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Induced p21^{waf} expression in H1299 cell line promotes cell senescence and protects against cytotoxic effect of radiation and doxorubicin

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The CDK inhibitor p21^{waf} is a principal mediator of p53 function but can also be transactivated by many p53-independent stimuli leading to cell growth arrest or differentiation. In order to study the function of p21^{waf} in a p53-deficient environment, we established an inducible expression of p21^{waf} in the p53-null lung cancer cell line H1299, based on the muristerone-regulated system. Overexpression of p21^{waf} led cells to growth arrest which after several days became irreversible and the arrested cells acquired a senescent phenotype as judged by cell shape, the senescence-associated β-gal marker and inhibition of colony formation. The effect of p21^{waf} overexpression, in the absence of p53, on the cytotoxicity caused by irradiation, doxorubicin and taxol was studied. Expression of p21^{waf} provided protection against the cytotoxic effect of radiation and doxorubicin but not of taxol. These results are relevant to treatment of cancer when p53 is inactive.

Keywords: inducible p21^{waf}; senescence; anti-cancer drugs; radiation

Introduction

The cellular response to stress and DNA damage is largely controlled by the tumor suppressor p53 and its target genes. The p21^{waf} protein functions as a mediator of p53 activity in controlling cell growth arrest, since it is a universal cyclin-dependent kinase (CDK) inhibitor (Xiong *et al*., 1993). p21^{waf} was first discovered as a gene overexpressed in senescence (Noda *et al*., 1994), a CDK2-associated protein (Harper *et al*., 1993) and a p53-activated gene (El-Deiry *et al*., 1993). This was soon followed by the demonstration that p21^{waf} can be induced by several p53-independent signaling pathways which may result in growth arrest or differentiation (Michieli *et al*., 1994; Harper and Elledge, 1996; Steinman *et al*., 1994; Halevy *et al*., 1995). p53-dependent activation of p21^{waf} is particularly important for cell cycle arrest after DNA damage due to radiation or chemotherapy (Brugaloras *et al*., 1995; Deng *et al*., 1995). In such cases p21^{waf} halts cell cycle progression, possibly providing a time window for repair of DNA damage prior to resumption of DNA replication. In addition to growth arrest the damaged cell can also undergo apoptosis; the relative contribution of apoptosis versus growth arrest to prevention of tumorigenesis may vary among different tissues and cell types (Hansen and Oren, 1997). Recent studies on colon cancer in mice of p53−/− and p53+/+ background indicate that p53 may retard tumor progression by yet another mechanism based on irreversible growth arrest which may lead to senescence (Fazeli *et al*., 1997) and it was also shown that induced p53 expression in EJ cells was shown to promote cell senescence (Sugrue *et al*., 1997). The choice between reversible growth arrest, apoptosis and senescence as a result of p53 function is under extensive study and may depend on cell type and developmental stage (Bates and Vousden, 1996). The outcome of any of the above may be an important factor in escaping from tumorigenesis and in providing an explanation for the mechanism of p53 action as a tumor suppressor.

Although the role of p21^{waf} in cell growth suppression as a CDK inhibitor is well established, its function in promoting or inhibiting cytotoxic effects and apoptosis is not completely known. In several studies upregulation of p21^{waf} was observed in cases of p53-dependent apoptosis (Kondo *et al*., 1997; Waldman *et al*., 1996), whereas in other cases p21^{waf} was found to protect cells from apoptosis or drug cytotoxicity (Poluha *et al*., 1996; Gorospe *et al*., 1996, 1997).

Mice deficient in p21^{waf} are more sensitive to radiation than normal mice (Brugaloras *et al*., 1995; Wang *et al*., 1997) and human fibroblastic cell lines that were made deficient for p21^{waf} bypass cellular senescence (Brown *et al*., 1997). Inactivation of p21^{waf} in colorectal cancer cells renders them prone to apoptosis (Polyak *et al*., 1996). Obviously, the function of p21^{waf} will be better understood if studied in the absence of p53, which has many other functions besides p21^{waf} transactivation. Therefore, we chose the p53 deficient human cell line H1299 in order to establish an ectopic regulatable system of p21^{waf} expression, using the ecdysone induction system (No *et al*., 1997). We studied the role of p21^{waf} in the control of cell growth and in protection against radiation and several anticancer drugs. We show that in this system p21^{waf} protects against the cytotoxic effect of radiation and doxorubicin and that overexpression of p21^{waf} facilitates cell senescence.

