Oncolytic Activity of the E1B-55 kDa-deleted Adenovirus ONYX-015 Is Independent of Cellular p53 Status in Human Malignant Glioma Xenografts

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ABSTRACT

Treatment of malignant gliomas remains a major challenge in adults and children because of high treatment failure. The E1B 55 kDa-gene deleted adenovirus, ONYX-015 (ONYX Pharmaceuticals), was demonstrated to replicate selectively in and lyse tumor cells. Currently ongoing clinical trials of ONYX-015 in head and neck tumors are promising.

Here, we demonstrate ONYX-015-mediated cell lysis and antitumor activity in three of four s.c. human malignant glioma xenografts deriving from primary tumors. Intratumoral injections of ONYX-015, 1 × 10^8 plaque-forming units daily for 5 consecutive days, yielded significant tumor growth delay in the p53 mutant xenografts IGRG98 and the p53 wild-type IGRG93 and IGRG121 treated at an advanced tumor stage. The p53 wild-type tumors IGRG93 and IGRG121 experienced 45% and 82% complete tumor regressions. Four and 8 of 11 animals, respectively, survived tumor free 4 months after treatment. Widespread intratumoral adenoviral replication was observed in tumor cells of these two xenografts compared with only scattered replication in the p53-mutant tumors. In addition to a fast tumor growth rate, wild-type p53 status was associated with increased antitumor activity of the E1B-attenuated virus, and induction of functional p53 may therefore determine adenoviral cytolysis in tumor cells.

In conclusion, ONYX-015 displayed a major antitumor activity in human xenografts derived from primary malignant glioma supporting its development in the treatment of these highly malignant tumors.

INTRODUCTION

Anaplastic astrocytoma and glioblastoma, the most common primary brain tumors in adults, are quite refractory to current treatment such as chemotherapy and radiation therapy, with median survival rarely exceeding 1 year (1). About 30–50% of gliomas harbor a p53 mutation; others reveal MDM2 amplification/overexpression or deletions of the CDKN2A/p14ARF tumor suppressor gene that may render the gliomas resistant to therapeutic approaches (2–4). Treatment failure in patients with brain tumors is a multifactorial process involving intrinsic resistance of these tumors to irradiation and chemotherapy, development of acquired treatment resistance, and limitations of drug delivery to the tumor site because of poor tumor vascularization and/or blood-brain barrier restrictions (5, 6). Local recurrence of a brain tumor represents the most common feature of treatment failure. Hence, the identification of new therapeutic agents or strategies with high intrinsic activity against brain tumors remains a challenge.

Conditionally replicative adenoviruses that can selectively replicate in and cause lysis of tumor cells but spare normal cells have been introduced recently as new therapeutic strategies. The E1B 55 kDa-gene defective adenovirus ONYX-015 (ONYX Pharmaceuticals) is currently undergoing clinical trials for the treatment of head and neck cancers with apparently promising results (7–9). This virus has an 800-bp deletion in the E1B region encoding the 55 kDa protein in infected cells, which binds and inactivates cellular p53. Therefore, the E1B-attenuated adenovirus is thought to replicate efficiently and cause cytotoxic effects in tumor cells lacking functional p53 (10, 11). Nevertheless, conflicting discussion has been raised about the selectivity of ONYX-015, because it has been shown to replicate in and lyse tumor cells irrespective of their p53 status (12–16). Mechanisms underlying the replication of ONYX-015 in tumor cells expressing wild-type p53 still remain to be defined. Sensitivity to lytic activity of ONYX-015 in p53-wt cells might be attributable to gene mutations outside exons 5–9 or inactivation of p53 by other mechanisms, such as loss of p14ARF, expression of human papilloma virus E6, E4orf6 protein, or MDM2 amplification (17–21). Although ONYX-015 has shown antitumor activity in vitro and in vivo against a wide spectrum of different tumor cells, only a little is known about its activity in brain tumor cell lines (11, 12). However, cell lines and tumor xenografts derived from cell lines differ from patient tumors because of prolonged in vitro selection and clonal origin. Human tumor xenografts directly deriving from primary tumors show stability in cytogenetic marker to the initial tumor and are, therefore, advantageous in their use for preclinical in vivo evaluation of antitumor treatments (22, 23).

