B-deleted, replication-competent ONYX-015 (dl1520) adenovirus was originally described as being able to selectively kill p53-deficient cells due to a requirement of p53 inactivation for efficient viral replication. This hypothesis has become controversial because subsequent in vitro studies have demonstrated that the host range specificity of ONYX-015 is independent of p53 gene status. Using a pair of isogenic cell lines that differ only in their p53 status, we demonstrate here that although ONYX-015 can replicate in both p53 wild-type and mutant cells in vitro, the virus demonstrates significantly greater antitumor activity against mutant p53 tumors in vivo. Moreover, ONYX-015 viral therapy can be combined with radiation to improve tumor control beyond that of either monotherapy. The results demonstrate that ONYX-015 can discern in vivo between tumors having a different p53 status and that it may be an effective neoadjuvant to radiation therapy.

Introduction

Successful anticancer strategies require a differential response between tumor and normal tissue (i.e., a therapeutic index). Replication-competent, E1B-attenuated adenoviruses represent a means of achieving a therapeutic index by selectively destroying tumor cells with minimal toxicity to normal cells (1–3). The prototype virus, ONYX-015 (dl1520), is deleted for the M, 55,000 E1B protein that binds to and inactivates cellular p53 in a complex with E4orf6 (1, 4). As a result, this virus was initially found to selectively replicate in and lyse tumor cells with p53 mutations (1). This finding was considered significant because p53 is the most frequently mutated gene in human cancer, and p53 mutation often correlates with resistance to conventional therapies (5, 6).

However, subsequent studies have called into question the host range specificity originally described for ONYX-015 (2, 7–12). Multiple groups have recently demonstrated in vitro that ONYX-015 can lyse tumor cells having a wt or mutant p53 status. Indeed, one study even argued that the cytolytic activity of ONYX-015 required normal p53 function (10). Because most of these studies used heterogeneous cell lines having diverse genetic backgrounds, it is possible that these conflicting observations were, in part, attributable to differences other than p53 status. In an attempt to clarify this issue, we reexamined the replication properties of ONYX-015 in vitro using a pair of isogenic cell lines that differ only in their p53 status. Moreover, for the first time, the antitumor activity of ONYX-015 against isogenic tumors with wt or mutant p53 status was examined in vivo. The merit of using ONYX-015 as a neoadjuvant to RT was also investigated.

Materials and Methods

Virus and Cell Lines. The M, 55,000 E1B gene-deleted, replication-competent ONYX-015 (dl1520) adenovirus has been described previously (1, 2). ONYX-015 was titered and propagated using HEK293 cells. The RKO human colon carcinoma (p53 wt) and RKO.p53.13 subclone expressing a dominant-negative form of p53 (Ref. 13; obtained from M. Kastan, Department of Hematology-Oncology, St. Jude Children’s Research Hospital, Memphis, TN) were maintained in MEM supplemented with 10% fetal bovine serum and nonessential amino acids (growth medium). To maintain selective pressure for the mutant p53 gene, RKO.p53.13 cell medium also contained 500 μg/ml G418.

Assays for ONYX-015 Viral DNA Replication. Cells (2 × 10⁶ cells/T-25 flask) were either mock-infected or infected with ONYX-015 at a MOI of 1 at 1 ml of MEM with 2% fetal bovine serum. After 1 h, 4 ml of growth medium were added. Cells were harvested 24–72 h postinfection for isolation of viral DNA. DNA from an equal number of cells was digested with HindIII and analyzed by Southern blotting as described previously (3). For radiation studies, mock- or ONYX-015-infected cells were exposed to a single dose of 137Cs γ-irradiation (2 or 20 Gy) 1 h postinfection. Cells were harvested 48 h postinfection for isolation of viral DNA.

