Selective Replication and Oncolysis in p53 Mutant Tumors with ONYX-015, an E1B-55kD Gene-deleted Adenovirus, in Patients with Advanced Head and Neck Cancer: A Phase II Trial

John Nemunaitis, Ian Ganly, Fadlo Khuri, James Arseneau, Joseph Kuhn, Todd McCarty, Stephen Landers, Phillip Maples, Larry Romel, Britta Randlev, Tony Reid, Sam Kaye, and David Kirn

ABSTRACT

ONYX-015 is an E1B-55kDa gene-deleted adenovirus engineered to selectively replicate in and lyse p53-deficient cancer cells. To evaluate the selectivity of ONYX-015 replication and cytopathic effects for the first time in humans, we carried out a Phase II clinical testing of intratumoral and peritumoral ONYX-015 injection in 37 patients with recurrent head and neck carcinoma. Patients received ONYX-015 at a daily dose of $1 \times 10^{10}$ plaque-forming units (pfu) via intratumoral injection for 5 days during week 1 of each 3-week cycle ($n = 30$; cohort A), or $1 \times 10^{10}$ pfu twice a day for 10 days during weeks 1 and 2 of each 3-week cycle.

INTRODUCTION

The p53 tumor suppressor gene is mutated in roughly 50% of all human cancers (1), including non-small cell lung (60%), colon (50%), breast (40%), head and neck (60%), and ovarian (60%) cancers in the advanced stages (2–6). Loss of p53 function is associated with resistance to chemotherapy and/or decreased survival in numerous tumor types, including breast (7), colon (8), bladder, ovarian (9), and non-small cell lung cancers (10). Therefore, effective therapies for tumors that lack functional p53 are clearly needed.

Many of the same critical regulatory proteins that are inactivated during carcinogenesis are also inactivated by adenoviral gene products during replication (10–13). Because of this convergence, the deletion of viral genes that inactivate these cellular regulatory proteins may also reduce the ability of p53 to block ONYX-015 replication in tumors that lack functional p53.

The p53 gene product can cause p53 induction through p14arf, the loss of p14arf and p14arf during immortalization. Because the adenoviral E1A gene product can cause p53 induction through p14arf, the loss of p14arf may also reduce the ability of p53 to block ONYX-015 replication in some p53 wild-type tumor cells.

One approach to studying the mechanism of ONYX-015 selectivity was to compare its behavior in cell lines that are identical except for p53 function. Four matched pairs of cell lines have confirmed that ONYX-015 replication and/or CPEs are significantly inhibited by functional p53: RKO (21), H1299 (30), A2780, and U343 (5). In contrast to these four cell lines, however, a fifth (U2OS) cell line did not become sensitive to ONYX-015 after transfection with dominant-negative p53 (31). Although it is possible that as-yet-undetermined complementing mutations within tumor cell lines may account for these differences in ONYX-015 effects, most data confirm the selective replication capacity of ONYX-015. Some of the controversy regarding ONYX-015 selectivity to p53 mutant targets may be related to the multiplicity of infection used, the study end points, and the time elapsed from infection to assessment of CPEs (32).

Overall, selectivity of ONYX-015 replication appears to be most consistent at low multiplicities of infection ($\leq 1.0$ pfu) after prolonged observation of CPE assays.

We carried out initial clinical testing of ONYX-015 in patients with recurrent squamous cell carcinoma of the head and neck. Abnormal-
ities in p53 function are common as a result of gene mutation or protein degradation attributable to human papillomavirus E6 protein expression (33, 34). Phase I investigation indicated good tolerability of ONYX-015 administered as a single intratumoral injection (doses up to $10^{11}$ pfu), and after a series of five daily consecutive intratumoral injections (doses up to $10^{10}$ pfu per injection (35). Injections were done and the tumors were biopsied in an outpatient setting, which allowed clinical and histological assessment over time after tumor inoculation. Local-regional tumor progression was the cause of morbidity and death in the majority of cases. We, therefore, could obtain valuable biological data on viral presence, necrosis, and inflammation while attempting to benefit patients through local tumor control in Phase II testing.

