Persistent Replication of the Modified Chimeric Adenovirus ONYX-015 in both Tumor and Stromal Cells from a Patient with Gall Bladder Carcinoma Implants

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Introduction

ONYX-015 (CI-1042, dl1520) is a type 2, type 5 chimeric adenovirus that has been genetically modified by a deletion of the E1B portion of the viral genome (1). The virus has been used therapeutically as an anticancer agent with the goal of restricting replication to p53-mutated neoplasias, sparing p53 wild-type human tissues (reviewed in Ref. 2). Because virtually half of all malignant neoplasias have deregulated p53 pathways (3), this strategy appears feasible.

Preclinical studies have confirmed the expected anticancer activity for the ONYX-015 virus (4). A Phase I study demonstrated the tolerability of this agent in patients (5), and a Phase II study has demonstrated clinical activity in patients with head and neck cancer receiving single agent therapy with ONYX-015 (6). In a subsequent study in patients with head and neck cancer who received conventional chemotherapy with or without intraslesional injections of ONYX-015, greater clinical benefits were observed in the patients receiving combination therapy (7). Currently, a multicenter confirmational trial is ongoing in patients with squamous cell carcinomas of the head and neck.

The pharmacokinetics and mechanism of action of the ONYX-015 virus remain poorly understood. Recently, there has been some controversy as to whether prolonged detectable circulation of ONYX-015 genome demonstrates the presence of viral replication (8). Therefore, it would be useful to have a clearer understanding of ONYX-015 viral replication in tumor tissues to optimize its use in the clinic, especially when used in combination with other therapeutic modalities.

In a recent clinical trial investigating the clinical activity of ONYX-015 administered intrasesionally in patients with refractory tumors of the upper gastrointestinal tract (9), we were able to study the presence of virus in two adjacent abdominal wall implants from a primary gall bladder carcinoma at 37 h and 7 days after intraslesional injection. Samples were obtained for IHC analysis of viral presence; however, this did not provide evidence as to whether the virus was replicating.

The adenoviral genome is a linear, double-stranded molecule varying from 34–36 kb in most serotypes with inverted terminal repeats that contain the origins of DNA replication (10). There are eight transcription units transcribed from both strands, and each transcription unit produces multiple transcripts via alternate splicing and multiple polyadenylation sites. The adenoviral genome is transcribed in a complex temporal manner by RNA polymerase II; viral gene products are divided into two groups based on the sequence in which they are transcribed (3).

Abstract

Purpose: ONYX-015 is a chimeric, E1B-deleted adenovirus designed to replicate preferentially in p53-deficient tumor cells; however, little is understood about its actual replication potential in human tumors. We hypothesized that replication of a late viral gene, hexon, would demonstrate replication of virus in human tissues.

Experimental Design: In the course of a clinical trial, a patient with paired abdominal wall implants from a primary gall bladder carcinoma was injected with ONYX-015, 1 × 10^{10} viral particles/lesion, followed by sequential excision of the lesions at 37 h and 7 days. Tissue sections were analyzed for evidence of viral replication.

Results: In situ Reverse transcription-PCR was used to measure expression of hexon. Strong signals were observed in gland-forming tumor cells both at 37 h and at 7 days. Signal was predominantly observed in the cytoplasm. The signal was also observed in adjacent normal stromal cells. Analysis of p53 status of the tumor by immunohistochemistry and Affymetrix Genechip demonstrated an inactivating mutation in p53. Routine H&E staining of the tumor sections revealed no evidence of necrosis at 37 h or 7 days after injection of virus. Presence of viral protein at both 37 h and 7 days was confirmed by immunohistochemistry using antibodies directed against hexon, penton, and fiber proteins.

Conclusions: Evidence for replication of hexon confirms that ONYX-015 is not only present but capable of replicating in tumor cells up to 1 week after intraslesional injection and that replication is not confined to p53-mutated tumor cells.

