Inactivation of p21\textsuperscript{WAF1} Sensitizes Cells to Apoptosis via an Increase of Both p14\textsuperscript{ARF} and p53 Levels and an Alteration of the Bax/Bcl-2 Ratio*

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p21\textsuperscript{WAF1} appears to be a major determinant of the cell fate in response to anticancer therapy. It was shown previously that HCT116 human colon cancer cells growing \textit{in vitro} enter a stable arrest upon DNA damage, whereas cells with a defective p21\textsuperscript{WAF1} response undergo apoptosis. Here we report that the enhanced sensitivity of HCT116/p21\textsuperscript{−/−} cells to chemotherapeutic drug-induced apoptosis correlates with an increased expression of p53 and a modification of their Bax/Bcl-2 ratio in favor of the pro-apoptotic protein Bax. Treatment of HCT116/p21\textsuperscript{−/−} cells with daunomycin resulted in a reduction of the mitochondrial membrane potential and in activation of caspase-9, whereas no such changes were observed in HCT116/p21\textsuperscript{+/−} cells, providing evidence that p21\textsuperscript{WAF1} exerts an antagonistic effect on the mitochondrial pathway of apoptosis. Moreover, the role of p53 in activation of this pathway was demonstrated by the fact that inhibition of p53 activity by pifithrin-α reduced the sensitivity of HCT116/p21\textsuperscript{−/−} cells to daunomycin-induced apoptosis and restored a Bax/Bcl-2 ratio similar to that observed in HCT116/p21\textsuperscript{+/−} cells. Enhancement of p53 expression after disruption of p21\textsuperscript{WAF1} resulted from a stabilization of p53, which correlated with an increased expression of the tumor suppressor p14\textsuperscript{ARF}, an inhibitor of the ubiquitin ligase activity of Mdm2. In accordance with the role of p14\textsuperscript{ARF} in p53 stabilization, overexpression of p14\textsuperscript{ARF} in HCT116/p21\textsuperscript{−/−} cells resulted in a strong increase in p53 activity. Our results identify a novel mechanism for the anti-apoptotic effect of p21\textsuperscript{WAF1} consisting in maintenance of mitochondrial homeostasis that occurs in consequence of a negative control of p14\textsuperscript{ARF} expression.

Stress stimuli such as cytotoxic drugs stabilize the tumor suppressor p53 (1–3). Depending on the cellular context, activation of p53 promotes either cell cycle arrest to enable DNA repair or apoptosis to eliminate defective cells (4). Recent studies have addressed the question of how p53 triggers either one or the other of these two pathways, and the protein p21\textsuperscript{WAF1} has been implicated as a major determinant of cell fate.

p21\textsuperscript{WAF1} is responsible for the p53-dependent checkpoint that results in G1 arrest after DNA damage (5, 6). Cells deficient in p21\textsuperscript{WAF1} display a markedly different response to DNA damaging agents compared with cells with an intact p21\textsuperscript{WAF1}-dependent checkpoint. HCT116 human colon cancer cells growing \textit{in vitro} enter a stable arrest following DNA damage by γ-irradiation or chemotherapeutic drugs, whereas cells with a defective p21\textsuperscript{WAF1} response undergo apoptosis (6–9). Moreover, whereas irradiation of xenograft tumors established from cells with the intact p21\textsuperscript{WAF1} gene resulted in no cure, a significant fraction of tumors generated from p21\textsuperscript{WAF1}-deficient cells were completely eliminated (10). Similarly, p21\textsuperscript{WAF1} antisense therapy of wild-type HCT116/p21\textsuperscript{−/−} cells led to loss of G1 arrest, and increased apoptosis after ionizing radiation exposure both \textit{in vitro} and in tumor xenografts in nude mice (11). An anti-apoptotic effect of p21\textsuperscript{WAF1} on p53-dependent or p53-independent cytostatic stimulation has also been observed in several other cell types such as melanoma cells (12) or lung cancer cells (13).

The mechanisms by which p21\textsuperscript{WAF1} inhibits cell cycle progression has been found to involve its binding to G1 cyclin/cyclin-dependent kinase complexes and to the proliferating cell nuclear antigen proliferating cell nuclear antigen (20). In consequence, overexpression of p21\textsuperscript{WAF1} sensitizes cells to apoptosis via an increase of p53 levels that resulted from an enhanced transcription factor NF-κB during treatment of Ewing sarcoma cells with TNF-α (14). Expression of p21\textsuperscript{WAF1} was also shown to play a key role during differentiation of several cellular systems by conferring cell survival. Indeed, inhibition of p21\textsuperscript{WAF1} expression participates in the anti-apoptotic effect of the transcription factor NF-κB during treatment of Ewing sarcoma cells with TNF-α (14).