Here we demonstrate adenoviral replication and cytolytic efficiency of ONYX-015 in human malignant glioma xenografts derived from primary tumors independent of their cellular p53 status. Moreover, wild-type p53 was associated with increased antitumor activity of the E1B-attenuated virus, and induction of functional p53 may determine adenoviral cytolysis in tumor cells. Our results support strongly the use of this attenuated replicative adenovirus in the treatment in malignant gliomas.

MATERIALS AND METHODS

Adenovirus. ONYX-015 is a chimeric human group C adenovirus (Ad2 and Ad5) that contains a deletion between nucleotides 2496 and 3323 in the E1B region encoding the 55-kDa protein (24). In addition, a C→T transition at position 2022 in E1B generates a stop codon at the third codon position of the protein. The virus was generously provided by ONYX Pharmaceuticals. Virus was stored at −80°C and aliquoted at the time of usage. During experiments, the virus was kept in LabTopcooler (Nalgene) performance at 20°C.

Animals. Female SPF-Swiss nude mice were bred in the Animal Experimentation Unit at the Institut Gustave-Roussy (Villejuif, France). The strain...
was obtained from Carl Hansen (NIH, Bethesda, MD) in 1976. Animals were housed in sterile isolators and fed with irradiated nutrients and filtered water ad libitum. Experiments were carried out under the conditions established by the European Community (Directive 86/609/EEC).

**Xenografts.** All four of the malignant glioma xenografts used in this study were derived from primary tumors by s.c. transplantation of small fragments in athymic mice irradiated previously (23). IGRG82 was established from a malignant glioma in a 7-year-old girl. IGRG88 was established from a primary hemispheric malignant glioma in a 60-year-old female (25). The xenograft was a hypertelaplped tumor with the classical histological features and chromosomal alterations of a malignant glioma and the following karyotype: 90–106, XXXX, +1, +7 × 2, −9, der9t (Refs. 9, 22; p21q11) × 3, −10 × 2, −11, −14 × 2, +16 × 2, +17, +18 × 2, +19 × 2, +21, −22 × 2. IGRG93 was established from a glioblastoma brain tumor in a 69-year-old woman. The diploid tumor had the karyotype: 47, XX, +7, der(3;10; q25;q11), +double minutes. IGRG121 was established from a hemispheric glioblastoma in a 59-year-old man. This diploid tumor had the following karyotype: 47–48, XY, 1p−, +7,i(9q), −16, +2 mars. All of the xenografts were maintained in vivo by sequential passaging from s.c. implants with an engrafment success rate >75%.

**Experimental Design.** Antitumor activity against unilateral advanced stage tumors was evaluated as described previously (26). Tumor fragments (30 mm³) were xenotransplanted s.c. in 60–100 athymic mice 6–8 weeks of age. On day 0 of treatment, animals bearing s.c. tumors of 100–300 mm³ were pooled and randomly assigned to treatment groups. Two tumor perpendicular diameters were measured three times weekly with a caliper. Each tumor volume was calculated according to the following equation: V (mm³) = width² (mm³) × length (mm)/2. The experiments lasted until tumor volumes reached 1500–2000 mm³ or were stopped after 120 days if animals were tumor-free.

**Adenoviral Treatment.** Animals were placed in a special device system with constant air renewal and negative air pressure. Animals were anesthetized with ketamine/xylazine i.p. ONYX-015 was administered with constant air renewal and negative air pressure. Animals were anesthetized for the injection procedure using ketamine/xylazine i.p.

**In Situ Hybridization of Adenoviral DNA.** In situ hybridization was performed on formalin-fixed, paraffin-embedded tissue cut into 5-μm sections as described (36). In brief, tissue sections were digested with proteinase K and fixed in 4% paraformaldehyde. Hybridization was carried out overnight at 37°C with 0.5 μg/ml biotinylated adenovirus DNA probe (Enzo Diagnostics, Madison, WI) and a Lumat LB 9507 luminometer (EG&G Berthold, Bad Eggingen, MA) and purified on G50-Sephadex columns. Hybridization was run overnight in Church buffer (1 mM EDTA, 0.5 mM NaHPO₄, 7% SDS) with 100 mg of salmon sperm DNA.

