

# ONYX-015 Enhances Radiation-Induced Death of Human Anaplastic Thyroid Carcinoma Cells

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**ONYX-015 is a genetically modified adenovirus with a deletion of the E1B early gene and therefore is designed to replicate preferentially in p53-mutated cells causing their death. We previously demonstrated that the ONYX-015 virus kills anaplastic thyroid carcinoma (ATC) cells and enhances the antineoplastic effects of doxorubicin and paclitaxel. Here we report that ONYX-015 increased the cytopathic effect of radiotherapy in three ATC cell lines. In fact, ONYX-015 and radiation induced a significant cytopathic effect on ATC cells.**

**DNA fragmentation analysis showed that ATC ONYX-015-treated cells were very sensitive to radiation-induced apoptosis. In addition, low doses of ONYX-015 associated with a single radiation dose of 10 Gy delayed the growth of a xenograft tumor induced by ARO cells in athymic mice. Our results suggest that the combination of ONYX-015 and radiotherapy should be considered for experimental trials in patients with anaplastic thyroid carcinoma. (*J Clin Endocrinol Metab* 88: 5027–5032, 2003)**

**A**NAPLASTIC THYROID CARCINOMA (ATC) accounts for about 2% of all human thyroid neoplasias (1, 2) and, being resistant to conventional antineoplastic therapy (3), it is one of the most lethal human neoplasms. ONYX-015 is an attenuated adenovirus that does not replicate efficiently in cells bearing a normal p53 gene; however, it replicates efficiently in tumor cells that lack either functional p53 or p14 Arf, so causing their death (4–6). Because mutations in the p53 gene are a feature of ATC (7–12), ONYX-015 seems a prime candidate as an antineoplastic agent in this type of tumor. The ONYX-015 virus has already been tested in phase II and III clinical trials for the treatment of squamous cell cancers of the head and neck (13, 14), in phase I and II trials for primary and secondary liver tumors (15–17), and in a phase I trial for primary carcinoma of the pancreas (18).

We previously showed that the ONYX-015 virus kills ATC cells and reduces the growth of ATC tumor xenografts (19). We also showed that ONYX-015 enhances the antineoplastic effects of doxorubicin and paclitaxel in ATC cells. These results suggested that ONYX-015 might be envisaged for the treatment of ATC. However, although the ONYX-015 virus has proven to be biologically active and safe in cancer patients (20), it is unlikely to be effective as monotherapy because few cancers are cured with a single agent. For example, treatment with ONYX-015, cisplatin, and 5-fluorouracil resulted in a higher number of complete responses *vs.* ONYX-015 alone in patients with head and neck cancer (13). In addition, ONYX-015 enhanced the effect of standard chemotherapy in primary lung cancer cells and in nude mice human tumor xenografts (21, 22).

The aim of our study was to investigate the effect of ONYX-015 plus radiotherapy, which is frequently used to

control local tumor growth, on the proliferation of human ATC cell lines. Here we report that ONYX-015 therapy enhanced radiation-induced cell death. The combination of ONYX-015 and radiotherapy also significantly delayed the growth of xenograft tumors induced in athymic mice by the injection of ATC cells.

## Materials and Methods

### Viruses

ONYX-015 (a gift from Dr. A. Balmain, University of California San Francisco Cancer Center and Cancer Research Institute, San Francisco, California; and Dr. I. Ganly, Cancer Research UK Beatson Laboratories, Bearsden, UK) is a chimeric human group C adenovirus (Ad2 and Ad5) that has a deletion between nucleotides 2496 and 3323 in the E1B region that encodes the 55-kDa protein. In addition, there is a C to T transition at position 2022 in region E1B that generates a stop codon at the third codon of the protein. These alterations prevent the expression of the 55-kDa protein in ONYX-015-infected cells (4).

Ad5 CMV *lacZ* (Quantum Biotechnology, Carlsbad, CA) is a non-replicating E1-deleted adenovirus in which the reporter construct *lacZ* has been inserted in the E1 region under the control of the cytomegalovirus (CMV) promoter. We used the Ad5 CMV *lacZ* adenovirus as a control in clonogenic assays and *in vivo* experiments. Viral stocks were expanded in the human embryonic kidney 293 cell line, which expresses the E1 region of Ad2, and purified, as previously reported (19). Stocks were stored at –70 °C after the addition of glycerol to a concentration of 50% vol/vol. Virus titer was determined by plaque-forming units (pfu) on the human embryonic kidney 293 cells.

