

ONYX-015, an E1B Gene-Defective Adenovirus, Induces Cell Death in Human Anaplastic Thyroid Carcinoma Cell Lines

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Being one of the most lethal human neoplasms and refractory to such conventional treatment as chemo- and radiotherapy, anaplastic thyroid carcinoma is a prime target for innovative therapy. *p53* gene inactivation is a constant feature of this neoplasia. Therefore, we evaluated a therapeutic approach based on an E1B 55-kDa gene-defective adenovirus (ONYX-015) that replicates only in cells with impaired *p53* function and leads to cell death. Here we report that the ONYX-015 virus induces cell death in three human thyroid anaplastic

carcinoma cell lines (ARO, FRO, and KAT-4). In addition, we found that the growth of xenograft tumors induced in athymic mice by the injection of ARO cells was drastically reduced by ONYX-015 treatment. The ONYX-015 virus worked synergistically with two antineoplastic drugs (doxorubicin and paclitaxel) in inducing ARO and KAT-4 cell death. These results suggest that ONYX-015 may be a valid tool in the treatment of the human thyroid anaplastic carcinoma. (*J Clin Endocrinol Metab* 87: 2525–2531, 2002)

THYROID FOLLICULAR CELL-DERIVED neoplasias comprise a broad spectrum of tumors, *i.e.* papillary, follicular, and anaplastic carcinomas, that have different molecular, biological, and clinical features (1, 2). Anaplastic thyroid carcinoma (ATC) accounts for about 5% of all human thyroid neoplasias and usually arises as terminal dedifferentiation of unrecognized long-standing differentiated carcinoma of the thyroid gland (1, 2). Cervical metastases are present in most cases at diagnosis. Tracheal invasion is present in 25% of patients, and lung metastases are found in 50% of patients at the time of presentation; survival time is 2–6 months after diagnosis (3).

Alterations of the *p53* tumor suppressor gene are a peculiar feature of anaplastic carcinomas (4–7). A G:C to A:T transition leading to an Arg to His substitution at codon 273 is the most frequent mutation in ATC (5). Moreover, the accumulation of *p53* protein, which is frequently caused by *p53* mutations, characterizes tumoral areas with a less differentiated phenotype (7, 8). Conversely, *p53* gene mutations are rare in differentiated thyroid carcinomas (9). Accordingly, the introduction of mutated *p53* genes into thyroid cells blocks the expression of thyroid differentiation markers and suppresses the thyroid transcription factor PAX-8 (10). Interestingly, *p53* gene function is impaired in all thyroid carcinoma cell lines established from both differentiated and undifferentiated histiotypes (11). This strongly suggests that *p53* inactivation is required for the establishment of human thyroid carcinoma cell lines.

ATC is refractory to chemotherapy and radiotherapy (3). Although drug resistance is multifactorial, *p53* inactivation

could be partly responsible for this event, because *p53* function induces growth arrest and apoptosis after the administration of DNA-damaging agents (12, 13). Gene therapy targeting ATC cells carrying a mutated *p53* gene seems a promising avenue of research. Indeed, transfer of the wild-type *p53* gene in undifferentiated thyroid carcinoma cell lines that harbor *p53* mutations inhibits proliferation and impairs colony formation in soft agar, thereby partially restoring the thyroid differentiated phenotype (14, 15). Moreover, the adenovirus-mediated transduction of a wild-type *p53* gene in ATC cell lines induced sensitivity to doxorubicin (16, 17).

We have examined the possibility of exploiting the loss of *p53* function and ONYX-015 in the treatment of ATC. This E1B 55-kDa gene-defective adenovirus contains an 827-bp deletion in the E1B region and a point mutation at codon 2022 that generates a stop codon (18). It replicates in tumor cells lacking functional *p53* and causes cell death; it does not replicate in normal cells. ONYX-015 kills cervical carcinoma, colon carcinoma, glioblastoma, and pancreatic adenocarcinoma cells, which lack functional *p53*, with an efficiency comparable with that of the wild-type adenovirus (19, 20). Moreover, it works synergistically with chemotherapy in lung cancer cell lines (21). It is being tested in phase II clinical trials for the treatment of squamous cell cancers of the head and neck (22–25) and a phase I trial for primary carcinoma of the pancreas (26).

