Mutant p53 Rescues Human Diploid Cells from Senescence without Inhibiting the Induction of SDII/WAF1

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ABSTRACT

Although the cyclin-dependent kinase inhibitor p21SDI1 (WAF1/CIP1) has been proposed as the mediator of p53-induced cell cycle arrest following DNA damage, several stimuli now appear to induce SDII independent of p53 function. We have examined the behavior of p53 and SDII in an isogenic model by manipulating p53 status in normal diploid human fibroblasts using an amphotrophic retroviral vector. Following DNA strand break damage induced by bleomycin, both SDII and G1/S cell cycle arrest are p53 dependent, consistent with SDII being the key mediator. In contrast, in cellular senescence (and following UV irradiation), induction of SDII occurs independent of p53 function yet growth arrest is still p53 dependent. We conclude (a) that redundant pathways exist for induction of SDII, but that (b) SDII, while perhaps necessary, is not sufficient for inhibition of cell cycle progression, requiring the cooperation of an additional factor (possibly another cyclin-dependent kinase inhibitor) whose expression, at least in the case of senescence, is strictly p53 dependent.

INTRODUCTION

Originally identified by its increased expression in senescing cells, p21SDI1 (1) is identical to the p53-inducible gene WAF1, which has been shown to be induced by DNA-damaging agents in the presence of wt3 p53 (2). The SDII protein inhibits the activity of several cdk-cyclin complexes and thereby may contribute to arrest of the cell cycle in G1 (3, 4). Furthermore, cells which lacked wt p53 function, failed to show induction of SDII in response to a variety of DNA-damaging agents and also failed to arrest in G1 (5, 6). Thus SDII has been suggested as a key downstream effector of wt p53-mediated growth arrest. However recent evidence has indicated that induction of SDII in a number of situations may not always be strictly p53 dependent. A variety of DNA-damaging agents such as UV irradiation and etoposide (but not γ-irradiation; Refs. 7 and 8), serum and individual growth factors (9), and terminal differentiation-promoting agents such as retinoic acid (10, 11) were all able to induce SDII transcription in cells lacking wt p53 function. Similarly, SDII expression is greatly increased in SV40-transformed cells in crisis, despite very low or nonexistent levels of p53 activity (12).

Our laboratory has been investigating the role of p53 in senescence-induced growth arrest using the novel approach of introducing mutant p53 by means of an amphotrophic retroviral vector into HDFs which have been grown to near senescence. By removing much of the intrinsic variability in the HDF life span which blurred previous studies using “young” cells, this enabled us to show conclusively that expression of mutant p53 (ala143) extends the life span of HDFs by over 15 pds (13), demonstrating that at least in this cell type wt p53 is an essential mediator of the growth arrest associated with cellular senescence, the M1 block in the Shay and Wright model (14). Since telomere shortening is a likely trigger for M1 arrest, we speculated that this is the signal which activates p53 in senescent cells. Given that SDII was also originally identified as a candidate effector of cell cycle arrest in senescence (1), we have examined this further by determining whether SDII expression is down-regulated in senescent cells which have been “rescued” by mutant p53. We have also compared the p53 dependence of SDII induction in senescence with that following DNA damage (induced by the radiomimetic agent, bleomycin, or by UV irradiation), again making use of the ability to manipulate p53 in an isogenic normal human diploid context.

MATERIALS AND METHODS

Cell Culture

Normal diploid (foreskin) fibroblasts were grown in DMEM (GIBCO) containing 10% FCS (GIBCO) and used either at population doubling level ~15 (young) or after continuous culture to population doubling level ~55 (near senescent). To obtain the latter, HDFs were repeatedly passaged in 75-cm² flasks, splitting 1:8 when confluent. The passage interval increased from ~ 3 days to ~14 days by the end of the aging period, which was reached after ~20 weeks.

Retroviral Gene Transfer

Two previously described high-titer amphotropic retroviral vectors were used, both based on the pHABeeno dual promoter system and produced by psi-CRIP packaging lines. Psi-CRIP-SCX encodes neo plus a human mutant p53 (codon 143 val → ala); psi-CRIP-neo provides a neo-only control (15). Gene transfer was carried out as described previously (15). Rescued cells were obtained by infecting near-senescent cells with psi-CRIP-SCX, passing at a split ratio of 1:4 into G418 selection (400 μg/ml) and either isolating individual, or pooled, colonies. HDF-neo and HDF-scx populations were obtained by infecting young cells with psi-CRIP-neo and psi-CRIP-SCX, respectively, passing at a split ratio of 1:2 into G418 selection and isolating colonies as above.

