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Report

Efficient induction of apoptosis by ONYX-015 adenovirus in human colon cancer cell lines regardless of p53 status

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The ONYX-015 virus is a mutated adenovirus that in theory selectively replicates and induces cytolysis in tumor cells lacking functional p53. The present study investigated whether ONYX-015 viral infection alone or in combination with conventional chemotherapeutic agents could significantly increase apoptosis in human colon cancer cell lines, regardless of p53 status, compared to untreated cells. A pair of colon cancer cell lines that differ only in their p53 status (RKO with wild-type p53 and RKOp53 with deficient p53) was tested. Two chemotherapeutic agents, 5-fluorouracil (5-FU) and CPT-11, were tested in combination with ONYX-015. Final concentrations of these agents corresponded to peak plasma levels achievable in patients. ONYX-015 concentration was 10 p.f.u./cell. In RKO and RKOp53 cell lines, ONYX-015 viral infection alone or in combination with 5-FU or CPT-11 induced a significant increase in apoptosis compared to chemotherapeutic agents alone, regardless of p53 status. Moreover, the combination of ONYX-015 and chemotherapeutics induced more apoptosis than chemotherapeutics alone in the two colon cancer cell lines independently of their p53 status. We conclude that ONYX-015 virus infection alone or in combination with 5-FU or CPT-11 induced apoptosis in human colon cancer cell lines, independently of p53 status. [© 2002 Lippincott Williams & Wilkins.]

Key words: Apoptosis, ONYX-015 adenovirus, p53.

Introduction

To allow efficient viral replication, adenoviruses must stimulate cell cycle and inactivate p53 in infected cells[1] The E1A region of the adenovirus genome encodes a 19-kDa protein which binds RB protein and forces human cells into S phase.[2] The E1B region encodes a 55-kDa protein which binds to the p53 protein and shackles it.[3] The ONYX-015 is a mutant adenovirus, with an 827-bp deletion in the EIB region and a point mutation at codon 2022 generating a stop codon that eliminates expression of the E1B gene.[4,5] It was hypothesized that the ONYX-015 virus would not be able to inactivate p53 and replicate in normal cells, but would replicate efficiently in tumor cells lacking functional p53.

The anti-tumor activity of ONYX-015 virus has been widely studied in *in vitro* studies, demonstrating cytopathic effects in human tumor cells lacking normal p53 function.[5] Recently, controversial results have been reported concerning the relationship between the cytopathic effects of ONYX-015 and the p53 status of the cells infected by the adenovirus. A report showed that that p53 was required for productive ONYX-015 infection and viral cytopathic effect.[6]

As induction of apoptosis is a major target for chemotherapeutic agents in tumor cells, the present study investigated whether ONYX-015 viral infection could significantly increase apoptosis in human colon cancer cell lines, regardless of p53 status, compared to untreated cells. As adenovirus EIA gene expression was previously demonstrated to increase cellular sensitivity to chemotherapy in a p53-independent manner, additional experiments tested the potential synergistic activity of ONYX-015 and conventional chemotherapy in human colon cancer cell lines with different p53 status.[7]

Methods

Human tumor cell lines

Two human colon cancer cell lines provided by Onyx Pharmaceuticals (Richmond, CA) were tested. This matched pair of colon cancer cell lines differed in the

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expression status of p53 (RKO with wild-type p53 and RKOp53 with deficient p53). These cell lines were chosen because preliminary results have demonstrated ONYX-015 activity in colon cancer.[8] Cell lines were grown according to the instructions provided by the supplier. The infection media was the same as regular growth media.

Adenovirus preparation

The preparation of adenovirus ONYX-015 was done by Onyx Pharmaceuticals. Stocks were aliquotted in small batches and stored at −70°C. Infection of the cells with ONYX-015 was done according to the protocol provided by Onyx Pharmaceuticals. Concentration used in the present study was 10 p.f.u./per cell. This concentration represents the ratio of virus to cells and is referred to as multiplicity of infection. Appropriate precautions were taken in the laboratory as outlined by Institutional Safety Protocols.[9]

Chemotherapeutic agents

CPT-11 and 5-fluorouracil (5-FU) were tested alone or in association with ONYX-015 infection in the two colon cancer cell lines. Final concentrations of these agents corresponded to peak plasma levels achievable in patients.[10–12]

