p14<sup>ARF</sup> Modulates the Cytolytic Effect of ONYX-015 in Mesothelioma Cells with Wild-type p53

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Abstract

ONYX-015 has been reported to kill selectively tumor cells lacking functional p53. Genetic alterations of INK4a/ARF locus, which is a predominant event in malignant pleural mesothelioma, may result in loss of p14<sup>ARF</sup> and subsequent disruption of p53 pathway in cancer cells. In the present study, ONYX-015 was able to kill three mesothelioma cell lines (H28, H513, and 211H) with wild-type p53 but lacking p14<sup>ARF</sup>. In contrast, MS-1 mesothelioma cells, which expressed both p53 and p14<sup>ARF</sup>, were resistant to ONYX-015. Introducing p14<sup>ARF</sup> gene into the H28 cell, a mesothelioma cell without p14<sup>ARF</sup> expression, significantly increased the resistance of this cell line to the cytolytic effect of ONYX-015. Our results suggest that human mesotheliomas with wild-type p53 yet lacking p14<sup>ARF</sup> are potential candidates for ONYX-015 therapy.

Introduction

MPM<sup>2</sup> is an asbestos-related malignancy characterized by rapidly progressive and diffusely local growth, late metastases, and poor prognosis. Approximately 3,000 patients are diagnosed with MPM in the United States annually. The incidence of this disease worldwide is rising and is expected to peak in the next 2 decades (1). Chemotherapy or radiation therapy, alone or in combination, is not effective, and the majority of patients with MPM die within 2 years regardless of treatment (1). Considering the uniformly fatal outcome and lack of effective treatment, the development of novel treatment strategies for MPM given the lack of response with conventional treatments is therefore needed. Replicating oncolytic virus is a promising new modality for cancer treatment. The strategy of this therapy is to develop viruses capable of selectively infecting malignant tumors which can spread and destroy malignant tumors without deleterious effects in normal tissues. The key to the development of such viruses is the identification of viral genes whose deletion or modification enables tumor-specific cell killing. ONYX-015, a conditional replication-competent adenovirus lacking E1b 55 kDa gene, is a promising agent based on this strategy. ONYX-015 contains an 827-bp deletion in the E1b region and a point mutation at codon 2022 that generates a stop codon. This genetic design takes advantage of the fact that adenovirus E1b 55k binds and thus inactivates wild-type p53 protein. This binding/inactivation is essential to virus replication. ONYX-015 cannot replicate in normal cells but can replicate in tumor cells lacking functional p53 (2). ONYX-015 has been shown to kill cervical, colon, pancreatic carcinoma cells, glioma cells, and non-small cell lung cancer cells lacking functional p53 with comparable efficiency to wild-type adenovirus (2, 3). In mesothelioma, unlike other adult malignancies, genetic alterations in p53 are rare (4). Homozygous deletions of INK4a/ARF locus, however, have been shown to be the predominant events which occur at a frequency of >70% in this malignancy (5, 6). The INK4a/ARF locus (7) on human chromosome 9p21 plays an important role in both the pRB and p53 tumor suppressor pathways by encoding two distinct proteins translated from alternatively spliced mRNAs. p16<sup>INK4a</sup> has been biochemically characterized as a protein that specifically binds to and inhibits cyclin-dependent kinase-4/6. Thus, p16<sup>INK4a</sup> regulates pRB phosphorylation and induces cell cycle arrest in G<sub>1</sub> phase (8), p14<sup>ARF</sup> plays a role as a negative regulator of MDM2, interfering with MDM2-mediated shuttling and degradation of p53 (9–11). A single mutational event at the INK4a/ARF locus therefore has the potential to disrupt both pRB and p53 tumor suppressor pathways. In addition, the p14<sup>ARF</sup> gene promoter is a CpG island that can be silenced by DNA methylation (12). These genetic alterations may result in a loss of negative regulation of MDM2. Because MDM2 has functional similarity to the adenoviral E1B 55K protein, lack of p14<sup>ARF</sup> may thus prevent a normal p53 response to viral infection, thereby allowing ONYX-015 replication. In the present study, we showed that ONYX-015 killed effectively mesothelioma cells with normal p53 gene but lacking p14<sup>ARF</sup> expression. Introducing the p14<sup>ARF</sup> gene into human mesothelioma cells lacking p14<sup>ARF</sup> expression by use of an adenoviral vector significantly increased the resistance of cells to the CPE of ONYX-015. This study suggests that ONYX-015 may be an effective treatment for tumors retaining wild-type p53 but lacking p14<sup>ARF</sup>, such as MPM.

