Expression of p14ARF Overcomes Tumor Resistance to p53

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Abstract

Tumors without p53 mutation are often resistant to p53 gene therapy. We examined the mechanism using p53-resistant A549 cells and p53-sensitive H1299 cells. We found that p53 delivered by adenovirus is poorly expressed in A549 (ARF-null) cells but efficiently expressed in H1299 cells (ARF-positive). Strong p53 expression and apoptosis can be achieved in A549 cells using a p53 mutant resistant to degradation by MDM2 or by coexpression of ARF. The results suggest that enhanced MDM2 activity attributable to loss of ARF contributes to p53 resistance. Surprisingly, tumor cell lines with MDM2 gene amplification are still deficient for ARF expression, suggesting that MDM2 amplification does not substitute for ARF inactivation during tumor development.

Introduction

Mutation of p53 occurs in 50% of human tumors (1), suggesting that it is a rate-limiting factor in tumor development. Tumors without p53 mutation may also have other defects in the p53 pathway that allow them to develop in the presence of p53. Recent gene therapy experiments using replication-defective adenovirus vectors to express p53 (Ad-p53) revealed that tumor cells with wt p53 are highly resistant to the apoptotic effect of Ad-p53 both in culture and in tumor xenograft models (2, 3). Because p53 adenoviruses are being tested in the clinic, understanding the mechanism of resistance is important for the application of p53 gene therapy. The p53-inducible gene MDM2 functions as a feedback regulator of p53 by binding and promoting p53 ubiquitination and degradation (4). MDM2 gene amplification or overexpression are frequently observed in sarcomas, breast tumors, and leukemias (5), suggesting that it contributes to the functional inactivation of p53. MDM2 activity is regulated by ARF, which is encoded by a reading frame that partially overlaps with p16INK4a (6). ARF binds to MDM2 and inhibits the ubiquitin E3 ligase function of MDM2 (7). ARF expression is stimulated by several oncogenes (c-myc, E2F1, and EIA), forming a surveillance mechanism against abnormal cell proliferation (6). A significant number of human tumor cell lines show loss of ARF expression attributable to deletion or promoter methylation (8). Loss of ARF or amplification of MDM2 is associated with lack of p53 mutation (9), suggesting that they are alternative mechanisms for p53 inactivation during tumor development. Because ARF regulates p53 by binding to MDM2, loss of ARF expression should result in hyperactive MDM2 and enhanced degradation of p53. Therefore, we examined whether loss of ARF contributes to p53 resistance in tumor cells expressing wt p53.

Materials and Methods

Cell Lines and Adenoviruses. H1299 (non-small cell lung carcinoma, p53-null), SJSJA (osteosarcoma, wt p53, MDM2 amplification), JAR (choriocarcinoma, wt p53, amplified MDM2), NGP (neuroblastoma, wt p53, amplified MDM2), and CCF-STTG1 (astrocytoma, wt p53, amplified MDM2) were provided by Dr. Arnold J. Levine. A549 (non-small cell lung carcinoma, wt p53) was provided by Dr. Ruwien Zhang. Cells were maintained in DMEM with 10% fetal bovine serum. Adenovirus expressing human ARF was kindly provided by Dr. Yue Xiong (UNC, Chapel Hill, NC). Recombinant adenoviruses expressing wt and p5314/19 (14Q/19S) mutant were described previously (10). The viruses were amplified using 293 cells and purified by CsCl density gradient centrifugation. Viral titer was determined by serial dilution and detection of cytopathic effect on 293 cells in 96-well plates. Cells were infected in medium containing 10% fetal bovine serum using titers indicated in the figure legends. Cell death was determined using the CellTiter 96 kit (Promega).

Western Blot. Cells were lysed in RIPA buffer [1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 50 mM Tris (pH 7.4), 150 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride] centrifuged for 5 min at 10,000 × g, and the insoluble debris were discarded. Cell lystate (100 μg of protein) was fractionated with SDS-PAGE and transferred to Immobilon P filters (Millipore). The filter was blocked for 1 h with PBS containing 5% nonfat dry milk, 0.1% Tween 20. MDM2 was detected by monoclonal antibody 39G (11), ARF was detected by monoclonal antibody 14P02 (NeoMarkers), and p16 was detected by monoclonal antibody G175-405 (PharMingen). The filter was developed using ECL-Plus reagent (Amersham).

Immunofluorescence and TUNEL Staining. Cells cultured on chamber slides were fixed with acetone:methanol (1:1) for 3 min at room temperature, blocked with PBS + 10% NGS for 20 min, and incubated with anti-ARF antibody 2A9 hybridoma supernatant (1:100 dilution) or anti-ARF antibody 14P02 (0.5 μg/ml) in PBS + 10% NGS for 2 h. The slides were washed with PBS + 0.1% Triton X-100, incubated with FITC-goat-antimouse IgG in PBS + 10% NGS for 1 h, washed with PBS + 0.1% Triton X-100, and mounted. ApopTag was confirmed by TUNEL staining using the ApopTag kit (Intergen).

