A Phase I Study of Onyx-015, an E1B Attenuated Adenovirus, Administered Intratumorally to Patients with Recurrent Head and Neck Cancer

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ABSTRACT

An E1B 55 kDa gene-deleted adenovirus, Onyx-015, which reportedly selectively replicates in and lyses p53-deficient cells, was administered by a single intratumoral injection to a total of 22 patients with recurrent head and neck cancer. The objectives of this Phase I study were to determine the safety, feasibility, and efficacy of this therapy and determine any correlation to p53 status. Six cohorts were investigated with a dose escalation from $10^7$–$10^{11}$ plaque-forming units. Toxicity was assessed using NCIC criteria. Tumor response was assessed by clinical and radiological measurement. Blood samples were taken to detect adenovirus DNA and neutralizing antibody to adenovirus. Tumor biopsies were taken to detect adenovirus by in situ hybridization. Treatment was well tolerated, with the main toxicity being grade 1/2 flu-like symptoms. Dose-limiting toxicity was not reached at the highest dose of $10^{11}$ plaque-forming units. Twenty-one of the 22 patients treated showed an increase in neutralizing antibody to adenovirus. In situ hybridization showed viral replication in 4 of 22 patients treated, all of whom had mutant p53 tumors. Using conventional response criteria, no objective responses were observed. However, magnetic resonance imaging scans were suggestive of tumor necrosis at the site of viral injection in five patients, three of whom were classified using nonconventional criteria as partial responders, and two of whom were classified using nonconventional criteria as minor responders. Of these five cases, four had mutant p53 tumors. The response duration for the three partial responders was 4, 8, and 12 weeks. An additional eight patients had stable disease in the injected tumors lasting from 4–8 weeks. These preliminary results show that intratumoral administration of Onyx-015 is feasible, well tolerated, and associated with biological activity. Further investigation of Onyx-015, particularly with a more frequent injection protocol and in combination with systemic chemotherapy, is warranted.

INTRODUCTION

Locoregional recurrence is the most common cause of failure after head and neck cancer surgery (1). It is a disease that causes significant morbidity, especially on speech and swallowing. There are many different treatments available including surgery (2), reirradiation (3), and chemotherapy (4–7). However, prognosis for these patients remains poor, and the average survival is between 3 and 6 months. Because of this, there has been considerable interest in the development of new biological therapies such as gene therapy and immunotherapy for this disease. These new therapies often use human nonreplicating adenoviruses as delivery vehicles for genes to treat cancer (8, 9).

An alternative approach to cancer therapy involves the use of replication-competent adenoviruses that can selectively replicate in and cause lysis of cells that lack wild-type p53 function (10). The E1B 55 kDa gene-deleted adenovirus Onyx-015 has an 800-bp deletion in the E1B region encoding the 55 kDa protein of E1B. The normal function of this protein is to bind to and inactivate p53 protein in infected cells. Because Onyx-015 lacks this protein, it is unable to replicate efficiently in cells with functional p53 because it cannot inactivate p53. However, it can replicate effectively in cells lacking functional p53, causing cytopathic effects. This approach has potential advantages over the traditional therapies of chemotheraphy and radiotherapy because of the possibility of targeting tumors at a molecular level while leaving normal tissue relatively unaffected.

p53 is the most commonly mutated tumor suppressor gene in human cancer, and it plays an important role in the regulation of the cell cycle (11). Its normal function is to recognize and respond to DNA damage induced by radiation and other cytotoxic agents, causing either cell cycle arrest or apoptosis (12). p53 mutations are present in over 50% of human tumors (13). In addition, tumors with a normal p53 gene sequence can have p53 inactivation through other mechanisms, e.g., mdm2 overexpression. Therefore, a therapy that targets cancers that lack functional p53 would be attractive because it would be applicable to a wide range of different cancers.

The reported incidence of p53 mutation in primary
HNSCC\textsuperscript{2} varies from 25–77\% (14, 15). In recurrent HNSCC, the incidence may be even higher; therefore, this tumor type may be a suitable target for E1B 55 kDa gene-deleted adenoviral therapy. Direct injection of a virus that targets these tumors should be a safe and feasible method of treating patients with recurrent tumors. Therefore, a Phase I dose escalation trial using Onyx-015 was carried out on patients with recurrent squamous cell cancer of the head and neck. The primary objectives of the study were to determine the feasibility, safety, and efficacy of this therapy. However, we also wanted to determine whether there was any correlation of tumor p53 status to viral replication and tumor response.