Evaluation of apoptosis induction by ONYX-015 virus and chemotherapeutics agents

The degree of apoptosis was determined by terminal deoxynucleotidyl transferase activity assays on day 4.[13] This time point was chosen because previous experiments demonstrated that apoptosis increased over time and the maximum changes in apoptosis were observed over 80 h after treatment (data not shown). The cells were plated on day 0 in six-well tissue culture plates (one plate for each day of harvest). On day 1, ONYX-015 virus at the appropriate concentration (10 p.f.u./cell) in 400 μl of infection media was added to the appropriate wells (400 μl infection media was added to all other wells) and incubated at 37°C with 5% CO_2 for 3 h. At the end of 3 h, the virus was removed, and the appropriate chemotherapeutic agent in 4 ml infection media was added and incubated for 1 h at 37°C with 5% CO_2. Then the drug was removed and replaced with 4 ml infection media. Viral exposure was chosen prior to chemotherapy because efficacy was demonstrated to be highly dependent on the sequential exposure of cells to these agents. Treatment with ONYX-015 prior to chemotherapy was significantly superior to chemotherapy followed by viral infection.[14] On day 4, one plate was harvested and the cells were pelleted in 15 ml conical tubes. The media was removed and the cells were resuspended in 1 ml 4% neutral buffered formalin for 10 min at room temperature. The cells were centrifuged at 1000 r.p.m. for 5 min, all formalin removed and the cells resuspended in 2 ml ice-cold 70% ethanol. Cells were stored at 4°C in 70% ethanol until the assay was performed.

On the day of the assay, the cells were repelleted by centrifugation at 1000 r.p.m. for 5 min. All ethanol was removed and the cells were resuspended in 100 μl 4% neutral buffered formalin. Seventy-five microliters of cell suspension were pipetted and spread onto one end of a microscope slide (Fisherbrand Superfrost/Plus; Fisher Scientific, Pittsburgh, PA) and allowed to dry.

When dry, the slides were placed in a rack and washed in a Wheaton jar with 2 changes of 1 × PBS for 5 min each wash. The slides were blotted around the edges and 75 μl of equilibration buffer (Apoptag Plus apoptosis detection kit; Intergen, Purchase, NY) was placed directly on the sample. A plastic coverslip was applied and the slides were placed in a humidified chamber at 37°C for 1 h. The coverslips were removed, and the slides were placed in a rack in a Wheaton jar containing working strength stop/wash buffer and incubated for 10 min at room temperature. The slides were then washed in 3 changes of 1 × PBS for 3 min each. Then 52 μl of working strength anti-digoxigenin–fluorescein (Intergen kit) was added to each slide, a plastic cover slip was placed on top of the slide and the slides were incubated in a humidified chamber for 30 min at room temperature. Again the slides were washed in 3 changes of 1 × PBS for 5 min per wash. The slides were blotted dry at the edges and stained with 15 μl of mixture of 499 μl Vectashield and 1 μl propidium iodide. A glass coverslip was placed on top of the slide and the slides were stored at 4°C until they were counted (after not more than 1 or 2 days). The apoptotic cells were counted on an Olympus Bx60 microscope at 400-fold magnification using a Texas Red rhodamine filter.

Statistical comparisons of the percent of apoptotic cells were performed using the χ^2 test. A p value of <0.05 was considered to indicate statistical significance.

Results and discussion

Onyx-015 viral infection induced a statistically significant increase in apoptosis from day 4 in RKO and RKOp53 cells, compared to untreated cells ($p=0.0008$ and $p=0.001$, respectively) (Figures 1 and 2).

In RKO cells, the combination of 5-FU (10 μg/ml) and ONYX-015 viral infection induced a statistically significant increase of apoptosis compared to 5-FU alone ($p<0.001$). The combination of CPT-11 (3 μg/ml) and ONYX-015 viral infection also induced a statistically significant increase of apoptosis compared to CPT-11 alone ($p=0.01$) (Figures 1 and 2).

In RKOp53 cells, the amount of apoptosis was increased by the combination of 5-FU and ONYX-015 virus ($p<0.001$) or the combination of CPT-11 and ONYX-015 ($p=0.01$), compared to the amount of apoptosis induced by 5-FU or CPT-11 without virus infection (Figures 1 and 2).

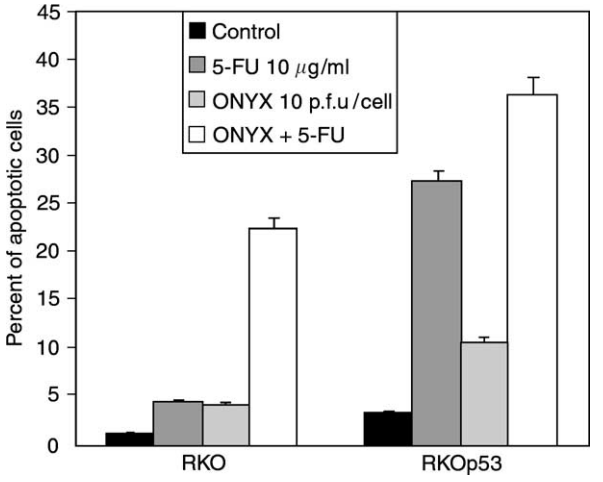

Figure 1. Apoptosis in RKO and RKOp53 cells on day 4 after treatment with 5-FU and ONYX-015.