In Vivo Studies. Female athymic mice [nu/nu (CD-1); Charles River Laboratories] were used in all studies. RKO and RKO.p53.13 tumors were established by injecting 2 × 10⁶ cells prepared in 0.9% NaCl and 25% Matrigel (Collaborative Biomedical Products) into the right gastrocnemius muscle (i.m.). On reaching 200–300 mm³, tumors were infected with 10⁶ pfu of ONYX-015 or PBS (50 μl) for 5 consecutive days (days 0–4). Animals in the radiation treatment groups were anesthetized by i.p. injection of 60 mg/kg Nembutal and received a single 20-Gy dose of 137Cs γ-irradiation to the tumor-bearing leg as described previously (4). Volumes of i.m. leg tumors were determined using the following formula (15): volume (cm³) = d³/2 × (0.6)d/2, where d is the average diameter of the tumor-bearing leg (cm), and the product (0.6)d/2 is the correction factor for normal leg volume. Animals were followed until death (euthanasia) from tumor burden or for at least 90 days after cessation of treatment. Federal and institutional guidelines for animal care were followed.

Results

Effect of p53 Status and Radiation on Replication of ONYX-015. To evaluate the effect of cellular p53 function and radiation on the replicative properties of ONYX-015, a pair of isogenic cell lines that differed only in their p53 status were used (13). Human RKO colorectal carcinoma cells, which are wt for p53, and the RKO.p53.13 subclone, which expresses an inactivating mutant of p53, were infected with ONYX-015, and the amount of viral DNA present in cells was determined at various times thereafter. Although the kinetics of viral replication were similar between the two cell lines (both reached a steady state by day 2), the final level of ONYX-015 viral DNA was ~3-fold less in wt p53 cells (Fig. 1A). These results were reproduced in three independent experiments (see Fig. 1B) and suggest that the...
therapy in vivo therapy could be successfully combined with RT. The lack of any significant radiation-mediated inhibition of viral DNA replication suggested that ONYX-015 could be resistant to ONYX-015, yielding an insignificant tumor growth delay relative to the controls (1 day; \( P = 0.63 \)), intratumoral injection of ONYX-015 resulted in significant growth inhibition of RKO.p53.13 tumors, producing a growth delay of 19 days (\( P < 0.001; \) Fig. 2A; Table 1).

**ONYX-015 Is an Effective Neoadjuvant to RT with p53 Mutant Tumors.** RKO and RKO.p53.13 tumors exhibited similar sensitivities to radiation, with 20 Gy producing growth delays of 16 and 22 days (\( P = 0.9 \)), respectively (Fig. 2B; Table 1). Consistent with the apparent resistance of RKO tumors to ONYX-015, combining ONYX-015 viral and radiation therapies produced a growth delay essentially identical to that obtained with radiation alone (Fig. 2C; Table 1; ONYX-015 + RT versus RT, \( P = 0.92 \)). In dramatic contrast, treatment of RKO.p53.13 tumors with ONYX-015 and radiation together produced an antitumor effect that was significantly greater than that achieved with either monotherapy (Fig. 2D; Table 1; ONYX-015 + RT versus RT, \( P = 0.001; \) ONYX-015 + RT versus ONYX-015, \( P = 0.0003 \)). At day 23, when RKO.p53.13 tumors treated independently with ONYX-015 and 20 Gy averaged 875 and 604 mm\(^3\), respectively, those within the combined treatment group averaged only 140 mm\(^3\). The combination of ONYX-015 viral and radiation therapies produced a tumor growth delay of 48 days (an approximately additive response) and a 33% cure rate, the latter of which was 3-fold greater than that achieved with ONYX-015 viral therapy. The results demonstrate that the replication-competent ONYX-015 virus is an effective neoadjuvant to RT.