Results

Regulation of p21^{waf} expression by muristerone

The human lung cancer cell line H1299 was chosen to generate the p21^{waf}-inducible cell line because it is null for p53 due to gene truncation, it is easily transfected and was shown to be growth arrested by transfection

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of p21waf (El-Deiry *et al*., 1993; Chedid *et al*., 1994; Zakut and Givol, 1995). Figure 1a shows the expression of p21waf in different selected clones after muristerone induction; Figure 1b shows the induction of p21waf expression in clone W-65 by various concentrations of muristerone and Figure 1c depicts the time course of induction in the clone W-65 using 2 μM muristerone. The results show that although H1299 express small basal amounts of endogenous p21waf the ectopic expression of p21waf is not leaky. Maximum expression is obtained at 2 μM muristerone (Figure 1b) and higher levels of muristerone did not change the expression level of p21waf (data not shown). The results also indicate that the p21waf inducible system is highly responsive to muristerone, and that levels of the expression of the inducible gene are easily controlled by adding increasing amounts of muristerone to the medium. The maximum levels of p21waf is reached 24 h after induction, although p21waf protein can be detected already as early as 3 h after induction (Figure 1c). The expression level of muristerone-induced p21waf is similar to that obtained in the temperature-sensitive cell line H1299Val135#3 (a gift from M Oren). Hence the transcriptional induction of p21waf by the ts p53Val135 upon shifting to 32° and the p53-independent muristerone induction result in a similar p21waf expression level (Figure 1a). Because our p21waf-inducible system is in the p53 null background, it offers the opportunity to directly assess the role of p21waf in cell cycle arrest, in promoting senescence as well as in response to genotoxic agents.

The stability of the inducible p21waf was analysed by withdrawal of muristerone from the medium and analysing the decrease of p21waf expression in a time-dependent manner, which depends both on mRNA and protein stability. Figure 2 shows that the half-life time of p21waf after muristerone withdrawal is 9 h which include both protein synthesis and degradation. However, in the presence of cyclohexamide which inhibits protein synthesis, the half-life time of p21waf in H1299 cells is about 90 min consistent with recently published results (Maki and Howley, 1997).

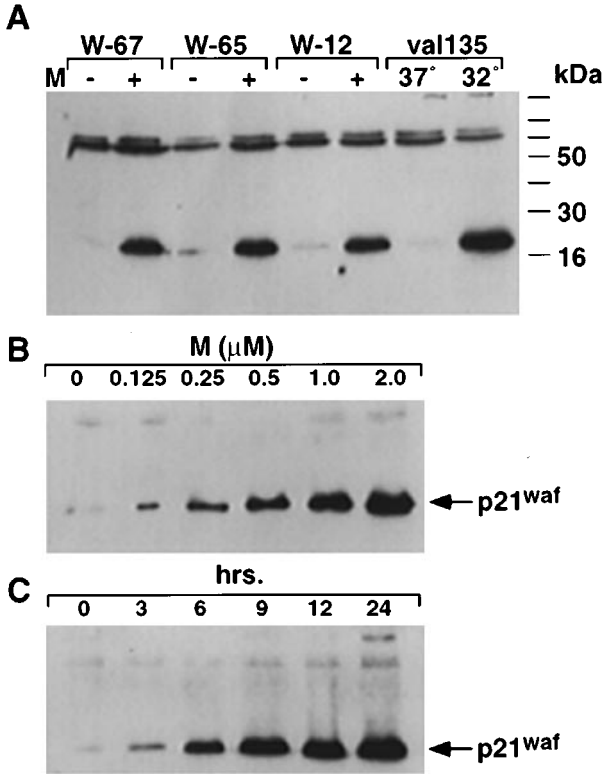

Figure 1 Muristerone-induced expression of p21waf in H1299 cells. Immunoblot analysis of cell lysates. (**a**) p21waf levels in some selected clones incubated for 24 h in the presence or absence of muristerone (2 μM), and compared with p21waf level in the temperature-sensitive cell line H1299Val135 incubated at 37°C or switched to 32°C 6 h before harvest. (**b**) p21waf levels in W-65 cells incubated for 24 h in the presence or absence of the indicated muristerone (2 μM) concentrations. (**c**) p21waf levels in W-65 cells incubated in the presence or absence of 2 μM muristerone for the indicated periods