In this study we investigated the mechanisms that are implicated in the enhanced sensitivity to therapeutic agents of p21\textsuperscript{WAF1} gene-deleted colorectal carcinoma cells. We report that abolition of p21\textsuperscript{WAF1} protein expression induced a post-transcriptional increase of p53 levels that resulted from an enhanced expression of the tumor suppressor p14\textsuperscript{ARF}. The consequence of the increased activity of p53 was a modification of the balance...
between the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax in favor of Bax.

**EXPERIMENTAL PROCEDURES**

**Cells**—Wild-type HCT116 human colon carcinoma cells (HCT116/p21\(^+/−\)), which express normal p53 and p21\(^{WAF1}\), and a derivative in which both p21\(^{WAF1}\) alleles have been deleted through homologous recombination (HCT116/p21\(^−/−\)) were kindly provided by Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD) and have been described previously (13). Plasmids and Reagents—The pp53-TA-luc vector and the pRSV-β-galactosidase (pRSV-βgal) vector were from Clontec and Promega, respectively. The pcDNA vector encoding p14\(^{ARF}\) was a gift from Dr. S. Gazzeri (INSERM EMI 9924, Institut Albert Bonniot, La Tronche, France).

The following antibodies were used for Western blot analysis: monoclonal antibodies against p53, Mdm2, c-Ab1, cLAP1, and Bcl-x\(_L\) (Pharmigen), caspase-9 (Upstate Biotechnology), poly(ADP-ribose) polymerase (Alexis Biochemicals), XIAP (Medical and Biological Laboratories Co.), p21\(^{WAF1}\) (Transduction Laboratories), p14\(^{ARF}\) (Oncogene), Bcl-2 (Dako), actin (ICN), rabbit serum against phospho-JNK and Bad (New England Biolabs), and JNK1, cLAP2, and Bax (Santa Cruz).

Mouse monoclonal antibody anti-CD95, clone B.G27, was purchased from Biomol. Reconstituant human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or TNF-α were from R&D Systems and Bender Wien, respectively. Pifithrin-α (PFTα) was purchased from Alexis Biochemicals. Daunomycin, adriamycin, and etoposide were from Sigma.

**RESULTS**

**p21\(^{WAF1}\) Protects HCT116 Cells from Chemotherapeutic Drugs, but Not from TRAIL-induced Apoptosis—**p21\(^{WAF1}\) has been reported to exert a protective effect against radiations and various chemical apoptosis inducers (7–14). Here we investigated whether p21\(^{WAF1}\) similarly influences the cellular response to chemotherapeutic drugs and to death receptor ligands. For this purpose, we used wild-type HCT116/p21\(^+/−\) human colon carcinoma cells and HCT116/p21\(^−/−\) cells in which both p21\(^{WAF1}\) alleles have been deleted by homologous recombination (6). Cells from both cell lines were exposed to the chemotherapeutic drugs daunomycin, adriamycin, and etoposide, and to the death receptor ligands TNFα, anti-CD95 (Fas), and TRAIL. The percentage of apoptotic cells was determined by staining with the DNA binding dye Hoechst 33258, which allows visualization of condensed chromatin and fragmented nuclei. As shown in Fig. 1, we observed a drastic increase in the chemosensitivity to daunomycin in cells lacking the p21\(^{WAF1}\) gene. Similar results were obtained for adriamycin and etoposide, in accordance with previously reported observations (8, 9). Whereas HCT116/p21\(^+/−\) and HCT116/p21\(^−/−\) cells were insensitive to the killing effect of TNFα and anti-CD95, both cell lines undergo cell death to almost similar extents following treatment with TRAIL. This demonstrates that in HCT116 cells, p21\(^{WAF1}\) does not extensively interfere with the death pathway triggered by TRAIL, in contrast to its strong inhibitory effect on chemotherapeutic drug-induced cell death.