**PATIENTS AND METHODS**

**Enrollment Criteria**

A total of 22 patients with recurrent head and neck cancer were entered into the trial from two centers in Glasgow, Scotland and San Antonio, Texas. Eligibility requirements included histologically confirmed squamous cell carcinoma of the head and neck that was recurrent and refractory to radiotherapy and/or chemotherapy. The tumor had to be amenable to direct injection and measurement, either clinically or radiographically. The tumors had abnormal p53 by immunohistochemistry. All patients had a Karnofsky performance status of \(\geq60\%\) and a life expectancy of 3 months and were over 18 years of age. All patients had adequate hematological, renal, and hepatic function. The maximum allowed creatinine was 1.5 mg/dl, the maximum allowed level of aspartate transaminase and alanine transaminase was 2.5-fold the upper limit of normal, the minimum allowed hemoglobin was 9 g/dl, the minimum allowed WBC count was 100,000/\(\mu\)l, and the minimum platelet count was 100,000/\(\mu\)l. Patients had not received any chemotherapy or radiotherapy within 4 weeks of study entry. All patients gave written informed consent. The protocol was approved by the United States Food and Drug Administration, the United Kingdom Gene Therapy Advisory Committee, and the local institutional review board ethics committees.

**Onyx-015**

Onyx-015 is a chimeric human group C adenovirus (Ad2 and Ad5) that has a deletion between nucleotides 2496 and 3323 in the E1B region encoding the 55 kDa protein. In addition, there is a C to T transition at position 2022 in E1B that generates a stop codon at the third codon position of the protein. These alterations eliminate the expression of the 55 kDa protein in Onyx-015-infected cells (16). Sterile purified lots of virus were produced for human clinical use by Magenta Corp. (Rockville, MD) and tested for titer, sterility, and general safety by Microbiological Associates using United States Food and Drug Administration-approved test methods.

**Treatment Protocol**

Pretreatment evaluation included complete blood cell count with differential, coagulation screen, routine biochemistry profile, urinalysis, chest X-ray, and electrocardiogram. All patients had a core biopsy of the recurrent tumor for p53 evaluation by immunohistochemistry and gene sequencing. CD4 lymphocyte counts were carried out to determine the immune status of the patients. The size of the recurrent tumors was measured clinically and also by ultrasound, computed tomography scan, or MRI scan.

The volume of the injected tumor was determined by either clinical measurement or radiological measurement, depending on the site of the tumor. Vials of Onyx-015 were then thawed and diluted with diluent (electrolyte 48 solution) to a volume equivalent to 30\% of the estimated tumor volume. The tumor to be injected was mapped into 1-cm\(^2\) areas, and then equal volumes of solution were injected into each area. A s.c. injection of local anesthetic was used in some patients to prevent pain on injection. Patients then had vital signs recorded every 20 min for 2 h, every 2 h for the next 22 h, and then every 6 h for the next 24 h. Patients were then discharged home if their vital signs were normal.

Follow-up was twice weekly. Blood counts and biochemistry were carried out weekly. Blood samples were taken weekly for PCR for adenoviral DNA and to determine neutralizing antibody titers to adenovirus. Swabs of the injection site and oropharynx taken pretreatment and 8 days posttreatment were assessed for adenovirus by a direct immunoassay for evidence of adenoviral hexon protein. Tumor core biopsies were taken at days 8 and 22 and examined for adenoviral replication by in situ hybridization and for evidence of necrosis. Tumor measurement was carried out clinically and radiographically at 4 weeks. Patients were eligible for retreatment with virus injections at 4 weeks (up to a maximum of five cycles) if (a) measurements indicated a response or stable disease in the injected tumor (see below), (b) there was no DLT (see below), and (c) there was no evidence of disease progression at other sites. Ethical approval was given for an additional injection of diluent alone into separate lesions using the same injection technique as described for Onyx-015. The aim was to assess the volume effect of control intratumoral injection, and this was carried out in three patients.

**Evaluation of Toxicity and Response**

Toxicity was assessed using NCIC Toxicity Criteria. The maximum tolerated dose was defined as the dose at which two patients experienced a DLT after the first treatment with Onyx-015. DLT was defined as either grade 4 toxicity for flu-like symptoms due to Onyx-015, grade 4 toxicity for local reaction at the Onyx-015 injection site, or any other toxicity of grade 3 severity due to Onyx-015. A maximum of three patients were treated at each dose level. If one of the three patients had a DLT, a total of six patients would be treated for that cohort. An escalation scheme was devised to permit a rapid but safe increase in dose with a maximum of \(10^{11}\) pfu. The \(10^{11}\) pfu limit was based on manufacturing limits. No intrapatient dose escalation was permitted.