**Gene Copy Dosage by Real-Time Quantitative PCR for p14 Gene Deletions.** Nucleic acids were extracted from frozen tumor samples using Qiagen DNeasy Tissue kit (Qiagen GmbH, Berlin, Germany), according to the manufacturer’s instructions. Quantification of p14 gene copy numbers was done by real-time quantitative PCR using the ABI PRISM 7700 Sequence Detection System (PE Biosystems). p14 gene copy numbers in the sample were normalized by copy number of two internal control genes, glyceraldehyde-3-phosphate dehydrogenase and albumin. The normalized gene dose, N, was obtained by calculating the ratio of the starting copy number of target gene:starting copy number of reference gene (31). All of the samples were performed in triplicates. PCR primer sequences are available on request.

**Analysis of p14ARF Coding Sequence.** We screened for mutations in CDN2A exon 18 and exon 2 by denaturing high performance liquid chromatography analysis, an automated heteroduplex detection method (32).

**CAR Expression.** Expression of human CAR was determined by cytometry using the monoclonal antibody RmcB (33), as described previously (34). Briefly, short-term cultures of tumor cells isolated from xenografts by mechanical dissociation were incubated with the primary antibody 10 μg/ml for 1 h. Controls were incubated with buffer or mouse immunoglobulin only. Subsequent detection was performed with a FITC-conjugated rabbit antirabbit antiserum (Dako, Glostrup, Denmark). Cells were fixed in 1% formaldehyde/PBS and analyzed on a FACScan (Becton Dickinson, Erembodegem-Aalst, Belgium).

**Monitoring of Infection Using Adluciferase Virus.** Short-term cultures of glioma cells (10⁵/well) in triplicates were incubated with AdCMVLuc at a concentration of 10 or 100 pfu/cell for 1 h. Luciferase activity in the cells was assayed 24 h after infection using the Luciferase Assay System (Promega, Madison, WI) and a Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany). Values were normalized per number of viable cells (trypan blue exclusion). The infectability of glioma cells was defined by the amount of luciferase activity measured after adenoviral-mediated gene transfer in the tumor cells.

**Statistical Analysis.** Statistical significance between treatment groups and controls in their time to reach five times initial tumor volume was estimated by the two-tailed nonparametric Mann-Whitney test. Tumor regression was defined as red colonies. p53 cDNA was extracted from mutant colonies and sequenced. The plasmid pCMVβ-LacZ, containing the LacZ gene under the ubiquitous CMV promoter, was used for positive control. The plasmid pGEM3z f- missing the LacZ gene was used for negative control.

**Northern Assay of MDM2 mRNA Expression.** Total RNA from frozen tumor samples was extracted using a chemical technique (Ref. 30; RNAble; Eurobio, Les Ulis, France). Total RNA (10 μg) from each sample was separated by gel electrophoresis 3-morpholinopropanesulfonic acid buffer and transferred overnight with standard techniques. The DNA probes were labeled with the Prime-It Random Primer Labeling kit (New England Biolabs, Beverly, MA) and purified on 50% Sephadex columns. Hybridization was run overnight in Church buffer (1 mM EDTA, 0.5 mM NaHPO₄, 7% SDS) with 100 mg of salmon sperm DNA.

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Inc. Farmingdale, NY). Detection was performed with an alkaline phosphatase-conjugated antibody (Vector Laboratories) and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. Slides were counterstained with nuclear fast red.

**IHC for Adenoviral Hexon Protein and Human p53.** The primary polyclonal antibody AB 1055 (Chemicon International, Temecula, CA) is specific for hexon protein of adenovirus type 2. Cellular p53 was detected by the antihuman p53 monoclonal antibody (DO-7, Dako). Formalin-fixed, paraffin-embedded tumors cut into 4-μm thick sections and rehydrated were incubated for 1 h at 35°C with an antibody dilution of 1:300 and 1:50, respectively. Detection was performed by a biotinylated rabbit secondary antibody to goat and mouse immunoglobulin, respectively, streptavidin-horseradish peroxidase conjugate (Dako), and the chromogen diaminobenzidine. p53 was detected by Inc. Farmingdale, NY). Detection was performed with an alkaline phosphatase-conjugated antibody (Vector Laboratories) and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. Slides were counterstained with nuclear fast red.