### Cell lines

ARO (23) and FRO (8), human thyroid anaplastic carcinoma cell lines, were obtained by Dr. G. Juillard (University of California Los Angeles); ARO and FRO cell lines are a kind gift of Prof. J. A. Fagin (University of Cincinnati College of Medicine, Cincinnati, OH); and KAT-4 (24) cells were obtained from Dr. K. B. Ain (University of Kentucky, Lexington, KY). Cells were grown in DMEM supplemented with 10% fetal calf serum and ampicillin/streptomycin.

ARO and KAT-4 harbor a mutated *p53* gene, *i.e.* 273 Arg->His,

Abbreviations: ATC, Anaplastic thyroid carcinoma; CMV, cytomegalovirus; MOL, multiplicity of infection; pfu, plaque-forming units.

whereas FRO cells express very low levels of p53 but do not have a p53 gene mutation (19).

### Clonogenic assay

Cells were seeded at a density of  $2 \times 10^5$  in 60-mm plates and infected with ONYX-015 or with a nonreplicating E1-deleted adenovirus with the lacZ reporter construct (Ad5 CMV lacZ virus) at multiplicity of infection (MOI) of 0.1 and 0.5 per cell for ARO, 0.5 and 1 per cell for KAT-4 cell line. FRO cell line was infected with a MOI of 0.5 and 2.5. An uninfected dish was used as a control. Cells were harvested 24 h later, plated at a density of 200 cells/plate (three replicates), and irradiated at a dose of 2, 4, or 6 Gy. Cells were incubated for 12 d to allow colony formation to occur. The colonies were stained with crystal violet, and colonies constituted by more than 40 cells were counted. The mean colony count in the three different plates per MOI, Gy dose, and both was calculated and expressed relative to the uninfected and untreated control. The results shown are the average of three different experiments

### DNA fragmentation

To analyze the time dependency of apoptotic DNA fragmentation, ARO cells were plated in 96-well plates ( $1 \times 10^3$  in 100- $\mu$ l medium) and treated with ONYX-015 (0.1 or 0.5 pfu/cell), radiation, or both. Cells were left for 48 h and then lysed. Apoptosis was measured in triplicate samples using the Cell Death Detection ELISA plus kit (Roche, Mannheim, Germany). This photometric sandwich enzyme-immunoassay determines cytoplasmic histone-associated DNA fragments (mono and oligo nucleosomes) upon induction of cell death. Briefly, a mixture of biotin-labeled antihistone and peroxidase-conjugated anti-DNA antibodies was added to the cell lysates and placed in a streptavidin-coated microtiter plate. During incubation, the antihistone antibody binds to the histone-competent apoptotic nucleosomes and links the immunocomplex to the streptavidin-coated plates via its biotinylation. The amount of nucleosomes by peroxidase activity was determined spectrophotometrically at 405 nm with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) as a substrate (Microplate reader, Bio-Rad, Munich, Germany). Results were expressed as relative amounts of apoptosis, being the results of the negative control equalized to 1.

### Tumorigenicity assay

All experiments were performed with 6-wk-old male athymic mice (Charles River, Calco, Lecco, Italy). ARO cells ( $5 \times 10^5$ ) were injected into the right flank of 60 athymic mice. After 15 d, the animals were divided into four groups (15 animals/group), and tumor volume was evaluated. ONYX-015 ( $5 \times 10^6$  pfu) was injected in the peritumoral area in two groups for a consecutive 4 d. On d 5, at the end of viral treatment, a control group and a virus-treated group were anesthetized, and a single radiation dose of 10 Gy was administered on the tumor volume at a distance of 80 cm with a bolus interposition to avoid lower doses at the tumor external edge. Tumor diameters were measured with calipers every second day by two blind and neutral observers until the animals were killed. No mouse showed signs of wasting or other indications of toxicity.

Tumor volumes (V) were calculated with the rotational ellipsoid formula:  $V = A \times B^2/2$  (A, axial diameter; B, rotational diameter).

The same schedule was used for a control experiment with the replication-defective virus Ad5 CMV lacZ virus. Briefly, 40 athymic mice were inoculated sc with  $1 \times 10^6$  ARO cells, and 15 d later, when tumors became detectable,  $5 \times 10^6$  pfu of Ad5 CMV lacZ virus were injected peritumorally for a consecutive 4 d in 20 animals. Ten animals treated with Ad5 CMV lacZ virus and 10 untreated animals were then irradiated with a single dose of 10 Gy.