We have analyzed the sensitivities of three human thyroid carcinoma cell lines (ARO, FRO, and KAT-4) to the ONYX-015 virus. All three cell lines, but not a normal rat thyroid cell line, responded to the cytopathic effect of ONYX-015. Moreover, ONYX-015 acted in synergy with two antineoplastic drugs (doxorubicin and paclitaxel) in two ATC cell lines (ARO and KAT-4).

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

Materials and Methods

Preparation of the ONYX-015 adenovirus

Viral stocks were expanded in the human embryonic kidney cell line HEK 293, as previously reported (18–21). Virus titer was determined by plaque-forming units on the HEK293 cells.

A control adenovirus was obtained by inactivating ONYX-015 with UV and was used as a control in the viability assay experiments. The virus was exposed to three cycles of 12,000 μ J.

TABLE 1. Infectivity of CMV *lacZ* adenovirus-infected cells

Cell line	500 pfu/cell	100 pfu/cell	10 pfu/cell
ARO	85 \pm 7.9	78 \pm 6.5	28 \pm 3.4
KAT-4	59 \pm 6.2	28 \pm 3.4	5 \pm 1.4
NPA	45 \pm 5.9	32 \pm 5.5	15 \pm 2.2
FRO	53 \pm 6.4	12 \pm 3.7	8 \pm 1.5
PC Cl 3	91 \pm 8.9	74 \pm 7.1	34 \pm 3.6
PC MPSV	88 \pm 7.9	76 \pm 8.1	29 \pm 6.6

Infectivity: percentage of β -galactosidase-positive cells at 24 h (mean \pm SE). Infectivity was evaluated at 500, 100, and 10 pfu/cell.

Cell lines

The human thyroid carcinoma cell lines used were described previously (15, 16, 27). They were grown in DMEM containing 10% FCS. PC Cl 3 cells are normal thyroid cells of Fischer rat origin (28) and were grown in Ham's F-12 medium, modified according to Coon (Sigma), and supplemented with 5% calf serum (Life Technologies, Inc., Gaithersburg, MD) and a mix containing six growth factors (6 H): 10 nM TSH, 10 nM hydrocortisone, 100 nM insulin, 5 mg/ml transferrin, 5 nM somatostatin, and 20 mg/ml glycyl-histidyl-lysine. The PC MPSV cell line was obtained by infecting PC Cl3 cells with MPSV, a retrovirus containing a *mos* oncogene (28). PC MPSV were grown in Ham's F-12 medium, modified according to Coon (Sigma), and supplemented with 5% calf serum.

Viability assay

For evaluation of the cytotoxic effects of the ONYX-015 virus, cells were seeded in 96-well plates, and 24 h later increasing concentrations of ONYX-015 or UV-irradiated control adenovirus were added to the incubation medium. Seven days later, the media were fixed with 10% TCA and stained with 0.4% sulforhodamine B in 1% acetic acid (29). The