**RESULTS**

**p53 Pathway in the Human Malignant Glioma Xenografts.** We first characterized the four xenografts used in this study concerning their cellular functional p53 status. We performed the FASAY transactivation function test with the pRGC-Δfos-LacZ reporter plasmid and sequenced the mutations of the p53 gene that were detected by the FASAY. The well-described p53 target proteins p21/WAF1 and MDM2 were investigated in the xenografts at baseline and 16 h after 5 Gy total body irradiation of the mouse. Furthermore, we determined p14ARF gene deletions and MDM2 expression, both described to have the ability to abrogate p53 function (37).

Table 1 gives a summary of the p53 pathway in the malignant glioma xenografts. In the xenografts IGRG82 and IGRG88, the FASAY revealed mutated p53. IGRG82 had a mutation in intron 4 with a G to A transition introducing an alternative splicing site and consequently a deletion of the 21 first nucleotides of exon 5 (codons 126 to 132). IGRG88 had a mutation in exon 4, with a T to A transition at codon 113, transforming phenylalanine to isoleucine. Only red colonies were observed demonstrating unizygocity and, therefore, loss of the wild-type allele. No other mutations were found in the tumors suggesting loss of heterozygosity at the other p53 locus. Absence of transactivation function of the protein was documented in primary culture from xenografts after transfection with pRGC-Δfos-LacZ reporter plasmid in IGRG82 and IGRG88. Expression of the p53 protein was high in both xenografts and increased moderately after irradiation (Fig. 1). The p21/WAF1 and MDM2 proteins were not induced after irradiation as expected from the p53 status. No overexpression of MDM2 mRNA or protein was found by Northern and Western blot analysis. Sequencing of the p14ARF gene (1β and 2 exons) revealed a wild-type p14 gene in both tumors. No deletion of the p14 gene was found in the IGRG121 xenograft by gene copy dosage real-time PCR. The IGRG93 tumor cells had a loss of one allele. We can conclude that the wild-type p53 protein is functional in IGRG121; however, it is impaired in IGRG93.

**Wild-type p53 Is Associated with Increased Oncolytic Activity of ONXY-015 in Glioblastoma Xenografts.** ONXY-015 was administered intratumorally at doses of 1 × 10⁶ pfu/injection on 5 consecutive days to mice bearing advanced stage s.c. tumor xenografts of the p53 wild-type IGRG121 and IGRG93, and the p53 mutant IGRG88 and IGRG82 (Table 2; Fig. 2). Intratumoral injections of ONXY-015 into p53 wild-type IGRG121 tumors yielded 82% complete and 9% partial tumor regressions, and resulted in significant tumor growth retardation of >111 days compared with controls (P < 0.001; Fig. 2, A and B). Eight of 11 animals survived tumor-free dosage carried out by real-time PCR. We could conclude that there is no functional p53 in these two xenografts.

In the xenografts IGRG93 and IGRG121, the FASAY did not detect any mutated p53. Presence of transactivation function of the p53 protein was established in primary cultures from xenografts after transfection with pRGC-Δfos-LacZ reporter plasmid in IGRG121. By contrast, transactivation function of the protein was impaired in the IGRG93 tumor. Stabilization and increased expression of the p53 protein was induced in both xenografts by irradiation (Fig. 1). Whereas radiation induced expression WAF1/p21 and MDM2 protein in the IGRG121 tumor, this could not be detected in IGRG93 (Fig. 1). MDM2 was not overexpressed in the tumors, as determined by Northern and Western blot analysis. Sequencing of the p14ARF gene (1β and 2 exons) revealed a wild-type p14 gene in both tumors. No deletion of the p14 gene was found in the IGRG121 xenograft by gene copy dosage real-time PCR. The IGRG93 tumor cells had a loss of one allele. We can conclude that the wild-type p53 protein is functional in IGRG121; however, it is impaired in IGRG93.