Response to therapy was assessed after each cycle by clinical and radiological tumor measurement. All radiological
measurements were made by the same radiologist. Because Onyx-015 caused substantial tumor necrosis centrally, rather than uniform tumor shrinkage peripherally, the nonnecrotic tumor area was determined and used to assess injected tumor response. The injected tumor response was then classified as a PR if there was a 50% reduction in the treated tumor, as a MR if there was a 25% but 25% increase in the injected tumor or the appearance of new lesions. Detailed responses are reported on a case-by-case basis because of the use of this nonconventional measurement methodology. To control for the effect of diluent, three patients who had more than one tumor had one tumor injected with Onyx-015 and another tumor injected with diluent only.

Evaluation of p53 Status

Immunohistochemistry. Immunohistochemistry was performed on formalin-fixed paraffin-embedded tumors cut into 5-μm sections. Slides were deparaffinized in xylene; hydrated through 100%, 90%, and 70% ethanol and then H2O2; and washed in PBS. Antigen retrieval was carried out by microwaving the slides in citrate buffer (pH 6.0) at 500 W for 25 min and then allowing them to cool over 20 min. The slides were washed in PBS for 5 min, and then the endogenous peroxide activity was blocked with 3% v/v hydrogen peroxide in methanol for 10 min. After washing in PBS for 5 min, the slides were blocked with Universal blocking solution (Biogenex) for 10 min, and then primary antibody (DO-1; Oncogene Science) at a dilution of 1:1000 in DAKO antibody diluent solution was added for 1 h at room temperature. Antigen detection was done using a biotinylated second antibody followed by streptavidin as supplied in the Biogenex link/label kit. We used the chromogen diaminobenzidine (Vector Laboratories) for detection for 3–10 min. The sections were counterstained with hematoxylin, dehydrated in graded alcohols followed by xylene, and then mounted in DPX mounting medium (BDH Chemicals). The percentage of brown-stained cells (positive for p53) was determined by counting the cells using light microscopy at a high-power magnification (×40). The average of three high-power field assessments was then expressed as a percentage. Tumors that had greater than 10% of cells stained positively were considered to have abnormal p53 and were entered into the trial.

Gene Sequencing. Exons 5–9 of the p53 gene were sequenced on pretreatment tumor biopsies by Oncormed Corp. (Gaithersburg, MD). This analysis was carried out retrospectively and was not a criterion for entry into the study.

In Situ Hybridization

In situ hybridization was performed on formalin-fixed paraffin-embedded tumors cut into 5-μm sections. Slides were deparaffinized in xylene and hydrated through 100%, 90%, and 70% ethanol and H2O2. The tissue was 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 antibody (Vector Laboratories) was applied. Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate was used as the chromagen, and slides were counterstained with nuclear fast red (Vector Laboratories).

Adenoviral PCR

The presence of adenovirus in plasma samples from patients was determined by PCR using primers of the E1A region of the adenoviral genome. This analysis was carried out by Onyx Pharmaceuticals.

Direct Immunofluorescence Assay for Hexon Protein

Swabs from the injection site and oropharynx were placed in viral culture medium. Human embryonic kidney cells (HEK293) were grown to 70–90% confluence and then inoculated with this medium. After 72 h, the cell sheet was scraped into the medium and pelleted by centrifugation at 2000 revolutions/min for 10 min. The cell pellet was washed in PBS and then resuspended in a small volume of PBS. Aliquots (25 μl) were pipetted into 6-mm wells on Teflon-coated microscope slides (DAKO; product code S6114), air-dried, and fixed in acetone at room temperature for 10 min. Cells were then stained with 25 μl of IMAGEGEN adenovirus reagent containing a FITC-labeled mouse monoclonal antibody to adenovirus hexon protein for 15 min at 37°C in a moist chamber, washed with PBS, and air-dried, and then one drop of IMAGEGEN mounting fluid and a coverslip were added. The 6-mm well was then scanned using a fluorescence microscope. Positive hexon staining was indicated by bright green fluorescence in the cytoplasm and/or nucleus of the cells.