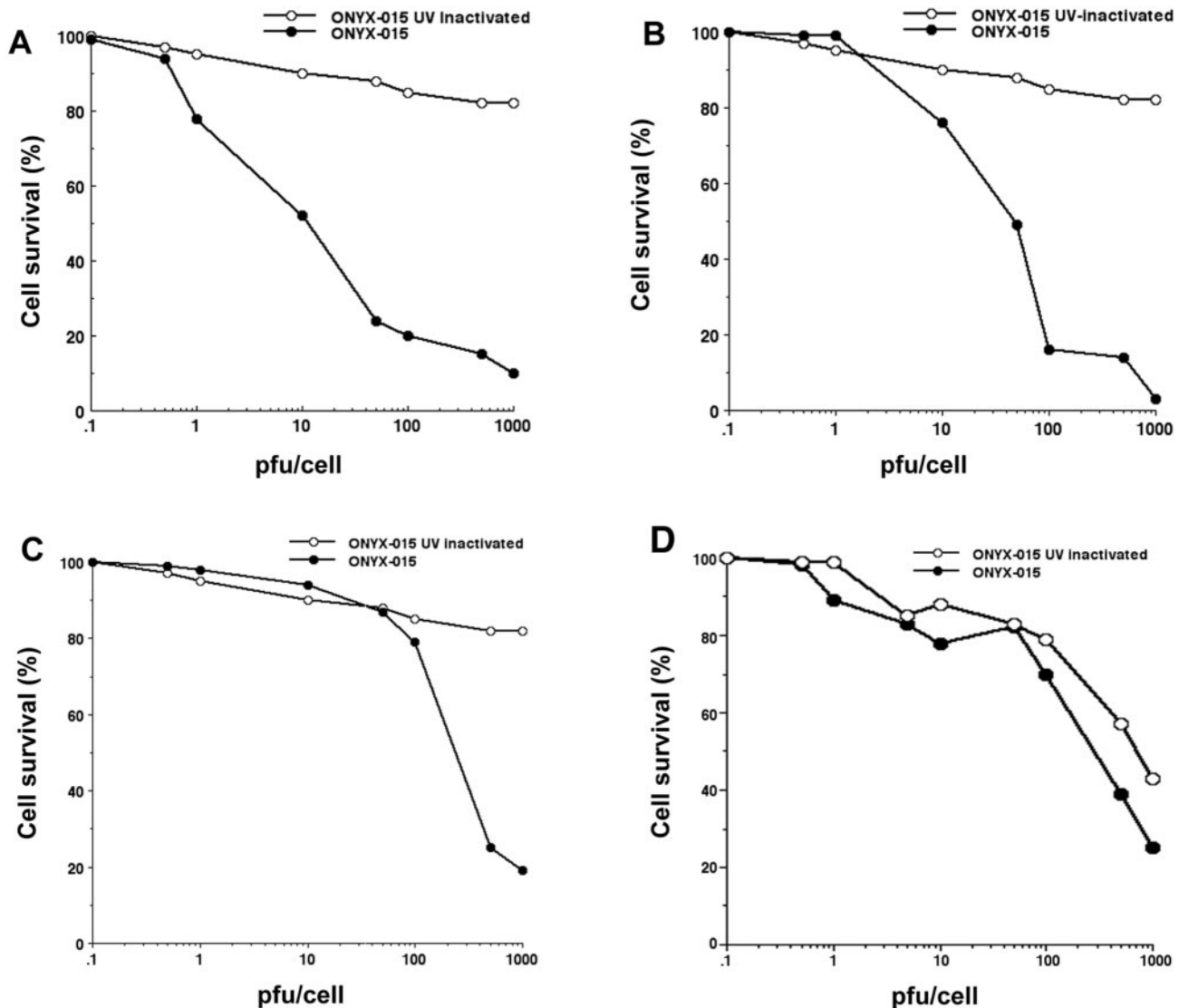


FIG. 1. Cell growth of ONYX-015-infected ATC cell lines. A, ARO cells exposed to increasing concentrations of ONYX-015. B, KAT-4 cells exposed to increasing concentrations of ONYX-015. C, FRO cells exposed to increasing concentrations of ONYX-015. D, PC Cl3 cells exposed to increasing concentrations of ONYX-015.