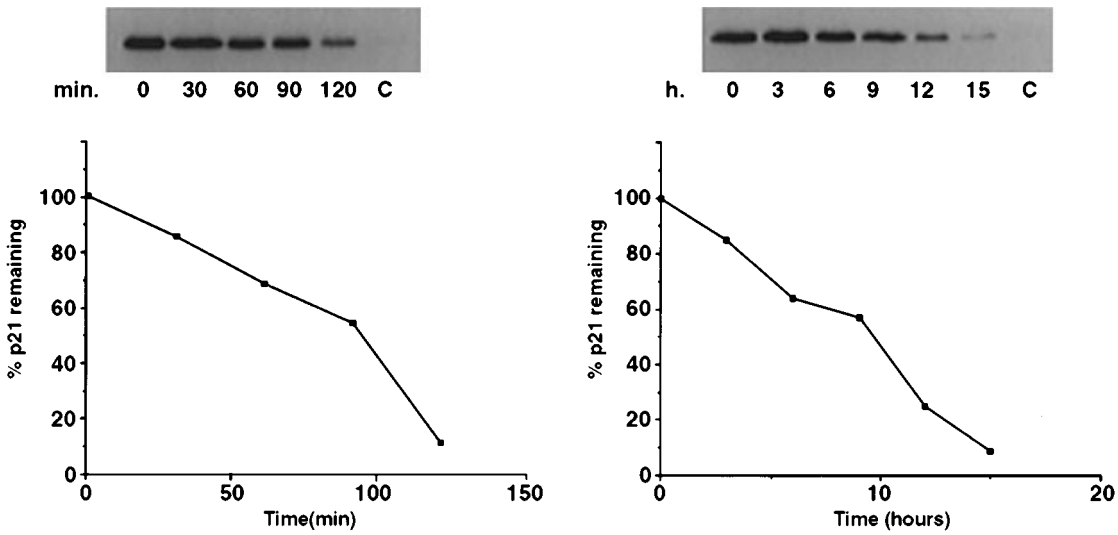

Figure 2 Stability of the inducible p21waf. Cells were incubated for 12 h with muristerone (2 μM), washed three times and incubated in the medium with (left panel) or without (right panel) cycloheximide (25 μg/ml). Cells lysate were prepared at indicated time and examined by Western blot analysis for p21waf. The levels of p21waf protein at each time point were quantitated by densitometric scanning of the resulting autoradiograms

Inhibition of colony formation and promotion of senescence by overexpression of p21^{waf}

The effect of induced p21^{waf} on H1299 cell growth was measured by a cell proliferation assay. Cells were cultured under the same conditions with or without muristerone for 5 days and the number of cells was counted every day. Cell growth rate was significantly reduced in the presence of muristerone, whereas no effect was found on the growth rate of the parental cell line R-38 (Figure 3a). Cell cycle analysis reveals that p21^{waf} induction arrested the cells primarily at the G1 phase, which increased from 45.7–62.5% within 48 h in the presence of muristerone. However, the most significant effect of p21^{waf} expression in H1299 cells is the reduction of the S phase fraction from 18.4% to 9.5% (Figure 3b). Cell cycle analysis was also performed with R-38 cells transfected with PIND (empty vector) treated similarly with muristerone. No significant change was observed at the various time points. No increase in the number of cells with sub-G1 DNA content was observed, even in the presence of muristerone for 5 days (data not shown). Hence, p21^{waf} induction alone does not promote apoptosis in H1299.

We also analysed the reversibility of the growth inhibition by p21^{waf} using the colony inhibition assay. This was performed by seeding approximately 100 cells of clone W-65 on a 60 mm plate in either the presence or absence of muristerone for various time periods, after which the medium was replaced by normal medium and the cells were allowed to multiply and form colonies for an additional 10 days. The parental R-38 clones served as a control. As shown in Figure 4, p21^{waf} induction by muristerone resulted in inhibition of colony formation even after muristerone withdrawal. The presence of muristerone for 4 days reduced by 60% the number of colonies formed after muristerone withdrawal, whereas after 10 days in the presence of