MATERIALS AND METHODS

Enrollment Criteria. All of the head and neck cancer patients participating in Phase II studies of intratumoral injection with ONYX-015 as a single agent were analyzed. Patients had histologically confirmed squamous cell carcinoma of the head and neck (excluding nasopharyngeal) that had recurred after surgery and/or radiotherapy for the primary tumor and had progressed at or within 8 weeks after completion of chemotherapy and/or radiotherapy (i.e., tumors were refractory). Tumors could not be Surgically curable. The tumor mass to be treated with ONYX-015 had to be adequately injectable (as defined below) and measurable (radiographically or by physical examination). Patients had to be ≥18 years old, had to have a Karnofsky performance status of ≥70%, and life expectancy of ≥3 months. Normal hematological and renal function were also required. This investigation was performed after approval by the local Institutional Review Board at US Oncology (Dallas, TX). An informed consent was obtained from each patient or from the patient’s legal guardian prior to enrollment. The p53 gene status was not used as an enrollment criteria. Institutional Review Board approval of the protocol and consent form was required.

Baseline Assessment. Baseline assessments were made prior to treatment, but these results were not used as enrollment criteria. A biopsy sample was obtained for p53 gene sequencing from the tumor to be injected (see methods below). Baseline blood tests were performed as follows: complete blood counts; CD3, CD4, and CD8 lymphocyte counts; electrolytes; blood urea nitrogen; creatinine; and liver function tests. In addition, baseline neutralizing antibody titers to ONYX-015 were determined (most adults have neutralizing antibodies to the adenovirus type 5 coat proteins that are present on ONYX-015). In addition, delayed-type hypersensitivity skin testing (Mericouex) and plain chest radiographs were performed.

ONYX-015. ONYX-015 (dl1520) is a chimeric human group C adenovirus (Ad2 and Ad5) that does not express the product of the E1B-55kDa gene; the virus was constructed in the laboratory of Arnold Berk (Barker and Berk, Ref. 11). The virus contains a deletion between nucleotides 2496 and 3323 in the E1B-55kDa region encoding the protein. In addition, a C to T transition at position 2202 in E1B generates a stop codon at the third codon position of the protein. These alterations eliminate expression of the E1B-55kDa gene in ONYX-015 infected cells. ONYX-015 was grown and titrated on the human embryonic kidney cell line HEK293 as described previously (14).

ONYX-015 Handling and Processing. ONYX-015 is formulated as a sterile viral solution in TRIS buffer [10 mM TRIS (pH 7.4), 1 mM MgCl2, 150 mM CaCl, and 10% glycerol]. The solution is supplied frozen (−20°C) in single-use, plastic screw-cap vials. Each vial contains 0.5 ml of virus solution at a specified viral titer. Vialased virus solution was thawed and diluted to the appropriate titer for dosing, and was then further diluted to a final volume equivalent to 30% of the volume of the tumor to be injected. Tumor volume was estimated by taking the product of the maximal tumor diameter, its perpendicular and estimated depth, and dividing by two. Vials of ONYX-015 were opened and diluted immediately prior to injection in biological safety cabinets at the patient treatment area. All of the waste items were disposed of in hazardous containers and autoclaved or incinerated.

Treatment Regimen. To ensure uniform dosing to the injected tumor in each patient, a single tumor was identified for ONYX-015 injection in each patient. If more than one injectable tumor was present, the most symptomatic and/or largest tumor mass was injected with ONYX-015. The tumor was mapped into five equally spaced and equally sized sections. Local anesthesia was applied to the skin as needed. The tumor was injected with $10^{10}$ pfu following the template displayed in Fig. 1. The suspension volume of saline used for ONYX-015 administration was normalized to 30% of the estimated volume of the tumor mass to be injected (see above). During each treatment session, one puncture of the skin was made at a site approximately 80% of the distance from the tumor center out to the tumor periphery. Six to eight needle tracts were made radially out from the puncture site; virus was administered equally along the length of the needle tracks (25-gauge needle). This approach was carried out each day from puncture sites that were equiradially spaced out and that encompassed the entire tumor mass. The majority of the viral dose was administered at the tumor periphery and at the interface between normal tissue and tumor tissue. This administration approach was used for two reasons. First, prior studies have suggested improved efficacy with this administration approach. Second, this technique allowed for assessment of the effects of ONYX-015 injection on both normal tissues and tumor tissues in the same patients.

