The dl1520 Virus Is Found Preferentially in Tumor Tissue after Direct Intratumoral Injection in Oral Carcinoma

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ABSTRACT

Purpose: **dl1520 (also known as Onyx-015) is an E1B-deleted adenovirus designed to selectively lyse p53-deficient cancer cells. Clinical trials involving patients with recurrent squamous cell carcinoma of the head and neck have shown clinical efficacy, but no direct evidence as to the tumor or p53 selectivity of the virus was demonstrated. We wanted to determine whether dl1520 is selective for survival and replication within tumor tissue after direct injection and whether this is determined by p53 status of the tissues. We also wanted to ascertain whether the virus has any macroscopic effect on normal tissue.**

Experimental Design: **An open-label Phase II trial was devised in which a fixed dose of the virus was administered to 15 patients via a direct intertumoral injection before surgery for untreated oral squamous cell carcinoma. The agent was also delivered into an area of adjacent normal buccal mucosa. Specimens of the excised tumor and of biopsies of the injected normal tissue were assessed for viral presence and p53 status.**

Results: **We demonstrated that the virus replicates selectively in tumor as opposed to normal tissue after this direct injection. It was not possible to determine whether this selectivity was p53 related. It was found that dl1520 triggers an early rise in apoptosis levels in injected normal tissues. No adverse effects of viral injection were noted.**

Conclusions: **This is the first report of injection of dl1520 into previously untreated squamous cell cancer. The data support the concept that dl1520 is replication deficient in normal, compared with cancerous, tissues and has potential as a selective anticancer agent against tumor tissues.**

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INTRODUCTION

Improved treatments for recurrent and metastatic cancer refractory to conventional therapies are urgently required. This is particularly so in squamous carcinoma of the head and neck, in which the primary tumor can be treated effectively with surgery and radiotherapy when localized but which is very difficult to treat, once recurrent or metastatic lesions develop. Gene therapy treatments offer the hope that genetic abnormalities within cancer cells can be specifically targeted allowing selective tumor cell destruction (1). One agent that has shown promise in this field is the attenuated adenovirus dl1520 (also known as Onyx-015).

The virus is E1B gene deleted and is designed to be cytolytic to cancer cells after viral replication but to cause little harm to normal tissues (2, 3). This is proposed to be due to the selective replication of the virus leading to cytolysis in mutant p53-expressing cells (4). It is purported that wild-type p53-expressing cells, such as certain tumor cells and normal cells, will not support viral replication and, instead, undergo apoptosis after viral inoculation. Because abnormalities of the *p53* gene are the commonest mutations in human solid malignancy, this agent can potentially treat a wide range of human cancer (5). The virus has been used previously, both as an individual agent and in combination with chemotherapy agents, to treat patients with recurrent squamous carcinoma of the head and neck (6–8). When dl1520 has been tested in other tumor types, it again has given encouraging results (9–12).

Detailed information as to levels of viral replication and spread is lacking. Representative biopsies of tissues have demonstrated evidence of viral survival and replication within tumors, but, thus far, it has been impossible to analyze large segments of tissue. Little information on the effect of virus on normal tissue has been obtained during these trials because only tumor tissue has been treated and no biopsies of surrounding or adjacent normal tissues have been taken for analysis.

We designed a clinical study with three objectives: firstly, to determine whether the virus is selective for survival and replication in tumor tissue as opposed to normal tissue, and whether this relates to p53 status of the tissues; secondly, to determine the level of viral spread and replication within these tissues; thirdly, to determine the levels of virus-induced apoptosis in normal and tumor tissues after virus injection.

PATIENTS AND METHODS

dl1520. dl1520 is a chimeric group C adenovirus (Ad2 and Ad5) that has a deletion between nucleotides 2496 and 3323. This deletion lies within the region coding for the E1B M_r 55,000 protein. In addition, there is a stop codon generated by a C to T transition at the 2022 nucleotide within the E1B region. The virus, therefore, does not express the E1B protein after inoculation into cells. The virus is produced by Magenta Corp (Rockville, MD) and is tested for titer, sterility and safety by

Microbiological Associates using United States Food and Drug Administration-approved tests.