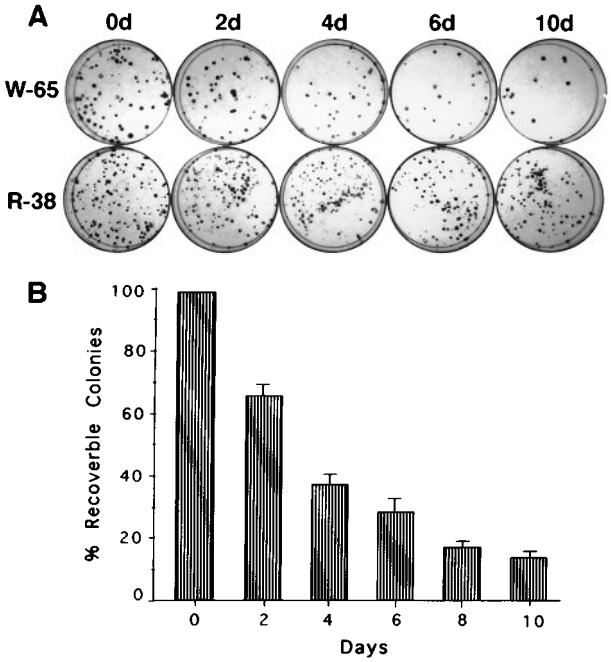

Figure 4 Colony growth inhibition by transient induction of p21^{waf} in W-65 cells. (**a**) W-65 and R-38 cells were seeded at a density of 100 cells per 60 mm dish. Muristerone was added 12 h later and maintained for the indicated times followed by 10 days of culture in the absence of muristerone. Colonies were stained with crystal violet. (**b**) Graphic representation of the colony formation efficiency data in **a**. The percentage of recovered colonies was normalized relative to the control dishes without muristerone

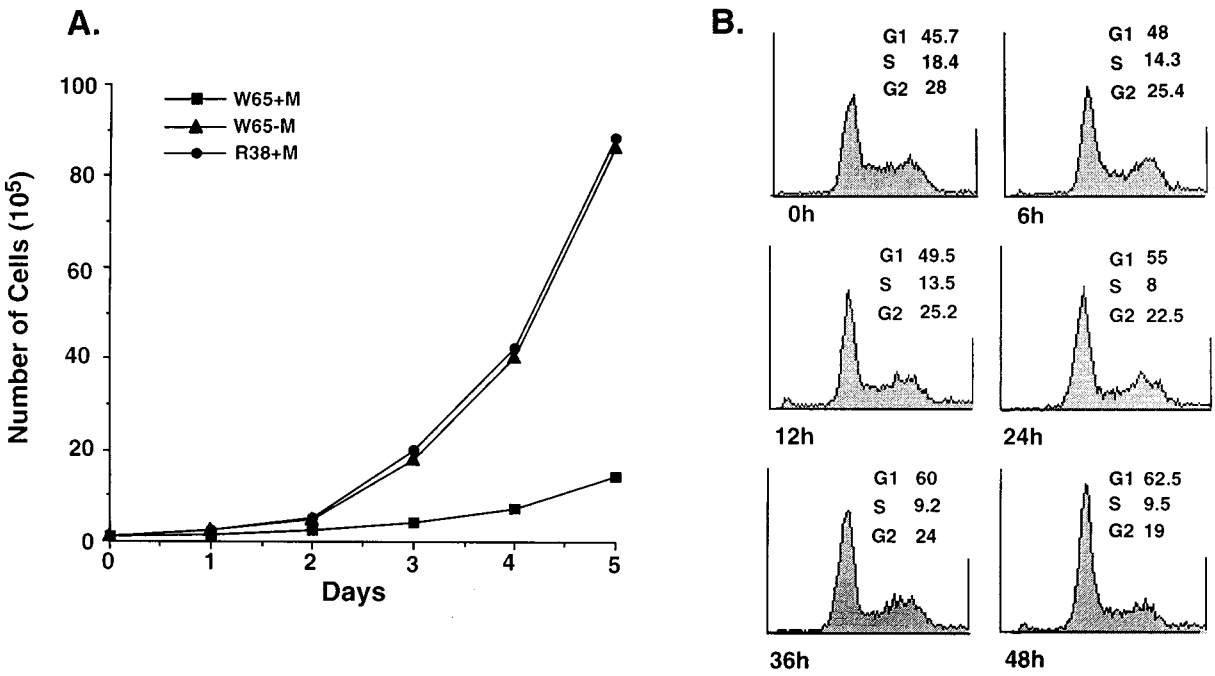

Figure 3 Growth curves and cell cycle analysis of W-65 in the absence or presence of muristerone. (**a**) Log-phase cells were seeded at 10^5 cells per 100 mm dish. After 12 h the medium was changed to a medium containing either 2 μM muristerone or no muristerone. R-38 cells (H1299 cells transfected with pVgRXR alone) grown in the presence of muristerone, served as a control. (**b**) 2.5×10^5 cells were seeded on 100 mm dish. After 24 h the medium was replaced with fresh medium containing 2 μM muristerone. Cells were harvested and prepared for cell cycle analysis at the indicated times