Materials and Methods

Cell Lines and Cell Culture. H28 (ATCC CRL-5820), H513 (ATCC CRL-5830), and MSTO-211H (ATCC CRL-2081, 211H) mesothelioma cells and Met5A (ATCC CRL-9444) transformed mesothelium cells were obtained from American Type Culture Collection. MS-1 mesothelioma cell line has been described previously (13). All were cultured in RPMI 1640 complete media containing 10% FCS.

CPE Assay. ONYX-015 and WTD were supplied by ONYX Pharmaceuticals (Richmond, CA). WTD is identical to ONYX-015 except in the E1B, 55-kDa gene region where the original, wild-type sequence is present. Cells (1 × 10<sup>5</sup> cells/well) were plated in replicate 6-well plates, incubated overnight at 37°C, and then infected with ONYX-015 or WTD at increasing MOI, i.e., 0, 0.01, 0.1, and 1 plaque-forming unit per cell, in RPMI 1640 containing 2% FCS for 4 h at 37°C. Then cells were incubated in complete medium containing 10% FCS, and plates were monitored daily for CPE. The assay was terminated when essentially total cytolyis was observed in cultures infected with WTD at an MOI of 0.1. The plates were then stained with crystal violet (0.5% in 20% methanol; Sigma Chemical Co., St. Louis, MO) for analysis.

Adenoviral Vectors. The adenoviral vectors for gene expression studies were constructed by using the AdEasy System (14). The p14<sup>ARF</sup> and p16<sup>INK4a</sup> cDNA were kindly provided by Dr. K. Vousden (National Cancer Institute, Frederick, MD) and Dr. D. Beach (Institute of Child Health, London, England, United Kingdom). The adenoviral vectors containing target cDNA were introduced into the packaging cells by calcium phosphate precipitation. An adenoviral vector expressing the tetracycline responsive promoter for human p14<sup>ARF</sup> was constructed by using the AdEasy System (14). The p14<sup>ARF</sup> and p16<sup>INK4a</sup> cDNA were kindly provided by Dr. K. Vousden (National Cancer Institute, Frederick, MD) and Dr. D. Beach (Institute of Child Health, London, England, United Kingdom). The adenoviral vectors containing target cDNA were introduced into the packaging cells by calcium phosphate precipitation.

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2 The abbreviations used are: MPM, malignant pleural mesothelioma; pRB, retinoblastoma protein; ARF, alternate reading frame; CPE, cytopathic effect; WTD, wild-type D; MOI, multiplicity of infection; CMV, cytomegalovirus; GFP, green fluorescent protein.
United Kingdom). Briefly, the gene of interest was first cloned into a shuttle vector, pAdTrack-CMV, which contained CMV promoters and a GFP gene. The resultant plasmid was subsequently cotransformed into Escherichia coli BJ5183 cells with an adenoviral backbone plasmid, pAdEasy-1. p14ARF and p16INK4A recombinant adenoviruses (Adp14 and Adp16, respectively) were then generated in 293 cells (E1-transformed human embryonic kidney cells) and identified by PCR analysis of the DNA samples prepared from the cell culture supernatant. The recombinant adenovirus, AdCtrl, which carries a GFP gene regulated by the CMV promoter, was constructed with pAdTrack and pAdEasy-1 and used as a control in these experiments.

**Immunoblotting.** Cells (1 × 10^6) were plated in 10-cm dishes and incubated for 4 h at 37°C. Cells were then mock infected or infected with either AdCtrl or Adp14 (MOI of 50 each) and incubated for 72 h. Cell samples were washed twice with PBS, scraped off the plates, and lysed in cell lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS]. Whole cell lysates were boiled, and the protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA). Balanced amounts of cell proteins (20–40 μg) were boiled again after the addition of mercaptoethanol and bromphenol blue and fractionated by SDS-PAGE in 4–20% linear gradient acrylamide gel (Ready-Gel; Bio-Rad). After transferring the proteins onto Immobilon-P (Millipore, Bedford, MA), the membranes were blocked in 5% nonfat milk powder, 0.2% Tween 20 in TBS overnight at 4°C, and incubated with the primary antibody for 1 h at room temperature. Membranes were then washed in TWEEN in TBS for 5 min three times. Primary antibodies for p14ARF (C-18), p21WAF1 (C-19), and p53 (DO-1) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Depending on the experiment, goat antimusorse and donkey antigoathorse radish peroxidase were used as secondary antibodies (Santa Cruz Biotechnology). Antibody binding was visualized using Chemiluminescence Luminol reagents (Santa Cruz Biotechnology).