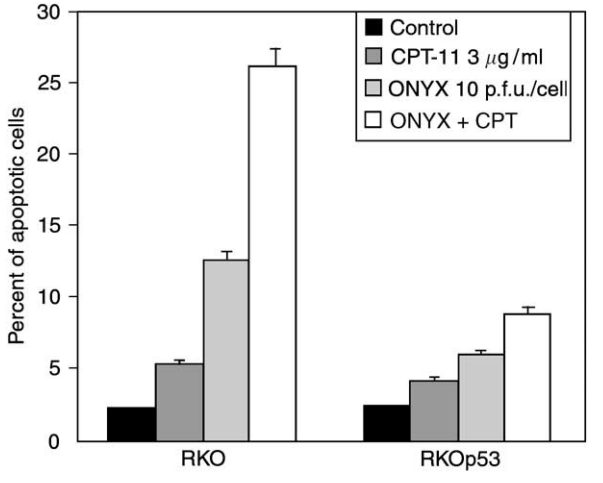

Figure 2. Apoptosis in RKO and RKOp53 cells on day 4 after treatment with CPT-11 and ONYX-015.

The present study showed that Onyx-015 viral infection (10 p.f.u./cell) significantly increased apoptosis in two colon cancer cell lines, regardless of p53 status, compared to untreated cells. The association between chemotherapeutic agents and ONYX-015 virus increased apoptosis over that caused by chemotherapy without concomitant viral infection.

The anti-tumor activity of ONYX-015 virus has been widely studied *in vitro* and *in vivo*. *In vitro* studies demonstrated cytopathic effects in human tumor cells lacking normal p53 function (e.g. cervical carcinoma cell lines expressing human papillomavirus E6, colon adenocarcinoma, glioblastoma and pancreatic adenocarcinoma).[5] The cytopathic effect of ONYX-015 on cell lines was demonstrated to be dose-dependent.[4] Complete cytolysis was seen in normal cells treated with wild-type adenovirus with a concentration of 0.01 p.f.u./cell, while this was not achieved with ONYX-015 at the same concentration. Cytolysis was induced by ONYX-015 at much higher concentration (>10 p.f.u./cell) in these cells. On the contrary, ONYX-015 was able to induce cytopathic effect in p53-deficient cell lines with a lower concentration (0.01 p.f.u./cell).

Recently, controversial results have been reported concerning the relationship between the cytopathic effects of ONYX-015 and the p53 status of the cells infected by the adenovirus. One study showed that p53 was required for productive ONYX-015 infection and viral cytopathic effects.[6] To explain this apparent contradiction, it was suggested that selectivity of ONYX-015 activity was dependent on the presence of the E1B/p53 complex.[15] Binding of E1B to p53 is required for activation of rapid cell death pathway, whereas viruses lacking E1B like ONYX-015 kill cells in a delayed manner independent of p53. In our study, we used a high concentration of ONYX-015 (10 p.f.u./cell) and we could demonstrate apoptosis induction in colon cancer cell lines, independently of p53 status.

Synergistic activity on cytolysis induction was demonstrated with the combination of ONYX-015 and chemotherapy agents in lung cancer cell lines with non-functional p53.[16] In the present study, viral infection had synergistic activity with chemotherapy for apoptosis induction both in p53 wild-type and p53-deficient cell lines.

Antitumor activity of ONYX-015 was also demonstrated in human tumor xenografts.[4] Subcutaneous xenografts of cervical carcinoma cell lines with inactivated p53 were reduced by intra-tumoral

injection of ONYX-015. Intra-tumoral delivery of the virus resulted in systemic release of virus and infection of distant tumor site.[17,18] In the same models, i.v. injection of ONYX-015 caused tumor cell infection and antitumor activity. In the HLaC human tumor xenograft model, the combination of cisplatin or 5-FU with ONYX-015 was more effective than chemotherapy or virus treatment alone.[4] Combination therapy with cisplatin and ONYX-015 led to improved survival compared to either agent alone in human ovarian tumor xenograft, independently of p53 functional status.[14] Combination therapy efficacy was highly dependent on the sequencing of the agents, with improved activity upon prior ONYX-015 treatment. ONYX-015 was also an efficient adjuvant treatment to radiation therapy, with a greater activity in p53-deficient tumors.[19]

In summary, ONYX-015 virus infection with high concentration (10 p.f.u.) induced apoptosis in two human colon cancer cell lines, independently of p53 status. The association of ONYX-015 virus infection and chemotherapeutic agents induced more apoptosis than each modality of treatment alone in these cell lines. These results provide a rational basis to evaluate the association of chemotherapy and ONYX-015 infection in clinical studies.

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