p53-independent Induction of Apoptosis in Human Melanoma Cells by a bcl-2/bcl-xL Bispecific Antisense Oligonucleotide

Mary Strasberg Rieber, Uwe Zangemeister-Wittke, and Manuel Rieber

IVIC, Tumor Cell Biology Laboratory, Center for Microbiology and Cell Biology Apartado 21827, Caracas 1020 A, Venezuela [M. S. R., M. R.], and Division of Oncology, Department of Internal Medicine, University Hospital, Zurich, CH-8044, Zurich, Switzerland [U. Z-W.]

ABSTRACT

Mutations in the p53 tumor suppressor gene are implicated in defective apoptotic response of tumors to genotoxic damage and, thus, are major determinants of resistance to a variety of anticancer agents. Because even melanomas harboring wild-type (wt) p53 show an abnormal response to radiation and p53 mutations occur late during melanoma progression, we investigated whether the effect of the bcl-2/bcl-xL bispecific antisense oligonucleotide 4625 is dependent on the p53 status in human C8161 melanoma cells. Upon treatment with oligonucleotide 4625, p53-mut C8161 cells showed earlier DNA damage, which occurred concomitantly with the reduction of bcl-2 and bcl-xL expression and the increase in the expression of proapoptotic bax. Loss of cell viability, bcl-2 down-regulation, and poly(ADP-ribose) polymerase cleavage, indicative of apoptosis, also occurred in wt p53 C8161 cells on treatment with oligonucleotide 4625. These effects, however, were mediated by strong induction of p53 without changes in p21 WAF1 expression in wt p53 cells, whereas a 70% decrease in p21 WAF1 expression was observed in mut p53 cells. In contrast to many other anticancer agents to which the apoptotic response is decreased because of p53 mutations, our data suggest that the bcl-2/bcl-xL bispecific antisense oligonucleotide 4625 effectively induces p53-independent apoptosis in human C8161 melanoma cells.

INTRODUCTION

Recent studies in which the contributions of defective mismatch repair and mut1 p53 to cisplatin resistance of human tumor cells were analyzed, revealed that defective p53 status is a major determinant of cisplatin resistance. In contrast, there is no comparable contribution of mismatch repair defects to a predictable degree of resistance (1). Moreover, the emergence of mut p53 cisplatin-resistant ovarian carcinoma cells was shown to occur on extended drug exposure. The survival advantage of mut p53 cells in the presence of genotoxic agents could be related to a loss of susceptibility to p53-dependent apoptosis and to defects in checkpoint control pathways (2). In paclitaxel resistance primarily conferred by tubulin mutations, the loss of functional p53 has been identified in many different tumor cell clonies (3). Recently, in human prostate cancer cells a mechanism tying the accumulation of p53 mutations to the multidrug resistance phenotype, which is frequently found in this disease, was described (4). Also, an abnormality in the p53 pathway in response to γ-irradiation is detected even in many wt p53 human melanoma lines (5) that retain susceptibility to camptothecin (6). However, although p53 mutations are not frequent in benign nevi and primary melanomas, mutations in the p53 gene occur in 25–30% of metastatic melanomas (7, 8) and reduce camptothecin-induced apoptosis in this tumor type (6). On the other hand, besides p53 status, response to anticancer treatments is also counteracted by the expression of antiapoptotic bcl-2 and bcl-xL, and it was recently demonstrated that the bcl-2/bcl-xL bispecific antisense oligonucleotide 4625 facilitates apoptosis in lung cancer cells (9). Because the role of p53 mutation in the response of melanoma cells to this bispecific antisense oligonucleotide is unknown, we used an isogenic C8161 human melanoma system with differing p53 status, to investigate whether the bispecific antisense treatment is effective against C8161 melanoma cells harboring mut p53.