All mice were maintained at the Dipartimento di Biologia e Patologia Animal Facility. The animal experimentations described herein were conducted in accordance with accepted standards of animal care and in accordance with the Italian regulations for the welfare of animals used in studies of experimental neoplasia, and the study was approved by our institutional committee on animal care.

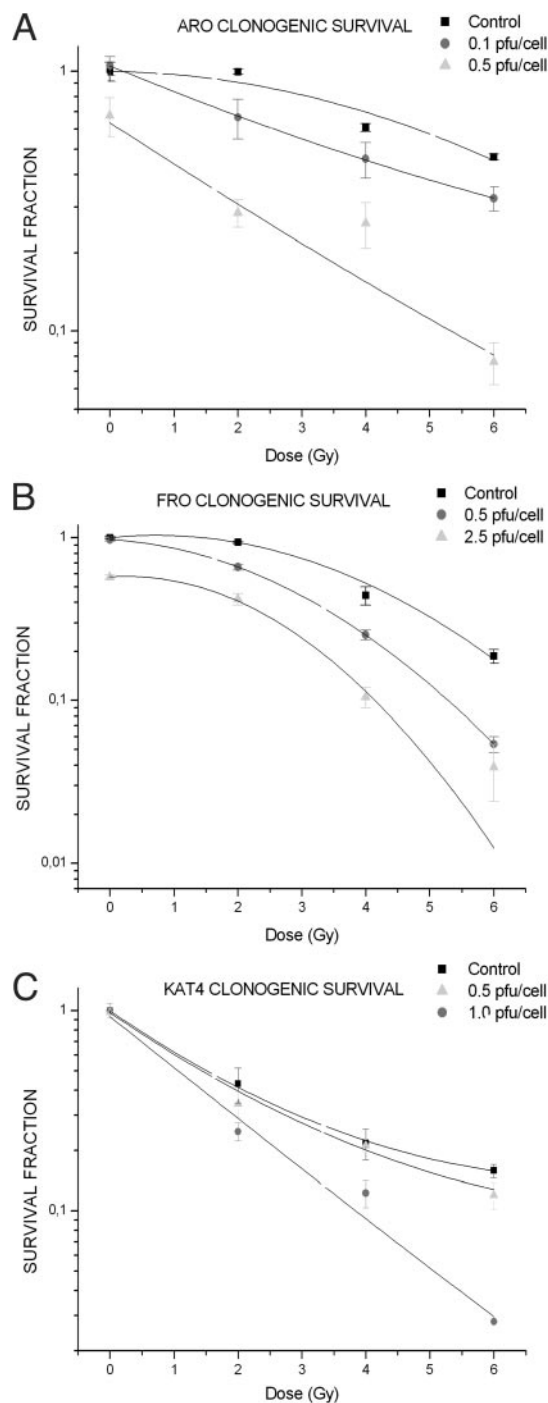


FIG. 1. ONYX-015 and radiation induced cell killing of human anaplastic thyroid carcinoma cell lines. ARO cells were infected with a MOI of 0.1 and 0.5 per cell, KAT-4 with a MOI of 0.5 and 1 per cell. FRO cell line was infected with a MOI of 0.5 and 2.5 per cell. After 24 h cells were irradiated with 2, 4, or 6 Gy. Control cells were irradiated at the indicated doses. Cells were incubated for 12 d to allow colony formation to occur, and colonies greater than 40 cells were counted. A, Clonogenic survival of ARO cells treated with ONYX and radiation. B, Clonogenic survival of FRO cells treated with ONYX and radiation. C, Clonogenic survival of KAT-4 cells treated with ONYX and radiation. Data are presented as mean  $\pm$  SE of three replicate cultures. The lines are statistically significant bi-exponential fit.

### Statistical analysis

Comparison among different treatment groups in the experiments was made by the ANOVA method and the Dunnett's *post hoc* test using a commercial software (SPSS Inc., Chicago, IL). For each cell line, comparison among cell survivals at different doses was made using ONYX-015 concentration as the grouping variable. Assessment of differences among rate of tumor growth in mice was made for each time point of the observation period; treatment groups were control, ONYX-015, radiotherapy, and ONYX-015 + radiotherapy.