Determination of Neutralizing Antibody Titers

Patient and control samples were incubated at 55°C for 30 min to inactivate complement. Clinical plasma samples previously determined to produce high, mid-range, and negative titers were designated as plasma controls. Each dilution was mixed with adenovirus stock at a titer prequalified to produce 15–20 plaques/well in a 12-well dish in DMEM growth medium. The patient’s samples and controls were inoculated for 1 h at room temperature and applied to 70–80% confluent JH393 cells in 12-well dishes. After 2 h of incubation at 37°C, 5% CO2 plasma-virus mix was removed, and 2 ml of 1.5% agarose in DMEM were added to each well. Plates were read on day 7 after inoculation by counting the number of pfu/well. The titer of neutralizing antibody for each sample was reported as the dilution of plasma that reduced the number of plaques to 60% of the number of plaques in the virus control without antibody.

RESULTS

Patient Characteristics. The characteristics of the patients treated are shown in Table 1. All 22 patients treated were evaluable for toxicity and efficacy. Seventeen patients were male, and five were female. The mean age was 63 years, and all patients had Karnofsky performance status equal to or above 60%, with a median of 80%. Twenty-one patients had had prior therapy. Nineteen patients had received radiotherapy alone or in combination with surgery and/or chemotherapy. Eight patients had received chemotherapy in combination with either radiotherapy or radiotherapy and surgery. Two patients had refused radiotherapy for the primary tumor (one patient had no prior therapy, and one patient had previous surgery). Both of these patients were enrolled into the study due to their refusal of forms of therapy other than Onyx-015.
The most common site of recurrence was in the neck (regional recurrence) in the cervical area. Other sites included intraoral, preauricular, clavicular, facial, and lip recurrences. The size of the recurrent tumors, expressed as the surface area in cm², varied over a range from 2.2–20 cm², with a median of 11.8 cm².

A total of 22 tumors were positive for p53 by immunohistochemistry. However, not all tumors had mutant p53 on gene sequencing. Sixteen tumors had mutant p53 sequence, 5 tumors had wild-type p53 sequence, and one tumor was noninterpretable. The pretreatment immune status of each patient, as assessed by CD4 lymphocyte count, showed that most patients were immunocompromised, with 19 of 22 patients having a CD4 count of less than 500, and only 3 patients having a count greater than 500. Thirteen of the 22 patients had preexisting neutralizing antibodies to adenovirus.

Toxicity and Safety. Table 2 summarizes the treatments given. Of the 22 patients, 14 patients received one treatment cycle, 6 patients received two treatment cycles, 1 patient received three treatment cycles, and 1 patient received four treatment cycles. Therefore, a total of 33 treatment cycles were given. Intratumoral injection of Onyx-015 was well-tolerated, with no DLT being observed. Toxicities probably or possibly related to Onyx-015 are shown in Table 3 and are all of grades 1/2. The most frequent symptom was grade 1/2 fever. Two patients experienced discomfort during the injection that subsided within 1 h both patients. One patient had grade 2 symptoms of tracheal obstruction that may have been related to Onyx-015. Serial blood counts showed no evidence of myelosuppression. Lymphocytopenia (grade 2–4) was seen in five patients, but this predated virus injection and was presumably related to preexisting immunosuppression. There was no evidence of any other hematological or biochemical abnormality attributable to therapy. Patients who were retreated did not experience any further toxicity, suggesting that neutralizing antibody levels did not sensitize the patients to added toxicity.

All patients had blood plasma samples collected before and after virus injection (days 0, 3, 8, 15, 22, and 29). DNA was isolated from these plasma samples, and PCR analysis was carried out using primers specific for adenovirus. All of these samples were negative, suggesting that Onyx-015 was not shed or did not persist in the circulation. All patients had swabs taken from the oropharynx and the injection site to detect virus shedding on days 0, 8, 15, 22, and 29. These swabs were analyzed by a direct fluorescence hexon protein assay, and all were negative.