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scribed. The immediate early genes, E1A, transactivate other early genes, E1B, E2, E3, and E4, which are mainly concerned with regulation of viral gene expression, DNA replication, and regulation of the host immune response to the viral infection. In contrast, late genes, L1 to L5, are transcribed from the major late promoter and mainly encode proteins composing the viral particle, which include the hexon proteins, the major virion coat protein accounting for 240 capsomers of the 252 subunits that comprise the capsid. Therefore, we reasoned that if there were evidence of late viral gene transcription in the injected tumor tissues, which we subsequently resected, this would be presumptive evidence of successful replication of ONYX-015.

Using in situ RT-PCR to measure expression of the late viral hexon gene, we were able to establish evidence for viral replication at both time points. Thus, these studies demonstrate in a metastatic human gall bladder carcinoma that ONYX-015 administered intralesionally is able to infect and replicate in human tumor cells in the clinical setting and that this replicative ability persists for at least 7 days. However, replication was not confined solely to the p53-mutated tumor cells but occurred in normal stromal cells as well.

**Materials and Methods**

**Cell Culture.** HT-29 cells (American Type Culture Collection, Manassas, VA), from a human colon carcinoma cell line, were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, and L-929 cells, from a murine fibroblast cell line which does not support replication of type 2 adenovirus, were grown in Eagle’s Minimum Essential Medium with 2 mM l-glutamine and Earle’s Balanced Salt Solution adjusted to contain 1.5 g/liter sodium bicarbonate.
Fig. 2 RT-PCR and Southern analysis of primers/probes. A, in HT-29 human colon cancer cells infected with ONYX-015, primers for hexon and for GAPDH generated unique transcripts at the expected sizes. B, Southern analysis of blots generated from the PCR products using a digoxigenin-labeled fluorescent probe for hexon or GAPDH mRNA generated unique bands at the expected sizes.

0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 10% fetal bovine serum, and 1% penicillin-streptomycin. All cells were maintained in a humidified atmosphere with 5% CO2 at 37°C. All cell culture products were purchased from Life Technologies, Inc. (Grand Island, NY).

**Tissue Acquisition.** Tissue was obtained from a 61-year-old female patient with metastatic gall bladder carcinoma enrolled in T98-0013, a Phase II study of ONYX-015 in primary hepatobiliary carcinomas, which was sponsored by the Cancer Therapy Evaluation Program, National Cancer Institute and approved by the Montefiore Medical Center Institutional Review Board and the Albert Einstein Comprehensive Cancer Center Protocol Review Committee. The results of this trial will be reported separately. Informed consent was obtained from the patient for participation in the protocol for surgical removal of tumors and for use of tissues for genetic studies. Two adjacent abdominal wall implants from a primary gall bladder carcinoma (Fig. 1) were each injected with ONYX-015, 1010 viral particles using a single direct injection into the center of the tumor. The implants were removed sequentially at 37 h and at 7 days under local anesthesia. After excision, tissue was immediately frozen and sections placed on slides suitable for in situ RT-PCR (Perkin-Elmer, Boston, MA) and fixed in 10% buffered formalin for 16 h. Sections were also obtained for routine H&E staining, for IHC studies, for viral coat proteins, and for p53 status. In addition, a tissue sample was obtained for genetic studies of p53 by Affymetrix Genechip microarray analysis.

**In Situ RT-PCR and Primers.** The methodology for in situ RT-PCR has been described previously (11, 12). Slides were rehydrated in graded ethanol solutions. Protease digestion was performed by treatment with 0.2% pepsin (Sigma Chemical, St. Louis, MO) in 0.01 M HCl for 45 min at room temperature, followed by DNase digestion with RNase-free DNase (Boehringer Mannheim, Indianapolis, IN), 0.1 M sodium acetate, 5 mM magnesium sulfate, and 15 units of DNase at 37°C overnight. First-strand cDNA was synthesized in intact cells under a coverslip using 60 μl of room temperature mixture (PCR buffer containing 5 mM MgCl2, 1 mM each of dATP, dTTP, dGTP, and dCTP, 50 units of RNase inhibitor, 125 units of Moloney murine leukemia virus reverse transcriptase (2.5 units/μl), and 5 pmol/μl of pdN6 as the primer for RT-PCR at 37°C for 1.5 h.