Results

An MDM2-resistant p53 Variant Induces Apoptosis in A549 Cells. Previous studies showed that adenovirus expressing wt p53 efficiently induces apoptosis in p53-deficient tumor cells but not in cells with wt endogenous p53 (2, 3, 12). H1299 (p53-null) and A549 (p53-wt) lung tumor cells represent examples of such differential responses (2). We found previously that inhibition of MDM2 expression in A549 using antisense oligonucleotide induces growth arrest and accumulation of p53 (13), suggesting that although MDM2 is not overexpressed in this cell line, it is still important for regulating endogenous p53. Therefore, we tested the possibility that MDM2 may also play a role in causing resistance to exogenous p53 expression using a previously characterized p5314/19 mutant. This p53 mutant carries two point mutations (14Q/19S) in the MDM2-binding site of p53, abrogating interaction with MDM2 without significant effect on the transcription activation potential (14).

Consistent with the phenotype described in previous reports, infection of H1299 and A549 with identical titers of Ad-p53 (100 pfu/cell) resulted in strong apoptosis in H1299 but not in A549 cells (Fig. 1). However, infection with Ad-p5314/19 resulted in similar levels of...
apoptosis in both cell lines (Fig. 1), suggesting that MDM2 binding is important for inhibition of apoptotic response in A549 cells.

**Efficient Degradation of wt p53 in A549 Cells.** Resistance to p53-mediated apoptosis in A549 may be attributable to tolerance to high-level p53 expression or efficient degradation of exogenous p53. Immunofluorescence staining of p53 in infected cells showed that wt p53 and p5314/19 were both efficiently expressed in H1299 cells, ~80% of cells were stained positive for p53 after infection with 100 pfu/cell of viruses (Fig. 2A). However, infection of A549 with Ad-p53 at the same titer resulted only in strong expression in a small percentage of cells; the majority of the population showed only a weak increase compared with the basal p53 level (Fig. 2B). In contrast, p5314/19 is more efficiently expressed in A549 cells after infection at 100 pfu/cell (Fig. 2B). Further increasing Ad-p53 infection multiplicity to 200 pfu/cell resulted in stronger expression in a subset of A549 cells but was still not able to achieve uniform expression (data not shown). It is unlikely that this is attributable to an inability of the adenovirus to infect a large fraction of A549 cells, because Ad-p5314/19 or Ad-lacZ control virus was able to express in most cells using the same infection procedure (Fig. 2 and data not shown).

Furthermore, inhibition of p53 degradation using MG132 resulted in a higher p53 level in most infected A549 cells compared with MG132-treated control (data not shown), suggesting that p53 was being expressed from the adenovirus but rapidly degraded in most cells.

**Restoration of ARF Expression Overcomes p53 Resistance.** A key regulator of MDM2 is the ARF tumor suppressor, which is often deleted or silenced in tumor cell lines expressing wt p53 (6). Previous studies showed that H1299 expresses high levels of ARF, whereas A549 has a deletion of the ARF locus (8, 15). Therefore, it is possible that loss of ARF expression resulted in hyperactive MDM2 and efficient degradation of exogenous p53. To test whether expression of ARF can restore the expression and apoptotic response to wt p53, Ad-p53 and Ad-ARF were used to coinfect A549 cells. Infection with Ad-ARF alone (40 pfu/cell) resulted in a weak but uniform increase of endogenous p53 level in A549 cells. Coinfection with Ad-p53 significantly increased the percentage of A549 cells that express high-level p53 (Fig. 3A). This correlated with strong cell death in the double-infected cells (Fig. 3, B and D). An increase of p53 expression by coinfection of Ad-ARF was also confirmed by Western blot analysis (data not shown).

The cell cycle status of infected cells was determined by FACS analysis. Ad-ARF or Ad-p53 infection alone induced significant reduction in the S-phase population in A549 cells with minimal apoptosis, suggesting that the level of p53 accumulation achieved by the single infections can only induce cell cycle arrest. However, coinfection with Ad-p53 and Ad-ARF resulted in strong apoptosis and generation of sub-G1 cell debris (Fig. 3E). The apoptotic nature of cell death in the coinfected cells was also confirmed by TUNEL staining (Fig. 3C). Therefore, ARF expression overcomes p53 resistance in A549 cells.

**Selective Loss of ARF Expression in Cells with MDM2 Amplification.** The results described above suggest that loss of ARF expression is an important mechanism of p53 resistance. Efficient degradation of p53 by MDM2 is an important step in this process, despite absence of MDM2 overexpression. In addition to cell lines with wt p53 and normal levels of MDM2, tumor cells with amplified MDM2 are also highly resistant to growth inhibition by exogenous p53 (10, 16). The cause of this resistance can be readily explained because MDM2 overexpression is thought to be sufficient to functionally inactivate p53. However, one would also expect that these tumors should continue to express ARF because there would be no selection pressure against ARF. To test this hypothesis, we examined ARF expression in four tumor cell lines known to contain amplified MDM2 (JAR, SJSA, NGP, and CFF-STTG1). Overexpression of MDM2 in these cells was confirmed by Western blot. The result showed that all four cell lines were also devoid of ARF expression (Fig. 4A). As reported previously, H1299 cells express high levels of ARF (8). MDM2 immunofluorescence staining in JAR cells showed a diffused localization in the nucleus and exclusion from the nucleoli (Fig. 4B). In contrast, endogenous MDM2 or transfected MDM2 in...
ARF-positive H1299 cells showed nucleolar accumulation. Because MDM2 is targeted to the nucleolus by ARF, a diffused localization pattern is consistent with absence of ARF expression. Because ARF is encoded by the p16INK4a locus, selection against p16 can simultaneously affect ARF expression. However, Western blot showed that p16INK4 was expressed in these cells, thus eliminating the possibility of co-deletion with p16. These results show that ARF expression is selectively repressed despite MDM2 amplification. Therefore, MDM2 amplification and loss of ARF expression are not mutually exclusive events. Inactivation of ARF may still be a necessary step in tumor development, even in cells with MDM2 overexpression.