### Table 1 Baseline patient characteristics

| Age (yrs) | Median 63 |
| Range | 32–81 |

| Sex | Male 17 |
| Female | 5 |

| Karnofsky performance status | 90% | 3 |
| 80% | 11 |
| 70% | 5 |
| 60% | 3 |

| Prior therapy | Surgery + XRT<sup>a</sup> | 10 |
| Chemo + XRT | 4 |
| Surgery + XRT + Chemo | 4 |
| Radiotherapy alone | 2 |
| Surgery alone | 1 |
| None | 1 |

| Location of recurrence | Cervical | 10 |
| Supraclavicular | 2 |
| Facial | 2 |
| Tongue | 2 |
| Oral | 2 |
| Other | 4 |

| Tumor size (cm²) | Median 11.8 |
| Range | 2.2–20 |

| Exons 4–9 | Mutant | 16 |
| Wild type | 5 |
| Noninterpretable | 1 |

| Baseline neutralizing antibody levels | Positive | 13 |
| Negative | 9 |

| CD4 counts (range, 152–1050) | <500 | 19 |
| >500 | 3 |

<sup>a</sup> XRT, radiotherapy; Chemo, chemotherapy.

### Table 2 Patients treated per cohort and target tumor response at >4 weeks using nonconventional measurements

<table>
<thead>
<tr>
<th>Cohort</th>
<th>No. of patients</th>
<th>No. of cycles</th>
<th>Target tumor response</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁶ pfu</td>
<td>5</td>
<td>2 1 1</td>
<td>PR 0 1 3</td>
</tr>
<tr>
<td>10⁷ pfu</td>
<td>4</td>
<td>4 0 0</td>
<td>MR 0 2 2</td>
</tr>
<tr>
<td>10⁸ pfu</td>
<td>4</td>
<td>2 0 0</td>
<td>SD 2 1 1</td>
</tr>
<tr>
<td>3 × 10⁹ pfu</td>
<td>3</td>
<td>2 1 0</td>
<td>PR 0 1 1</td>
</tr>
<tr>
<td>10¹⁰ pfu</td>
<td>3</td>
<td>2 1 0</td>
<td>MR 0 2 1</td>
</tr>
<tr>
<td>10¹¹ pfu</td>
<td>3</td>
<td>2 1 0</td>
<td>SD 1 1 1</td>
</tr>
</tbody>
</table>

Total | 22 | 14 6 1 | PR 3 2 8 9
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suggesting that Onyx-015 was not readily shed from treated tumors, even when the tumor was ulcerated.

**Tumor Response and Correlation to p53 Status.** All patients were evaluable for response, and the data are summarized in Table 2. Using conventional criteria, no objective responses were observed because all patients in the study either progressed at the site of tumor injection, developed other neck lesions, or developed distant metastases in the lung, liver, or bone. However, if we consider only the tumors that were injected, then there was evidence of antitumor activity. A common finding after injection was that the injected tumor became soft and fluctuant, usually within 8 days after injection. The overlying skin often became erythematous. MRI scans of injected tumors often showed a change in the signal from the tumor center, in keeping with liquefaction of solid tumor, i.e., necrosis. Using nonconventional criteria for measuring the degree of tumor shrinkage (i.e., subtracting the central necrosis), three patients showed a PR in the treated lesion, and two patients showed a MR. An additional eight patients had SD. There was no correlation between the viral dose injected and tumor response.

Details of the three cases showing a substantial tumor shrinkage in the treated lesion are as follows:

(a) Patient 1003 had a primary tongue tumor treated with surgery, radiotherapy, and chemotherapy. He developed a recurrence in the right submandibular area with severe trismus and pain. This area was injected with 10^7 pfu of virus. A 50% reduction in the size of the tumor occurred, with three cycles of treatment given over 12 weeks. The MRI scans pre- and post-treatment showing changes suggestive of necrosis are illustrated in Fig. 1. Symptomatically, the patient improved, with increased jaw mobility and reduced pain. This response was of 12 weeks duration before the patient was removed from the study due to the development of neck metastases.

(b) Patient 1007 had a left temple tumor treated initially with surgery and radiotherapy. He developed a recurrence in the left preauricular area, extending deep into the pterygoid fossa. This was injected with 10^9 pfu of virus, and a 75% reduction in the size of the tumor, with radiological changes in keeping with necrosis, occurred after two cycles of treatment. This response was of 8 weeks duration before the patient was removed from the study due to the development of orbital disease requiring radiotherapy.

(c) Patient 2006 had a primary tongue tumor treated with radiotherapy. He developed a tongue recurrence, and the right third of the tongue was injected with 10^9 pfu of virus. A large portion of tumor, measured as 80% of the original injected tumor, sloughed off on day 8. This response was of 4 weeks duration before the patient died from bacterial pneumonia (believed to be unrelated to virus injections).