PCR was performed in 60 μl of PCR mixture containing PCR buffer with 5 mM MgCl2, 200 μM each of dATP, dTTP, dGTP, and dCTP, 12 units TaqDNA Polymerase (Perkin-Elmer), and 1.6 mM upstream and downstream primers (Table 1). All primers were designed by our laboratory and synthesized by Sigma Genosys Facility (Woodlands, TX). RT-PCR was carried out on an In Situ RT-PCR System 1000 (Perkin-Elmer) with initial denaturation at 94°C for 2 min followed by 30 cycles at 94°C for 1 min and annealing at 60°C for 2 min, and extension at 72°C for 2 min.

**In Situ Hybridization.** PCR products were detected as previously described (11, 12) by hybridizing with a region-specific E1B probe labeled with digoxigenin, 5′-TTGAGTCA-CACACTAAGATATT GCT-3′, a region-specific hexon probe labeled with digoxigenin, 5′-CACCCTGGCACCTGA CGACG-3′, and a region-specific GAPDH probe labeled with digoxigenin, 5′-CATGTTCGTCATGGGTGTGAA-3′ (Genosys, Woodlands, TX), respectively. Hybridization was performed at 42°C overnight in hybridization buffer [10% deionized formamide (Sigma Chemical), 10% (wt/vol) dextran sulfate (Sigma Chemical), 2× SSC, 5 pmol/100 μl probe]. After hybridization, slides were washed in 1× SSC and 0.2% BSA for 10 min at 54°C, followed by washes in Buffer A [0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl] and then incubated at room temperature for 30 min in 1:50 diluted antidigoxigenin-AP Fab fragments (Boehringer Mannheim) in Buffer A. After a rinse in Buffer B [0.1 M Tris-HCl (pH 9.5), 0.15 M NaCl, 50 mM MgCl2] at room temperature for 5 min, the slides were incubated in nitroblue...
tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim) at a ratio of 1:400:1 for nitroblue tetrazolium:buffer:5-bromo-4-chloro-3-indolyl phosphate.

Internal controls on each slide included a tissue section treated with reverse transcriptase, but not DNase (positive control), and a tissue section treated with DNase, but not reverse transcriptase (negative control). External controls, used with each experiment, used in situ RT-PCR analysis for expression of the housekeeping gene, GAPDH, which also included its own internal positive and negative controls.

**IHC and Antibodies.** IHC was performed on formalin-fixed, paraffin-embedded tissue sections. The antibodies for IHC, antifiber, antihexon, and antipenton antibodies were described previously (13, 14). IHC staining was performed using a TechMate 500 automated immunostainer (Ventana Medical Systems, Tucson, AZ), according to a modified macrophage inflammatory protein protocol from the manufacturer. Briefly, paraffin tissue sections (4-μm thick) were deparaffinized in a series of xylexes and rehydrated in descending concentration of alcohols. Before staining, slides were pretreated with 0.1% trypsin (pH 7.8) at 37°C for 10 min. All three polyclonal rabbit antibodies (hexon, penton, and fiber) raised against the individual viral proteins purified from Ad2-infected HeLa cells were used at 1:1000 dilution, using ChemMate ABC peroxidase sec-