**Discussion**

ONYX-015 viral therapy was initially regarded as a potentially significant advancement in cancer treatment because of its ability to destroy p53-mutated tumors, which comprise approximately 50% of all human cancers (5). By virtue of its inability to express the p53-inactivating E1B \( M_0 \), 55,000 protein, viral replication and accompanying cytolysis effects were hypothesized to be restricted to cells lacking functional p53 (1). Although subsequent studies have demonstrated clearly that ONYX-015 replication and cytolysis in vitro are not limited to cells with mutated p53, we demonstrate here that ONYX-015 antitumor activity is modulated by p53 in vivo. Our results agree well with the recent observations of Vollmer et al. (17), who found that ONYX-015 (dl1520) was efficacious against hepatocellular carcinoma with a null (Hep3B) but not wt (HepG2) p53 status. Thus, despite the confusion regarding the host range specificity of ONYX-015 (dl1520) in vitro, evidence supporting the selectivity and effectiveness of E1B-attenuated, replication-competent adenoviruses has been demonstrated independently in several preclinical tumor models.

Utilization of isogenic cell lines differing only in their p53 status, as opposed to using heterogeneous cell lines with widely varying genetic backgrounds, suggests that the observed differential effect is due to differences in p53 status. Our in vitro analyses demonstrate that ONYX-015 replicates to a greater level (by ~3-fold) in p53 mutant RKO.p53.13 cells compared with parental p53 wt RKO cells. Our observations agree well with the recent results of Harada and Berk (12), who found, using a cell line expressing a temperature-sensitive mutant of p53, that ONYX-015 (dl1520) viral DNA replication was reduced ~3-fold when p53 was functional. It is unlikely, however, that the modest differences in viral DNA replication observed in vitro.

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**Fig. 1.** A. temporal analysis of ONYX-015 viral replication. Cells were either mock-infected (\( M \)) or infected with ONYX-015 at a MOI of 10. Viral DNA was isolated every 24 h thereafter. DNA from an equal number of cells was digested with \( \text{HindIII} \) and analyzed by Southern blotting as described in A.

B. effect of radiation on ONYX-015 viral replication. RKO and RKO.p53.13 cells were either mock-infected (\( M \)) or infected with ONYX-015 at a MOI of 10. One h after infection, cells were either left untreated or exposed to a single dose of \( ^{137}\text{Cs} \) \( \gamma \)-radiation (2 or 20 Gy). Cells were harvested 48 h after infection for isolation of viral DNA. DNA from an equal number of cells was digested with \( \text{HindIII} \) and analyzed by Southern blotting as described in A.

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Presence of functional p53 restricts but does not abolish ONYX-015 viral replication.

As demonstrated previously, commonly used chemotherapeutics such as cisplatin and 5-FU augment the antitumor effects of ONYX-015 in vivo (2). In view of this, we wanted to determine whether another widely used anticancer therapy, ionizing radiation, could also enhance the therapeutic effects of the ONYX-015 virus. Because the cytotoxic effects of radiation result from DNA damage (16), we first examined the effect of ionizing radiation on viral DNA replication. RKO and RKO.p53.13 cells received a single dose of \( \gamma \)-radiation (2 or 20 Gy) shortly after infection with ONYX-015. Radiation produced no significant effect on ONYX-015 viral DNA replication (Fig. 1B). At a dose of 20 Gy, which is far greater than that typically used in the clinic (2 Gy), only a slight decrease in viral replication was evident in both cell lines (Fig. 1B). The lack of any significant radiation-mediated inhibition of viral DNA replication suggested that ONYX-015 therapy could be successfully combined with RT.

**ONYX-015 Demonstrates Greater Efficacy in Vivo against Tumors with Mutant p53.** To evaluate the efficacy of ONYX-015 viral therapy in vivo, i.m. leg tumors (200–300 mm\(^3\)) were injected with either PBS or ONYX-015 (\( 10^8 \) pfu) for 5 consecutive days. PBS-injected RKO and RKO.p53.13 tumors grew with nearly identical kinetics, attaining five times their initial volume in 9 days (Fig. 2A). ONYX-015 viral therapy, however, resulted in dramatically different responses for each RKO tumor. Whereas RKO tumors (wt \( p53 \)) proved to be resistant to ONYX-015, yielding an insignificant tumor growth delay relative to the controls (1 day; \( P = 0.63 \)), intratumoral injection of ONYX-015 resulted in significant growth inhibition of RKO.p53.13 tumors, producing a growth delay of 19 days (\( P < 0.001; \) Fig. 2A; Table 1).