Of the two patients with a MR to treatment, one patient was removed from the study at 4 weeks due to the development of a second locoregional recurrence, and one patient was removed at 4 weeks for radiotherapy to the target treated tumor (this patient had initially refused radiotherapy but then changed his mind after one cycle of virus therapy). Of the eight patients with SD, three were removed from the study due to the development of a second locoregional recurrence (one patient at 8 weeks and two patients at 4 weeks). The other five patients were removed from the study due to progression at the injected tumor at 4 – 6 weeks after virus injection. All other patients showed evidence of tumor progression locally and at other sites.

To control for the effect of diluent, three patients had satellite lesions injected with diluent alone. All of these tumors progressed, with no clinical or radiological signs suggestive of necrosis.

The p53 status of tumors versus the tumor response is shown in Table 4. Of the five tumors that showed evidence of response, four had mutant p53, and one had wild-type p53, suggesting that Onyx-015 may selectively replicate in mutant p53 tumors. However, if we compare the p53 status of tumors showing evidence of response (MR and PR) with those that did not (SD and Prog), statistical analysis using Fisher’s exact test showed no significant correlation (P = 0.53).

**Detection of Onyx-015 in Tumor and Correlation to p53 Status.** Adenoviral DNA was detected in tumor biopsies by *in situ* hybridization. A typical example of staining is shown in Fig. 2. Four of the 22 patients showed positive evidence of viral replication on the biopsies obtained. All four of these patients had mutant p53 on gene sequencing. No patients with a wild-type p53 gene sequence showed virus replication. In addition, adenoviral DNA was only detected in tumor cells, and normal skin and mesenchymal tissue within the biopsies were negative. This may suggest that viral replication was selective for tumors with mutant p53. However, statistical analysis using Fisher’s exact test (Table 5) showed no significant correlation (P = 0.53).

**Humoral Immune Response and Correlation to Tumor Response.** Thirteen of the 22 patients treated (59%) had pre-existing neutralizing antibodies to adenovirus, and all but one patient developed increased antibody levels after treatment. If we compare baseline neutralizing antibody levels of patients who had evidence of tumor response (MR and PR) with those who did not (SD and Prog), statistical analysis using Fisher’s exact test (Table 4) showed no statistically significant correlation (P = 0.96). Therefore, preexisting neutralizing antibody levels did not determine tumor response.

**DISCUSSION**

The primary objective of this Phase I study was to determine the safety of a single injection of Onyx-015 by intratumoral injection in patients with recurrent HNSCC. The results obtained showed that toxicity was very minor, with the main...
symptoms being mild flu-like symptoms. Two patients did experience mild pain on injection, and this was related to the volume of the injected solution. The pain resolved within 1 h and rarely required any further analgesia. The maximum dose injected was 10^{11} pfu, and this did not produce any serious adverse effects. The maximum tolerated dose was clearly not reached in this study, but dose escalation ceased because the limit of virus manufacturing capacity had been reached. This lack of toxicity is very encouraging and suggests that perhaps more potent replicating viruses can be explored in future trials.

Safety data were also encouraging because no virus was detected in either blood samples or the injection site and oropharyngeal swabs, and this suggested that virus shedding was not a major issue. This was in agreement with a previous study that involved the intratumoral injection of replication incompetent adenovirus Ad5p53 in patients with recurrent head and neck cancer (9). In this study, adenovirus was detectable in blood samples by PCR at 30–90 min after virus injection but was undetectable by 48 h. In our study, the earliest blood sample was at 72 h after injection, and all were negative for adenovirus. Thus, both studies show that the virus is undetectable 48–72 h after virus injection.

One of the secondary objectives of the study was to determine whether there was evidence of an antitumor effect. This was assessed both clinically and radiographically by either computed tomography or MRI scans. Using conventional criteria, no objective responses were observed. However, if we consider only the tumors that were injected, then there was evidence of antitumor activity. Using nonconventional measurements, five (23%) patients showed either a PR or MR, whereas an additional eight patients (36%) showed SD. All five responding patients had abnormal p53 on immunohistochemistry, and sequencing revealed that four patients had mutant p53. One patient had a wild-type p53 sequence, but it is possible that in this patient’s tumor, the p53 protein was inactivated by either human papillomavirus infection (17, 18), mdm2 overexpression (19, 20), or some other factors. Alternatively, a mutation of the p53 gene may have been present outside exons 4–9 or in the intron regions.