**Inactivation of p21\(^{WAF1}\) Alters the Cellular Bax/Bcl-2 Ratio**—It has been shown that chemotherapeutic agents can induce apoptosis through multiple pathways, mainly by targeting the mitochondrial pathway (25). This results in cytochrome c release and activation of the apoptosome complex that then activates downstream caspases. Binding of TRAIL to its cognate receptors DR4 and DR5 can activate a second pathway leading to direct caspase activation independently of mitochondria (26). This explains the fact that TRAIL-induced apoptosis has been reported to be independent of the expression of proteins of the Bcl-2 family in various cell types (27). As we observed that p21\(^{WAF1}\) exerted an anti-apoptotic effect against chemotherapeutic drugs but not against TRAIL-induced cell death, we analyzed the consequences of p21\(^{WAF1}\) gene deletion
on the expression of various members of the Bcl-2 family. The constitutive level of Bcl-2 was decreased in HCT116/p21/WAF1−/− cells compared with parental cells (Fig. 2A). Inversely, p21WAF1 deletion resulted in an increase in Bax protein expression, whereas levels of Bcl-xL and Bad were unchanged. As shown in Fig. 2B (upper), our results were obtained in the linear range of the assay. Quantitative analysis of the intensity of the bands revealed that the apparent Bax/Bcl-2 ratio was 4-fold higher in the absence of p21WAF1 (Fig. 2A and B, lower), thus demonstrating a change in relative amounts of Bax and Bcl-2 in favor of Bax. Treatment of both cell lines with daunomycin did not significantly change this ratio. As shown in Fig. 2A, the level of expression of other regulators of apoptosis, the IAP proteins (inhibitor of apoptosis protein) cIAP1, cIAP2, and XIAP known to control caspase activity, were similar in HCT116/p21/WAF1−/− cells.

During the mitochondrial pathway of apoptosis, mitochondria suffer specific damage such as loss of the mitochondrial membrane potential (Δψm), and one of the critical roles of the anti-apoptotic members of the Bcl-2 family is to protect mitochondrial homeostasis (28–30). This prompted us to investigate whether the increase of the Bax/Bcl-2 ratio was accompanied by a reduction of the mitochondrial membrane potential in HCT116/p21/WAF1−/− treated with daunomycin as compared with HCT116/p21/WAF1+ cells. Mitochondrial membrane potential (Δψm) was measured using DiOC6(3), a cyanine dye that accumulates in the mitochondrial matrix under the influence of the Δψm. Treatment of HCT116/p21/WAF1−/− cells with daunomycin resulted in a significant decrease in DiOC6(3) uptake as compared with untreated cells (Fig. 2C). Such a decrease was not observed in HCT116/p21/WAF1+ cells (Fig. 2C). This last result further establishes the correlation between the Bax/Bcl-2 ratio and the ability of daunomycin to disrupt the mitochondrial membrane integrity. As expected, treatment of HCT116/p21/WAF1−/− cells with daunomycin resulted in an activation of caspase-9, the apical caspase in the mitochondrial pathway (Fig. 2D).

Inactivation of p21WAF1 Results in an Increase of p53 Expression and Transcriptional Activity—Because Bax and Bcl-2 expression have been shown to be, respectively, up- and down-regulated by the tumor suppressor p53 (31), we compared p53 levels in HCT116/p21/WAF1−/− and HCT116/p21/WAF1+ cells. Deletion of the p21WAF1 gene resulted in a higher expression of p53 (Fig. 3A). To test whether this enhancement in p53 expression was...
accompanied by an increase in its transcriptional activity, cells were transiently transfected with a plasmid containing the luciferase reporter gene under the control of p53-binding elements. As shown in Fig. 3B, the luciferase activity measured in HCT116/p21−/− cells was 5-fold higher than that measured in HCT116/p21+/− cells.

To corroborate the inverse correlation between expression of p21WAF1 and p53 proteins and to eliminate the possibility that this reflects clonal selection of HCT116/p21−/− cells expressing high levels of p53, we used specific antisense oligonucleotides (p21AS) to inhibit p21WAF1 expression in wild-type HCT116 cells. Treatment of HCT116/p21−−/− cells with p21AS resulted in an inhibition of p21WAF1 expression, whereas control oligonucleotide had no effect (Fig. 3C). No change in actin expression was observed, thus confirming the specificity of action of p21AS. Inhibition of p21WAF1 expression by p21AS was accompanied by an increase in p53 level (Fig. 3C), and here again control AS were without effect. Thus, these results further demonstrate that inhibition of p21WAF1 expression results in an enhancement of p53 protein levels.