MATERIALS AND METHODS

Retroviral Transduction and Verification of p53 Status in Cells. These experiments were performed with C8161 human melanoma cells (10), which harbor a functional wt p53, as evidenced by its high inducibility on radiation- (10) or drug-induced DNA damage (11). To obtain mut p53 cells, parental C8161 cells were transduced with retroviruses packaged into Phoenix retroviral packaging cells (kindly provided by Dr. Garry P. Nolan, Department of Molecular Pharmacology, Stanford University, CA). We used calcium chloride transfection of Phoenix cells with pWZL-Hygro plasmid harboring a human p53–175 histidine mut gene, which is a dominant-negative mut p53 plasmid, as previously demonstrated elsewhere (12). Control cells were retrovirally transduced with the empty pWZL-Hygro plasmid. Both plasmids were kindly provided by Dr. Scott Lowe, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. Verification of p53 status was achieved by immune precipitation of mut p53 with monoclonal antibody Pab 240(SC-99) which is mut p53-specific under nondenaturing conditions. wt p53 was identified by its lack of immune precipitation with Pab 240(SC-99) under nondenaturing immune precipitation and.
reactivity with monoclonal antibody (Pab DO-1-SC-126) under comparable conditions. Immune precipitation was aided by the addition of Protein A/G Plus agarose (SC-2003). Subsequently, immune precipitated proteins were subjected to SDS-PAGE, bidirectionally blotted onto nitrocellulose membranes (11), and p53 identified by the DO-1 (SC126) monoclonal antibody, which recognizes both wt and mut p53 in denatured form after SDS-PAGE. Both of these antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

**Antisense Oligonucleotides.** These studies were performed with a recently reported bcl-2/bcl-xL bispecific 2'-O-methoxy-ethoxy-modified phosphorothioate antisense oligonucleotide 4625, which recognizes a region of homology shared by the bcl-2 and bcl-xL mRNA and has the potential to downregulate both of the antiapoptotic genes (9) at the same time. The antisense 4625 and the scrambled control 4626 sequences were as previously described: 4625, 5'-AsAsGsGsCsAsTsCsCsGsGsCsTsAsTsAsTsT-3'; 4626, 5'-CsAsGsGsTsCsAsGsGsCsAsCsTsAsTsT-3'. Oligonucleotides were delivered as 1:1 complexes with the lipofectin transfection reagent (Life Technologies, Inc., Gaithersburg, MD) into subconfluent C8161 cells 20 h after seeding. Lipofectin-oligonucleotide complexes, prepared to provide a final oligonucleotide concentration of 600 nM, were added for 8–16 h in serum- and antibiotic-free medium. Subsequently, cells were washed and incubated in complete serum-containing medium for 20 or 36 h prior to assay of oligonucleotide activity, unless otherwise indicated.

**Immune Blotting.** Cell lysates were obtained as described recently (10), followed by SDS-PAGE and bidirectional immune blotting (11) using 70 μg of protein, including prestained Mr markers (Life Technologies, Inc.) during electrophoresis to help in identifying specific changes in protein expression (10, 11). After blocking the nitrocellulose membranes with 5% skim milk in Tris-buffered saline (pH 7.5) for 2 h at room temperature, membranes were reacted overnight with specific antibodies in the same blocking solution. Antibodies used for specific immune blotting included: p53 (DO-1), p21WAF1 (SC-397), PARP (SC-7150), bcl-2 (SC-492), bcl-xL (SC-7195), bax (SC-526). All of these antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). After extensive washing with Tris-buffered saline containing 0.05% Tween 20, membranes were reacted with antimouse IgG-peroxidase for DO-1-mediated p53 detection or with Protein A-peroxidase for detection of all other proteins. Finally, blots were assayed by SuperSignal- (Pierce Chemical, Rockford, IL) mediated chemiluminescence.

**Densitometric Quantitation.** Analysis of differential protein expression was achieved by digitalization of images with a Fluor-S Imager (Bio-Rad) followed by quantitation of specific bands with the Gel Pro Analyzer software (Media Cybernetics, Silver Spring, MD).

**RESULTS**

**Induction of Growth Arrest, PARP Cleavage, and Melanoma Cell Death Independent of p53 Status.** Initial morphological studies demonstrated that 36 h after lipofectin-mediated uptake of the bcl-2/bcl-xL-bispecific antisense oligonucleotide 4625 at a dose of 600 nM, C8161 melanoma cells underwent growth arrest, rounded up, and detached from their substrate. This occurred irrespective of the p53 status, and no comparable effect was observed in control cultures treated with lipofectin and the scrambled control oligonucleotide 4625 (Fig. 1). Because PARP cleavage is indicative apoptosis (13), we also investigated whether the morphological changes in cells occurring within 36 h posttreatment with oligonucleotide 4625 correlated with PARP cleavage. These experiments, which included another lipofectin-only control also, confirmed the p53-independent cleavage of PARP only in C8161 melanoma cells.
treated with antisense oligonucleotide 4625 (Fig. 2). PARP cleavage also correlated with a loss of cell viability, because the bispecific 4625 oligonucleotide induced trypan blue uptake in most wt or mut p53 cells shown in Fig. 1, with no comparable result after exposure of the cells to the scrambled control oligonucleotide 4626 (not shown).

