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Inhibition of E6 induced degradation of p53 is not sufficient for stabilization of p53 protein in cervical tumour derived cell lines

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The E6 proteins derived from tumour associated papillomavirus types target the cellular tumour suppressor protein p53 for ubiquitin mediated degradation. In cell lines derived from cervical tumours the p53 protein is present in very low amounts, but it can be activated by appropriate DNA damaging agents, indicating that functional p53 is present within these lines. Recent studies have also shown that different polymorphic forms of the p53 protein are differentially susceptible to E6 mediated degradation. Therefore we have been interested in analysing the effects of different HPV E6 proteins upon p53 levels in a variety of cervical tumour derived cell lines. We show that inhibition of E6 mediated degradation of p53 frequently results in increased levels of p53 expression. However, there are notable exceptions to this where increased p53 levels are only obtained following DNA damage and proteasome inhibition. We also show in E6 expressing cells, that as well as p53 being targeted for degradation, the localization of p53 to the nucleus is also inhibited, consistent with previous observations which indicate that degradation of p53 is not essential for E6 mediated inhibition of p53 function. These results have important implications for any potential therapies which might aim to block E6 mediated degradation of p53.

Keywords: HPV; E6; p53; proteasome

Introduction

A subset of human papillomavirus types, in particular types 16 and 18, are intimately associated with the development of cervical tumours (zur Hausen and Schneider, 1987). These viruses encode two principal oncoproteins, E6 and E7, both of which are continually expressed in cervical tumours and cell lines derived therefrom (Smotkin and Wettstein, 1986; Androphy *et al*., 1987; Banks *et al*., 1987). Inhibition of the expression of either protein results in a cessation of transformed cell growth (von Knebel Doeberitz *et al*., 1988; Crook *et al*., 1989; Storey *et al*., 1994); hence both proteins represent ideal therapeutic targets. Both oncoproteins exert their transforming activity through interactions with key components of the cellular regulatory machinery. E7 binds to the cellular tumour suppressor pRb and the related pocket proteins (Dyson *et al*., 1989; Davies *et al*., 1993), resulting in a release of free E2F and stimulation of proliferation related genes (Bandara *et al*., 1991; Phelps *et al*., 1991). The viral E6 protein's principal target is the cellular tumour suppressor p53 (Werness *et al*., 1990); as a consequence of this interaction, p53 is labelled for ubiquitin mediated degradation, leading to an inhibition of p53's growth regulatory functions (Scheffner *et al*., 1990; Huibregtse *et al*., 1991, 1993; Crook *et al*., 1991; Pim *et al*., 1994).

Previous studies have shown that, although p53 protein levels are invariably quite low in cell lines derived from cervical tumours, consistent with the notion of E6 induced degradation, the pathways leading to induction of p53 are nonetheless intact (Butz *et al*., 1995). Thus, treatment with DNA damaging agents often results in increased p53 protein levels and an increase in p53 mediated transcriptional activation, regardless of the fact that E6 protein is present (Butz *et al*., 1995). We have also recently shown that the presence of a common polymorphism within the p53 protein, at position 72, can affect the susceptibility of p53 to E6 mediated degradation. Thus, the p53Arg polymorphic variant is more susceptible to the effects of HPV E6 than the p53Pro (Storey *et al*., 1998). Interestingly, this polymorphism lies within the polyproline region of p53 which has also been recently shown to be involved in the induction of p53 mediated apoptosis (Walker and Levine, 1996; Sakamuro *et al*., 1997). An obvious question which arises from these data is whether inhibition of E6 mediated degradation of p53 in these tumour derived cell lines will result in an activation of p53. This is particularly important from a therapeutic point of view, since this will determine whether the appropriate signals are already present within these cell lines to activate p53 function.

We show that in a number of cell lines proteasome inhibition does indeed give rise to an increase in p53 levels, consistent with the notion that p53 is continually activated within these lines but that the levels are low due to the effects of E6. However, several cell lines failed to induce high levels of p53 following proteasome inhibition, indicating that in these cases the signals required for p53 activation are lacking. In such cell lines a significant increase in p53 levels is only seen upon treatment with DNA damaging agents.