Inhibition of p53 Activity by Pifithrin-α Reduces the Sensitivity of HCT116/p21−/− Cells to Daunomycin-induced Apoptosis and Alters Their Bax/Bcl-2 Ratio—PFTα is a small molecule that was isolated for its ability to reversibly block p53-dependent transcriptional activation (32). We used this compound to investigate whether the higher p53 activity present in HCT116/p21−/− cells as compared with HCT116/p21+/− cells participated in higher sensitivity to daunomycin-induced apoptosis. As shown in Fig. 4A, treatment of HCT116/p21−/− cells with 30 μM PFTα resulted in an inhibition of p53 transcriptional activity. This reduction of p53 activity was accompanied by a reduction of the sensitivity of these cells to daunomycin-induced cell death (Fig. 4B). Furthermore, as shown in Fig. 4, C and D, PFTα-induced inhibition of p53 activity resulted in both an increase in Bcl-2 expression and a decrease of Bax expression, thus further establishing the correlation between p53 activity and the Bax/Bcl-2 ratio.

Enhancement of p53 Expression Consequently to Inactivation of p21WAF1 Occurs at a Post-transcriptional Level and Correlates with an Increase in p14ARF Level—p53 expression is tightly regulated through several mechanisms, the most effective one being regulation of protein stability (1–3). To investigate whether p21WAF1 affects p53 expression at a transcriptional or a post-transcriptional level, constitutive p53 mRNA expression was analyzed in HCT116/p21−/− and HCT116/p21+/− cells by reverse transcriptase-PCR. As shown in Fig. 5A, no difference in p53 mRNA expression was observed between the two cell lines, indicating that regulation of p53 expression by p21WAF1 occurs at a post-transcriptional level.

Several mechanisms have been shown to be implicated in the regulation of p53 stability. A complex pattern of regulation of p53 stability by the stress kinase JNK has been described. Nonphosphorylated JNK targets p53 ubiquitination and degradation (33) whereas stress-activated JNK stabilizes and activates p53 (34). Because p21WAF1 has been shown to inhibit JNK activation in various cells, we investigated whether absence of p21WAF1 expression in HCT116/p21−/− cells resulted in an enhanced level of phosphorylated JNK that could participate in p53 stabilization. As shown in Fig. 5B, such a mechanism was not implicated in p53 stabilization that occurs in the absence of p21WAF1 because no difference in JNK expression or activation was observed between HCT116/p21−/− and HCT116/p21+/− cells.

One of the key regulators of p53 expression is the oncogenic protein Mdm2 (35). This protein can transport p53 from the nucleus to the cytoplasm where its ubiquitin ligase activity contributes to the proteasome-mediated degradation of p53. Inversely, the nonreceptor tyrosine kinase c-Abl and the tumor suppressor p14ARF up-regulate p53 levels by preventing its Mdm2-driven nuclear export and ubiquitination (36–38). As
shown in Fig. 5B, p53 stabilization in HCT116/p21−/− cells was not correlated with a decrease of Mdm2 or an increase of c-Abl levels. In contrast, disruption of p21WAF1 resulted in a strong increase in the basal level of p14ARF expression (Fig. 5B). Also, inhibition of p21WAF1 expression in wild-type HCT116 cells with specific antisense oligonucleotides resulted in an increase in p14ARF expression without affecting JNK, phosphorylated JNK, Mdm2, c-Abl, or p14ARF. C. HCT116/p21−/− cells were left untreated or were treated with p21WAF1 antisense oligonucleotides as described under “Experimental Procedures.” Whole cell extracts were prepared 48 h after the last treatment and analyzed by Western blot for expression of the indicated proteins. D. cells were transfected with pp53-TA-luc or pTA-luc together with pRSV-β-gal vectors in the presence of 4 μg of either pcDNA or p14ARF encoding pcDNA vector. Results, presented as the ratio of luciferase activity in pp53-TA-luc- to pTA-luc-transfected cells, are the mean ± S.E. of three independent experiments.

DISCUSSION

Apoptosis is a tightly regulated process essential to maintain tissue homeostasis. Acquisition of a resistance to apoptosis plays a pivotal role in tumorigenesis by disrupting the balance between cell proliferation and cell destruction and by allowing cancer cells to resist radiation and chemotherapy. Identification of mechanisms that can antagonize apoptosis is essential to devise therapeutic strategies aimed at enhancing the efficiency of cancer treatment (39). Recent technological advances have allowed the successful targeting of individual genes in human somatic cells, thus permitting studies of activation or inactivation of a particular gene on the response of cancer cells to therapeutic agents.