muristerone the number of colonies went down to only 13% of the control. This suggested that p21waf, originally discovered as a senescence associated gene (Noda *et al*., 1994), induces senescence in H1299 cancer cells by suppressing their ability to form colonies even after removal of muristerone. In the presence of muristerone, the majority of the cells were clearly visible as well-isolated single cells or clusters of few cells. They exhibited an increased size, flattened shape and a morphology similar to that of senescent cells. Senescent, but not quiescent cells have been shown to express a specific β-galactosidase (β-Gal) with activity detected by X-gal at pH 6.0 (Dimri *et al*., 1995). We therefore analysed muristerone treated H1299 W-65 cells for the expression of this senescence-specific marker. After 5 days in the presence of muristerone approximately 50% of the cells stained blue with X-gal at pH 6.0. In contrast, no positive staining for SA-β-gal was observed in W-65 cells grown in the absence of muristerone (Figure 5). The senescence effect of p21waf and the inhibition of colony formation 10 days after removal of muristerone are consistent with the notion that p21waf confers replicative senescence on H1299 cells. Taken together, our results indicate that p21waf induction in the absence of p53 causes a preferential G1 arrest and a reduction in the fraction of cells at S phase, leading to irreversible growth arrest with characteristics of senescence.

Effect of p21waf overexpression on the cellular response to γ-irradiation and anti-cancer drugs

Over 50% of cancers contain p53 mutations which render the protein inactive as a transcription factor and unable to transactivate p21waf. In many cases, this inactivation of p53 leads to a decreased sensitivity to DNA damaging agents. We therefore wanted to analyse the effect of the p53-independent activation of p21waf on the response of cells to genotoxic agents. H1299 cells are p53−/− and induction of p21waf in such cells can provide insight into the direct effect of p21waf on the cytotoxicity of radiation and anti-cancer drugs. First, we wished to know whether such treatment can induce p21waf in a p53-independent manner. As seen in Figure 6, γ-irradiation or taxol did not induce p21 expression in H1299 cells. Doxorubicin, however, induced a moderate elevation in p21waf expression, but far below that achieved by muristerone induction (Figure 6).

We then analysed the cytotoxic effect of irradiation, doxorubicin and taxol on H1299 in the presence or absence of muristerone-induced p21waf. Cells were seeded at a density of 2.5×10^5 per 100 mm plate. Twenty-four hours later muristerone (2 μM) was added together with either doxorubicin (0.5 μg) or taxol (12.5 nM) for 72 h. Similarly, cells were irradiated with 10 Gy (1.5 Gy/min) after addition of muristerone. Cells were harvested at 1 day intervals and subjected to FACS analysis and counting of dead cells by trypan blue uptake.

The results obtained 48 h after exposure to treatment are shown in Figure 7. In the presence of muristerone the cytotoxic effect of radiation was markedly reduced, as reflected by a reduction of the dead cell fraction from 29 to 9%. This was correlated with a similar reduction in the subG1 fraction (from 26 to 7.2%). A protective effect was also exerted against doxorubicin cytotoxicity, with a reduction of the dead cell count from 14.6 to 4% and the subG1 fraction from 10.2 to 3.6%. It should be noted that doxorubicin induces also the expression of endogenous p21waf in a p53-independent manner (Figure 6), which may reduce its cytotoxic efficacy. In contrast, in the case of taxol no significant difference was observed between the number of dead cells in the presence or absence of muristerone. A consistent outcome of p21waf over expression was the increase in the G1 fraction in all cases.

Discussion

We have used the muristerone-regulated expression system in order to study the effect of p21waf on the human lung adenocarcinoma cell line H1299. This p53-deficient cell line was employed to analyse the effect of the p53-independent transcriptional activation of p21waf

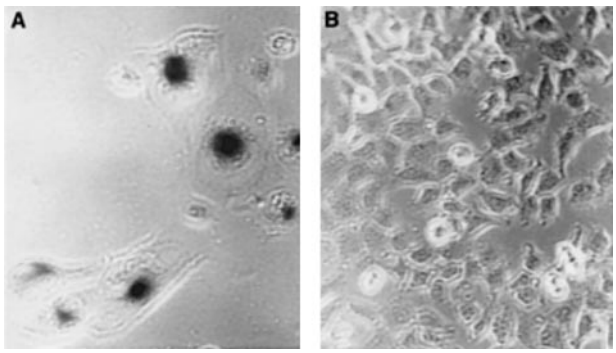

Figure 5 SA-β-gal staining of W-65 cells induced to express p21waf. W-65 cells were seeded at a density of 2×10^5 cells per 100 mm plate and after 12 h the medium was replaced with a medium containing either 2 μM muristerone (**a**) or no muristerone (**b**). This led to growth inhibition of the muristerone treated cells (**a**) due to p21waf induction. Five days later the cells were washed, fixed and stained with x-gal at pH6 and 37°C without CO_2 for 16 h to detect senescence-associated β-gal (Dimri *et al*., 1995). Cells were photographed using a Zeiss axiophot microscope. Approximately 50% of the muristerone treated cells were stained blue (**a**)