In the initial phase of the study, tumor injections were performed once daily for 5 consecutive days; these injections were repeated every 3 weeks or until tumor progression. After documentation of safety with this regimen, a more aggressive injection regimen was pilot-tested in subsequent patients; identical injections were performed twice daily for 5 days during each of the first 2 weeks on study. After a 1-week rest period, this regimen was repeated. Following this induction regimen, maintenance treatment cycles were given by the same schedule as was used in the initial patient cohort (every 3 weeks as described above). No significant complications related to injection were observed (36). The injections were given in the outpatient clinics including Beatson Cancer Institute (Glasgow, Scotland), M. D. Anderson Cancer Center (Houston, Texas), Mary Crowley Medical Research Center at Baylor University Medical center (Dallas, Texas), and Albany Regional Cancer Center (Albany, New York), which are investigative sites within US Oncology (Houston, Texas). Patients’ vital signs were taken 15 min prior to and after each treatment for a minimum of 30 min. Patients were eligible for repeat treatment cycles at the same dosage every 3 weeks if no grade 4 toxicity (National Cancer Institute Common Toxicity Criteria) occurred with the prior treatment cycle of ONYX-015 and no progression of the injected tumor was observed.

Tumor Assessments. Tumor masses were measured serially by either physical examination or radiographic scanning (computed tomography or magnetic resonance imaging), whichever the principal investigator deemed most accurate for the measurement of the injected tumor mass. In general, very superficial lesions were measured by physical examination, and deeper tumors were measured most accurately by radiographic scanning. Tumor measurements were determined either every 3 weeks (physical examination) or every 6 weeks (computed tomography/magnetic resonance imaging scans) while patients were on active study treatment. After treatment completion, patient’s tumor(s) were assessed every 8 weeks or sooner if signs/symptoms of progression became evident. Radiographic scans were assessed by independent radiologists who were not investigators on the study.
The degree of response within injected tumors was categorized as follows: complete regression, complete disappearance of measurable tumor; partial regression, ≥50% but <100% decrease in cross-sectional tumor area; minor response, <50% but ≥25% decrease in tumor area; stable disease, <25% decrease and [int/25%] increase in tumor area; progressive disease, ≥25% increase in tumor area versus the baseline area. The time-to-injected tumor progression was defined as the time from treatment initiation to an increase of ≥25% in the nonnecrotic cross-sectional tumor area. To adequately assess the correlation between the effects of ONYX-015 injection within the injected tumor and predictive factors (e.g., p53 status), patients who received less than two cycles of treatment because of either development of comorbid medical conditions (n = 6) or progression at noninjected sites (n = 7) were not evaluable for this analysis. Investigators and radiologists were blinded to the final p53 gene status and neutralizing antibody titer of the patients at the time of tumor assessment.

Additional Follow-Up after Treatment Initiation. Neutralizing antibody titers were repeated every 4 weeks. Injection site biopsies between days 5 and 22 of the first treatment cycle were optional, based on patient consent because of ethical considerations. These biopsies were analyzed for EIA protein expression (the earliest gene product expressed) and viral replication by in situ hybridization. Routine blood testing (complete blood count, electrolytes, blood urea nitrogen, creatinine, and liver function tests) was repeated every 3 weeks.

p53 Gene Sequencing. Pretreatment tumor biopsies were taken for p53 sequencing from the recurrent tumor mass that was to be injected. Exons 5–9 were sequenced completely during the first two-thirds of the trial. Mutations were considered functionally significant if present in the Soussi database. Exons 2–11 were assessed by p53 gene chip technology during the final one-third of the trial. Because certain gene deletions can be missed by gene chip analysis (i.e., a wild-type sequence is reported despite a functionally significant mutation), wild-type p53 gene sequences by gene chip analysis required confirmatory sequencing to be validated.