We have also analysed the effects of E6 upon the nuclear localization of activated p53 protein. In cell lines lacking E6, proteasome inhibition coupled with treatment with a DNA damaging agent results in a rapid nuclear localization of the p53 protein. In contrast, in cells containing E6 protein similarly treated, the majority of p53 is found in a perinuclear location. These results demonstrate that inhibition of E6 mediated degradation of p53 alone is not sufficient in all cases for reactivation of p53 function. Rather,

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complete inhibition of the E6–p53 interaction would seem to be necessary to fully realize the therapeutic potential of targeting the E6–p53 association.

Results

Comparison of lactacystin and N-acetyl-leu-leu-norleucinal protection of p53

It has previously been shown that DNA damaging agents can induce functional p53 protein in cell lines derived from cervical tumours, which indicates that the pathways leading to activation of p53 are functional in these lines (Butz *et al*., 1995). However, a vital question for designing strategies for blocking E6-mediated degradation of p53 is whether or not those same pathways are intrinsically active within these transformed cells. Since p53 is targeted for degradation by E6 through the ubiquitin pathway (Scheffner *et al*., 1990; Huibregtse *et al*., 1991, 1993) an obvious means of addressing this question is to monitor p53 levels following proteasome inhibition in E6 expressing cell lines. A number of proteasome inhibitors of varying specificity have been described (Rock *et al*., 1994), therefore we first determined the effects of lactacystin (LC) and N-acetyl-leu-leu-norleucinal (LL) upon p53 levels in HPV-16 (CaSKi) and HPV-18 (HeLa) containing cervical tumour derived cell lines. Cells were treated for 2 h with the proteasome inhibitors in the presence or absence of mitomycin C, after which the cells were harvested and p53 levels determined by Western blot analysis with a pool of anti p53 monoclonal antibodies. The results obtained are shown in Figure 1. As can be seen, treatment of CaSKi cells with either LC or LL results in similar levels of induction of p53 protein. Interestingly, the slower migrating form of p53, corresponding to the p53Pro variant is protected to a greater extent and this is consistent with our previous observations (Storey *et al*., 1998). Treatment with mitomycin C alone results in no significant increase in p53 levels. In marked contrast, addition of either LC or LL to the HeLa cells has very little effect on the steady state level of p53 within these cells. Even mitomycin C treatment induces only a modest increase in p53 levels and this is most readily detected if LL is also present. These results demonstrate that inhibition of E6 mediated degradation of p53 in CaSKi cells will result in a significant upregulation of the p53 protein levels, whereas this is not the case in HeLa cells. Additional stimulation of the DNA damage induction pathway is required before a significant induction of p53 protein levels can be detected in HeLa cells.

Differential induction of p53 following inhibition of E6 mediated degradation of p53

Having shown that differences exist in the induction of p53 protein between the CaSKi and HeLa cells, we

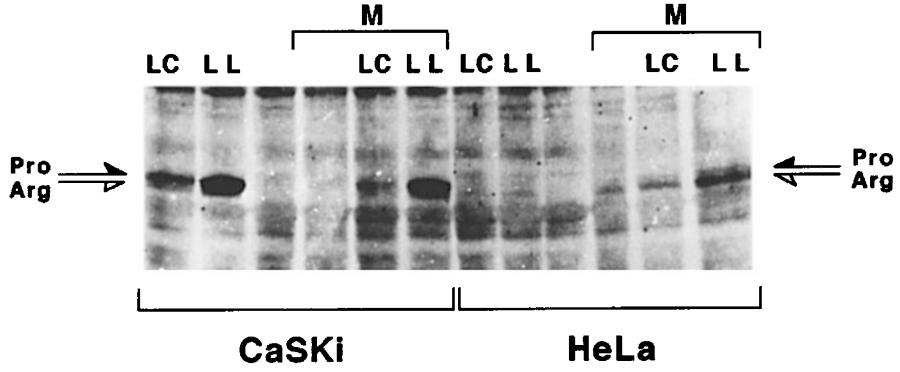

Figure 1 Comparison of lactacystin and N-acetyl-leu-leu-norleucinal protection of p53 in CaSKi and HeLa cells. HPV-16 positive CaSKi and HPV-18 positive HeLa cells were either untreated or treated for 18 h with mitomycin C (M) and with proteasome inhibitors lactacystin (LC) or N-acetyl-leu-leu-norleucinal (LL) for 2 h before harvesting; p53 levels were then determined by Western blotting with a pool of anti p53 monoclonal antibodies. Pro and Arg polymorphic forms of p53 are indicated by the arrows. Background staining confirms equal levels of protein loading