Fig. 3  In situ RT-PCR studies of HT-29 cells infected with ONYX-015. Cells were assayed for expression of GAPDH (a, c, e) or hexon (b, d, f) at 48 h after infection. Positive controls (a and b) demonstrate both strong nuclear and cytoplasmic staining. In cells treated with DNase to eliminate genomic DNA, both GAPDH (c) and hexon (d) mRNA are detected primarily in the cytoplasm. Negative controls for both (treated with DNase and without reverse transcriptase) demonstrate no signal (e and f).
ondary detection system (Ventana Medical Systems). The per-
oxidase reaction was developed using liquid 3,3'-diaminoben-
zidine substrate chromogen provided in the kit. A positive
control was included with paraffin sections of formalin-fixed
section made from cultured HT-29 cells infected with the
same adenovirus. The sections were stained with a mouse an-
tiadenovirus monoclonal antibody (blend of clones 20/11 and
2/6) raised against adeno 3 (MAB805; Chemicon International,
Temecula, CA) with specificity for the 41 adenoviral serotypes
tested at 1:1000 dilution (data not shown). Negative controls
with nonimmune rabbit IgG or mouse IgG were run in parallel.

HIC staining with p53 antibody (diluted 1:50; DAKO) was
performed on the DAKO Autostainer Universal Staining System
and developed using the DAKO EnVision™, mouse peroxidase
kit with DAKO 3,3'-diaminobenzidine chromogen.

p53 Analysis by Microarray. Qiagen DNA Mini kits
(Qiagen, Inc., Valencia, CA) were used to extract genomic DNA
from tumor samples. The genomic DNA was amplified using the
GeneChip p53 primer set (Affymetrix, Santa Clara, CA) and
AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster
City, CA) according to the Affymetrix instructions for p53
target preparation. The coding regions of the human p53 gene
were amplified as 10 separate amplicons in a single multiplex
reaction. The DNA amplicons were then fragmented using the
GeneChip Fragmentation Reagent (Affymetrix) according to
manufacturer’s instructions. Fragmented DNA amplicons were
labeled at their 3' ends with a fluorescein-dideoxynucleotide
fluorescein-ddCTP) and the BioArray terminal labeling kit for
dNA probe array assays (Enzo Diagnostics, Farmingdale, NY)
according to manufacturer’s instructions. Labeled fragments
were then placed in hybridization buffer (6× saline-sodium
phosphate-EDTA, 0.05% Triton X-100 (Sigma Chemical), 2
mg/ml acetylated BSA (Life Technologies, Inc.), and 2 nt
control oligonucleotide F1 (Affymetrix). GeneChip p53 probe
arrays (Affymetrix) were hybridized to labeled fragments and
washed on the GeneChip Fluidics Station 400 according to
manufacturer’s instructions. The probe arrays were scanned
(GeneArray Scanner 2508; Affymetrix) and analyzed using the
Microarray Suite software version 5.0 (Affymetrix).

Results

Specificity of the in Situ RT-PCR Methodology. As
shown in Fig. 2A, RT-PCR analysis of ONYX-015 infected cell
extracts using primers for hexon and GAPDH resulted in unique
transcripts of the expected molecular weights. As shown in Fig.
2B, digoxigenin-labeled probes for hexon and GAPDH trans-
scripts identified unique transcripts of the expected molecular
weights.

Human colon carcinoma HT-29 cells were infected with
ONYX-015 in vitro at a multiplicity of infection of 1, which
results in infection in nearly all cells. As shown in Fig. 3, in situ
RT-PCR demonstrated strongly positive staining in positive
control cells (treated with reverse transcriptase), positive stain-
ing in cells treated with both DNase and reverse transcriptase,
et and essentially no staining in negative controls (treated with
DNase but not reverse transcriptase). In the cells treated with
DNase and reverse transcriptase, staining was confined, as ex-
pected to the cytoplasm, whereas in the positive controls, both
cytoplasm and nucleus were strongly positive, as expected.

To confirm the specificity of the method, in situ RT-PCR
studies were performed in murine L929 cells, which allow entry
of adenovirus and therefore contain viral DNA but which do not support transcription of viral late genes (15). As shown in Fig. 4a, in positive controls, treated with reverse transcriptase, high levels of the viral hexon transcripts were detected, whereas no evidence of transcription was detected in the cells treated with DNase with (Fig. 4b) or without (negative controls, Fig. 4c) reverse transcriptase, indicating that all transcripts in the positive controls were from the hexon DNA and not hexon mRNA. In Fig. 4a, staining was confined to the nucleus, additional confirmation that DNA, but not mRNA, was transcribed.