**Discussion**

Resistance to p53-induced apoptosis is an important phenotype of tumor cells expressing wt p53 and may hamper cancer gene therapy using wt p53. Cellular response to p53 expression has been investigated previously using colorectal cancer cell lines (17). The results showed that certain cell lines such as DLD1 express a dominant-acting factor that confers sensitivity to p53. The identity of key regulators of p53 sensitivity and the mechanism of resistance has not been determined. Our results suggest that a major defect that contributes to p53 resistance is lack of ARF expression. In the absence of ARF, the MDM2 feedback loop may function at a hyperactive state that not only neutralizes endogenous wt p53 but also has the additional capacity to inactivate exogenous p53 by promoting its efficient degradation. We should emphasize that this is unlikely to be the only mechanism of p53 resistance. Other changes in the p53 pathway, such as altered expression of apoptosis-regulating genes, may also play a role.

Given its ability to promote p53 degradation and inhibit p53 activity, MDM2 amplification has been described as an alternative mechanism to p53 mutation. Consistent with this notion, several studies found that MDM2 amplification and p53 mutation rarely occur in the same tumor (5). The relationship between MDM2 overexpression and ARF has not been addressed in previous studies. In the absence of MDM2 overexpression, loss of ARF is essential for allowing low levels of MDM2 to efficiently degrade p53. Overexpression of MDM2 should remove selection pressure for both p53 mutation and ARF inactivation. However, we found
Fig. 3. Restoration of ARF expression sensitizes A549 to p53. A and B, A549 cells were incubated with Ad-p53 (100 pfu/cell) and Ad-ARF (40 pfu/cell) alone or in combination. Cells were stained for p53 expression with Pab1801 24 h after infection or photographed 96 h after infection. C, apoptosis was detected by TUNEL staining 96 h after infection. Arrows, TUNEL-positive apoptotic cells. D, A549 cells were incubated with Ad-p53 (100 pfu/cell), Ad-ARF (40 pfu/cell), and Ad-lacZ (100 pfu/cell) alone or in combination for 96 h. Cell survival was quantitated using the 3-(4,5-di methylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonil)-2H-tetrazolium assay. Bars, SD. E, A549 cells were incubated with Ad-p53 (100 pfu/cell) and Ad-ARF (40 pfu/cell) for 72 h. Cell cycle status was determined by staining with propidium iodide and FACS. Brackets, sub-2N apoptotic cells.
that four tumor cell lines with amplified MDM2 still suffer specific loss of ARF expression. This observation suggests that MDM2 amplification alone is not sufficient to inactivate p53 in the presence of ARF. Alternatively, both events facilitate tumor development through distinct mechanisms.

At different stages of tumor development, p53 is activated by a variety of stress signals through diverse mechanisms involving phosphorylation, ARF expression, and suppression of MDM2 expression (18). It is possible that loss of ARF provides an initial growth advantage to tumor cells by preventing mitogenic activation of p53. Loss of ARF may also decrease genomic stability and facilitate subsequent amplification of MDM2. MDM2 overexpression further inhibits p53 and protects cells against other stress during later stages of tumor development. Alternatively, MDM2 amplification may be the early event but is not sufficient to inactivate p53 during late-stage tumor development. Subsequent loss of ARF expression further increases the potency of MDM2 and prevents p53 activation. Furthermore, MDM2 overexpression or loss of ARF expression may promote transformation through mechanisms unrelated to inactivation of p53, because both proteins have other p53-independent functions (19, 20). In either case, the importance of ARF in tumor development is underscored by the finding that ARF expression is lost even in tumors harboring amplified MDM2.

Our results suggest that use of MDM2-resistant variants of p53 or coexpression of ARF will be more efficient in gene therapy of tumors that harbor wt p53. However, experiments using MDM2-null mouse fibroblasts suggest that normal cells may also be susceptible to p53-mediated apoptosis when MDM2 function is inhibited or bypassed, which will create nonspecific toxicity (21). Despite this potential caveat, a recent study using a different MDM2-resistant p53 mutant suggested that such a mutant still retains selectivity against tumor cells versus nontransformed human cells (22). Therefore, other intrinsic sensitivity of tumor cells to induction of apoptosis by p53 may provide a useful therapeutic window for this treatment strategy.

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References


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