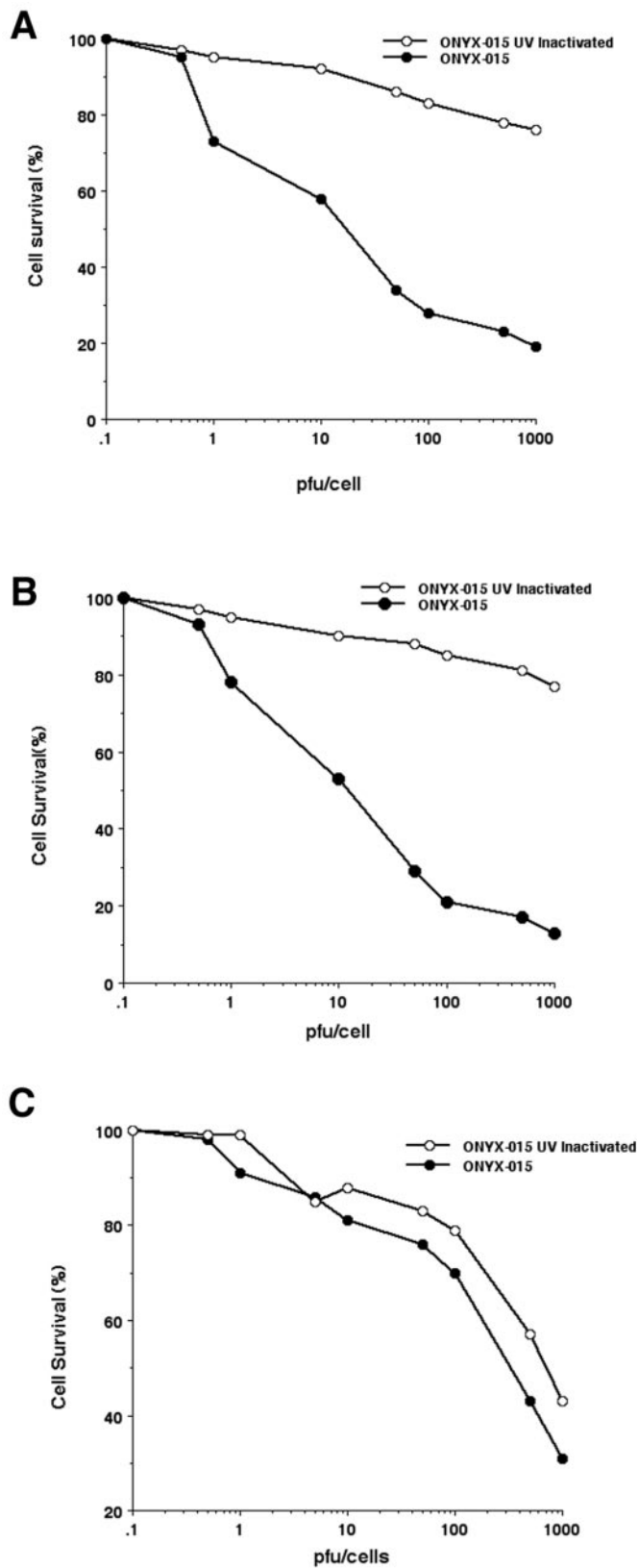


FIG. 2. Cell growth of ONYX-015-infected thyroid carcinoma cell lines. A, NPA cells exposed to increasing concentrations of ONYX-015. B, WRO cells exposed to increasing concentrations of ONYX-015. C, PC MPSV cells exposed to increasing concentrations of ONYX-015.

bound dye was solubilized in 100–200 μ l 10 mM unbuffered Tris solution, and OD was determined at 540 nm in a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA). The percent survival rates of cells exposed to adenovirus vectors were calculated by assuming the survival rate of untreated cells to be 100%.

For the evaluation of synergy between ONYX-015 and chemotherapeutic agents in killing ARO cells, doxorubicin (Sigma-Aldrich Corp., St. Louis, MO) or paclitaxel (Sigma-Aldrich Corp.) was added to the plates together with ONYX-015 at different concentrations. The drugs were dissolved in tissue culture grade dimethylsulfoxide before dilution in tissue culture medium. The final concentration of dimethylsulfoxide was 0.1%. Seven days later the percent survival rates of cells exposed to adenovirus and doxorubicin or paclitaxel were evaluated.

Infectivity assay

A non-replicating E1-deleted adenovirus with the *lacZ* reporter construct [cytomegalovirus (CMV) *lacZ* virus] was used to determine the infectivity of the cell lines used in the study. Cells were infected at multiplicities of infection (MOI) of 10 and 100 plaque-forming units (pfu)/cell and were incubated for 48 h. Cells were fixed in glutaraldehyde solution and incubated in X-galactosidase solution overnight (30). The percentage of β -galactosidase-positive cells was quantified by counting blue-stained cells in three randomly selected fields.