Treatment Protocol. Fifteen patients with previously untreated primary oral squamous carcinoma were entered into the study. The subjects consisted of 13 male and 2 female, with an average age of 62 years (range, 42–74). All of the patients had hematological, renal, and hepatic function within normal limits. Pretreatment evaluation included prothrombin time and international normalized ratio, partial thromboplastin time, electrocardiogram and chest X-ray. The protocol was approved by the Gene Therapy Advisory Committee and the local ethical committee. A tumor biopsy was removed for *p53* gene sequencing. Each tumor was divided into halves using Toludine blue tattooing to mark each area. Virus (10^{10} plaque-forming units) in a volume of diluent equal to 15% of tumor volume was injected into one of the halves, and an equivalent volume of diluent only was injected into the other half (control half). A standard injection protocol, whereby the needle was passed in turn into each quadrant of the hemitumor and the injection made as the needle was withdrawn, was followed in each case. The first author (S. M.), who is a surgical oncologist with a special interest in head and neck cancer, performed all of the injections. In a similar fashion after marking with Toludine blue tattooing, dl1520 (10^{10} pfu) was also injected into an area of normal buccal mucosa adjacent to the tumor. At surgery, the entire tumor was removed as per the normal surgical protocol (see Table 1 for primary tumor sites). The excised tumor was bisected into viral-injected and diluent-injected hemitumors, with care taken to orient the specimens correctly under the control of the pathologist who was to examine the tumors. Five patients underwent surgery 24 h (±6 h) post-dl1520 injection; five patients underwent surgery 3 days (±1 day) post-dl1520 injection, and five patients underwent surgery 14 days (±1 day) post-dl1520 injection. These time points were chosen to coincide with initial viral delivery at 24 h, the point of maximal viral replication at 72 h (13), and a later point was chosen to determine levels of ongoing viral replication and survival at 14 days. A separate biopsy was taken of the viral-injected normal tissue at the same time points.

Gene Sequencing. Tumor biopsies for gene sequencing were immediately snap-frozen in liquid nitrogen. Gene sequencing was performed using the GeneChip microarray (14) at Virco Laboratories, Baltimore, MD.

Immunohistochemistry Stain for p53. Microwave antigen retrieval was performed for 15 min. Vectastain ABC kits were used to provide link antibodies prior to the addition of primary antibody added at a dilution of 1/1000 for 45 min at room temperature (DO1, Oncogene Science, Cambridge, MA). Sections were then incubated with 100 μl of biotinylated antibody. DAB (3,3′-diaminobenzidine) was used as a chromogen. Immunohistochemistry scores for p53 expression were derived from assessing a combination of the percentage of cells that were positive within the sample and the intensity of the staining (see Table 2).

Table 1 Site of primary tumor

Tongue	5
Floor of mouth	5
Tonsil	5

Table 2 Immunohistochemistry scores for p53 expression

Percent cells staining positive	0–20%	20–80%	>80%	
Score	1	2	3	
Intensity of cell staining	Nil	Weak	Moderate	Strong
Score	0	1	2	3

Table 3 Gene sequencing for p53 in frozen tissue tumor biopsies

Patient no.	p53 sequencing
1	Wild-type
2	Mutation missense exon 6 644G>T
3	Mutation missense exon 7 746T>G
4	Mutation missense exon 5 488A>G
5	Inadequate sample
6	Wild-type
7	Mutation intron 6 IVS6 + 2T>G
8	Mutation missense exon 5 476C>T and mutation nonsense exon 10 1024C>T
9	Mutation missense exon 7 742 C>T
10	Mutation intron 5 IVS5–1G>C
11	Wild-type
12	Mutation intron 8 IVS8–2A>G
13	Mutation missense exon 5 524G>A
14	Wild-type
15	Mutation missense exon 5 536A>T

Hexon Stain for Adenoviral Protein Expression. Paraffin sections were dewaxed, rehydrated in ethanol, and then immersed in 3% hydrogen peroxide. The primary antibody used was mouse anti-adenovirus monoclonal antibody Ig1k isotype (Chemicon) at a dilution of 1:500, and sections were incubated for 45 min in a humidified chamber at 35°C. Subsequent antibodies were provided from the Biogenex Super Sensitive Immunodetection System, Biogenex. DAB was used as a chromogen.[7]

***In Situ* Hybridization for Adenovirus DNA.** DNA probe (Enzo AdV adenovirus probe, Enzo Diagnostics Ltd.) was used with hybridization carried out overnight at 37°C. Anti-biotin/alkaline phosphatase conjugate, diluted 1:200 with PBS with 0.1% Tween 20, was applied to each slide, and the slides were incubated for 2 h at room temperature. The slides were then rinsed in color development buffer and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) was used for detection (Vector Laboratories; Refs. 4, 16).