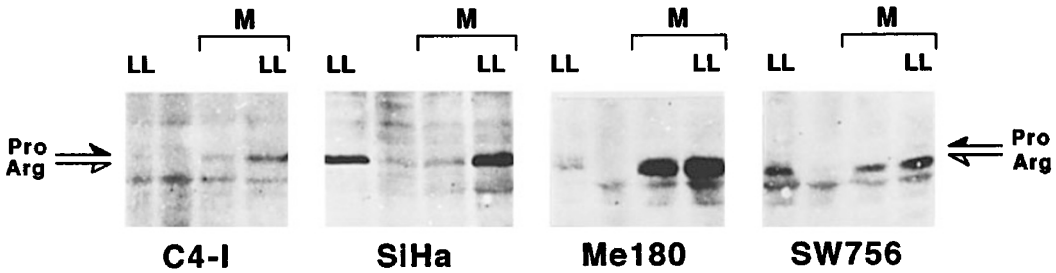

Figure 2 Inhibition of E6 mediated degradation of p53 in HPV positive cell lines. HPV-16 positive SiHa, HPV-18 positive C4-I and SW756, and HPV-68 positive Me180 cervical carcinoma cells were either untreated or treated for 18 h with mitomycin C (M) and with the proteasome inhibitor N-acetyl-leu-leu-norleucinal (LL) for 2 h before harvesting; p53 induction was then assessed by Western blotting with a pool of anti p53 monoclonal antibodies. Arrows show the Pro and Arg polymorphic forms of p53. Background staining confirms equal levels of protein loading

were next interested in examining the effects of proteasome inhibition upon p53 levels in a variety of other HPV DNA-containing cervical tumour derived cell lines. Since the highest level of p53 protection was obtained using LL as opposed to LC, the remaining studies were all performed using LL. We first compared the induction of p53 protein in four different cell lines derived from cervical tumours, C4-I, SiHa, Me180 and SW756. The cells were treated with LL plus or minus mitomycin C, and the p53 protein levels were ascertained as described above. The results obtained are shown in Figure 2. It is clear that the pattern of p53 protection/induction seen in the SW756 and SiHa cells is similar to that seen in the CaSKi cells. Inhibition of E6 mediated degradation of p53 gives rise to a dramatic increase in p53 protein levels, which is consistent with there being signals already present within these cells for activating and stabilizing the p53 protein. This increase in the p53 levels is augmented by the addition of DNA damaging agents and is in agreement with previous studies (Butz *et al*., 1995). In contrast, the C4-I and, to a slightly lesser extent, the Me180 cells both behave like the HeLa cells. Proteasome inhibition does not result in a significant increase in the p53 levels, indicating that the intrinsic pathways of p53 activation are not activated within these cells. Only following treatment with a DNA damaging agent is a significant increase in the levels of p53 protein detected.

For comparison we next analysed the effects of proteasome inhibition on p53 levels in two cell lines lacking E6 sequences. HT1080 cells contain wild type p53 protein and, as can be seen from Figure 3, proteasome inhibition gives rise to a modest increase in p53 levels, consistent with the observation that p53 is normally regulated via the proteasome (Haupt *et al*., 1997; Kubbutat *et al*., 1997). DNA damage induction results in a dramatic increase in the p53 levels and, in contrast to the E6 containing cell lines, this is not augmented by treatment with proteasome inhibitors. In contrast, in C33-I cells which have mutant p53 protein (Scheffner *et al*., 1991), the levels of p53 are completely