Tumorigenicity assay

All experiments were performed in 6-wk-old male athymic mice (Charles River, Lecco, Italy). Untreated ARO cells (2×10^6), or 2×10^6 ARO cells transduced with 10 pfu/cell ONYX-015 or with 10 pfu/cell UV-inactivated ONYX-015 were injected into the flanks of athymic mice. Another group of animals was injected with ARO cells (2×10^6) sc, and 3 d later 5×10^7 pfu ONYX-015 or 5×10^7 pfu UV-inactivated ONYX-015 were injected in the inoculation region. Tumor size was evaluated 20 d later. Tumor diameters were measured with calipers every other day until the animals were killed. Tumor volumes (V) were calculated by the formula of rotational ellipsoid: $V = A \times B^2/2$ (A is axial diameter, B is rotational diameter). No mouse showed signs of wasting or other visible indications of toxicity. All mice were maintained at the Dipartimento di Biologia e Patologia animal facility. The animal experiments described here 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.

Results

ONYX-015 induces cytolysis of human anaplastic thyroid carcinoma cell lines

We analyzed the sensitivity to ONYX-015 of three ATC-derived human cell lines (ARO, KAT-4, and FRO). ARO and KAT-4 harbor a mutated *p53* gene, *i.e.* 273 Arg[arrow]His (15, 16), whereas FRO cells express very low levels of *p53*, but no *p53* gene mutation (15). We evaluated the infectivity of the cell lines with a nonreplicating E1-deleted adenovirus with the *lacZ* gene under the control of a CMV promoter (CMV-*lacZ* virus). The thyroid carcinoma cells were infected at MOI of 10 and 100 pfu/cell and incubated for 48 h. At an MOI of 10 pfu/cell, 28% of ARO cells were infected (Table 1). KAT-4 and FRO cells were less susceptible to adenoviral infection (5% and 8% of infected cells, respectively, at an MOI of 10 pfu/cell).

All thyroid cells were sensitive to the cytopathic effect of ONYX-015. Sensitivity to ONYX-015 was correlated with the infectivity of each cell line. ARO and KAT-4 were sensitive even at an MOI of 5 pfu/cell (Fig. 1, A and B), whereas FRO cells were more resistant to ONYX-015 (Fig. 1C). No cytopathic effect was observed when the same cells were infected

with the UV-irradiated virus at MOI of 100 and 500 pfu/cells (Fig. 1, A–C), used as a negative control. It is important to note that UV inactivation does not reduce virus infectivity, as previously reported (31, 32). Conversely, the ONYX-015 virus did not have a cytopathic effect on normal rat thyroid differentiated cells, PC Cl 3 (Fig. 1D), even though these cells were infected by the *lacZ* control adenovirus at an efficiency comparable to that observed for the other cell lines (Table 1). This finding indicates that the cytopathic effect of ONYX-015 is specific to the thyroid carcinoma cell lines.

ONYX-015 induces cell death in papillary and follicular thyroid carcinoma cell lines carrying p53 gene mutations

p53 impairment is a feature of thyroid carcinoma cell lines established in culture regardless of histiotype (11, 15). Therefore, we also evaluated the sensitivity to ONYX-015 of two cell lines derived from thyroid differentiated carcinoma: NPA, which derives from a poorly differentiated papillary carcinoma, and WRO, which derives from a follicular car-

cinoma. Both cell lines carry a *p53* gene mutation (15). Both cell lines had a similar response to ONYX-015 as the ATC cell lines (Fig. 2, A and B). PC MPSV an *in vitro* transformed cell line, obtained by infecting PC Cl3 cells with a retrovirus containing a *mos* oncogene (MPSV), was used as a control to verify that ONYX-015 acts in a p53-dependent fashion (Fig. 2C).

Suppression of the ARO cell line tumorigenicity by E1B gene-defective ONYX-015 adenovirus

To evaluate whether ONYX-015 inhibited the growth of ARO cells *in vivo*, 30 athymic mice were inoculated sc with 1×10^6 ARO cells untreated or infected with ONYX-015 or UV-irradiated control virus. ONYX-015 inhibited tumor growth; the average tumor volume in ONYX-015-treated mice was about 60% less than those in the untreated and control virus-treated mice (Fig. 3A). To investigate whether ONYX-015 reduced the growth of an established ARO-induced tumor, ARO cells were injected into athymic mice,