Absence of Necrosis in the Metastatic Lesions after Viral Replication. An H&E-stained section of the gall bladder abdominal wall implant resected at 7 days after intratumoral injection of ONYX-015 is shown in Fig. 5. The representative section shows scant tumor cells, some in glandular patterns, surrounded by dense stromal tissue (Fig. 5A). There is no evidence of tumor necrosis or apoptosis and only scant evidence of an inflammatory reaction around the tumor cells at either time point.
p53 Analysis. Status of the p53 genome was assessed by IHC and Genechip analysis (16). Both nodules demonstrated overexpression of p53 by IHC (58 and 84% positive; data not shown), indicating the presence of a mutation in the genome. Affymetrix studies demonstrated a g→c (arginine to proline) mutation at codon 273, which is an inactivating mutation.

Fig. 6 In situ RT-PCR analysis of GAPDH expression in an abdominal wall implant at 37 h after ONYX-015 injection (×400). a, a representative section treated with reverse transcriptase but not DNase (positive control) demonstrates positive staining of both DNA and mRNA. b, there is staining of GAPDH mRNA in the sections treated with reverse transcriptase and DNase, which clearly differs from c in which the sections were treated with DNase alone (negative control). Fine, blue, granular, cytoplasmic staining is observed both in the larger tumor cells (white arrows) and in the adjacent stromal cells (black arrowheads). Identical findings were observed in tissues excised at day 7 (data not shown).

Fig. 7 In situ RT-PCR analysis of viral hexon expression in an abdominal wall implant from a primary gall bladder carcinoma at 37 h after injection of ONYX-015 (×400). a, positive controls (treated with reverse transcriptase, without DNase) show robust staining, indicating reverse transcription of hexon mRNA and/or hexon DNA in both tumor cells (white arrows) and stromal cells (black arrowheads). b, in sections treated with both DNase and reverse transcriptase, there is cytoplasmic staining, characterized by fine, blue granules, in both the larger tumor cells (white arrows) and the adjacent stromal cells (black arrowheads), indicating reverse transcription of hexon mRNA. c, negative controls (DNase only) demonstrate nearly complete absence of signal, indicating the efficacy of the DNase treatment.
Presence of Viral Replication in Gall Bladder Carcinoma Implants at 37 h and 7 Days. Expression of GAPDH, used as an external control for all experiments, was identified by in situ RT-PCR in all tumor sections studied (Fig. 6b). ONYX-015 was present in both stromal and tumor cells at both 37 h and 7 days after intratumor injection, as confirmed by the strong staining in the positive controls (treated with reverse transcriptase but not DNase, Fig. 7a) and by the weaker, but clearly positive staining shown in Fig. 8a. There is clear evidence of expression of hexon mRNA in sections treated with DNase and reverse transcriptase at both 37 h (Fig. 7b) and 7 days (Fig. 8b). At higher magnification, Fig. 9, replication of hexon mRNA can be observed in the cytoplasm of tumor cells forming a glandular pattern, as well as normal stromal tissues, which are represented by abundant spindle cells adjacent to the tumor cells. The less intense staining observed at 7 days (Figs. 8b and 9b) as compared with 37 h (Figs. 8a and 9a) is consistent with a lower viral burden at this time point.

As expected, negative controls (treated with DNase and without reverse transcriptase, Figs. 7c and 8c) demonstrated no evidence of signal. This confirmed the efficacy of the DNase treatment. Furthermore, in tissue sections from an irrelevant, viral-naive tumor, no signal was detected using this assay (data not shown).