Protocol for TUNEL *in Situ* Cell Death Detection. This protocol was used as per the manufacturer's instructions. Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) reagent was added, and slides were incubated in a humidified chamber for 60 min at 37°C. DAB was used as a chromogen (17).

RESULTS

Fifteen patients with primary oral carcinoma were treated with the dl1520 virus before surgical excision. The treatment was well tolerated and there were no side effects attributable to

[7] C. Heise, 1995; protocol obtained from Onyx Pharmaceuticals.

Table 4 Overall results for ISH[a] to detect adenoviral DNA: wild-type *versus* mutant p53 (Mut p53)

Sample type	*n*	Tumor dl1520 injected, density	Tumor saline injected, density
Mut p53 (ISH)	10	12.6 ± 6	1.3 ± 0.4
Wild-type p53 (ISH)	4	15.5 ± 13	0
Mut p53 (IHC)	10	9.9 ± 3.7	1.3 ± 0.4
Wild-type p53 (IHC)	4	7.5 ± 6	0

[a] ISH, *in situ* hybridization; IHC, immunohistochemical staining.

the dl1520 virus. No gross evidence of damage to the normal tissue or shrinkage of the injected tumor was noted.

The Status of p53 in Tumor and Adjacent Normal Tissue. Samples of each tumor were assessed for p53 status by gene sequencing (Table 3). Of 15 samples assessed, 10 had a mutant p53 sequence, 4 had wild-type gene sequences, and 1 sample was inadequate for assessment. All of the mutations found have been previously described and are known to cause significant dysfunction of the p53 protein (18, 19, 20). Immunohistochemistry staining (IHC) was also used to detect mutant p53, and we demonstrated a good correlation between this and gene sequencing with all of the samples with a wild-type gene sequence scoring zero for p53 expression (21, 22). Eight of the 10 samples with a p53 mutation on gene sequencing stained positive for p53, and two were negative. This could be explained by the possibility that the mutation observed had led to the loss of both alleles, and, as such, no detectable p53 can be produced (21). Of the normal tissue samples, 8 of 14 stained positive for p53, indicating a possible abnormality of p53 in these specimens.

Detection of dl1520. Samples were assessed for the presence and density of cells positive for viral detection per high-powered field (hpf) in areas in which virus was detected (Figs. 1 and 2). This is expressed as the average number of positive cells per hpf (Table 4). Adenoviral DNA was detected in 2 of 15 normal tissue biopsies using *in situ* hybridization (ISH) with a mean density of 1.4 ± 0.9. Six of 15 virus-injected tumor samples were positive (average density of cells positive, 12.5 ± 5.5). This demonstrates a significantly increased level of viral survival and replication in tumor samples than in normal tissue ($P = 0.03$). IHC detected adenoviral hexon protein in 2 of 15 normal tissue samples with a mean density of 0.6 ± 0.4. Eight of 15 virus-injected tumor samples were positive with a mean density of 8.6 ± 2. Again, this indicates an higher level of viral survival and replication in tumor samples than in normal tissue ($P = 0.009$). All of the normal tissue samples in which virus was detected stained positive for p53. From this study, we cannot determine whether injection of the virus itself affected p53 expression in normal tissue, because no control samples of normal tissue into which diluent alone was injected were available.