In Situ Hybridization for Adenoviral DNA. In situ hybridization for adenoviral DNA was carried out on biopsy samples to determine the extent of replication of ONYX-015 in both tumor and adjacent normal tissues as described previously (14). Briefly, in situ hybridization was performed on formalin-fixed, paraffin-embedded tissue, cut into 5-μm sections. Slides were deparaffinized in xylene, hydrated through ethanolis, digested with proteinase K, and postfixed in 4% paraformaldehyde. Hybridization was carried out overnight at 37°C with 0.5 μg/ml biotinylated adenovirus DNA probe (Enzo Diagnostics, Inc., Farmingdale, NY). After three successive washes in 1× SSC at 55°C, an alkaline phosphatase conjugated-antibiotin antibody (Vector Laboratories) was applied. NBT/BCIP was used as the chromagen, and slides were counterstained with nuclear Fast Red.

EIA Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and hydrated. Slides were subjected to antigen retrieval at 120°C for 10 min in citrate buffer and incubated with an adenovirus-type-2 E1A antibody (Clone M73; Calbiochem) for 90 min at room temperature. This was followed by incubation with a biotinylated goat antimouse secondary antibody, and streptavidin/horseradish peroxidase conjugate.

Determination of Neutralizing Antibody Titer. Patient and control samples were incubated at 55°C for 30 min to inactivate complement. Clinical plasma samples previously determined to produce high, midrange, and negative titers were designated as plasma controls. Each dilution was mixed with plasma samples previously determined to produce high, midrange, and negative titers were designated as plasma controls. On day 7 postinoculation by counting the number of plaque-forming units per well. The titer of neutralizing antibody for each sample was reported as the dilution of plasma that reduced the number of plaque-forming units per well. The titer of neutralizing antibody for plasma-virus mix was removed and 2 ml of 1.5% Agarose in DMEM were added. The patient's samples and controls were incubated at 55°C for 30 min to inactivate complement. Clinical plasma samples previously determined to produce high, midrange, and negative titers were designated as plasma controls. Each dilution was mixed with plasma samples previously determined to produce high, midrange, and negative titers were designated as plasma controls.

RESULTS

Patient Characteristics. A total of 37 patients were enrolled into one of two protocols to receive either 5 consecutive injections of ONYX-015 over 5 days or 10 consecutive twice-a-day injections of ONYX-015 over 5 days for 2 weeks. To adequately assess the correlation between the effects of ONYX-015 injection within the injected tumor and predictive factors (e.g., p53 status), patients who received <2 cycles of treatment because of either development of comorbid medical conditions (n = 6) or progression at noninjected sites (n = 7) were not evaluable for this analysis. This report focuses on the 24 patients evaluable for response. Baseline patient characteristics were similar for this end-stage patient population (Table 1). Most patients were male (71%). The median age was 58.5 years, and all of the patients had a Karnofsky performance status of ≥70.

Patients were heavily pretreated in most cases; 88% of patients had received two or more previous therapeutic modalities, and 54% had received three previous modalities. The most common site of the patients' injected recurrent tumor was the cervical area.Injected tumors had a median diameter of 3.38 cm (range, 1–7 cm) and a median cross-sectional area of 11 cm² (range, 1.1–39 cm²). Only one patient had a distant metastasis present outside of the head and neck region. Patients were relatively immunosuppressed. Delayed-type hypersensitivity skin-testing reactivity to common antigens was below the normal range in 70% of patients, and the median CD4 cell count was 339 (range, 126–1318).

Treatme...
Fig. 2. Evidence of selective intratumoral replication of ONYX-015. In situ hybridization for adenoviral DNA on a tumor biopsy specimen obtained on day 8 after treatment initiation with ONYX-015 for 5 consecutive days. The specimen stained with H&E demonstrates viral-induced CPEs (arrow, A) and neutrophil infiltration (arrow, B) within tumor tissue only. In situ hybridization for adenoviral DNA demonstrates replication of ONYX-015 within nests of tumor cells (short wide arrow(s), C and D) but not within normal tissues (thin arrow(s), C and D). Immunohistochemical staining for E1A protein was also demonstrated (E and F). Electron microscopy represented in previous publications (46) confirms the presence of intranuclear replicating viral particles (G) and pseudo-crystalline arrays (H) within tumor cells.
in situ hybridization for adenoviral DNA (specific nuclear staining was required) and by assessment of CPEs on H&E-stained slides (Fig. 2, A and B).