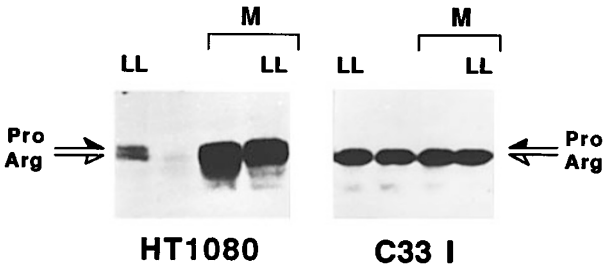

Figure 3 Effects of DNA damage and proteasome inhibition on p53 induction in cell lines lacking HPV E6. HPV-negative HT1080 human fibrosarcoma cells and C33-I cervical carcinoma cells were either treated or untreated with mitomycin C (M) for 18 h and with N-acetyl-leu-leu-norleucinal (LL) for 2 h before analysing p53 levels by Western blotting. The Pro and Arg polymorphic forms of p53 are indicated by the arrows

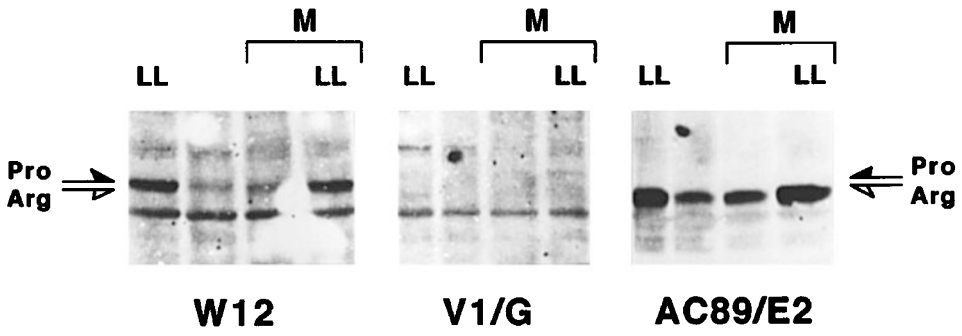

Figure 4 Effects of proteasome inhibition on p53 induction in HPV-16 immortalized keratinocyte cell lines. W12, V1/G and AC89/E2 HPV-16 immortalized cell lines were either treated or untreated with mitomycin C (M) for 18 h and treated (LL) or untreated with N-acetyl-leu-leu-norleucinal for 2 h before analysing p53 levels by Western blotting. The Pro and Arg polymorphic forms of p53 are indicated by the arrows. Background staining confirms equal levels of protein loading

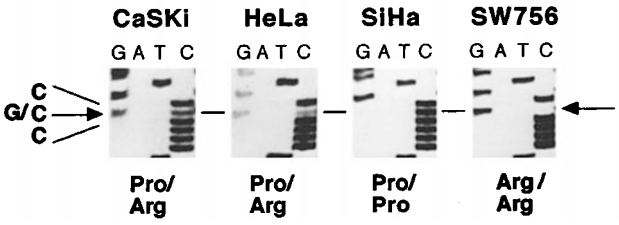

Figure 5 Sequence analysis of the Pro/Arg polymorphic status of p53 at codon 72 in different HPV DNA-containing cell lines. The region of p53 comprising codon 72 was amplified by RT–PCR from total RNA of different cell lines and subsequently analysed by cycle sequencing. Arrows indicate the polymorphic base at second position of codon 72: CCC = Pro, CGC = Arg. Cell lines heterozygote for the Pro and Arg alleles have both C and G at this position

Table 1 Polymorphic status of the p53 in the cell lines analysed

Cell line	*HPV type*	*p53 Pro/Arg status*
HeLa	18	Pro[a]/Arg
CaSKi	16	Pro/Arg
Me180	68	Pro/Arg
HT1080	−ve	Pro/Arg
SiHa	16	Pro
C4-I	18	Pro
W12	16	Pro
C33I	−ve	Arg
V1/G	16	Arg
Ac89 E2	16	Arg
SW756	18	Arg

[a]Pro allele detection consistently very weak

unaffected by either DNA damage or treatment with proteasome inhibitors.