Detection of Viral Protein in Gall Bladder Carcinoma Implants by IHC at 37 h and 7 Days after ONYX-015 Injection. Tissue sections were analyzed by IHC using antibodies against viral hexon, penton, and fiber proteins. As shown in Fig. 10, IHC analysis using antihexon antibodies of tissue sections both at 37 h and 7 days after ONYX-015 injection demonstrate presence of virus both in the larger tumor cells, which are forming a glandular pattern, as well as normal stromal tissues, which are represented by the abundant spindle cells adjacent to the tumor cells. IHC studies using antibodies to penton and fiber proteins confirmed this finding, although much lower levels of positivity were noted (data not shown).

Discussion
Preclinical studies using ONYX-015 activity have demonstrated effective infection of and replication in human tumor explants by the virus. In athymic nu/nu mice with bilateral s.c. C33A cervical carcinoma implants, i.v. injection of ONYX-015, 10⁹ pfu by intratumoral injection resulted in the presence of virus in the contralateral, as well as the injected tumor, as assessed by IHC staining (17). Remarkably, after tail vein injection of ONYX-015, 10⁹ pfu, high titers of virus initially detected in the liver, the main site of virus deposition after i.v. injection decreased by 1000-fold to undetectable levels at 72 h, whereas virus titers in tumors increased 150-fold between 3 and 72 h after injection. Finally, 10 daily injections of ONYX-015, 10⁹ pfu in nu/nu mice bearing HCT116 tumor xenografts resulted in some complete tumor regressions with extensive viral replication-associated cytopathology and necrosis and the presence of virus by in situ hybridization.

The clinical situation is somewhat different. In a controlled Phase II trial of fluorouracil, cisplatin, and ONYX-015 in patients with squamous cell carcinoma of the head and neck, metastatic lesions injected with the virus demonstrated necrosis and adenovirus-associated cytopathic changes as assayed by in situ hybridization that were considered indicative of adenoviral replication (7). In another recent Phase II trial of intralesional
injection of ONYX-015, $2 \times 10^{11}$ particles for 5 consecutive days or twice daily for 2 consecutive weeks, circulating ONYX-015 genome was detectable in 41% of patients on days 5 and 6 of cycle 1, in 9% of patients on day 11 of cycle 1 and in no patients at 22 days using a TaqMan assay designed to amplify an amplicon of 92 nucleotides that is specific for ONYX-015 DNA (18). The authors concluded that this was “suggestive of intratumoral replication.” In contrast to the animal studies in which extensive cytopathic effects and necrosis were observed after i.v. injection, only modest antitumor efficacy was observed in this study.

The suggestion that the presence of viral genome as detected by TaqMan assay is evidence for viral replication has been disputed (8). Specifically, among 190 subjects treated with the replication-incompetent RPR/INGN 201 adenovirus, vector-related sequences were detected in urine up to 28 days after the final injection of virus. This demonstrates that detection of viral genome, even persistently, is not equivalent to demonstrating viral replication in biological samples.

To address this problem, we used in situ RT-PCR methodology, modified from a method developed in our laboratory (11, 12), on tissue samples from metastatic lesions injected with ONYX-015, $1 \times 10^{10}$ pfu, at 37 h and 7 days. This method has two advantages as compared with alternative assays such as real-time PCR. First, it is capable of detecting the presence of virus in specific cells, thus distinguishing alterations in gene expression in tumor cells from gene expression in adjacent normal cells, as opposed to detecting viral genome in body fluids or in homogenized tumor or biopsy specimens. Second, it is capable of detecting, and in some cases quantifying, gene expression, as opposed to detecting the presence of viral genomic material.

Rigorous controls, including both positive and negative controls for hexon and for an external standard, GAPDH, were used. Furthermore, exhaustive in vitro studies validated this methodology. The mouse L cell experiments are particularly relevant in this regard as clearly distinguishing viral replication from presence of nonreplicating viral DNA (19).