The cause of the clinical and radiological changes, in

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**Table 4** Response to Onyx-015 related to p53 gene sequence and baseline neutralizing antibody level

| Baseline neutralizing antibody levels | Target tumor response | No. of patients | PR | MR | SD | Prog | P  
|--------------------------------------|-----------------------|----------------|----|----|----|------|---
| Positive                             |                       | 13             | 1  | 2  | 3  | 7    |    |
| Negative                             |                       | 9              | 2  | 0  | 5  | 2    | 0.96 |
| p53 gene sequence                    |                       |                |    |    |    |      |    |
| Mutant                               |                       | 16             | 2  | 2  | 4  | 8    |    |
| Wild-type                            |                       | 5              | 1  | 0  | 3  | 1    | 0.53 |
| Noninterpretable                     |                       | 1              | 0  | 0  | 1  | 0    |    |

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**Fig. 1** Patient 1003 had a primary tongue tumor and developed a local recurrence in the right submandibular region. The depth of the tumor is shown in the baseline MRI scan outlined in red. Fifty percent necrosis of the tumor occurred after three cycles of treatment. The site of injection is indicated with a white arrow. The area of necrosis is shown on the Week 12 MRI scan.
keeping with necrosis in responding patients, is most likely a result of the injection of Onyx-015 because diluent-injected tumors showed no evidence of necrosis. This was in agreement with preclinical nude mouse xenograft models (10). These changes may be due to viral replication and spread. However, it may also be possible that some of the observed response is due to an immune response against virally infected tumor cells.

Viral replication was detected in patient biopsies by in situ hybridization techniques. Positive evidence of replication was found in four patients, all of whom had mutant p53 tumors. No replication was detected in tumors with wild-type p53 or in surrounding normal tissue. This suggested that Onyx-015 may selectively replicate in mutant p53 tumors in vivo. However, other tumors with mutant p53 did not show evidence of virus by in situ hybridization, and statistical analysis showed no correlation between p53 status and viral replication. There are several possible explanations for this. It may be that Onyx-015 does not have rigid selectivity for p53 mutant tumors, as suggested by Bischoff et al. (10). Indeed, recent studies (20–22) have shown no correlation between p53 status and viral replication in cell lines with known p53 status. However, even these studies were controversial because the p53 functional status of the cell lines used had not been fully determined, and the mechanism of cell death (i.e., apoptosis versus viral replication) had not been fully established. The clinical study presented here on head and neck cancer patients has not resolved this controversial issue. It is also possible that the low detection rate of viral replication in mutant p53 tumors may also have been due to the sensitivity and specificity of the technique used. For example, the tumor biopsies were very small compared to the size of the injected tumors (<1%); therefore, the area of tumor that was injected may not have been biopsied in the majority of cases. In animal experiments, replication was evident in nude mouse human tumor xenografts in the cells at the watershed between necrosis and viable tumor (23). Because it is not possible to biopsy necrotic tissue effectively, it is not surprising that biopsies of viable tumor did not show replication. In addition, because this was a single-injection protocol, viral distribution may not have been as effective as a multiple-injection protocol because animal studies in nude mice have shown that distribution is more widespread with a multi-injection protocol (23). It is also possible that viral spread is limited by the fibrotic nature of these tumors because the majority of patients had previously been treated with external beam radiotherapy.

Lastly, it is possible that viral spread is affected by systemic or local immune effects. Adenovirus can produce two systemic immune effects: (a) cell-mediated immunity; and (b) humoral immunity. Cell-mediated immunity is mediated by CTLs and is stimulated by adenovirus antigens produced in the host cell and presented along with MHC moieties on the cell surface. This can cause early elimination of virus but may also be beneficial because it causes the immune-mediated killing of tumor cells. In this clinical study, all patients were immunosuppressed at baseline with low total lymphocyte counts and low CD4 counts. A total of 19 patients had a CD4 count of less than 500, with a median range of 200–300. To determine whether or

Table 5  Viral replication related to p53 gene sequence

<table>
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<th>p53 gene sequence</th>
<th>No. of patients</th>
<th>Positive</th>
<th>Negative</th>
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Fig. 2  Detection of adenoviral DNA in tumor core biopsies by in situ hybridization. A day 8 tumor biopsy in patient 2009, who showed SD in the injected tumor, is shown. A, an H&E-stained biopsy; B, the same area stained for adenoviral DNA. The dark blue-stained cells represent cells expressing adenoviral DNA.
not a cellular immune response occurred, biopsy specimens were stained for helper T lymphocytes (CD4), CTLs (CD8), and macrophage infiltration (CD68). However, because the biopsies were so small, it was not possible to make any conclusions regarding the relevance of a cellular response to overall tumor response. We are currently planning to carry out a preoperative study on patients with early-stage HNSCC in which patients receive a single intratumoral injection of virus within the first 2 weeks before definitive surgery. It will then be possible to examine whole tumor sections once the tumor is excised to determine the extent of viral spread and immune effector cell response.