Biopsies were positive for specific adenoviral DNA staining within the tumor as follows: 5 of 7 on days 1–3 after the last injection; 2 of 4 on days 7–10; and 0 of 10 on days 14–17. The intensity and distribution of staining varied between samples, with up to 25% of tumor cells showing evidence of viral presence in samples staining positive (Fig. 2, C and D). In contrast, normal skin and mesenchymal tissue within the biopsies were uniformly negative (n = 21) by in situ hybridization (Fig. 2, C and D). Comparison of ONYX-015 presence in tumor tissue before day 14 (7 of 11 positive) versus normal tissue (0 of 11 positive) was a significant difference (P = 0.01). Immunohistochemical staining for adenoviral E1A protein confirmed expression within in situ hybridization-positive tumor cells (Fig. 2, E and F). Viral presence was further confirmed by electron microscopy (Fig. 2, G and H). Areas of viral presence within the tumors were associated with areas of CPEs and necrosis as seen by neutrophil infiltrate (Fig. 2, A and B). CPEs were not seen within normal tissues. Thus, the high concentration of ONYX-015 in malignant tissue ≥24 h after the last injection is likely related to selective viral replication.

Abnormalities in p53 were detected in all of the tumors demonstrating viral presence. On days 1–3 after the last injection, four of five p53 mutant biopsies showed viral presence. Two tumors without p53 mutation were biopsied; one was negative for viral presence, whereas the other showed very focal viral presence within an otherwise negative tumor specimen. p53 immunohistochemical staining of this tumor sample documented focal elevated expression within a small nest of tumor cells (<5% of the total), consistent with a p53 abnormality; therefore, focal replication or infection may have occurred within a small focus of cells with abnormal p53. p53 mutations may not be detected by DNA sequencing if present in less than 25% of the cells in the biopsy sample.6

**Tumor-specific Response.** ONYX-015 injection induced a 25–100% response of the injected tumor mass in 8 (33%) of 24 cases (Table 2): two complete (8.3%), three partial (12.5%), and three minor (12.5%) regressions were observed. Normal peritumoral tissue did not appear affected by physical exam in any case, despite direct injection with ONYX-015 (Fig. 3). The intent-to-treat objective response rate (≥50%) for all of the 37 patients receiving any treatment was 14%.7

Of the eight tumor regressions observed, two partial and one minor regression were confirmed approximately 4 weeks later. Confirmation of the other five regressions at 4 weeks after initial response characterization was not possible because of injected-tumor progression (n = 2), death from unrelated causes (n = 1), patient decision to withdraw from the study (n = 1), or distant noninjected tumor progression (n = 1) just prior to the 4-week confirmatory evaluation.

**Correlation of Tumor Response with p53 Gene Status.** A significant correlation was demonstrated between the induction of tumor response after necrosis and the p53 gene status of the tumor (Table 2). Seven (58%) of 12 p53 mutant tumors underwent significant necrosis and achieved significant response, whereas none of the 7 p53 wild-type tumors achieved a response (P = 0.017). An evaluable p53 gene sequence could not be obtained from five tumors. Neither the baseline neutralizing antibody status (positive or negative), nor exposure to prior radiotherapy, nor the baseline tumor size (maximal diameter < or ≥3 cm.), nor Karnofsky performance status (≥90 versus <90) correlated significantly with response (Table 2).

**Time-to-Tumor Progression.** Tumor progression was rapid in most cases. The median time to progression at the injected tumor site was 51 days (range, 21–114 days). Six (25%) of 24 patients were without progression of the injected tumor after 3 months on study (Fig. 4). The time to progression of injected p53 mutant tumors (median, 56 days) was delayed compared with p53 wild-type tumors, although not significantly (median, 21 days; Fig. 4; P = 0.28).

**Humoral Immune Response.** Approximately 60% of patients had positive neutralizing antibody titers at baseline. Within 3 weeks of treatment initiation, all of the patients had positive neutralizing antibody titers and significant antibody titer increases (Table 3). There was no correlation between baseline neutralizing antibody titers and induction of tumor response (Table 2).