### Results

#### ONYX-015 increases the radiation-induced death of human thyroid anaplastic carcinoma cells

To evaluate the efficacy of ONYX-015 combined with radiation treatment, three ATC-derived human cell lines (ARO, KAT-4, and FRO) were used. ARO cells were infected with a MOI of 0.1 and 0.5 per cell, KAT-4 with a MOI of 0.5 and 1 per cell. FRO cell line was infected with a MOI of 0.5 and 2.5 per cell.

Twenty-four hours later, cells were irradiated with different doses (2, 4, or 6 Gy), and the cytotoxicity and the synergistic effects were evaluated. At these doses, radiation does not affect ONYX-015 DNA replication or DNA integrity. In fact, even a dose as high as 20 Gy does not alter virus integrity (25). A MOI of 1 or 2.5 was required to obtain an adjuvant effect of ONYX-015 on FRO cells. This is consistent with our previous finding that FRO cells are more resistant than ARO cells to adenovirus infection (19). However, in this previous study FRO cells were treated with 10 pfu/cell of ONYX-015 for 7 d, and a viability assay was used to assess the effects of the treatment yielding a survival fraction of about 90%. In the present study, FRO cells yield a survival fraction 55% at 2.5 pfu/cell of ONYX-015. The different treatment time and the different methods used to assess the effect of the replicating virus ONYX-015 can explain the discrepancy between these studies.

ONYX-015 enhanced the cytopathic effects of radiotherapy in all cell lines tested (Fig. 1). This enhancement was statistically significant at the doses of 2, 4, and 6 Gy for ARO cells (Dunnett's *post hoc* test,  $P < 0.01$ ) with a MOI of 0.5, and for FRO cells (Dunnett's *post hoc* test,  $P < 0.05$ ) with a MOI of 2.5. For KAT-4 cells a significant effect (Dunnett's *post hoc* test,  $P < 0.05$ ) was observed at a dose of 6 Gy with a MOI of 1.0. In particular, the enhancement factor for a radiation-induced clonogenic inhibition of 50% in ARO cells pretreated with 0.1 pfu/cell of ONYX-015 was 1.55. Conversely, infection with a replication-defective virus bearing deletions of E1A and E1B that contains a *lacZ* reporter gene does not induce any significant additive or synergistic effects upon radiation treatment (Table 1). These results indicate that

ONYX-015 could be used in association with radiotherapy to treat ATC because low viral concentration results in significant cell killing upon radiation.

#### DNA fragmentation of human thyroid anaplastic carcinoma cells infected with ONYX-015 and irradiated

We evaluated the capability of ONYX-015 to induce apoptosis in irradiated ATC cells using an ELISA-based assay to measure cytosolic apoptotic nucleosomes. ARO cells were infected with 0.1 or 0.5 pfu/cell of ONYX-015, and 24 h later the cells were irradiated with 2 Gy. Cells were left for 48 h and then lysed. The infection of ARO cells with 0.1 or 0.5 pfu/cell of ONYX-015 greatly increased the DNA fragmentation of ARO with respect of untreated or not infected control cells (Fig. 2).

#### Reduced growth of tumor xenografts of ATC cells treated with ONYX-015 and irradiated

To evaluate whether radiation treatment had a synergistic effect with ONYX-015 also *in vivo*, we inoculated 60 athymic mice sc with  $5 \times 10^5$  ARO cells, and 15 d later, when tumors became detectable, animals were randomly assigned to four groups. Two groups were injected with  $5 \times 10^6$  pfu of ONYX-015 peritumorally for a consecutive 4 d. One group not treated with ONYX-015 and one group treated with ONYX-015 were then irradiated with a single dose of 10 Gy. Tumor growth was then followed. In Fig. 3 tumor growth is expressed as a percentage of growth relative to the volume observed at radiation ( $T = 0$ ). As expected, in the group that received either ONYX-015 or radiation only, tumor growth was not significantly inhibited compared with the control group (Dunnett's *post hoc* test,  $P > 0.1$ ). Only the combination of ONYX-015 viral plus radiation therapy significantly delayed tumor growth compared with the control (Dunnett's *post hoc* test,  $P < 0.05$ ). Conversely, the combination of Ad5 CMV *lacZ* plus a single dose of 10 Gy did not significantly delay tumor growth (Fig. 4).