DNA Damage

Bleomycin (Lundbeck, Milton Keynes, United Kingdom) was prepared as a 7.5 mg/ml stock solution in deionized water, stored at ~20°C, and used at a final concentration of 250 μg/ml, which was previously determined as the minimal dose required to obtain maximum growth arrest in these cells (16). Pooled young HDF-neo and HDF-scx colonies in 100-mm dishes containing thermox coverslips (Flow) were treated with bleomycin for 1 h, and 3 h later coverslips were removed for immunocytochemical analysis. For UV irradiation, cells were washed twice in serum-free media, medium was removed, and the dishes, without lids, were placed under a UVG-11 mineralight lamp (UV Products, San Gabriel, CA) and exposed to 25 J/m² UVC (over a period of 25 s). Fresh complete medium was then added, and the cells were incubated for 24 h before removing coverslips for immunocytochemistry. The relative timings of analysis of SDII expression following bleomycin and UV treatment were chosen from the time of maximum induction of p53 expression, as determined by ourselves and others (17). Untreated controls were included for both bleomycin and UV treatments.

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2 To whom requests for reprints should be addressed.
3 The abbreviations used are: wt, wild type; cdk, cyclin-dependent kinase; HDF, human diploid fibroblast; ps1, population doubling; dThd, thymidine; LI, labeling index.
4 F. S. Wyllie, J. A. Bond, and D. Wynford-Thomas, unpublished observations.

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Growth Arrest Assay

The effect of bleomycin on DNA synthesis was determined by tritiated thymidine ([3H]dThd) autoradiography. Cells were plated in 35-mm dishes and 17 h after bleomycin treatment the proportion of nuclei in S-phase was determined by labeling cultures with 10 μCi/ml [3H]dThd (41 Ci/mmol⁻¹; Amersham, United Kingdom) for 1 h. Dishes were then fixed in methanol: acetic acid (3:1) and emulsion autoradiography was carried out as described (18). Results are presented as mean LI ± SE from a minimal count of 1000 nuclei/data point.

Immunocytochemistry

SDII. Cells growing on coverslips were fixed with 4% paraformaldehyde in PBS, washed three times with PBS, and processed immediately (storage caused a decrease in signal intensity). Coverslips were pretreated successively in: (a) 50 mM glycine in PBS for 10 min to quench free aldehyde groups, (b) 0.2% Triton X-100 in PBS for 10 min to permeabilize membranes, and (c) 0.3% H₂O₂ for 3 min to block endogenous peroxidase. Nonspecific binding was inhibited by incubation for 10 min with 2% horse serum in PBS before performing a standard indirect immunoperoxidase procedure using mAb 6B6H4 (PharMingen, Inc.) as primary antibody followed by rabbit anti-mouse immunoglobulin-peroxidase conjugate as a secondary antibody. Sites of antibody binding were visualized by the deposition of brown polymer following incubation in diaminobenzidine-hydrogen peroxide solution (19).

p53. Cells were fixed in methanol:acetone (1:1) and immunostained as described previously by an indirect immunoperoxidase procedure using mAb PAb240 (15).

RESULTS

Following DNA Damage Induced by Bleomycin, Both G₁-S Growth Arrest and SDII Induction Are Dependent on p53 Function. Previous studies have shown that SDII accumulation associated with G₁ arrest induced by ionizing radiation (and radiomimetic agents such as bleomycin) is lost in transformed cells lacking functional p53 (4–6). However, the presence of additional molecular abnormalities in such cells raises the possibility that loss of p53 function alone may not be sufficient for abrogation of SDII induction. Therefore, we first repeated these studies using a normal diploid cell type (HDF) as a model, to permit manipulation of p53 function in the absence of other abnormalities.

The p53 mutant ala¹⁴³, or a neo-only control, construct was introduced into young HDF cells by infection with the appropriate retroviral vector (15) and pooled colonies (at least 100 in each case) were selected in G418. Uniform, stable expression of the mutant p53 vector psi-CRIP-SCX was confirmed by immunocytochemical demonstration of high nuclear levels of p53 protein in > 99% of pooled HDF-scx cells using mAb PAb240 (Fig. 1).