**Cytotoxicity Assays.** Cells were plated in triplicate on 96-well culture plates (500 cells/well) and incubated overnight at 37°C. Cells were then exposed to varying concentrations of AdCtrl, Adp14, or Adp16 and incubated for 7 days at 37°C. A colorimetric assay was performed as described previously (15). Briefly, cells were fixed in 10% trichloroacetic acid for 1 h, washed five times with water, and allowed to air dry. Cells were then stained for 30 min with 0.4% sulforhodamine B (Sigma Chemical Co.), dissolved in 1% acetic acid, and rinsed five times with 1% acetic acid to remove unbound dye. Bounded dye was then solubilized with 10 mM unbuffered Tris-base (pH 10.5) for 5 min. Visual absorbance (A595) was obtained using a kinetic microplate reader (Vmax; Molecular Devices, Sunnyvale, CA), which was used as a measure of cell number. The IC_{50}, dose which inhibited cell growth by 50%, was calculated assuming the survival rate of uninfected cells to be 100%. The relative differences in IC_{50} were calculated by dividing the IC_{50} of cells infected with Adp14 or Adp16 by the IC_{50} of cells infected with AdCtrl for each cell line. See Figure 1 for an example of the results. A two-sided paired t test was used to determine the statistical significance of differences.

**Results**

CPE of ONYX-015 in Human Mesothelioma Cells. The four mesothelioma cell lines, H28, H513, 211H, and MS-1, used in the present study were examined previously by single-strand conformation polymorphism, and no mutations in exons 5–8 of the p53 gene were detected (data not shown). ONYX-015, however, can effectively lyse H28 at MOI of 0.1, and both H513 and 211H were lysed at MOI of 1 within 10 days. Only MS-1 was resistant to ONYX-015 up to MOI of 1 (Fig. 1).

p53, p21WAF1, and p14ARF Expression in Mesothelioma Cells. Previously, we have reported the absence of p14ARF and p16INK4A expression in H28, H513, and 211H cells (15). In the present study, expression of endogenous p14ARF was detected by immuno blotting in MS-1 cells (Fig. 2A). We have detected homozygous deletion of exons 1α and 1β of INK4a/ARF locus in both H28 and 211H cells. Loss of exon 1α only (but not 1β) was found in MS-1 cells (data not shown). The transcription of p14ARF (exon 1β) in MS-1 cells and the absence of p14 ARF transcripts in H28, H513, and 211H cells was confirmed by reverse transcription-PCR (data not shown). Moreover, the absence of p16 as well as p14 ARF expression in H28 cells was also revealed by Waheed et al. (16). To investigate adenovirus-mediated p14ARF expression in mesothelioma cells, the H28 cell line was infected with Adp14 or AdCtrl. Forty-eight h after transfection at MOI of 50, fluorescent microscopic examination revealed that nearly 100% of the cells became GFP positive. As illustrated in Fig. 2A, endogenous p14ARF expression was absent in H28 mesothelioma cells. After Adp14 infection, strong expression of p14ARF was detected by immuno blotting. Because p14ARF functions to stabilize p53 by inhibiting MDM2, we looked next for the effect of p14ARF overexpression on p53 expression. The expression of endogenous p53 in H28 was markedly lower than that in MS-1. Transfection with Adp14 at MOI of 50 increased markedly the amount of p14ARF expression and also increased the expression of p53 in H28 (Fig. 2A). Treatment with AdCtrl did not have significant effect on p14ARF or p53 expression in this cell line (15).

In a time course study, p14ARF, p21WAF1, and p53 expression in MS-1 cells at 0, 3, 6, 9, 12, and 24 h after ONYX-015 infection (MOI of 1) was determined by Western blot analysis (Fig. 2B). A significant increase of expression of p14ARF, p21WAF1, and p53 was noted at 12 h and 24 h after adenoviral infection. A decline of protein levels of p14ARF, p21WAF1, and p53 was observed at 24 h after the infection.