Our hypothesis was that expression of the hexon gene, a representative late gene (10), would demonstrate that the virus was replicating in tumor tissue, rather than merely being present in a nonreplicative state. One caveat is that although expression of a late viral gene is presumptive evidence of viral replication, nevertheless, we have not demonstrated that the viral protein assembles into virion in these cells, which would require transmission electron microscopy studies. Furthermore, the absence of cellular necrosis or cytopathologic changes observed in the explanted tumor models described above suggests that viral
replication may be occurring at a relatively low level. Nevertheless, the data presented does appear conclusive for viral replication occurring in a sustained fashion.

The proliferation of virus within tumor cells is not unexpected based on the hypothesis that ONYX-015 will replicate in p53-mutated cells (20). The data supporting a mutation in p53 in these tumors is quite strong. The IHC was strongly positive, indicating the presence of a mutation in the genome, which stabilizes the protein and increases the normally short half-life. In addition, a mutation from arginine to proline at codon 273 was observed. This is one of the amino acids that directly interacts with DNA at the protein-DNA interactive site of p53; therefore, it is likely that this mutation impedes the regulatory function of p53.

It is of considerable interest that viral replication was observed, not only in tumor cells, but in adjacent, normal, presumably p53 wild-type stromal cells in our tumor specimens. The necessity for p53 pathways to be nonfunctional in order for ONYX-015 replication to occur is another ongoing controversy. Despite the original design of the virus as selectively replication-competent only in p53-deficient cells, there seems to be very good evidence that the ONYX-015 virus is capable of replicating in p53 wild-type cells (4, 21, 22). Replication may not occur as efficiently in p53 intact cells both in vitro (23) and in the clinical setting (18). Furthermore, aberrations in the p53 pathway, including changes in other components of the p53 pathway, such as p14ARF or MDM2, may be functional surrogates for p53 mutation in cells with intact p53 (24, 25). Perhaps an inactivating mutation in p53 allows more rapid viral replication but is not required for viral replication, even in the absence of other mutations or alterations in the p53-Rb pathway. The presence of viral hexon mRNA in the spindled stromal cells adjacent to the tumor cells may indicate that ONYX-015 can replicate in normal p53 wild-type cells. Additional studies are required to demonstrate that these spindle cells are not in fact tumor cell variants and that they don’t have inactivated p53 pathways.

The absence of necrosis or cytopathic changes in the metastatic tumors used in this study, despite evidence of viral proliferation, is of interest and contrasts with the studies in head and neck cancer cited above (7, 18). Even in specific cells where viral replication was shown to be occurring, there did not appear to be any evidence of necrosis, either in those cells or in adjacent cells. One possibility is that the level of viral replication was too low to induce cellular necrosis. This is consistent with the dosages of ONYX-015 used in our study, which were ~100-fold lower than those used in the head and neck study (18). An alternative hypothesis is that ONYX-015 is acting by another mechanism, which does not induce cell death by replication of virus and cell lysis, e.g., by induction of apoptosis or immune stimulation to cancer or viral antigens on the tumor cell surface. A recent study suggests that the E1A portion of the adenoviral genome has cell cycle regulatory properties and specifically that...

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**Fig. 10**  IHC analysis of hexon protein at 37 h and 7 days (×400). Hexon protein is expressed in both the large, gland-forming tumor cells (white arrows) and adjacent spindled stromal cells (black arrows) at both time points.
it represses expression of cyclin D1 (26). Studies in the in vitro setting support such a hypothesis.4 Therefore, ONYX-015 replication at the tumoral level may result in multiple, cell type-dependent effects, including cell necrosis in some tissues and cytostasis in other tissues. This hypothesis remains to be confirmed.

In summary, this is the first demonstration of ONYX-015 replication in human tumor specimens both at 37 h and 7 days after injection using rigorous criteria, rather than presumptive evidence. The absence of tumor necrosis in sections, which clearly demonstrate viral replication, suggest that in these tumors, replication may be occurring at a low level. This differs from previous preclinical studies using human tumors explanted to nu/nu mice and also from previous studies in patients with squamous cell carcinoma of the head and neck. Furthermore, our data suggest that there may be multiple mechanisms by which ONYX-015 treatment results in an anticancer effect.

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