To further confirm the biological activity of our mutant p53 vector for subsequent experiments, we assessed the effect on cell cycle arrest following DNA damage induced by bleomycin. Pooled colonies were treated with bleomycin (250 μg/ml) and 17 h later the proportion of cells remaining in S-phase ([3H]dThd LI) was determined. In control (HDF-neo) cells, bleomycin treatment led to a decrease in LI from 45% to 4.3%, consistent with complete G₁ arrest. In contrast, in cells expressing mutant p53 (HDF-scx) cells, LI was only decreased from 43% to 20% in response to DNA damage (Fig. 2A), indicating loss of G₁-S checkpoint function. The partial reduction in LI in HDF-scx is explained by retention of the p53-independent G₂-M checkpoint in these cells as shown previously (16), which by blocking entry to G₂ indirectly causes a delayed fall in entry to S-phase. We conclude therefore that the exogenously derived mutant p53 is biologically active in these cells, as assessed by the abrogation of G₁ arrest following DNA damage.

To assess the accompanying changes in expression of SDII, intranuclear SDII protein levels were assessed immunocytochemically 4 h after the addition of bleomycin. Untreated control (HDF-neo) cells showed marked cell-cell heterogeneity in expression, with SDII being below detectability in the majority of cells (Fig. 2B). Bleomycin treatment led to a dramatic induction of expression, with over 95% of nuclei now strongly immunostained (Fig. 2C). Untreated HDF-scx cells showed a low level of immunostaining similar to HDF-neo cells (Fig. 2D). In contrast, however, in HDF-scx cells, bleomycin caused a much less marked induction, with still no more than 10% of nuclei showing detectable immunostaining in treated cultures (Fig. 2E).

We conclude that in a normal diploid context, as in transformed cells, both bleomycin-induced growth arrest and SDII induction appear to be highly p53 dependent. Using this result as a baseline, we next compared the behavior of SDII induction in senescence-induced growth arrest.

In Senescence, Growth Arrest Is Dependent on p53 Function, but SDII Induction Is Not. In order to determine directly the effect of mutant p53 on senescence-induced growth arrest, normal HDF cells were grown to a point very close to senescence before being infected with either the amphotropic retroviral vector, psi-CRIP-SCX, or with the psi-CRIP-neo control (15) and colonies subsequently selected in G418.

Introduction of neo alone led to flattened colonies which ceased proliferating after an average 3.3 pds (Fig. 3A), with the characteristic morphological features of senescence. In marked contrast, colonies expressing high levels of mutant p53 initially grew rapidly and showed an average extended life span of 16 pds when compared with HDF-neo (Fig. 3A). (After this extended life span they showed mor-
p53 AND SDII IN SENESCENCE

Fig. 2. Effect of mutant p53 expression on response to bleomycin-induced DNA damage in human diploid fibroblasts. A, proportion of nuclei undergoing DNA synthesis (LI ± SEM) in mutant p53-expressing HDF-scx fibroblasts (mp53) compared to controls (neo) in untreated cultures (D) and 17 h after exposure to 250 ng/ml bleomycin (T). B-D, expression of SDII assessed by immunoperoxidase assay in HDF-scx (D and E) compared to neo controls (B and C) in untreated cultures (B and D) and 4 h after bleomycin treatment (C and E). Note that no counterstain was used so that negative nuclei (the majority in B, D, and E) are not visible; representative fields were chosen so that they contained the same number of cells. Bar, 70 μm.

phological features consistent with crisis, rather than senescence.) As shown previously (13), growth arrest induced at senescence in normal HDF cells therefore appears to be dependent on intact p53 function.

To investigate whether SDII levels also show this dependency on p53, we compared expression of the protein as cells progressed toward senescence with that in cells which have been rescued from senescence by introduction of mutant p53. It can be seen from Fig. 3C that near-senescent cells show increased immunostaining for SDII protein both in terms of intensity and in the proportion of positive cells as compared with young cells (Fig. 3B), a result which is consistent with a previous assessment of SDII mRNA levels (1). Strikingly, near-senescent cells which had been rescued by introduction of mutant p53 maintained this elevated level of SDII protein. The frequency and intensity of nuclear immunopositivity in pooled clones of rescued cells was indistinguishable from that of near-senescent cells (compare Fig. 3, D with C), despite a rate of proliferation comparable to that of young cells. The ability of immunocytochemistry to analyze small numbers of cells was particularly valuable here in allowing assessment very soon after rescue, thereby minimizing the chances of missing any early, short-lived fall in SDII levels. Nevertheless Northern blot analysis was performed on later rescued cultures, and again SDII expression (mRNA level) was not reduced compared to near-senescent cells (data not shown). Uniform high level expression of mutant p53 in the rescued cells was again confirmed by immunocytochemical analysis (compare Fig. 3G with Fig. 3, E and F). We therefore conclude that mutant p53 can overcome senescence-induced growth arrest in normal HDF cells without lowering SDII expression.