**Down-Regulation of bcl-2 Protein Expression Preferentially Occurs in Melanoma Cells with Mutant p53.** To better define the effect of the bcl-2/bcl-xL bispecific antisense oligonucleotide 4625 on bcl-2 expression, we compared the expression levels of this antiapoptotic protein (9) in cells 36 h after treatment with either antisense oligonucleotide 4625 or the scrambled control oligonucleotide 4626. When quantitated with reference to loading proteins, treatment with antisense oligonucleotide 4625 in wt p53 cells reduced bcl-2 expression by 32% compared with the reduction in cells treated with control oligonucleotide 4626, whereas in mut p53 cells, treatment with oligonucleotide 4625 reduced bcl-2 expression by 69% compared with the reduction in control-treated cells (Fig. 3).

**DNA Fragmentation and Increase in the bax:bcl-xL Ratio Preferentially Occurs in Melanoma Cells with Mutant p53.** To determine whether DNA fragmentation differed with the unequal p53 status, we compared early DNA damage within 20 h of treatment with antisense and control oligonucleotide. As shown in Fig. 4, antisense oligonucleotide 4625 induced a preferential DNA damage in mut p53 cells. In parallel cultures, we investigated whether this DNA cleavage correlated with the induction of proapoptotic bax and the down-regulation of bcl-xL. Down-regulation of bcl-xL and increase in the bax:bcl-xL ratio was detectable in the mut p53 cells. Also, the lack of early DNA damage seen in wt p53 cells assayed within 20 h of exposure to antisense and scrambled oligonucleotides (Fig. 4, left) correlated with little change in the bax:bcl-xL ratio (data not shown). This suggests that DNA damage is required to increase the bax:bcl-xL ratio.

**Antisense Oligonucleotide-mediated Death of Mutant p53 Melanoma Cells Is Characterized by the Preferential Reduction of p21WAF1 Expression.** Using conditions identical to those showing apoptosis-associated PARP cleavage in both wt and mut p53 C8161 melanoma cells (Fig. 2), we investigated whether the p53 status influenced the expression of p53 and p21WAF1, a p53-activated gene (9) which can also be regulated in a p53-independent manner (14, 15). In agreement with the concept that wt p53 is poorly expressed in proliferating C8161 melanoma cells but inducible by DNA damage (10, 11, 15), and with the known overexpression of mut p53 in late-stage melanomas (7, 8), low p53 levels were detected in wt p53 cells treated with the control oligonucleotide 4626. In contrast, a 9-fold induction of p53 was observed on treatment of cells with oligonucleotide 4625. Induction of wt p53 did not significantly change the level of p21WAF1 (Fig. 5, left). As expected for cells harboring mut p53 in which p53-dependent activation of p21WAF1 is impaired (15), p53 protein was overexpressed and lower p21WAF1 basal levels were even more decreased in mut p53 cells undergoing death mediated by antisense oligonucleotide 4625.

**DISCUSSION**

In this study, we have investigated the effect of antisense oligonucleotide 4625 designed to inhibit simultaneously the expression of the antiapoptotic genes bcl-2 and bcl-xL (9) on isogenic C8161 melanoma cells expressing either wt or mut p53 (12). The efficacy of oligonucleotide 4625 was recently demonstrated against a variety of human lung cancer cell lines (9); however, little is known about the effect of this oligonucleotide on tumors harboring mut p53. The wt p53 protein plays a critical role in cellular events including cell-cycle arrest and cell death, in response to DNA damage induced by chemotherapeutic agents (6) and γ-irradiation (5). wt p53 dysfunction, as found in melanomas, (3) can result in
abnormal cell growth, increased cell survival, genetic instability, and drug resistance. p53 mutations occur in approximately one-half of all human cancers and are associated with poor treatment outcome and poor prognosis (1–4, 6, 7, 15). In contrast to results showing diminished susceptibility of cells with p53 mutations to cisplatin (1, 2) or paclitaxel (3), we have now demonstrated that in response to treatment with antisense oligonucleotide 4625, human C8161 melanoma

Fig. 3 Down-regulation of bcl-2 protein expression occurs preferentially in melanoma cells with mut p53. Cells treated as indicated in legend to Fig. 1 were lysed, and extracts were used for SDS-PAGE and immune blotting with rabbit antibody to bcl-2. Results quantitated with reference to the protein loading profile showed lower bcl-2 expression by 32% of cells in wt p53 cells treated with antisense oligonucleotide compared with the reduction in wt p53 cells treated with control oligonucleotide. In mut p53 cells, treatment with antisense oligonucleotide reduced bcl-2 expression by 69% compared with its control.