Having demonstrated differences in the intrinsic activation of p53 in cell lines derived from cervical tumours we were next interested in investigating the effects of proteasome inhibition in cell lines immortalized by HPV-16 but which are not fully transformed. To do this W12, V1/G and AC89/E2 cells were analysed as described above, and the results obtained are shown in Figure 4. In the case of V1/G cells no detectable p53 protein was found. However, in both the AC89/E2 and W12 cells, a strong induction of p53 protein was obtained following proteasome inhibition. These results indicate that, at least in the case of these

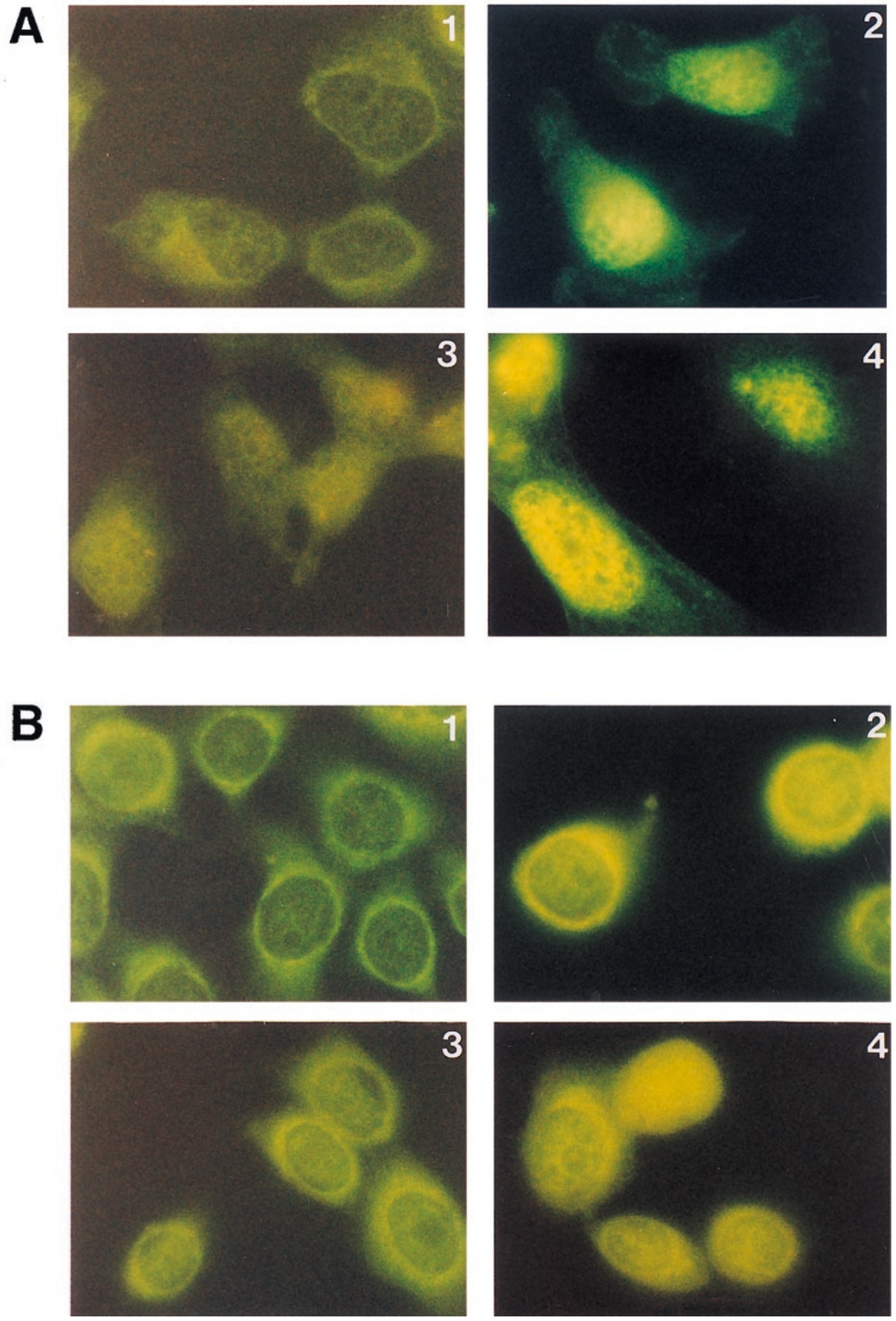

Figure 6 Comparison of p53 localization in HPV E6-containing and non-containing cell lines. (**a**) Immunofluorescence analysis of p53 in HPV-negative HT1080 human fibrosarcoma cells. The panels correspond to: 1, untreated cells; 2, cells treated with mitomycin C for 18 h; 3, cells treated with N-acetyl-leu-leu-norleucinal (LL) for 2 h; 4, cells treated both with mitomycin C for 18 h and with LL for 2 h prior to immunostaining. (**b**) Immunofluorescence analysis of p53 in HPV-positive Me180 human cervical carcinoma cells. The panels correspond to: 1, untreated cells; 2, cells treated with mitomycin C for 18 h; 3, cells treated with N-acetyl-leu-leu-norleucinal (LL) for 2 h; 4, cells treated both with mitomycin C for 18 h and with LL for 2 h prior to immunostaining

two immortalized keratinocyte lines, the intrinsic activation pathways to p53 are active in the absence of any additional stimulus.

The Pro/Arg polymorphism at position 72 of p53 is not responsible for differences in intrinsic activation of p53

Since we have recently shown that the p53 polymorphism at position 72 can affect the susceptibility of p53 to E6 mediated degradation (Storey *et al*., 1998), we were next interested in determining whether particular polymorphic forms of p53 were more likely to be intrinsically activated than others. To do this we determined the polymorphic status of the p53 protein in all the cell lines analysed. A representative sequence analysis is shown in Figure 5 and the results are summarized in Table 1. As can be seen, in the nine HPV containing lines analysed there was a broad spectrum of homozygous p53Arg, homozygous p53Pro and heterozygous p53 containing lines. Of those cell lines which gave rise to the weakest induction of p53 following proteasome inhibition, C4-I were homozygous p53Pro and both HeLa and Me180 were heterozygous, although the level of detection of the Pro allele in HeLa cells was consistently low, the significance of which remains to be determined. However, based on this analysis, the polymorphic status of p53 within these lines does not appear to be responsible for differences in the levels of intrinsic activation in the absence of additional stimuli.