**Table 1** Analysis of the p53 pathway in the four malignant glioma xenografts.

<table>
<thead>
<tr>
<th>Xenograft</th>
<th>FASAY% red colonies</th>
<th>p53 mutationa</th>
<th>p53 transactivation functionb</th>
<th>MDM2 expressionc</th>
<th>p14ARFd</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGRG82</td>
<td>100%</td>
<td>Deletion in intron 4</td>
<td>Negative</td>
<td>Normal</td>
<td>Deletion</td>
</tr>
<tr>
<td>IGRG88</td>
<td>100%</td>
<td>Transition G → A</td>
<td>Negative</td>
<td>Normal</td>
<td>Deletion</td>
</tr>
<tr>
<td>IGRG93</td>
<td>1%</td>
<td>Wild-type</td>
<td>Impaired</td>
<td>Normal</td>
<td>Heterozygotic</td>
</tr>
<tr>
<td>IGRG121</td>
<td>3%</td>
<td>Wild-type</td>
<td>Positive</td>
<td>Normal</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>

a Functional assay of separated alleles in yeast.  
b Gene sequencing.  
c RGC transactivation test: in vitro primary cell cultures derived from xenografted tumors were transfected with the pRGC-Δfos-LacZ reporter plasmid. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside was added 48 h after transfection. Blue colored cell were the intracellular synthesis of β-galactosidase and, therefore, a functioning protein able to transactivate the RGC.  
d Northern and Western blot analysis.  
e Quantification of gene copies by real-time PCR. All samples were measured in triplicates. Gene sequencing.
ONXY-015 IN MALIGNANT GLIOMA XENOGRAFTS

Table 2  Antitumor activity of ONXY-015 (intratumoral injection) in s.c. malignant glioma xenografts in athymic mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>DT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CR&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PR&lt;sup&gt;c&lt;/sup&gt;</th>
<th>5 × vol.</th>
<th>TGD&lt;sup&gt;d&lt;/sup&gt;</th>
<th>TFS&lt;sup&gt;e&lt;/sup&gt;</th>
<th>p&lt;sup&gt;f&lt;/sup&gt;</th>
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<tr>
<td>IGRG121 (wild-type p53)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>8</td>
<td>2.9</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>120</td>
<td>&gt;111</td>
<td>8</td>
</tr>
<tr>
<td>ONXY-015</td>
<td>11</td>
<td>4</td>
<td>1</td>
<td>&gt;120</td>
<td>&gt;111</td>
<td>8</td>
<td>&lt;0.001</td>
<td></td>
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<tr>
<td>IGRG93 (wild-type p53)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>8</td>
<td>5.0</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ONXY-015</td>
<td>11</td>
<td>4</td>
<td>4</td>
<td>47</td>
<td>36</td>
<td>4</td>
<td>&lt;0.001</td>
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<tr>
<td>IGRG88 (mutant p53)</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>5.1</td>
<td>0</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ONXY-015</td>
<td>12</td>
<td>9</td>
<td>38</td>
<td>24</td>
<td>0</td>
<td>0.001</td>
<td></td>
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<tr>
<td>IGRG82 (mutant p53)</td>
<td></td>
<td>9.6</td>
<td>0</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ONXY-015</td>
<td>12</td>
<td>9.6</td>
<td>0</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Median tumor doubling time.  
<sup>b</sup> Complete regression.  
<sup>c</sup> Partial regression.  
<sup>d</sup> Tumor growth delay.  
<sup>e</sup> Tumor-free survival day 120.  
<sup>f</sup> For each experiment, the 5 × volumes in the treatment group were compared with those of the control group using a two-tailed nonparametric Mann-Whitney test.

120 days after treatment. In the p53 wild-type IGRG93 tumors, ONXY-015 induced 45% complete and 36% partial tumor regressions and a significant tumor growth delay of 36 days compared with controls (P < 0.001; Fig. 2, C and D). Four of 11 animals survived tumor-free 120 days after treatment. Intratumoral injections of ONXY-015 into p53-mutant IGRG88 tumors achieved 8% complete and 75% partial tumor regressions, and a significant tumor growth delay of 24 days compared with controls (P = 0.0001; Fig. 2, E and F). However, no animal survived tumor-free. In contrast, no significant growth retardation was observed in the p53-mutant IGRG82 tumors after adenoviral treatment (9 days). Partial tumor regression was observed in only 1 of 12 animals, and tumors ultimately grew progressively despite treatment (Fig. 2, G and H).