Cytostatic Effect of Adenovirus-mediated p14ARF or p16INK4A Expression in H28 and MS-1 Mesothelioma Cells. Both p14ARF and p16INK4A function as the tumor suppressors, and they have been targeted for gene-replacement strategies for cancer therapy (7, 8, 15, 17, 18). We additionally studied the cytostatic effect of gene replacement with p14ARF or p16INK4A in H28, the cell lacking expression of p53.
both the p14ARF and p16INK4A proteins. We also studied MS-1, a cell line with p14ARF expression but deficient in p16INK4A, by examining the ratios of the IC50s after Adp14 or Adp16 infection relative to the IC50 of AdCtrl. Although the cytostatic effect of Adp16 varied between these two cell lines, the IC50 of Adp16 in both cells was 10-fold (8) lower than that of AdCtrl. The IC50 of Adp14 for H28 cells was 8-fold lower relative to that of AdCtrl, whereas the cytostatic effect of Adp14 was similar to that of AdCtrl in MS-1 cells (IC50 of AdCtrl/IC50 of Adp14 = 0.79 ± 0.41; Fig. 3).

Treatment with p14ARF Recombinant Adenoviruses Induced Resistance to the CPE of ONYX-015 in H28 Mesothelioma Cells.

To examine if the restoration of the p14ARF function in H28 cells would increase the resistance of the cell to ONYX-015-induced cytolytic effect, we infected H28 with combinations of either WTD or ONYX-015 (MOI of 1 each) and Adp14 or AdCtrl (MOI of 10 each). Although infection with Adp14 alone significantly inhibited the growth of the H28 cells compared with AdCtrl (41.5 ± 0.5 × 10⁴ cell/well, respectively; P = 0.001, in triplicate samples), the Adp14 attenuated the cytolytic effect of ONYX-015 (Fig. 4). When H28 cells were coinfected with WTD and AdCtrl, a complete cytolysis was observed at day 9, and there were ~5000 viable cells in the well coinfected with WTD and Adp14 (in triplicate) at the same time point. An additional 36 h of incubation was required (time course not shown) for the latter to reach the complete cell lysis. As shown in Fig. 4, ONYX-015 significantly reduced the viable cell numbers compared with mock infection in both samples coinfected with either Adp14 or AdCtrl (16.33 ± 2.75 versus 8.83 ± 0.76 10⁴ cell/well, respectively; P < 0.001 in both comparisons). The viable cell number in the sample treated with Adp14 and ONYX-015 was significantly higher than that treated with AdCtrl and ONYX-015 (16.33 ± 2.75 versus 8.83 ± 0.76 10⁴ cell/well, respectively; P = 0.01, on triplicate samples).

Discussion

Functional inactivation of pRB and p53 tumor suppressor pathways appears to be a fundamental requirement for the development of most
human cancers. In normal cells, pRB regulates cell proliferation by binding and sequestering E2F transcriptional factors essential for progression to S phase. These transcriptional factors are released in late G1 phase by the cyclin-D-dependent kinase-mediated phosphorylation of pRB, thereby allowing cells to enter S phase (7, 19). Defects in the pRB pathways, such as mutations in the RB gene or loss of p16INK4A, lead to the deregulation of the E2F transcription factors. Hyperactivation of E2F results in increased transcription of p14ARF, and p14ARF stabilizes p53 by promoting MDM2 degradation. Accumulation of p53 can cause growth arrest and induce apoptosis and, thus, prevent uncontrolled cell proliferation. Therefore, most tumors having defects in pRB pathways should harbor defects in p53 pathways as well.

The mechanisms involved in viral replication are somewhat similar to those in tumor cell proliferation. A major function of the early proteins of various DNA viruses is to provoke the infected cell to enter the cell cycle and progress to S phase. In this phase of the cell cycle, a virus can take advantage of the host’s DNA replication machinery to duplicate its own DNA. The E1a protein is necessary for adenovirus to drive cells toward S phase. E1a binds pRB and related proteins and, thus, liberates E2F. E2F is essential for S phase entry and is also able to activate transcription from the E2 region of the viral genome, a region that encodes a DNA polymerase and other proteins essential for viral replication (20). However, E2F also leads to increased transcription of p14ARF. p14ARF inhibits MDM2 activity and, thus, allows p53 to accumulate. This in turn can cause growth arrest, through induction of the cell cycle inhibitor p21WAF (15, 19), or it can provoke apoptosis through induction of Bax and by other means. Growth arrest and apoptosis early in infection would certainly reduce viral yield. On the other hand, adenovirus produces E1b 55K, the viral version of MDM2, to inhibit p53 and prevent transcriptional activation and exports p53 to the cytoplasm for degradation (20). ONYX-015, an E1b gene-attenuated adenovirus, should therefore replicate only in p53-deficient cells but not in normal cells. Six cancer cell lines (HCT116, A549, G401, HLA-C, McF-7, U87) in which the p53 gene is wild type, however, have been shown to support replication of ONYX-015 (21). Some of these cells express human papillomavirus E6, a protein known to target p53 for destruction (20). In the other cases, where ONYX-015 replicates efficiently even in cancer cells, retaining wild-type p53 gene may be attributable to the loss of p14ARF and subsequent functional inactivation of p53.