p53 localization is perturbed in E6 containing cells

Previous studies have shown that activation of p53 results in nuclear accumulation and activation of a variety of p53 responsive promoters (El-Deiry *et al*., 1993; Pietenpol *et al*., 1994; Miyashita and Reed, 1995; Knippschild *et al*., 1996; Crook *et al*., 1998). Since we have found very low levels of intrinsic activation in a number of the cell lines analysed, it seems probable that blocking E6 mediated degradation of p53 would not alone be sufficient to activate high levels of p53 activity. We were next interested in determining whether p53 can localize to the nucleus when E6 is present, particularly in those lines in which DNA damage and proteasome inhibition can induce high p53 levels. To address this question, we performed a series of immunofluorescence assays on HT1080 cells, which contain wild type p53 and lack E6 protein, and Me180 cells, which contain wild type p53 and harbor HPV-68 E6. Cells were treated with LL for 2 h in the presence or absence of mitomycin C, and were then fixed and probed for p53 protein. The results obtained are shown in Figure 6. As can be seen in Figure 6a, treatment of HT1080 cells with LL results in a weak accumulation of p53 within the nucleus, consistent with the Western blot analysis above. Induction with mitomycin C gives a strong nuclear localization, and a similar effect is seen when LL is also added to the cells. Similar results were also obtained with epithelial MCF7 cells (data not shown). In contrast, the treatment of Me180 cells with mitomycin C produces a marked increase in perinuclear staining with no apparent transport to the nucleus. Even following proteasome inhibition there is a dramatic increase in the level of p53 protein detection, but very little nuclear localization is detected within the time frame of this assay. Identical results were also obtained when HPV-16 containing SiHa cells were analysed in a similar manner (data not shown). These results demonstrate that the presence of E6 alone, in the absence of ubiquitin mediated degradation, is sufficient to inhibit p53's localization to the nucleus.