**CAR Expression in Human Glioblastoma Xenografts.** The CAR plays a crucial role in adenoviral infection and might be important for the sensitivity of these tumors toward ONXY-015. Expression of CAR in the four xenografts was examined by fluorescence-activated cell sorter analysis of *in vitro* cultured tumor cells. The tumor cells IGRG82, IGRG88, and IGRG93 expressed CAR (median fluorescence ratios: 2.16 +/- 0.05, 2.19 +/- 0.10, and 2.17 +/- 0.02, respectively). However, CAR was not expressed in the IGRG121 tumor cells (median fluorescence ratio: 1.04 +/- 0.12). The glioblastoma cell lines U373-MG and U118-MG were used as positive and negative controls (median fluorescence ratio: 7.59 +/- 2.5 and 0.89 +/- 0.25, respectively), as described elsewhere (34).

**Malignant Glioblastoma Cells Are Sensitive to Adenoviral Infection.** We next examined the infectability of the glioblastoma tumor cells using an adenovirus expressing luciferase under the CMV promoter. All four of the xenograft tumor cells, including the CAR-negative IGRG121 tumor, could be infected significantly by the virus at 10 and 100 pfu/cell, demonstrating sensitivity to adenoviral infection in these glioblastoma models (Fig. 3).

**ONYX-015 Infects and Replicates in Glioblastoma Tumors.** To determine infection and replication of the ONXY-015 virus in the glioblastoma tumors *in vivo*, we performed *in situ* hybridization for adenoviral DNA and IHC for adenoviral hexon protein on paraffin-embedded tissue sections of IGRG121, IGRG93, IGRG88, and IGRG82 xenografts. Tumors were harvested on day 5 after four or five adenoviral or PBS injection treatments. Nuclear staining of adenoviral DNA and hexon protein was detectable in all four of the xenografts injected with ONXY-015 (Fig. 4). The distribution of the positive cells within the tumor samples was diffuse; few tumors showed focal areas of positive staining. However, the amount of infected tumor cells differed significantly in the xenografts. In IGRG93 and IGRG121, 30% and 15% of tumor cells were detectable with adenoviral replication, whereas infected cells were scattered in the xenografts IGRG88 and IGRG82 with 5% and 2% positive-stained tumor cells.

**ONYX-015 Induces Expression of Cellular p53 Protein in Tumor Cells.** The EIA gene is a potent inducer of p53. To investigate whether ONXY-015 is able to induce cellular p53, we performed IHC for the p53 protein in tissue sections from tumors injected for 5 consecutive days with the adenovirus. The mutant p53 tumors IGRG88 (Fig. 5A) and IGRG82 showed at baseline a homogenous pattern of strong positive nuclear staining in nearly 100% of the tumor cells. p53 wild-type protein was expressed scattered and far less intense in tumor cells of the xenografts IGRG93 and IGRG121 (Fig. 5B shows IGRG121). However, after treatment with five intratumoral injections of ONXY-015, p53 protein expression was induced and/or stabilized in both tumors as shown in IGRG121 in Fig. 5C.

**DISCUSSION**

Replication-selective oncolytic viruses offer several advantages in the treatment of human tumors. The virus itself is capable of lysing...
Fig. 2. Antitumor activity of ONYX-015 (intratumoral injection $10^8$ pfu/day × 5) against p53 wild-type and p53-mutant s.c. glioblastoma xenografts. Mice bearing s.c. xenografts IGRG121, IGRG93, IGRG88, or IGRG82, were each randomly assigned to two groups: control animals received injections of PBS and the treated animals ONYX-015 $10^8$ pfu/day for 5 consecutive days. Figures on the left (A, C, E, and G) give the means of treatment groups for each experiment. Figures on the right (B, D, F, and H) show all individual tumors treated. Each line represents one individual tumor.
specifically the infected tumor cell to eradicate or reduce tumor mass, and importantly, replication leads to amplification of the viral input “dose” through virus release by virus-mediated lysis of the infected cell, and subsequent spread and infection of surrounding cells. ONYX-015 (dl1520 and CI-1042) is a replication-selective adenovirus lacking the viral gene E1B 55 kDa, of which the product is required to inactivate the cellular tumor suppressor p53. Therefore, substantial tumor specificity is thought to be achieved through virus replication and potential lysis of the tumor cells that lack functional p53. Antitumor activity of this adenovirus has been shown in a wide range of different human tumors in vitro and in vivo. Its use in clinical trials in head and neck cancer has produced promising results (7, 9, 38). A phase I clinical trial in recurrent malignant glioma is currently ongoing (NABTT-9701). ONYX-015 has been tested occasionally in brain tumor cell lines. Rothmann et al. (12) reported replication of the E1B-attenuated adenovirus in p53 wild-type U87-MG and p53-mutant U373-MG glioma cell lines in vitro. However, Heise et al. (11) described that U87-MG glioblastoma tumor xenografts were unaf-
fected by ONYX-015 injections. In vitro intracellular replication may not be predictive for adenoviral oncolytic activity in vivo.