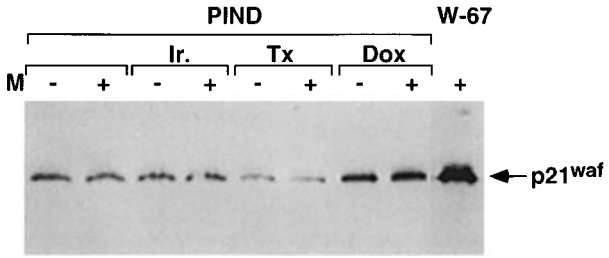

Figure 6 Expression of p21waf in PIND cells after γ-irradiation and anti-cancer drugs treatment. Log-phase PIND cells (R-38 cells transfected with the empty vector PIND) were exposed to irradiation (10 Gy, 1.5 Gy/min) or taxol (12.5 nM) or doxorubicin (0.5 μg/ml) and after 12 h. total cell lysates were prepared and 50 μg of protein were used for Western analysis as described in Materials and methods. W-67 cells induced by 2 μM muristerone for 12 h were used as a control. M-muristerone; Ir-Irradiation; Tx-taxol; Dox-doxorubicin

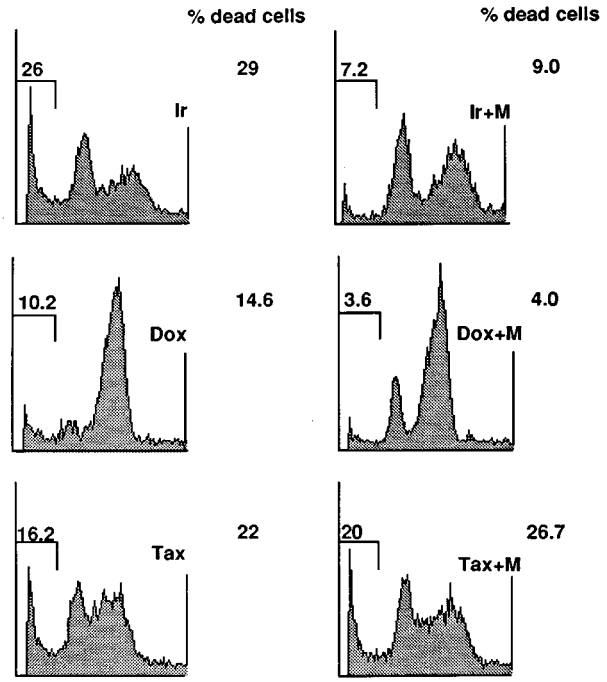

Figure 7 The effect of p21waf on the response of W-65 cells to irradiation and anti-cancer drugs. W-65 cells were seeded at 2.5×10^5 cells/100 mm dish. After 24 h the medium was replaced to a medium with or without muristerone (2 μM), and the cells exposed to irradiation or taxol or doxorubicin as described in Figure 6. After 48 h floating cells were collected, spun down and combined with the trypsinized adherent cells. The cells were resuspended in a total volume of 1 ml, of which 50 μl was used for trypan blue staining and dead cell counting while the rest was used for cell cycle analysis by using a Becton Dickinson FACScan flow cytometer. The percentage of cells with sub-G1 DNA content and dead cell counts are shown. Ir, γ-irradiation; Dox-doxorubicin; tax-taxol. M-muristerone