Discussion

The E6-induced degradation of p53 is an important factor in the development of HPV-associated cervical neoplasia. Indeed, individuals homozygous for the more susceptible polymorphic form of p53 have been estimated to be significantly more at risk of developing HPV associated neoplasia than heterozygote individuals (Storey *et al*., 1998). In the study presented here it is clear that, in many cell lines derived from cervical tumours, p53 is indeed being activated by intrinsic pathways within the cell but its accumulation is prevented by the presence of the HPV E6 protein. However, there are notable exceptions to this. In three tumour derived cell lines little or no p53 induction was observed following treatment with proteasome inhibitors alone, indicating that in these cases the endogenous p53 was not activated. Induction of p53 was observed only upon mitomycin C treatment, and this was further augmented by proteasome inhibition. In addition, although in a number of cell lines blocking E6 mediated degradation of p53 resulted in an accumulation of p53 protein, the subsequent nuclear localization was nonetheless disrupted by the presence of the HPV E6 protein. These results suggest that blocking E6 induced degradation of p53 will, at best, be only partially effective in blocking the deleterious effects of E6 upon p53.

Previous studies have shown that the E6 proteins from the tumour-associated HPV types can bind and target p53 for ubiquitin mediated degradation (Scheffner *et al*., 1990; Huibregtse *et al*., 1991, 1993). As a consequence, the growth suppressive activities of the p53 protein can be abrogated (Demers *et al*., 1994; Thomas *et al*., 1996). However, several studies have also shown that complex formation between E6 and p53 is alone sufficient for inhibition of p53 function (Lechner and Laimins, 1994; Pim *et al*., 1994; Thomas *et al*., 1995). *In vitro*, E6 has been shown to be able to inhibit p53 DNA binding activity independently of its ability to target p53 for degradation (Lechner and Laimins, 1994; Thomas *et al*., 1995). In addition, several *in vivo* studies have also shown that E6 mutants can abrogate the transcriptional transactivation and growth arrest activities of the p53 protein despite being defective in their ability to target p53 for degradation (Lechner *et al*., 1992; Pim *et al*., 1994; Thomas *et al*., 1996). In contrast, the inhibition of p53's apoptotic activities by E6 would appear to require the ability to target p53 for degradation (Thomas *et al*., 1996). It has also been reported that the signalling pathways, both upstream and downstream of p53, in cervical tumour derived cell lines are frequently intact (Butz *et al*., 1995), suggesting that therapeutic inhibition of the E6–p53 association might allow functional p53 protein to bring about growth arrest and the potential

cessation of tumour development. We were therefore interested in analysing whether intrinsic signals were present within HPV containing tumour derived cell lines which would stabilize the p53 protein if E6 mediated degradation was inhibited. To address this, proteasome inhibitors were assessed on a variety of tumour derived and HPV immortalized cell lines. In most cases proteasome inhibition indeed resulted in increased levels of p53 protein, suggesting that the intrinsic signals to activate the p53 were present. In three cases however, proteasome inhibition alone did not result in increased levels of p53 expression, indicating that in these cases the signals required to activate p53 were not present. Only after the additional insult of treatment with a DNA damaging agent was the p53 protein induced. This failure of induction is not related to the polymorphic status of the p53, since analysis showed two cell lines to be heterozygous and one to be homozygous Pro.

Activation of the p53 protein results in its stabilization and re-localization into the nucleus where it functions as a transcriptional activator of a variety of target genes (El-Deiry *et al*., 1993; Pietenpol *et al*., 1994; Miyashita and Reed, 1995; Knippschild *et al*., 1996; Crook *et al*., 1998). A key question in the therapeutic reactivation of p53 in HPV positive tumour cells is whether or not this process of p53 localization to the nucleus can efficiently take place in the presence of E6. To investigate this question we performed a series of studies on cell lines containing wild type p53, either with or without the HPV E6 protein. The accumulation and re-localization of the p53 protein was monitored following both proteasome inhibition and induction of DNA damage. The E6 containing cell line chosen for this analysis was Me180, which we have shown to induce very high levels of p53 expression following proteasome inhibition and treatment with a DNA damaging agent.