This investigation demonstrates significant antitumor activity of the E1B-deleted replicating adenovirus ONYX-015 in three of four human malignant glioma xenografts deriving from primary tumors. Repeated intratumoral injections of ONYX-015 10⁶ pfu for 5 consecutive days to mice bearing s.c. advanced-stage tumors induced significant tumor growth delays and tumor regression in one p53-mutant xenograft (IGRG88) and the two p53 wild-type tumors IGRG93 and IGRG121. Unexpectedly, the glioblastoma tumors with a p53 wild-type gene were more susceptible to ONYX-015-mediated cytolysis than the p53-mutant tumors used in this study. ONYX-015 treatment resulted in 45% and 82% complete tumor regressions and 4 of 11 and 8 of 11 tumor-free survivors at 4 months in IGRG93 and IGRG121, respectively, whereas none of the animals bearing p53-mutant tumors survived tumor-free. According to results from in vitro experiments in the literature, antitumor activity and cytolysis in vivo were mainly correlated with intratumoral adenoviral replication. Widespread viral replication was determined in 30 and 15% of the tumor cells within the highly sensitive tumor xenografts IGRG93 and IGRG121 compared with only scattered distribution in IGRG88 and IGRG82. Moreover, ONYX-015 treatment yielded a higher rate of complete tumor regressions and tumor-free survivors in the IGRG121 xenografts than in the IGRG93 tumors (Table 2 and Fig. 2), although a more intense viral replication throughout the tissue sections was seen in the latter (Fig. 4).

ONYX-015 was originally hypothesized to target only tumors of mutant p53 status by virtue of its inability to express the p53-inactivating 55 kDa E1B protein (10, 39). However, our results conflict with this tenet, and discussion has already been raised about the selectivity of ONYX-015 and even requirement of functional p53 for adenovirus infection, replication, or induction of cytopathic effects in cells. Although Harada and Berk (40) described that ONYX-015 had greater antitumor activity in a dominant-negative p53-mutant tumor cell line compared with its isogenic p53 wild-type cell line, the Ad5-E1B adenovirus has been shown to replicate in cells irrespective of their p53 status and to lyse efficiently tumor cells of mutant and wild-type p53 status in vitro and in vivo (12, 16, 41). Sensitivity to lytic activity of ONYX-015 in p53 wild-type cells was additionally suggested to be attributable to gene mutations outside exons 5–9 or inactivation of p53 by other mechanisms, such as loss of p14ARF, or expression of human papilloma virus E6, E4orf6 protein, or MDM2 amplification (17, 18, 20). The p14ARF protein encoded from the INK4a/ARF locus functions to promote MDM2 degradation and, thus, prevents the neutralization of p53 by MDM2 (37). The characterization of our glioblastoma xenografts in regard to their p53 functionality showed that the wild-type p53 protein in IGRG121 was functional with positive transactivation function and induction of the well-described target protein p21/WAF1 after radiation-induced DNA damage. In addition, the MDM2 and p14ARF genes were both expressed normally. However, in the second p53 wild-type xenograft IGRG93, p53 transactivation function was impaired. Heterozygosity of the p14ARF wild-type gene might determine an impaired p14 tumor suppressor function (42). Therefore, we conclude that ONYX-015 has the capability to replicate within p53 mutant and p53 wild-type tumor cells, and, in fact, the highly sensitivity of the IGRG121 tumors even suggests that functional p53 might be required for or support cytotoxicity of the E1B-attenuated adenovirus in glioma tumors. Nevertheless, these are observations in four xenografts including only one tumor with functional p53, and more tumors have to be studied. The infectability of p53 wild-type cells additionally raises the question of potential toxicity to normal tissue. However, these animal models do not represent adequate systems to address adenoviral toxicity. Treatment with this E1B-attenuated adenovirus induced expression and/or stabilization of the nuclear p53 wild-type protein, and induction of p53 pathway might, therefore, mediate efficient oncolysis, thereby increasing viral spread. Hall et al. (14) suggested an important role for p53 in mediating cellular destruction to allow a production adenovirus infection. Formation of a complex between p53 and the adenoviral E1B55k protein was necessary for the activation of the rapid cell death pathway, whereas cell death was delayed considerably in the absence of p53 or the absence of complex formation between p53 and E1B55k (43). Viruses lacking E1B55k, such as ONYX-015, were therefore suggested to kill in a delayed manner inducing growth arrest and not apoptosis (43). Investigations done in our study could not contribute to these findings. We determined terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling staining in the examined histological specimens 5 days after treatment start and found a high spontaneous apoptotic rate within the p53-functional IGRG121 xenografts compared with IGRG93, IGRG88, and IGRG82 tumors. However, a significant increase of terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling positive tumor cells or induction of apoptotic figures after adenoviral treatment could not be determined (data not shown). Nevertheless, this might be either attributable to the difficulty to determine significant changes in the amount of apoptotic cell death within tissue sections or the time point of detection, or even a characteristic of glioblastoma tumor cells. Shu et al. (44) described a lack of p21/Bax induction in glioblastoma cells in response to DNA damage by irradiation, irrespective of their functional endogenous p53. Additional investigations are currently ongoing.