A

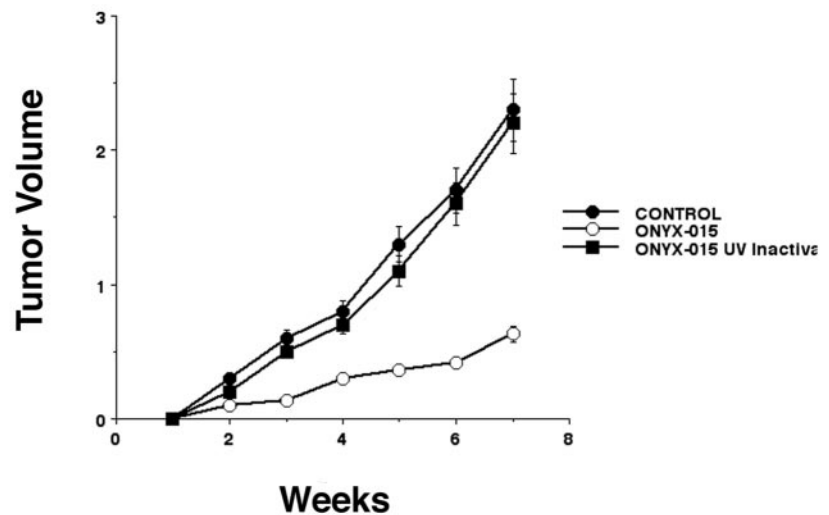
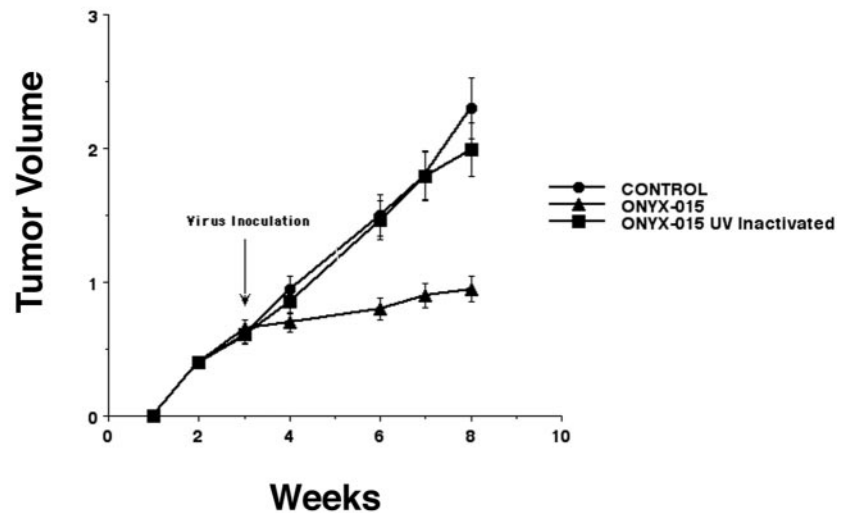


FIG. 3. Suppression of the ARO-induced tumor growth by ONYX-015. A, Tumor growth in athymic mice inoculated sc with 1×10^6 ARO cells untreated (control) or infected with ONYX-015 or UV-irradiated control virus. B, ARO cells were injected into athymic mice, and 20 d later, when tumors were clearly detectable, 5×10^7 pfu ONYX-015 or of UV-inactivated virus were intratumorally injected at weekly intervals. Tumor growth was significantly reduced in ONYX-015-treated animals.

B



and 20 d later, when tumors were clearly detectable, 5×10^7 pfu ONYX-015 were injected intratumorally at weekly intervals. Four weeks later the tumor was about 50% smaller in ONYX-015-treated animals (Fig. 3B) compared with those in animals untreated or treated with the control virus.

ONYX-015 enhances the cytotoxicity of two antineoplastic drugs (doxorubicin and paclitaxel) in two anaplastic thyroid carcinoma cell lines (ARO and KAT-4)

To determine the synergistic effect of ONYX-015 with drugs used to treat ATC, we evaluated the cytotoxic effects of doxorubicin and paclitaxel on ARO cells. The IC_{50} was 7 ng/ml for doxorubicin and 3 ng/ml for paclitaxel (Fig. 4A). Subsequently, ARO cells were exposed to increasing concentrations of doxorubicin or paclitaxel alone or combined with ONYX-015 (1 pfu/cell). The doxorubicin/ONYX-015 combination killed about 70% of cells even at a concentration of doxorubicin (1 ng/ml) that kills only 20% of ARO cells

when used alone. Analogous results were obtained with ONYX-015 combined with paclitaxel. At a paclitaxel concentration (0.5 ng/ml) that kills about 25% of cells (Fig. 4A), 65% of cells were killed when paclitaxel was associated with ONYX-015.