**Table 1:** ONYX-015 antitumor activity is modulated by p53 genetic backgrounds, suggests that the observed differential effect is due to differences in p53 status. Our in vitro analyses demonstrate that ONYX-015 replicates to a greater level (by ~3-fold) in p53 mutant RKO.p53.13 cells compared with parental p53 wt RKO cells. Our observations agree well with the recent results of Harada and Berk (12), who found, using a cell line expressing a temperature-sensitive mutant of p53, that ONYX-015 (dl1520) viral DNA replication was reduced ~3-fold when p53 was functional. It is unlikely, however, that the modest differences in viral DNA replication observed in vitro...
between p53 wt and mutant cells can fully account for the significant differences in tumor growth control observed in vivo. Along these lines, Harada and Berk (12) also found that expression of functional p53 suppressed the yield of infectious ONYX-015 virus 12-fold (after correcting for the effect of temperature), which correlated with a severe block in late viral protein synthesis. Thus, it appears as though p53 can suppress the production of E1B-attenuated adenoviruses at multiple levels. Although not examined here, such dramatic differences in ONYX-015 virus production between p53 wt and mutant tumors in vivo, together with the p53-mediated suppression of viral DNA replication, may account for the significant differences in tumor growth control observed here in vivo. Future studies will compare the relative efficiency of each step of the viral replication process in vivo using isogenic tumor lines with different levels of p53 function.

Although ONYX-015 viral therapy has demonstrated efficacy in the clinic (18, 19), it is unlikely to have widespread applicability as a monotherapy because few human cancers are curable with a single modality. Indeed, repeated intratumoral injections of ONYX-015 in head and neck cancer patients have resulted in few, classically defined, objective responses, although tumor-specific viral replication
and tumor necrosis were observed.\textsuperscript{4} This has prompted investigations of combining ONYX-015 with chemotherapy (2, 17) or RT (this study). Preclinical studies have demonstrated that the antitumor activity of ONYX-015 can be augmented by cisplatin and 5-FU (2, 17). More importantly, clinical trials using a combination of ONYX-015 with cisplatin and 5-FU chemotherapy have demonstrated a dramatic increase in durable responses over that expected with either ONYX-015 or chemotherapy.\textsuperscript{5} We have extended these observations by demonstrating that ONYX-015 is also an effective neoadjuvant to RT. Unlike other strategies (3, 14, 20, 21) that generate synergistic effects (\textit{i.e.}, radiosensitization), the combined effects of ONYX-015 viral and radiation therapies appear to be additive in the RKO/RKO.p53.13 tumor model. Nevertheless, radiation can be successfully combined with cytolytic adenoviral therapy because, as demonstrated here, it does not curtail viral replication, and its damaging effects are primarily limited to the irradiated host cell. Because of its small target size, the adenoviral genome (36 kb) is far less likely to sustain radiation-induced damage because it is 10\textsuperscript{5}-fold smaller than that of a human cell (3 \times 10\textsuperscript{6} kb). Indeed, combining cytolytic adenoviral therapy with RT could prove valuable in the clinic because the two modalities appear to be complementary. As demonstrated here, radiation proved effective against RKO tumors that were apparently resistant to ONYX-015 therapy. Thus, radiation may complement the clinical utility of E1B-attenuated, cytolytic adenoviruses by effectively targeting tumor cell populations that are, for one reason or another, resistant to these viruses. This may be important because many human tumors are comprised of a mixture of cells having varying genetic makeup, and intratumoral heterogeneity may be a major reason why most monotherapies fail to achieve a cure. Thus, E1B-attenuated, replication-competent adenoviruses may augment the efficacy of standard cancer modalities, and such novel therapeutic combinations may prove valuable in the clinic.

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\textbf{References}


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