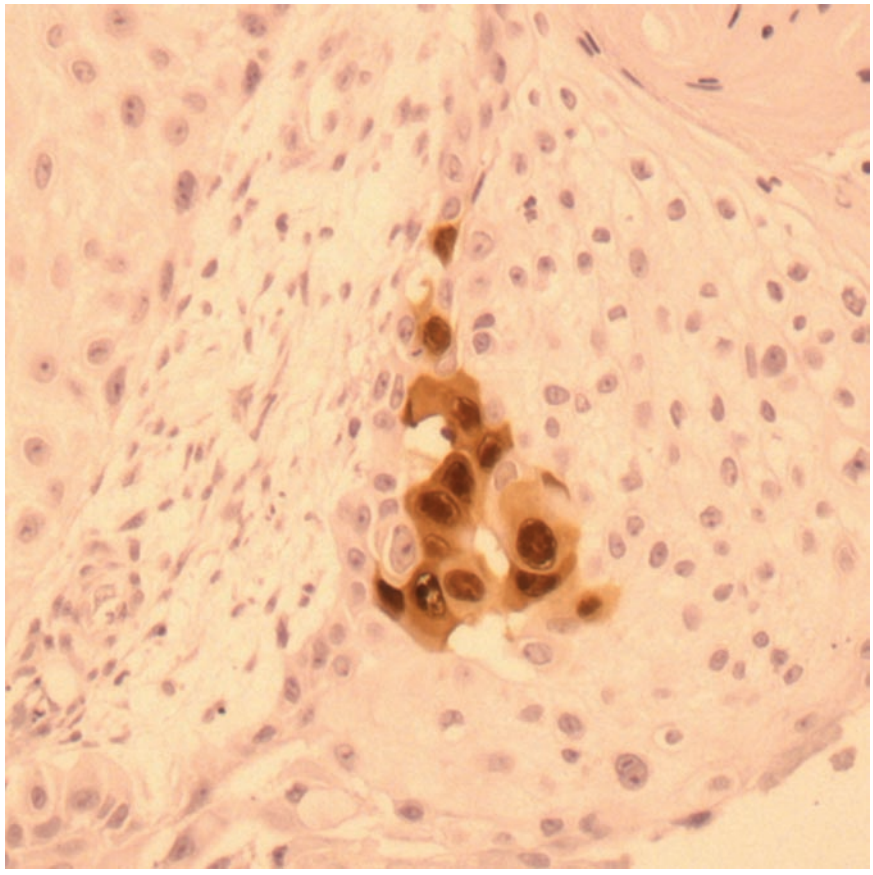

Fig. 1 Positive immunohistochemistry staining for adenoviral hexon protein in a dl1520-injected tumor sample.