Disruption of the p53 pathway in tumor cells, such as H28, HS13, and 211H, because of the lack of p14ARF expression promotes the inhibition of p53 by MDM2, thus allowing ONYX-015 replication regardless of the presence of wild-type p53 gene. The relatively higher titer of ONYX-015 required (2) for cytolysis suggests that p53 functions in these cells are not completely abrogated. In our previous work (15), we showed that the replacement of p14ARF gene in these mesothelioma cell lines restores p53 function and results in cell cycle arrest and/or apoptotic cell death. In the present study, MS-1 cells, which express the p14ARF protein, were resistant to both ONYX-015 and Adp14. Craig et al. (8) reported previously that effects of adenovirus-mediated p16INK4A expression on cell cycle arrest were determined by endogenous p16INK4A and pRB status in human cancer cells. p16INK4A-mediated cytostatic effect is tightly associated with the presence of functional pRB in human cancer cells. Although we did not examine the genetic status of pRB in our cells tested, the assay for p16INK4A-mediated cytostatic effect in MS-1 and H28 suggested that both cell lines have functional pRB. The difference in sensitivity to Adp14 between these two cells was not related to the status of RB gene. If the loss of p14ARF leads to the sensitivity of H28 cells to ONYX-015, the replacement of p14ARF gene should inhibit the CPE of ONYX-015 in this cell. To minimize the cytoplastic effect resulting from overexpression of p14ARF, a lower titer (MOI of 10) of Adp14 was used for gene replacement. At this titer, we found that Adp14, although still having minimal cytoplastic effect in H28 cells, was able to attenuate the CPE of ONYX-015 in the cell tested. These results, to a great extent, indicate that the p14ARF protein can modulate the CPE of ONYX-015 in a cell line with wild-type p53. Reintroduction of p14ARF into H28 cells did not appear to prevent wild-type virus replication, indicating that the cells are still dividing.

MS-1 appears to have higher levels of p14ARF and p53 protein (Fig. 2A), suggesting that there might be some defect in the p53 pathway in MS-1 cells. To explore the response of p53 pathway in MS-1 cells to ONYX-015 infection, we monitored the protein levels of p14ARF, p21WAF, and p53 in a time course study. The p14ARF, p21WAF, and p53 protein levels were found significantly increased at 12 and 24 h after the adenoviral infection, indicating that the p53 pathway in MS-1 cells responded normally to oncogene E1A (Fig. 2B). Furthermore, we checked MDM2 protein level in MS-1 cells by immunoblotting. MS-1 was found to contain higher levels of MDM2 than H28 cells (data not shown). We hypothesize that the higher MDM2 levels in MS-1 cells lead to the higher levels of p14ARF, and its inhibition of MDM2 ubiquitin ligase activity is sufficient to stabilize p53 protein (22). Thus, the levels of p14ARF and p53 protein are higher in MS-1 cells. Therefore, although the protein levels of p14ARF and p53 in MS-1 cells are higher than in normal cells, the p53 pathway in MS-1 cells responds normally to oncogenes such as E1A. MS-1 is a unique mesothelioma cell line which has only p16 (Exon 1a) deletion but still has intact and functional p14ARF. This phenomenon was confirmed by PCR, reverse transcription-PCR, and immunoblotting.

We have shown that ONYX-015 infection can lead to a normal response of p53 pathways in MS-1 cells. Previously, we and others have shown that normal cells are resistant to ONYX-015, whereas tumor cells with nonfunctional p53 are sensitive to this adenovirus (3, 21). Recently, it was shown that this protective effect against ONYX-015 by re-expression of p14ARF is p53 dependent, indicating that a functional p14ARF-p53 pathway is required for inhibiting ONYX-015 replication (23).

Mutations in p53 genes occur in ~60% of all human cancers. Loss of p14ARF may well account for a significant proportion of the remaining tumors. A recent survey of human tumor-derived cell lines revealed that all cell lines examined that retained wild-type p53 had lost expression of p14ARF (24). Our results suggest that these tumors, including MPM, may be potential candidates for ONYX-015 therapy. Because ONYX-015 has been reported to work synergistically with chemotherapy in tumor cells (3, 21), additional studies to test the effect of ONYX-015 in combination with conventional chemotherapeutic agent(s) in human mesothelioma cells are currently underway in our laboratory.

References

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