**DISCUSSION**

p53 mutation is the most common genetic abnormality identified in human cancer (37), and it is frequently associated with both a de-

---

6 Unpublished observations.

creased responsiveness to standard chemotherapeutic agents and a poor prognosis (6, 38, 39). Novel therapies that can target these resistant tumors are needed. We report that ONYX-015 can cause CPEs in recurrent and refractory p53 mutant squamous cell tumors of the head and neck. ONYX-015 presence was not detected in peritumoral normal tissue, despite direct injection. In addition, ONYX-015 induced p53 mutant tumor-specific response in association with necrosis. CPEs were not documented in normal tissue nor in tumors containing wild-type p53 genotype. ONYX-015 seems to be the first therapeutic agent specifically designed to target p53-deficient tumor cells that has demonstrated selectivity in patients. Future clinical trials will demonstrate whether this selectivity for p53 mutant tumor generalizes to other cancer patient populations. ONYX-015 is also the first genetically engineered replication-competent virus to demonstrate selective intratumoral activity in patients.

The role of the immune response to replicating viral agents is...
clear. This will best be answered in clinical trials because of the lack of an immunocompetent animal model that supports efficient adenoviral replication (40, 41). Neutralizing antibody titers either before or after treatment were not predictive for antitumor activity. Although encouraging, this finding does not rule out an inhibitory role for neutralizing antibodies during a longer-term treatment or after other routes of administration (e.g., i.v.). Future studies will be needed to better define the role of antibodies and whether their suppression would be beneficial. The role of cell-mediated immunity in either increasing or decreasing the antitumor activity in these patients is still unclear. These end-stage head and neck cancer patients were relatively immunosuppressed. CD4 cell counts were less than 500 (per μl) in 65% of the patients and less than 200 in 25%. Delayed-type hypersensitivity skin reactivity to common antigens was low in 70% of patients. Therefore, cell-mediated immunity may play less of a role than in more immunocompetent patient populations. The time course and magnitude of immune cell infiltration into tumors after injection will best be determined by histological assessment of the entire tumor mass (e.g., after surgical resection) at varied time points after treatment. On the basis of these results, immunomodulatory strategies might be developed. Finally, antiviral cytokines may also affect adenoviral replication and/or spread (42, 43).

Despite the encouraging biological activity demonstrated with ONYX-015 in this clinical trial, clinical benefit was not seen in the majority of patients. Tumor progression was rapid in the vast majority of patients, even for tumors that underwent substantial necrosis after treatment. These patients were heavily pretreated and were end-stage in most cases; the life-expectancy is 3–4 months in this patient population (44, 45). Additionally, because patients who progressed within two cycles at noninjected sites were excluded, these results cannot entirely rule out the possibility of a more beneficial response in patients with multiple slower-growing tumors. The true clinical benefit of intratumoral injection with ONYX-015 as a monotherapy will, therefore, need to be determined in randomized trials and, possibly, in earlier stage patients.

Future approaches also include the addition of therapeutic genes with antitumor effects to ONYX-015 (i.e., using ONYX-015 as a delivery vehicle), so-called “armed therapeutic viruses.” ONYX-015 has several favorable characteristics as a vector for gene delivery: inherent antitumoral activity and selectivity; potential amplification of the transgene leading to high level expression; and enhanced intratumoral spread versus nonreplicating vectors. If these approaches are successful, viral therapy with genetically engineered viruses may become a novel therapeutic platform for the treatment of cancer.

ACKNOWLEDGMENTS

We thank the following individuals for their important contributions: Dianne Davies, Sherry Toney, Deborah Hahn, Olga Diri, Ana Petrovich, Patrick Town, Amy Waterhouse, Brian Breitbard, Pia Roo, Kimberly Sultan, and Fran Kahane.

REFERENCES


Selective Replication and Oncolyis in p53 Mutant Tumors with ONYX-015, an E1B-55kD Gene-deleted Adenovirus, in Patients with Advanced Head and Neck Cancer: A Phase II Trial


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/60/22/6359

Cited articles
This article cites 43 articles, 21 of which you can access for free at:
http://cancerres.aacrjournals.org/content/60/22/6359.full#ref-list-1

Citing articles
This article has been cited by 49 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/60/22/6359.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.