Here we observed that disruption of p21WAF1 expression in human colon cancer cells, either by gene targeting or by the use of specific antisense oligonucleotides, increased p53 expression. This effect correlated with a sensitization of cells to chemotherapy-induced apoptosis. It also correlated with a change in the Bax/Bcl-2 ratio as a consequence of increased expression of the proapoptotic protein Bax and reduced expression of the antiapoptotic protein Bcl-2, respectively. The roles of the observed enhancement of p53 levels in the sensitization to daunomycin-induced apoptosis, and in the change in the Bax/Bcl-2 ratio were demonstrated by the fact that inhibition of p53 activity upon treatment of HCT116/p21−/− cells with PFTα reversed both effects.

The relative ratio of anti-versus pro-apoptotic Bcl-2 family proteins has been shown to dictate the ultimate sensitivity or resistance of cells to various apoptotic stimuli (28–30). Therefore, the observed modification of this ratio in favor of Bax very likely participates in the enhanced sensitivity of HCT116/p21−/− cells to chemotherapeutic drugs as compared with their normal counterparts. However, one cannot exclude the possibility that additional mechanisms may be implicated in the pro-apoptotic effect of p53. For example, p53 was also shown to induce expression of the pro-apoptotic proteins FAS/APO1, KILLER/DR5, NOXA, p53AIP1, PIDD, and PUMA (1–4) and to reduce expression of the anti-apoptotic protein, survivin (40).

We observed that disruption of p21WAF1 up-regulated p53 expression at a post-transcriptional level. A key player in the regulation of p53 stability is the Mdm2 protein that promotes the ubiquitination of p53 and its subsequent degradation by the proteasome (1–3, 35). The ability of Mdm2 to promote p53 ubiquitination can be modulated by the binding of other proteins. Among these proteins, p14ARF, the product of an alternative transcript of the INK4a tumor suppressor locus, has been shown to be involved in the response to oncogenic stress by up-regulating p53 expression (37, 38). p14ARF is very likely responsible for p53 stabilization that occurs after p21WAF1 disruption because we observed that it is expressed at a higher level in HCT116/p21−/− cells than in HCT116/p21+/+ cells. The ability of p14ARF to control p53 expression was demonstrated by the fact that transfection of HCT116/p21−/− cells with an expression vector encoding p14ARF strongly increased p53 activity. Altogether, our results provide evidence that repression of p14ARF that occurs in consequence of the inhibition of p21WAF1 expression is an initial step in the sensitization of HCT116/p21−/− cells to daunomycin-induced apoptosis.

The mechanism leading to an increased expression of p14ARF subsequently to p21WAF1 disruption remains to be established. It has been shown that a transcriptionally functional E2F DNA-binding site is located in the p14ARF promoter region (41). Control of G1/S transition by p21WAF1 occurs through inhibition of cyclin D-cdk4/6 complex activity in late G1 (20). Inhibition of such activity maintains the RB protein in an hypophosphorylated form that sequesters E2F1 and inhibits its transcriptional capacity (42). Thus, the mechanism by which inhibition of p21WAF1 expression increased the p14ARF level could be the activation of E2F1-dependent transcription. However, although as expected, inactivation of p21WAF1 in HCT116/p21−/− cells resulted in the disappearance of the hypophosphorylated form of RB, it did not correlate with an increased E2F1 transcriptional activity (not shown). One explanation could be a repression of E2F1 by p14ARF, because it was recently reported that p14ARF physically interacts with E2F1 and negatively regulates its activity (43). p14ARF expression was also shown to
be regulated by other transcriptional regulators such as c-Myc, β-catenin, and E1A and by the extent of p14ARF gene methylation (44–46). Whether control of p14ARF expression by p21WAF1 implies one of these mechanisms is presently under investigation.

Mutations in the p53 gene are the most frequent genetic alterations in cancer. Intensive efforts are presently aimed to offer new approaches to cancer therapy based on restoring mutant p53 function by small molecule drugs (47, 48). Our results, which show that in the absence of p21WAF1 p53 participates in the triggering of cell death induced by DNA damaging agents, suggest that during chemotherapy high benefits could result from strategies combining inhibition of p21WAF1 expression and restoration of p53 activity in p53-deficient cancer cells.

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