Strikingly, in comparison to the HT1080 cells in which p53 was found to localize efficiently to the nucleus, following proteasome inhibition and DNA damage induction, in Me180 cells p53 was found only in the cytoplasm. Similar results were also obtained when the HPV-16 positive cell line SiHa was used. These results demonstrate that even if p53 degradation by E6 is blocked by proteasome inhibition, the p53 protein fails to localize to the nucleus and this suggests that complex formation between E6 and p53 is sufficient to perturb p53 nuclear localization. This provides an explanation for previous studies which have shown that E6 induced degradation of p53 is not necessary for the inhibition of p53 transcriptional activation, since the failure of p53 to localize to the nucleus would certainly be sufficient to inhibit transcriptional activity. It should also be mentioned that the perinuclear accumulation of p53 is not due to the cells arresting in the G2 phase of the cell cycle (Butz *et al*., 1995; F Mantovani personal observations). At present we cannot formally exclude the possibility that the failure of p53 to localize to the nucleus is due solely to the presence of E6, but rather may be due to the presence of another viral protein. We are presently producing cell lines expressing inducible E6 protein in order to directly address this possibility.

In summary, we have shown that inhibition of E6 induced degradation of p53 in cell lines derived from cervical tumours frequently gives rise to an increase in the levels of p53 expression. However cases exist where induction of p53 is not obtained without additional genotoxic insult. In addition, even when p53 levels can accumulate following inhibition of E6 induced degradation, the localization of p53 to the nucleus is still perturbed. Taken together, these results suggest that inhibition of E6 mediated degradation of p53 as a therapeutic strategy may be only partially successful.

Materials and methods

Cell culture and genotoxic treatment

Human cervical carcinoma cell lines HeLa, SiHa, CaSKi, Me-180, SW756, C4-I and C33I, plus the human HT1080 fibrosarcoma and MCF7 mammary adenocarcinoma cell lines were maintained in DMEM supplemented with 10% foetal calf serum, at 37°C in the presence of 10% CO_2. Establishment of HPV-16 immortalized cell lines W12, V1/G and AC89/E2 has been described previously (Stanley *et al*., 1989; Bouvard *et al*., 1996; Storey *et al*., 1992). Keratinocyte cell lines were grown in a 3 : 1 mixture of DMEM and Ham F12 medium, supplemented with 10% foetal calf serum, 10 ng/mL epidermal growth factor, 10^{-10} M cholera toxin and 0.4 μg/mL hydrocortisone at 37°C and 10% CO_2.

Exponentially growing cells were incubated with 10 μg/ml mitomycin C for 18 h, and N-acetyl-leu-leu-norleucinal (50 μM) or lactacystin (25 μM) were added for 2 h where indicated, prior to harvesting the cells for subsequent analysis.

Western blotting

Cells were extracted in a solution of 50 mM HEPES pH 7.0, 250 mM NaCl, 0.1% NP40 and 1% aprotinin. Protein concentrations were determined using the BioRad protein assay system and equal amounts (200 μg) were run on 10% PAGE and transferred to nitrocellulose membrane. p53 protein was detected using a pool of the anti-p53 monoclonal antibodies pAb 1801, 1802 and 1803 (Banks *et al*., 1986) and developed using the Amersham ECL system.

Immunofluorescence assays

Cells were fixed for 20 min with methanol at −20°C, rehydrated with PBS, then reacted for 1 h with the same anti-p53 antibody pool used for Western blotting. After incubation for 30 min with biotinylated anti-mouse antibody (DAKO), diluted at 1 : 200, and for 20 min with streptavidin–FITCH diluted at 1 : 50 (Southern Biotechnology Associates) the cells were mounted with mounting medium for fluorescent microscopy (KPL).

RNA extraction, RT–PCR and cycle sequencing

Total cellular RNA was isolated from cultured cells with RNAzol B according to the manufacturer's instructions. 5 μg of total RNA were then reverse transcribed for 1 h at 39°C using 200 U of Moloney murine leukaemia virus reverse transcriptase (Gibco BRL) and the p53 specific antisense primer 5′-GGCAGGTCTTGGCCAGTT-3′. 30 cycles of amplification were then performed using oligonucleotides 5′-CTCTGAGTCAGGAAACATT-3′ and 5′-AAGGGACAGAAGATGACAG-3′, amplification conditions were: 1 min at 95°C, 1 min at 58°C and 1 min at 72°C. After purification, the RT–PCR products (259 bp) were then subjected to cycle sequencing with the internal oligonucleotide 5′-GATATTGAACAATGGTTC-3′, using the Amersham Thermosequenase kit and ^{33}P-labelled ddNTP terminators; cycling conditions were: 1 min at 95°C, 1 min at 55°C and 2 min at 72°C for 45 cycles.

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