ONYX-015 and the antineoplastic drugs also acted synergistically on KAT-4 cells. In fact, the IC_{50} values for doxorubicin and paclitaxel were 75 and 35 ng/ml, respectively, in the presence of ONYX-015 (10 pfu/cells), and decreased to about 30 ng/ml for doxorubicin and to 20 ng/ml for paclitaxel (Fig. 4B)

Discussion

ATC leads to death in a very short time and is resistant to treatment (3, 33). In a novel therapeutic approach to ATC, manumycin (a farnesyl:protein transferase inhibitor), alone and combined with drugs used to treat ATC, was shown to be effective in ATC cell lines (34). Similarly, we found that

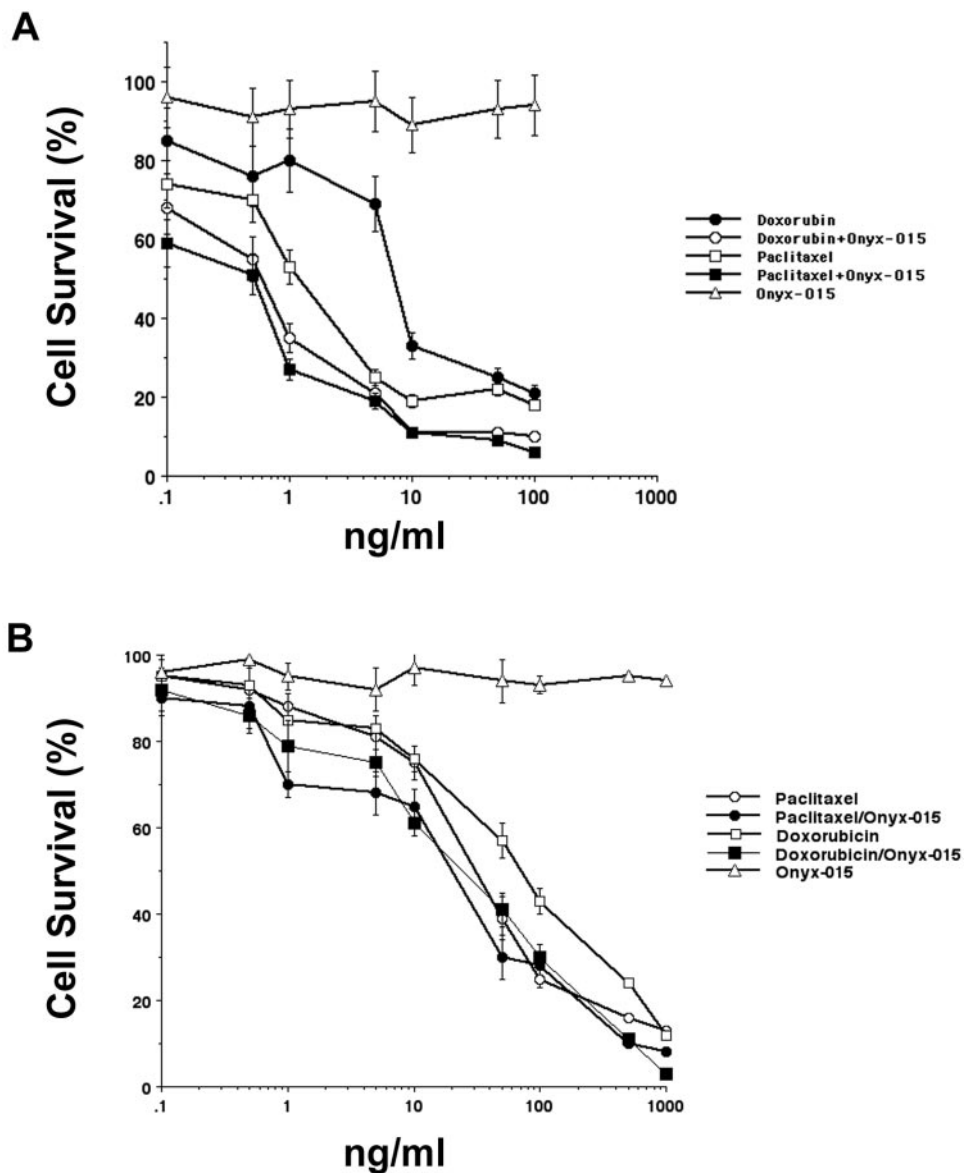


FIG. 4. Infection with ONYX-015 increases sensitivity to doxorubicin or paclitaxel. A, ARO cells exposed to increasing concentrations of doxorubicin or paclitaxel alone or combined with 1 pfu/cell ONYX-015 for 7 d. Combined doxorubicin/ONYX-015 treatment killed about 70% of cells at a doxorubicin concentration of 1 ng/ml. B, Paclitaxel at a concentration of 0.5 ng/ml killed 65% of cells when combined with ONYX-015. B, KAT-4 cells exposed to increasing concentrations of doxorubicin or paclitaxel alone or in combination of 10 pfu/cell ONYX-015.

an adenovirus carrying the HMGI(Y) gene in an antisense orientation (Ad-Yas) induced programmed cell death of the human ATC cell lines ARO and FB-1 (27). Another novel approach exploits the p53 impairment found in almost all ATC tumors and cell lines. It has been demonstrated that the infection of ATC cell lines with a wild-type p53-expressing adenovirus induced sensitivity to doxorubicin (16) and partial reversion of the differentiated phenotype.

Gene therapy aimed at repairing p53 function in ATC tumors seems promising. However, the viral vectors currently available do not transduce the p53 wild-type gene within the tumor sufficiently to block the growth of very aggressive neoplasias. Therefore, we envisaged using the ONYX-015 adenovirus that can replicate and then induce a cytopathic effect specifically in cells with impaired p53 function (18–21).