Following UV Irradiation SDII Induction Is Also Independent of p53. Evidence has already been presented that DNA damage in the form of UV irradiation may represent another stimulus for p53-independent induction of SDII (8). However, again this has only been
shown in transformed lines. To investigate this relationship in our normal isogeneic model, young HDF-neo or HDF-scx cells were exposed to UV irradiation and 24 h later SDH expression was assessed by immunocytochemistry. As seen previously after bleomycin treatment (Fig. 2), HDF-neo cells showed a dramatic increase in the proportion and intensity of nuclei immunopositive for SDH protein in response to UV irradiation (Fig. 4, A and B). In this case, however, expression of mutant p53 did not change the pattern or magnitude of induction of SDH by UV irradiation (Fig. 4, C and D). Therefore, in contrast to bleomycin, UV treatment apparently induces SDH expression in normal HDF cells in a manner independent of p53 function.

DISCUSSION

Using a simple immunocytochemical assay, we have shown that the extent to which induction of SDH is dependent on p53 function varies markedly according to the nature of the underlying stimulus. Thus, while the response to DNA damage induced by bleomycin is highly p53 dependent, consistent with a simple linear model (bold pathway, Fig. 5), the induction of SDH by UV irradiation and senescence is unaffected by expression of mutant p53 at levels sufficient to abrogate at least one of its biological functions, G1-S arrest. Furthermore, although SDH induction may be a necessary signal for growth arrest, the rescue of senescent cells by mutant p53 without any accompany-
Fig. 4. Effect of mutant p53 expression on SDII response to UV-induced DNA damage in HDFs. Expression of SDII assessed by immunoperoxidase assay in mp53-expressing HDFs (C and D) compared to neo controls (A and B) in untreated cultures (A and C) and 24 h after exposure to 25 J/m² UV radiation (B and D). No counterstain; bar, 70 μm.

Fig. 5. Role of p53 and SDII in cell cycle arrest. A simple linear model fits the response to bleomycin-induced DNA damage (bold arrows), but a more complex model is required to explain responses to UV and senescence. Key features are (a) the redundancy in signaling to SDII, with an alternative pathway through effector E1; and (b) the requirement for a second p53-dependent signal (via predicted effector E2), in addition to SDII, to bring about growth arrest.

These data add senescence to the growing list of conditions associated with p53-independent induction of SDII and provides further evidence for the redundancy of signals regulating expression of this gene. Whether the same signal pathway (E1, Fig. 5) mediates p53-independent induction in all cases is not known. Nor is it clear how the choice of pathway is determined by the underlying stimulus. From the known actions of the agents studied here, however, it can be inferred that DNA strand breaks (as produced by bleomycin) activate almost exclusively the p53 pathway, while bulky lesions (e.g., UV-induced 6–4 photoproducts and/or cyclobutane-pyrimidine dimers) can also signal (either directly or after processing) through the “by-pass” route (E1, Fig. 5). Interestingly senescence also activates both pathways. In this case we and others have speculated that the relevant signal is generated by telomere erosion, either directly as a consequence of the structural change being recognized as a form of DNA “damage” or indirectly through alteration in activity of subtelomeric genes (13, 20, 21). Given the key growth-limiting role of senescence in restraining development of potential tumor clones, it is logical that redundancy should be built into this system.

Given this reasoning, it is all the more surprising to find that SDII induction is insufficient to maintain senescent growth arrest. (Indeed there appears to be lack of redundancy at this key point in that maintenance of senescence is critically dependent on p53.) It can be inferred from the data that at least one additional downstream effector (E2, Fig. 5) is needed for p53-induced growth arrest in this situation. This result contrasts with reports that transfection of an SDII expression vector into HDF was sufficient on its own to inhibit proliferation (1, 3). It is highly likely however that the levels of expression achieved in these experiments were significantly in excess of that reached by the endogenous gene in our work, particularly since very strong viral promoters were used.

The simplest interpretation of our data is that the putative effector E2 represents cyclin-cdk inhibitors which normally cooperate with...
SDII to reduce the overall activity of cyclin-cdkks below the threshold required for cell cycle progression (22). Experimental manipulation of the expression of candidate cyclin-cdk inhibitors in this model should provide a biological assay for testing their role in senescence.

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