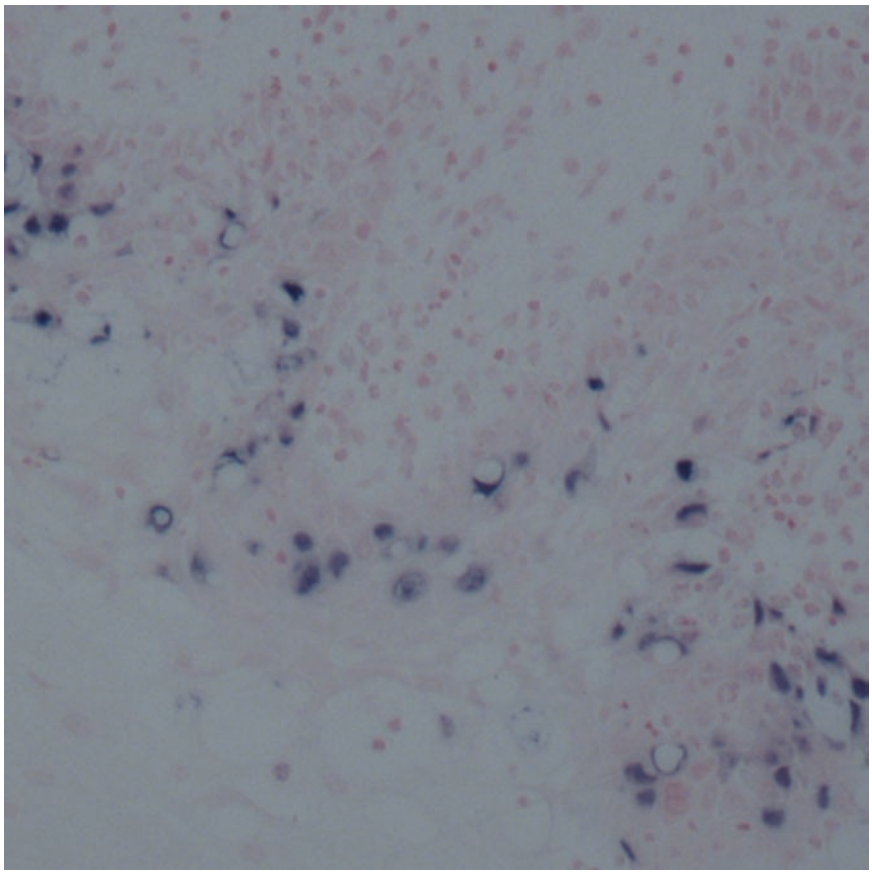

Fig. 2 Positive staining for adenoviral DNA using *in situ* hybridization in a dl1520-injected tumor sample.

Overall Viral Detection. Combining the results of the ISH and IHC, we were able to detect the virus in 10 of 15 tumors altogether. This includes 8 of 10 positive with mutant p53 and 2 of 4 with wild-type p53 (one sample was inadequate for gene sequencing). This compares with detection in 3 of 15 normal tissue biopsies taken after injection of the same dose of dl1520. This clearly shows that dl1520 exists preferentially in tumor tissue as opposed to normal tissue from a similar tissue type, *i.e.*, oral mucosa (Table 5).

Comparison of the p53 Mutant *versus* Wild-Type Tumor Samples. ISH detected adenoviral DNA in 5 of 10 samples with a mutation of p53 and 1 of 4 with a wild-type p53 sequence The average density of virus found was 12.6 ± 6 and 15.5 ± 13 cells per hpf for mutant and wild-type tumor respectively. Using IHC, we detected virus in 7 of 10 samples with a mutation of p53 and 1 of 4 with a normal p53 sequence. The average density of virus found was 9.9 ± 3.7 and 7.5 ± 6 cells per hpf for mutant and wild-type tumor, respectively. We detected virus more often in p53 mutant tumor samples using both ISH and IHC, although this difference was not statistically significant.

Detection of Virus in Saline-Injected Tumor Samples. ISH-detected adenoviral DNA was detected in 2 of 15 samples of saline-injected tumor, and IHC detected adenoviral hexon protein in 3 of 15 samples of saline-injected tumor. All of these had a mutation of p53. This implies that virus translocation from injected to control tumor is possible but minimal after a direct injection.

Cellular Effect of Virus Injection. Levels of apoptosis detected using the TUNEL test were expressed as an average of apoptotic cells per hpf (Table 6). In the tumors, fields were selected at the interface between invasive tumor and normal

Table 5 Overall results for dl1520 injection in tumor *versus* normal tissue

	Normal tissue, viral density	Tumor tissue, viral density
ISH[a]	0.6 ± 0.4	12.5 ± 5.5
IHC	1.4 ± 0.9	8.6 ± 2.9

[a] ISH, *in situ* hybridization; IHC, immunohistochemical staining.

Table 6 TUNEL[a] stain to detect apoptosis in differing tissue types: cells positive per high-powered field

	Tumor, dl1520 injected	Tumor, saline injected	Normal tissue
24 h	4.6 ± 1.1	1.8 ± 0.2	6.1 ± 2.2
72 h	1.7 ± 0.52	2.5 ± 0.5	5.3 ± 3
14 days	2.8 ± 1.2	2.5 ± 0.5	3.2 ± 1.6
All	3 ± 1.1	2.3 ± 0.42	4.9 ± 1.3

[a] TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

tissue. Levels of apoptosis in the virus-injected tumor samples were 4.6 ± 1.1/hpf at 24 h after injection, 1.7 ± 0.52/hpf at 72 h, and 2.8 ± 1.2/hpf at 14 days. In the saline-injected tumor samples, the values were 1.8 ± 0.2/hpf at 24 h after injection, 2.5 ± 0.5/hpf at 72 h, and 2.5 ± 0.5/hpf at 14 days. In the normal tissue samples, the values were 6.1 ± 2.2/hpf at 24 h after injection, 5.3 ± 3/hpf at 72 h, and 3.2 ± 1.6/hpf at 14 days. These results suggest that there is an increase in apoptosis in the normal, dl1520-injected tissues as compared with the dl1520 or saline-injected tumors at the 24-h and 72-h time points. This increased apoptosis is lost by the 14-day time point, a time point when there is no significant difference between the normal tissues and the tumor tissues. There was no difference in apoptosis between the dl1520-injected and saline-injected tumors. This suggests that the dl1520 virus can lead to high levels of apoptosis in normal tissue for the first 24–72 h after injection.