Fig. 4 DNA fragmentation and increase in the bax:bcl-xL ratio occurs preferentially in melanoma cells with mut p53. wt p53 or mut p53 cells were exposed for 8 h to either lipofectin plus 600 nM antisense oligonucleotide (Antisense) or to lipofectin plus 600 nM scrambled control oligonucleotide (Control). After 20 h in complete medium after exposure to the corresponding oligonucleotides, DNA fragmentation was detected after electrophoresis in 1.4% agarose gels (left), and bax:bcl-2 levels were assessed by immune blotting (right). The bispecific oligonucleotide increased preferentially DNA damage (left) in mut p53 cells, correlating with down-regulation of bcl-xL and increase in bax (right).

In contrast to results showing diminished susceptibility of cells with p53 mutations to cisplatin (1, 2) or paclitaxel (3), we have now demonstrated that in response to treatment with antisense oligonucleotide 4625, human C8161 melanoma
cells with mut p53 show earlier DNA fragmentation compared with their wt p53 isogenic counterparts. As expected from the DNA damage data and sequence specificity (9), antisense treatment down-regulated the antiapoptotic bcl-xL and bcl-2 in mut p53 C8161 melanoma cells. This bispecific effect was previously found to correlate with caspase 3 activation in lung tumor cells (9). In the present study, we demonstrated that antisense oligonucleotide 4625 induced PARP cleavage, which is mediated by activated caspase 3 (16). However, although apoptosis-associated PARP cleavage occurred in both wt and mut p53 tumor cells, there was a different requirement for p53 (13). A loss of cell viability in wt p53 cells occurred through a 9-fold induction of p53 and no significant reduction of p21WAF1 expression. In contrast, on treatment with antisense oligonucleotide 4625, C8161 melanoma cells with mut p53 revealed no increase in their overexpressed p53 (7, 8), but there was nearly a 70% decline in the lower basal expression level of p21WAF1, compared with that in wt p53 cells.

Because the antisense oligonucleotide 4625 specifically targets the expression of bcl-2 and bcl-xL (9), it must indirectly down-regulate p21WAF1 in mut p53 cells (Fig. 5), which show earlier DNA damage in response to antisense treatment (Fig. 4). Recently, it was demonstrated by others that p21WAF1 acts in synergy with bcl-2 to prevent apoptosis in human lung cancer cells (17). In this test system, cotreatment with p21WAF1 and bcl-2 antisense oligonucleotides restored drug sensitivity in camptothecin-resistant cells more than either antisense alone (17). Because p21WAF1 is lower and does not increase with DNA damage in p53-mut cells (Fig. 5; Ref 15), this may allow for unrestricted proliferation in the absence of death-promoting agents like antisense oligonucleotide 4625. However, p21WAF1 is indirectly decreased in p53-mut cells exposed to this antisense oligonucleotide and, therefore, the loss of p21WAF1 perhaps results in checkpoint failure leading to apoptosis in response to DNA damage (15) and down-regulation of bcl-2 and bcl-xL (9). The direct effect of oligonucleotide 4625 on bcl-2 and bcl-xL expression that results in increased apoptosis (9) and its indirect effect on p21WAF1 that results in a defective checkpoint may partly explain its efficacy in p53-mut cells.

In contrast to the early DNA-damage induced in mut p53 C8161 melanoma by the bcl-2/bcl-xL bispecific antisense oligonucleotide 4625 (Fig. 4), no comparable effect was seen in parallel experiments with an antisense oligonucleotide 4259 against bcl-xL (18). This greater efficacy of oligonucleotide 4625 (9) compared with that of oligonucleotide 4259 (18) is compatible with results that show a 10-fold increase in caspase activity in SW2 lung cancer cells treated with oligonucleotide 4625, which show no such increase in the same cells treated with oligonucleotide 4259 (18).

In summary, although earlier studies have implicated p53 mutations in a defective apoptotic response to various forms of genotoxic damage (1-4, 6, 15), this is the first report demonstrating apoptosis of human melanoma cells overexpressing mut p53–175 (Arg to His; Refs. 7, 8) in response to DNA damage (15) and down-regulation of bcl-2 and bcl-xL (9). The susceptibility of C8161 melanoma with mut p53–175 (Arg to His) to the bcl-xL bispecific antisense oligonucleotide contrasts with the decreased camptothecin-mediated apoptosis versus mut p53 melanoma cells (6). Additional studies based on the use of this promising antisense strategy, either by itself or in combination with other therapies, are warranted because, compared to a variety of conventional anticancer treatments, it seems to be more effective against mut p53 tumors (1, 3, 5, 6, 15).
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