We now show that ONYX-015 induced cell death in three human thyroid anaplastic cells. Conversely, normal thyroid cells bearing a wild-type p53 gene were not affected by ONYX-015. Moreover, ONYX-015 drastically reduced the size of a xenograft tumor in athymic mice in two different experimental conditions (incipient tumors and established tumors). The local injection of ONYX-015 in established tumors led to a reduction of neoplastic growth; however, complete eradication of the tumors was not observed. It is very difficult to completely eradicate an established tumor of a significant size using intratumoral injection of ONYX-015 alone. In fact, it is almost impossible to deliver the virus to all the tumoral cells; therefore, the uninfected tumoral cells will continue to grow. In this way we can get a significant reduction in the size of the tumor, but not a complete regression. Consistently, in previous studies tumor regression was obtained by the association of ONYX-015 with chemotherapeutic drugs (21).

ONYX-015 virus acted synergistically with two antineoplastic drugs (doxorubicin and paclitaxel) in killing ARO and KAT-4 cells. Similar results have been obtained in lung cancer primary cultures and cell lines treated with ONYX-015 alone or combined with paclitaxel or cisplatin (35). Enhanced sensitivity to chemotherapeutic regimens in ONYX-015-infected cells may depend on E1A gene expression. In fact, E1A expression activates the cell cycle and increases cellular sensitivity to p53-independent apoptosis (36, 37). Therefore, the apoptotic effects of anticancer agents can be enhanced by the E1A adenoviral gene product (38).

In a phase II trial with patients affected by recurrent head and neck carcinoma, ONYX-015 resulted in significant tumor regression in evaluable patients (22–24). These results indicate that ONYX-015 could be beneficial in the treatment of locally aggressive neoplasias. Advanced or recurrent ATC poses a difficult therapeutic problem because the tumor soon invades the trachea and causes obstruction or compression, thereby reducing patient survival even further. Because ONYX-015, alone or combined with therapeutic agents, is able to kill ATC cell lines, it is reasonable to assume that local administration of ONYX-015 could reduce the local growth of ATC.

In conclusion, the results described herein could contribute to the development of new therapeutic protocols for the treatment of ATC.

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