DISCUSSION

We have shown evidence from a clinical setting to suggest that the dl1520 adenovirus is selectively found in tumor tissue as opposed to normal tissue after direct injection. Of the tumor samples, it was possible to detect the virus in 10 of 15 cases when combining the results of the ISH and IHC for hexon protein. This includes 8 of 10 positive with mutant p53 and 2 of 4 with wild-type p53 (one sample being inadequate for gene sequencing). This compares with detection in 3 of 15 normal tissue biopsies. The virus was more commonly detected in p53 mutant tumors as opposed to wild-type p53, but, with only small numbers involved, this difference does not reach statistical significance. Importantly, direct injection of the virus into normal tissues does not cause any significant side effects or adverse events. Clearly there are other differences between normal tissue and tumor, such as the presence of gap junctions and inflammatory cells that may differ between normal and tumor tissue and affect the movement of virus. We did not detect any evidence that these differences significantly affected the dl1520 virus in this study, but it could be that the differences in viral detection were due to viral movement out of normal tissue as well as replication within tissue. It is known that inflammatory cells respond to gene therapy adenoviral vectors in both normal and tumor tissues (23, 24). There is no evidence that this effect is stronger in reducing viral levels in normal, as opposed to tumor, tissue either in this study or from previous studies. We feel that these data do indicate that dl1520 is selective for tumor tissue as opposed to normal tissue, although the mechanism for this may relate to viral movement as well as replication.

Increased apoptosis was noted in the normal tissue biopsies at both the early time points assessed, *i.e.,* 24 and 72 h postinjection. Previous studies have demonstrated low levels of apoptosis in normal oral mucosa, with apoptosis playing a crucial role in the eventual pathogenesis of oral cancer (25–27). It would be expected that injection of the virus into normal tissues with a functional p53-dependent apoptotic pathway would stimulate apoptosis to a relatively high level (28). This effect would lead to a containment of the viral infection with little viral replication.

Further information relating to the effect on normal tissue of viral injection would have been obtained had normal tissue biopsies been taken as a baseline prior to injection. It was outside the ethical approval obtained for this study to take such biopsies. The investigators considered that approval for injection and biopsy of one normal tissue site was as much as could reasonably be proposed in this study, but we accept that the lack of this baseline has limited the conclusions than can be drawn from the study.

Some authors have recently cast doubts on the mechanism of action of the virus, in particular the level of p53 selectivity. Hall *et al.* (29) reported results from tissue culture experiments involving dl1520 showing that tumor cell lines expressing both functional and mutant p53 allowed viral replication. Some results suggested that wild-type p53 was, in fact, required for cell death after this infection. We have clearly demonstrated that dl1520 exists preferentially in tumor tissue, especially tumor tissue with mutant p53. Other workers have also shown that the virus can replicate within some tumor cells of wild-type p53 status (30). These workers further demonstrated that the virus could infect and replicate within some primary human cell lines including keratinocytes and fibroblasts. Our results in the clinical setting suggest that the virus can exist only in very small amounts in normal tissues.

Our data do indicate the relatively limited capacity of dl1520 to spread to any significant extent to areas within a treated tumor distant from the injection site. This emphasizes the importance of combination with other treatment modalities, as well as exploring techniques for enhancing viral spread. Additional studies are ongoing to examine the potential for systemic delivery of dl120, including the use of liposomes.

The aim of the study was not to demonstrate a tumor response; a low dose of the virus was used and only with a single injection. Previous studies have demonstrated the ability of the virus to shrink tumors, either as a single agent or in combination. This trial gives additional evidence that the virus cannot significantly harm normal tissues. Our data support the concept of the capacity of the dl1520 virus to target tumor tissue. This could be related to p53 function, although other factors are also important. These results should encourage further research using this agent, which continues to show promise as a selective form of viral gene therapy.

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