The neutralizing antibody response (humoral response) to adenovirus occurs later. Theoretically, this should reduce the ability to reinfect host cells with adenovirus after the first inoculation. In this study, all patients developed a rising neutralizing antibody response despite being immunosuppressed. Statistical analysis showed no correlation between pretreatment antibody levels and tumor response. In addition, patients who were retreated continued to show a response despite rising antibody levels, and this would suggest that neutralizing antibody to adenovirus may not be clinically relevant to intratumoral efficacy. There is also some evidence that suggests that antibody penetration into these tumors may be minimal (24, 25), in which case a rising antibody would have no effect in a repeat intratumoral administration procedure and would only be of importance in repeat systemic viral injection. If humoral immunity proves to be troublesome, it is possible to suppress this with IFN-γ and interleukin 12 (26) by using viruses with non-cross-reactive serotypes (27) or by creating viruses that are encapsulated in poly(lactic-glycolic) acid copolymer to evade the immune system (28).

Local immunity, as well as systemic immunity, is also a factor that may limit viral spread. Tumors secreting IFN may neutralize viral spread. It has recently been shown that adenovirus infection stimulates activation of the transcription factor nuclear factor κB, which results in a downstream inflammatory response (intercellular adhesion molecule 1 up-regulation; Ref. 29). It has also been shown that adenovirus infection causes stimulation of the Raf/mitogen-activated protein kinase pathway and interleukin 8 secretion (30). Experiments to investigate the role of the immune system are planned with a preoperative study of patients with early-stage HNSCC.

Recent work in nude mouse xenograft models has suggested that multiple injections improve viral distribution and efficacy (23), and a multiple-injection Phase I study has recently been carried out. This study involved five daily intratumoral injections of Onyx-015 at a dose of 10⁶ or 10⁷ pfu/day. The results of this study will be reported separately. Recent work has also shown that combination therapy with Onyx-015 and chemotherapeutic agents such as cisplatin and 5-fluourouracil was superior to treatment with either agent alone when tested in nude mouse xenograft tumors (31). It is likely that many head and neck tumors are heterogeneous in their p53 status. In this case, combination therapy with Onyx-015 and chemotherapy may be beneficial because Onyx-015 will kill cells with mutant p53, and chemotherapy will kill cells with wild-type p53. A clinical trial of cisplatin/5-fluourouracil combined with Onyx-015 has recently been completed in patients with recurrent head and neck cancer, and the results are very promising (32).

In summary, we have shown that intratumoral injection of the E1B-attenuated replicating adenovirus Onyx-015 is feasible and safe with very limited toxicity. Although no objective tumor responses were observed, evidence for biological activity was observed. However, response did not correlate significantly with the p53 status of injected tumor. In addition, although viral replication was found only in tumors with mutant p53 and not in normal cells, the relationship between p53 status and viral replication was not statistically significant. This may have been due to limitations in the techniques used to assess replication but does not exclude the possibility that the selective replication of Onyx-015 for p53 mutant tumors is not as stringent as first thought. Additional studies in patients are therefore required to resolve this issue. Nevertheless, the observation of biological activity of this agent in recurrent head and neck cancer patients is encouraging. Current available therapies for recurrent head and neck cancer, such as tumor debulking surgery, further radiation, and chemotherapy have all produced poor responses of limited duration. All of these therapies also produce significant morbidity. An agent such as Onyx-015, which has very little toxicity and which can be given on an outpatient basis without the need for hospitalization, may be an attractive alternative to these therapies, but only if responses are comparable. Additional studies are therefore warranted to further evaluate this form of therapy for this disease.

REFERENCES

Correction

In the article by I. Ganly et al., which appeared in the March, 2000 issue of Clinical Cancer Research (pp. 798–806), the author name “Gail I. Rodriguez” should read “Gladys I. Rodriguez.”
A Phase I Study of Onyx-015, an E1B Attenuated Adenovirus, Administered Intratumorally to Patients with Recurrent Head and Neck Cancer

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