on cell growth and resistance to DNA damaging agents. This is particularly relevant for understanding the response of the many cancer cell types in which p53 is non-functional but expression of p21waf can nevertheless be modulated by a variety of means. Our results demonstrate that the muristerone-regulated system is not leaky and shows rapid response to induction as well as time-dependent expression of p21waf which can be either up or down regulated. The major effect of p21waf overexpression is the cell growth arrest due to reduction in the number of cells in the S fraction and an increase in the G1 phase fraction. We also show that the p21waf effect resulting in growth arrest is to a great extent irreversible leading to a senescent phenotype. The induction of senescence by p21waf takes 4–6 days (Figure 4) and results in loss of colony formation capability. Previous work on overexpression of p53 in the EJ cell line and our results on overexpression of p53 in H1299 cells (Wang *et al*., 1998) also demonstrated that the p53-dependent growth arrest resulted in senescence (Sugrue *et al*., 1997). This raised the possibility that senescence may be an important mechanism which prevents tumorigenesis and may be an alternative to apoptosis. Our results show that overexpression of p21waf has a similar potential to that of p53 in driving cells to senescence, and suggest that perhaps p53-driven senescence may also be due to the p53-induced p21waf expression. Recent work on cell senescence demonstrated that p16INK4A, a tumor suppressor gene that is not activated by p53, also plays a major role in cell senescence (Alcorta *et al*., 1996). In human fibroblasts replicative senescence is a multistep process, where p21waf expression is elevated at the initial stages of senescence (Noda *et al*., 1994) and later on there is a marked elevation of p16 expression and a decrease in p21waf expression (Alcorta *et al*., 1996). This is correlated with the results of inducible p16 expression in glioma cells U-1242 MG (Uhrbom *et al*., 1997). Removal of tetracyclim and induction of p16 expression in these cells resulted in reversal of the immortal phenotype and entrance into senescence as indicated by the SA-β-gal staining. Thus both p16INK4A and p21waf which are CDK inhibitors and control cell cycle checkpoints play a major role in cell sensecence. In the case of the glioma cells the p16-dependent senescence is somewhat reversible (Uhrbom *et al*., 1997), whereas in the study reported here no regain of colony formation by W-65 cells was observed after removal of muristerone. Because of the importance of senescence as a mechanism to avoid cancer further work is required to determine the relative role of p21waf and p16 in this process.

We next wished to examine the effect of p21waf overexpression on the cellular response to γ-irradiation and the anti-cancer drugs doxorubicin and taxol. The mechanism of action of the two drugs is different; doxorubicin like ionizing radiation, leads to double strand breaks of the DNA, whereas taxol causes tubulin aggregation and prevents cell division. All three agents lead to a cytotoxic effect resulting in cell death. Our results show that p21waf protects H1299 cells against the cytotoxic effects of radiation and doxorubicin but has no significant effect on taxol cytotoxicity. This highlights the possibility that some drugs, like taxol, may be more effective than others even in the presence of elevated level of p21waf. This matter is of importance in making cancer chemotherapy decisions since many tumors contain wt p53 which may elevate p21waf expression.

It is of interest that doxorubicin has a p53-independent ability to elevate p21waf expression and this may be one of the reasons for the relatively low cytotoxic effect of this drug in H1299 cells. The induction of p21waf by muristerone further reduces this cytotoxicity. The anti-cytotoxic effect of p21waf in cases of radiation and doxorubicin treatment is consistent with results with cells deficient for p21waf, which show increased sensitivity to radiation and various anti-cancer drugs (Brugardas *et al*., 1995; Waldman *et al*., 1996; Wouters *et al*., 1997). These results are also in line with the observation that p21waf protects against apoptosis (Gorospe *et al*., 1996, 1997; Polyak *et al*., 1996) and that loss of p21waf enhances the apoptotic effect of ionising radiation (Wang *et al*., 1997). Our results are also consistent with a previous study showing that p21waf expression increases the clonogenic survival after irradiation (Waldman *et al*., 1997).

The protection from radiation or doxorubicin cytotoxicity by p21waf on the one hand, and the promotion of apoptosis by p53 on the other hand bring up the question of the relevance of these antagonistic effects to cancer treatment, since p21waf is

a mediator of the growth arrest signaling by p53. Several lines of research have demonstrated that the p53-driven apoptosis and growth arrest are due to different biochemical pathways (Hansen and Oren, 1997; Bates and Vousden, 1996). Recently it was also implied that the anti-apoptotic effect of p21^{waf} may classify it as an 'oncogene' which may enhance lymphomagenesis and that drugs which inhibit p21^{waf} function should be considered for cancer therapy (Deng *et al*., 1995). The interpretation of our results may reconcile these possibly antagonistic views by showing that the growth arrest caused by p21^{waf} in certain cancer cell lines may be irreversible and lead to senescence which can result in escape from tumorigenicity. Since p21^{waf} can be activated through many p53-independent pathways, this may open new avenues for consideration in cancer treatment.

Materials and methods

Plasmid construction

We used the recently described ecdysone-induced system (No *et al*., 1997) to achieve inducible p21^{waf} expression in H1299 cells. Plasmids pVgRXR, encoding the receptor subunits, PIND which contains muristerone response elements and PIND/LacZ were from Invitrogen (San Diego, CA, USA). PIND/p21^{waf} was prepared by cloning the 0.5 kpb *Bam*HI/*Eco*RI fragment of human p21^{waf} cDNA (Chedid *et al*., 1994) into the multiple cloning site of PIND. The ecdysone analog muristerone was from Invitrogen.