### Discussion

ATC is one of the most aggressive solid tumors, and patients with ATC have a very poor prognosis (3, 26). Consequently, several novel therapeutic approaches have been proposed. Manumycin, a farnesyl:protein transferase inhibitor, has proved to be effective against ATC cells alone and in combination with paclitaxel (27, 28). Moreover, there is evidence that suppression of HMGA1 protein synthesis leads to ATC cell death (29). More recently, we found that the replication defective ONYX-015 virus is highly efficient in

**TABLE 1.** Effects of infection of ATC cells with Ad5 CMV *lacZ*, an adenovirus lacking E1A and E1B regions, and radiation

Cell line	Untreated	Ad5 <i>lacZ</i>	2 Gy	Ad5 <i>lacZ</i> /2 Gy	4 Gy	Ad5 <i>lacZ</i> /4 Gy	6 Gy	Ad5 <i>lacZ</i> /6 Gy
ARO	285 ± 21	267 ± 27	215 ± 26	204 ± 31	175 ± 28	160 ± 19	110 ± 13	94 ± 11
KAT-4	305 ± 32	292 ± 28	273 ± 33	280 ± 27	215 ± 31	196 ± 25	138 ± 17	125 ± 23
FRO	324 ± 35	305 ± 28	286 ± 29	295 ± 32	210 ± 22	219 ± 28	157 ± 21	146 ± 19

ARO and KAT-4 cells were infected with a replication-defective virus at an MOI of 0.5 pfu/cell, whereas FRO cells were infected at an MOI of 2.5 pfu/cell. After 24 h, cells were irradiated with 2, 4, or 6 Gy and incubated for 12 d to allow colony formation to occur. Control cells were irradiated at the indicated doses. The total number of colonies obtained are indicated. No additive or synergistic effects of Ad5 CMV *lacZ* virus and radiation were observed on the different cell lines.

FIG. 2. DNA fragmentation of ARO thyroid anaplastic carcinoma cells infected with ONYX-015 and irradiated. ARO carcinoma cells were treated with ONYX-015 (0.1 or 0.5 pfu/cell) and irradiated. DNA fragmentation was measured after 48 h. Results are the means  $\pm$  SD of duplicate determinations of three independent experiments. Results were expressed as relative amounts of apoptosis, being the results of the negative control equalized to 1.

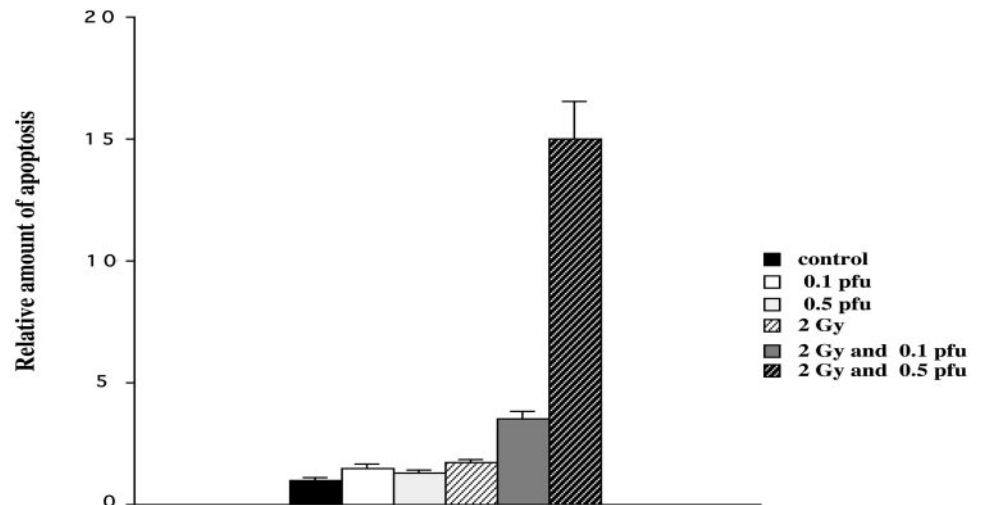
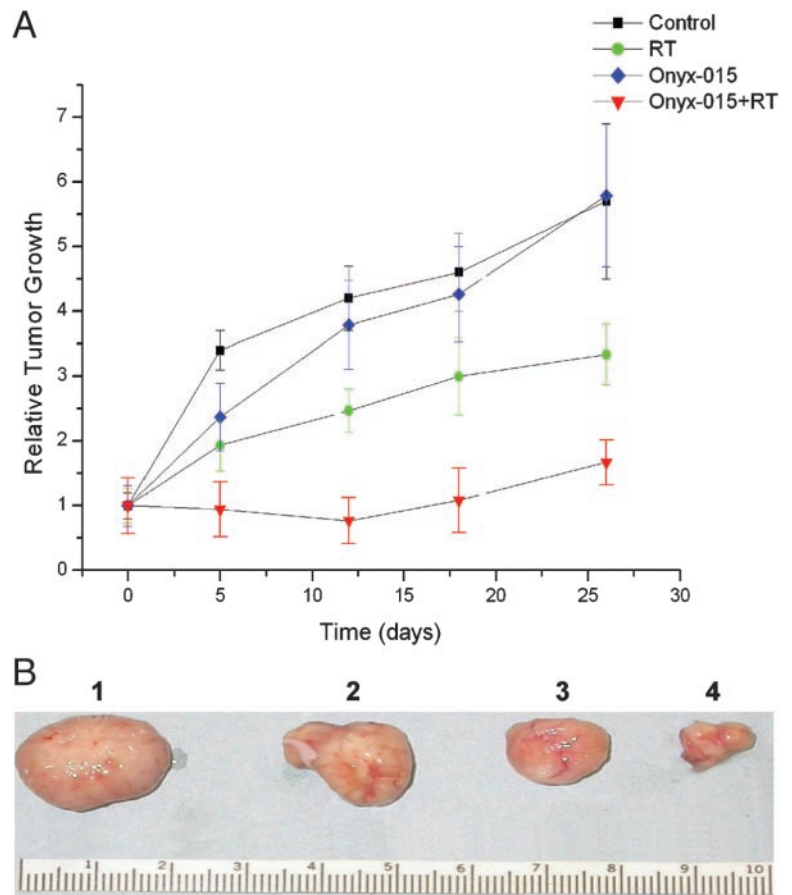