In addition to p53 function, other cellular factors may play important parts in determining the sensitivity of a particular cell type to a viral agent. Adenovirus entry is dependent on the expression of the fiber receptors, such as CAR and the αv integrins on the target cells for binding and internalization, respectively (33, 45). Significant variations in transduction efficiency and therapeutic outcome after adenovirus-mediated gene transfer have been observed among several cancer cell lines relating to their expression of CAR (bladder cancer cell lines, head and neck squamous cell carcinoma, melanoma, and glioma cell lines; Refs. 34, 35, 46–48). In the glioblastoma xenografts used in this study, adenovirus receptor CAR was expressed in IGRG82, IGRG88, and IGRG93 tumors in a significant amount. However, the ONYX-015-mediated cytolysis highly sensitive tumor IGRG121 did not express CAR. Nevertheless, sensitivity to adenoviral infection was demonstrated by Ad5CMVLuc in all four of the xenografts. Infection of the CAR-negative IGRG121 was insignificantly lower than those of receptor-positive tumors. Nevertheless, lack of CAR expression might be responsible for the lower replication of ONYX-015 seen in the IGRG121 tumor compared with the second p53 wild-type xenograft, IGRG93. Other factors that enhanced adenoviral replication probably overcame the block in infection because of CAR deficiency in IGRG121. Inside the tumor after local injection, viral titers are high enough that CAR deficiency may not play a major role anymore in governing adenoviral infection and subsequent spread.

In addition, adenoviral replication has been shown to be dependent on S phase fraction of the tumor cells (49). Cell cycle status analysis performed in the p53 wild-type tumors showed 2–3% more tumor cells in S phase and 3–8% less resting cells in G0/G1 compared with the p53-mutant tumors (data not shown). Importantly, however, the xenograft IGRG121 is a fast proliferating tumor. Median tumor doubling time was 2.9 days compared with 5.0, 5.1, and 9.6 days in the IGRG93, IGRG88, and IGRG82 tumors, respectively (see Table 2). Therefore, a high cellular proliferation rate may additionally significantly contribute to the sensitivity toward virus-mediated cell lysis.
In summary, we demonstrated the efficacy of local treatment of the replication-competent E1B-mutant adenovirus ONYX-015 in human malignant glioma xenografts independent of their cellular p53 status. Unexpectedly, viral replication and oncolysis was highest in p53 wild-type and fast proliferating tumors. Whereas it is difficult to make a definite conclusion as to the relative rate of susceptibility on the basis of data from only four tumors, only one of which has demonstrable wild-type p53 activity, we clearly showed that wild-type p53 does not influence ONYX-015 from lysing tumor cells. Although it remains to be determined whether ONYX-015 exerts any toxicity to normal brain tissues, this therapeutic approach is highly promising for the treatment of malignant gliomas.

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