Cell line and cell culture, and transfection

The human lung adenocarcinoma cell line H1299 (Takahashi *et al*., 1992) was grown in RPMI medium supplemented with 10% fetal bovine serum in a 37°C incubator with 5% CO_2. Transfections were performed using the calcium phosphate method (Sambrook *et al*., 1989). To generate a cell line expressing inducible p21^{waf} protein, a two-step procedure was used. We first generated stable cell lines expressing only pVgRXR in H1299 cells. To test for clonal cell lines capable of inducing expression from the muristerone response element in PIND about 40 cell clones expressing pVgRxR were isolated, transiently transfected with PIND/lacZ and maintained in the presence of muristerone A for 24 h and then stained with X-gal. Two of the 40 clones (R-37 and R-38) were found to be the best expressors of lacZ and were used for generating the p21^{waf} expressing clone by transfection with PIND/p21^{waf}, followed by G418 selection (400 μg/ml). We previously used the same clones (R-37 and R-38) to establish a p53-inducible system in H1299 cells (Wang *et al*., 1998.) Western blot analysis with anti- p21^{waf} antibody (C-19, Santa Cruz) was employed to screen for inducible expressors. Cells were maintained under double selection with zeocin (100 μg/ml) and G418 (400 μg/ml).

Immunoblot analysis

Whole-cell extracts were prepared by lysing cells with NP40 lysis buffer (150 mM NaCl, 50 mM Tris (pH 7.5), 1 mM DTT, 0.5% NP40, 25 ng/ml aprotinin, 25 ng/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) for 15 min on ice with occasional low speed vortex. Protein content was determined with the Bio-Rad protein assay Kit (Bio-Rad). Fifty μg total protein mixed with equal amounts of 2 × sample buffer was boiled for 5 min and then subjected to 15% SDS/PAGE. Following electrophoresis, proteins were transferred to a nitrocellulose membrane (Schleicher and Schnell). Blots were blocked in PBS containing 0.1% Tween 20 and 2.5% non-fat milk (PBS-T-milk) for 30 min at room temperature followed by incubation with the first antibody in PBS-T-milk for 2 h at room temperature then washed three times with PBS-T, and incubated with a second antibody (goat anti rabbit-Ig) conjugated to horseradish peroxidase for 1 h at room temperature. The filter was washed three times with PBS-T, developed by chemiluminescence (Amersham) and exposed to X-ray film (Fuji) for 5–60 s.

Inhibition of colony formation

Cells were seeded at a density of 100 cells per 60 mm plate and maintained in the presence or absence of muristerone A (2 μM) for varying time periods. After withdrawal of muristerone at the indicated time, cells were subsequently maintained in medium without muristerone for 10 more days. For staining, 0.5 ml of crystal violet in ethanol solution was added to 2.5 ml medium of adherent cells and after 30 min the plates were washed with water, air-dried and photographed.

Senescence-associated β-gal (SA-β-gal) staining

2×10^5 cells were seeded per 100 mm plate. Cells were allowed to attach for 12 h and maintained with or without muristerone A (2 μM) for 5 days. Cells were washed with PBS and fixed with 3% formaldehyde for 5 min at room temperature, and stained with X-gal (pH 6.0) as described (Dimri *et al*., 1995).

Growth rate and cell cycle analysis

To determine the rate of cell growth, 1×10^5 cells were seeded on 100 mm plate and 12 h later the medium was replaced with muristerone-containing medium and changed with the same fresh medium every 48 h. At indicated time, cells were trypsinized and collected separately from at least two plates followed by cell counts with a hemacytometer. For cell cycle analysis, 2×10^5 cells were seeded per 100 mm plate. After 24 h, fresh medium with 2 μM muristerone was added to the cells. At the indicated times, cells were trypsinized, collected and fixed with methanol for 30 min at $-20°$. Fixed samples were washed, resuspended in PBS containing 50 μg/ml propidium iodide (Sigma) and 50 μg/ml of RNAse. The stained cells were then analysed on a Becton Dickinson FACScan flow cytometer. Data were analysed using the Cellfit program.

Cells response to radiation and anti-cancer drugs

5×10^5 cells were seeded on a 100 mm plate and 24 h later the medium was replaced by medium with or without muristerone (2 μM) and the cells were exposed to 0.5 μg/ml doxorubicin or 12.5 nM paclitaxel (taxol) or 10 Gy irradiation using a Co_{60} source with a dose rate of 1.5 Gy/min. At different time points, both floating and adherent cells were collected and used for immunoblot or cell cycle analysis as previously described.

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