FIG. 3. Tumor growth delay induced by ONYX-015 and radiation. A, Relative tumor growth of animals treated with ONYX-015 and radiation, and control groups. Data are presented as mean  $\pm$  SE. B, Representative tumors excised from mice after treatment: 1) control; 2)  $5 \times 10^6$  pfu ONYX-015; 3) 10 Gy radiotherapy; and 4)  $5 \times 10^6$  pfu of ONYX-015 and 10 Gy radiotherapy. RT, Radiotherapy.



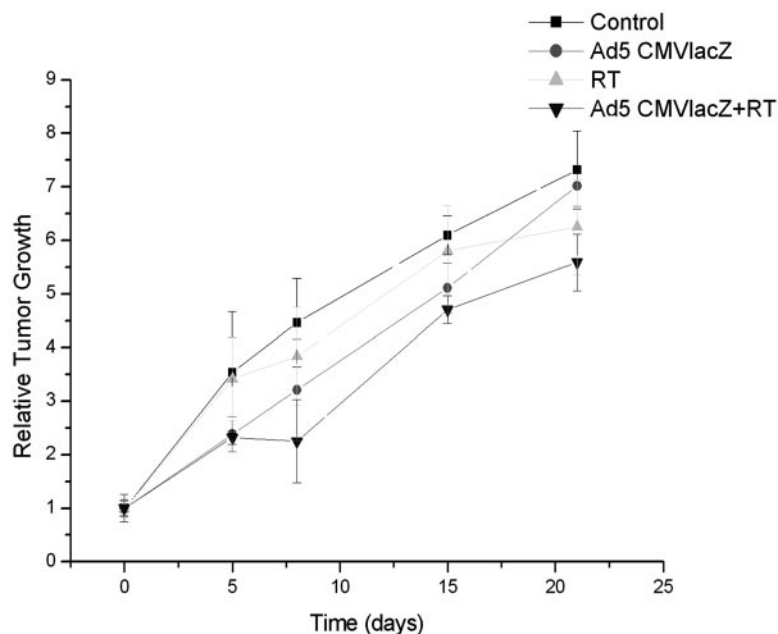
killing human ATC cell lines either alone or in combination with doxorubicin and paclitaxel (19), and that this effect results from impairment of p53 function, which is a general feature of ATC cells (7–11).

The ONYX-015 plus radiation combination was used in a human colon carcinoma cell line carrying a wt p53 gene (RKO) and in a subclone that expresses an inactivating mutation of p53 gene (RKO p53.13). In this study, the authors have shown that ONYX-015 exerted a neoadjuvant effect to radiation against xenograft induced by the cell line carrying

a mutant p53 gene (25). These findings prompted us to evaluate the effects of combined ONYX-015/radiation treatment on ATC cells.

Here, we report that ONYX-015 at a low MOI is an effective neoadjuvant to radiotherapy in ATC treatment. Our finding confirms that ONYX-015 could be beneficial in the treatment of this very aggressive disease. We found that very low viral concentrations (from 0.1–2.5 MOI), which are suitable for the clinical setting, enhanced the radiation-induced killing of ATC cells. Moreover, low doses of ONYX-015 associated

FIG. 4. Ad5 CMV *lacZ* does not modify the growth of xenograft tumors. Growth of xenograft tumors induced in nude mice treated with Ad5 CMV *lacZ* and radiation and control groups. RT, Radiotherapy.



with a single 10 Gy dose of radiation drastically delayed the growth of established xenograft tumors in athymic mice. Conversely, infection of ATC cells with Ad5 CMV *lacZ* virus, a replication-defective virus bearing deleted E1A and E1B sequences and treated with various doses of radiation, did not have any additive or synergistic cell killing; furthermore Ad5 CMV *lacZ* virus associated with a single 10 Gy dose of radiation was not able to delay the growth of established xenograft tumors in athymic mice. Consequently, the anti-neoplastic effects reported herein are specific for ONYX-015.

A DNA fragmentation assay showed that ATC ONYX-015-treated cells were very sensitive to radiation-induced apoptosis, which explains the synergistic effects observed in this study. The enhanced sensitivity to anti-neoplastic drugs or radiation observed in ONYX-015-infected cells has been attributed to the expression of E1A (30). Ad5 E1A expression has been shown to sensitize cells to radiotherapy and anti-cancer agents by inducing apoptosis (31–34), and E1A mediates apoptosis by inducing p53 accumulation (35, 36). However, E1A *per se* is sufficient to induce apoptosis regardless of p53 (36). Further experiments are required to elucidate how ONYX-015 and radiation activate apoptosis in ATC cells.

The necessity for p53 pathways to be nonfunctional in order for ONYX-015 replication to occur is an ongoing controversy. Despite the original design of the virus as selectively replication-competent only in p53-deficient cells, there are several reports indicating that the ONYX-015 virus is capable of replicating in p53 wild-type cells (37, 38). However, inactivation of the p53 pathway can be induced by changes in other components of the pathway, such as p14ARF or MDM2, and these alterations are functional surrogates for p53 mutation. Recently, in abdominal wall implants from a primary gall bladder carcinoma injected with ONYX-015, replication of the virus has been observed, not only in tumor cells, but in adjacent stromal cells presumably normal and bearing 53 wild type (39). Further studies are

required to clarify whether these cells represented tumor cell variants or normal stromal cells, and the p53 status of these cells needs to be assessed.

The treatment of advanced or recurrent ATC is complex because the tumor invades the trachea and causes death by asphyxiation. Monotherapy usually fails to control local and regional ATC, and a multidisciplinary strategy consisting of surgery, chemotherapy, and radiotherapy is preferred (26). Clinical trials indicate that treatment with ONYX-015 helps to control locally aggressive neoplasms. Moreover, ONYX-015 administered *iv* has been found to be clinically safe and effective (20). Therefore, we propose that our results indicate that ONYX-015 plus radiation should be attempted to control local and regional ATC without increasing the toxic effects of radiation.

### Acknowledgments

We are indebted to Dr. A. Balmain (University of California San Francisco Cancer Center and Cancer Research Institute, San Francisco, CA) and Dr. I. Ganly (Cancer Research UK Beatson Laboratories, Bearsden, UK) for providing the ONYX-015 adenovirus. We are indebted to S. Sequino and J. A. Gilder (Dipartimento di Biologia e Patologia Cellulare e Molecolare Università Federico II Napoli) for excellent technical assistance and for editing the text, respectively.

Received March 5, 2003. Accepted July 14, 2003.

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This study was supported by the Associazione Italiana per la Ricerca sul Cancro, by the Programma Italia-USA sulla Terapia dei Tumori coordinated by Prof. C. Peschle.

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