

The human papilloma virus 16E6 gene sensitizes human mammary epithelial cells to apoptosis induced by DNA damage

(p53/p21/mitomycin C/staurosporine/ubiquitinylation)

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ABSTRACT Programmed cell death (apoptosis) is a normal physiological process, which could in principle be manipulated to play an important role in cancer therapy. The key importance of p53 expression in the apoptotic response to DNA-damaging agents has been stressed because mutant or deleted p53 is so common in most kinds of cancer. An important strategy, therefore, is to find ways to induce apoptosis in the absence of wild-type p53. In this paper, we compare apoptosis in normal human mammary epithelial cells, in cells immortalized with human papilloma virus (HPV), and in mammary carcinoma cell lines expressing wild-type p53, mutant p53, or no p53 protein. Apoptosis was induced with mitomycin C (MMC), a DNA cross-linking and damaging agent, or with staurosporine (SSP), a protein kinase inhibitor. The normal and HPV-transfected cells responded more strongly to SSP than did the tumor cells. After exposure to MMC, cells expressing wild-type p53 underwent extensive apoptosis, whereas cells carrying mutated p53 responded weakly. Primary breast cancer cell lines null for p53 protein were resistant to MMC. In contrast, two HPV immortalized cell lines in which p53 protein was destroyed by E6-modulated ubiquitinylation were highly sensitive to apoptosis induced by MMC. Neither p53 mRNA nor protein was induced in the HPV immortalized cells after MMC treatment, although p53 protein was elevated by MMC in cells with wild-type p53. Importantly, MMC induced p21 mRNA but not p21 protein expression in the HPV immortalized cells. Thus, HPV 16E6 can sensitize mammary epithelial cells to MMC-induced apoptosis via a p53- and p21-independent pathway. We propose that the HPV 16E6 protein modulates ubiquitin-mediated degradation not only of p53 but also of p21 and perhaps other proteins involved in apoptosis.

Considerable excitement has been engendered by the growing evidence of a normal biological process by which chosen cells undergo a programmed cell death program, apoptosis, not only during development but also in adult tissues (1–3). These findings open the possibility that apoptosis might be manipulated clinically to destroy tumor cells (4, 5). p53 is the most commonly mutated or deleted gene identified in mammary carcinoma (6, 7). Recent studies have shown that p53 plays an important role in inducing apoptosis after ionizing radiation, most clearly seen in comparing thymocytes (8–10) and fibroblasts (11) from p53 +/+ and –/– “knockout” mice. In mouse mammary epithelial cells transfected with a temperature-sensitive p53 gene, mitomycin C (MMC) was effective in inducing apoptosis only at the permissive temperature, demonstrating a p53 requirement in this system (12). However, p53 –/– thymic lymphoma cells were killed by apoptosis induced by MMC, cisplatin, etoposide, dexamethasone, and UV or γ -irradiation (10). Thus, the requirement for wild-type p53 in

apoptosis induced by DNA damage is not invariant, and furthermore it may operate through a different pathway from the effect on G₁ growth arrest (13).

The adenovirus E1A protein, which binds to the Rb protein, can elevate the sensitivity of cells to apoptosis induced by ionizing radiation, 5-fluorouracil, etoposide, and adriamycin (11) as well as under conditions of low serum or high cell density (14) in the presence of wild-type p53 (15). On the other hand, the human papilloma viruses (HPVs), which encode E6 and E7 proteins, are anticipated to protect cells from apoptosis induced by DNA damage because E6 protein triggers the destruction of p53 protein through the ubiquitin pathway (16).

p53 activation leads to upregulation of p21, resulting in growth arrest through inhibition of cyclin-dependent kinases (17, 18). However, p53-independent pathways have also been reported (19–21). Furthermore, it is not established whether p53 modulates apoptosis through p21 activity.

The work reported here was initiated as a study of apoptosis in a series of breast cancer cell lines and their normal and immortalized counterparts. Two prototypic inducers of apoptosis have been used: staurosporine (SSP), a protein kinase inhibitor (22, 23), and MMC, a DNA cross-linking and damaging agent that exerts its toxic effects through cross-linking of DNA by alkylation and through generation of oxygen radicals (24, 25).

We show here that wild-type p53 is required for effective MMC-induced apoptosis in breast cancer cells, but in the HPV 16E6 immortalized normal cells, MMC-induced apoptosis occurs in the absence of p53 and p21 protein, demonstrating that HPV 16E6 sensitizes cells to MMC-induced apoptosis through a unique pathway.

MATERIALS AND METHODS

Cells and Cell Culture. 70N and 76N are normal human mammary epithelial cell (HMEC) strains established in this laboratory (26). They express wild-type p53, as does the mammary tumor cell line MCF-7. MDA-MB-435 and MDA-MB-231 are mammary carcinoma cell lines carrying mutant p53; 21NT and 21PT (together 21T) are primary tumor cell lines expressing no p53 protein (27). 16E6 and 16E6E7 are cell lines of 76N cells immortalized with the E6 gene of HPV 16 or with 16E6E7 (28). They express no p53 as a result of E6-activated ubiquitinylation of p53 leading to its degradation (16). 70N cells and HPV immortalized cells were grown in DFCl-1 medium (D medium) (26, 27). MCF-7 and MDA-MB-231 were grown in α -MEM plus 10% fetal calf serum (FCS) supplemented with insulin (1 μ g/ml), 2.8 μ M hydrocortisone, and epidermal growth factor (25 ng/ml). MDA-MB-435 was cultured in α -MEM plus 5% FCS. All breast tumor cells used

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Abbreviations: HPV, human papilloma virus; MMC, mitomycin C; SSP, staurosporine; HMEC, human mammary epithelial cell; PI, propidium iodide.

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for apoptosis experiments were preadapted for one passage in D medium.

Apoptosis Induction and Assay. For microscopy, cells were plated in D medium at $3\text{--}5 \times 10^4$ cells per 35-mm dish and incubated at 37°C in 6.5% $\text{CO}_2/93.5\%$ air for 48 h before drug treatment. The monolayer cultures were incubated with $30 \mu\text{M}$ MMC or $0.2 \mu\text{M}$ SSP. Plates were washed twice with solution A (phosphate-buffered saline supplemented with 10 mM glucose/30 mM Hepes). The cells were then fixed, stained with Hoechst 33258 dye (Sigma), and examined by fluorescence microscopy (29).

For flow cytometry, cells were plated in D medium at $2\text{--}3 \times 10^5$ cells per 60-mm dish, incubated at 37°C in 6.5% $\text{CO}_2/93.5\%$ air for 48 h, and then treated with agents as described above. After treatment, cultures were harvested, washed, centrifuged, and resuspended in propidium iodide (PI) as described (30). Fluorescence was measured with a Coulter EPICS Elite flow sorter.

Western Blot Analysis. The detection of p53 and p21 proteins by immunoblotting analysis was performed essentially as described (17). Monolayer cultures at 70–80% confluence were harvested using 2 mM EDTA/solution A for 10–15 min. Cells (10^6 cells per $40 \mu\text{l}$) were counted, centrifuged, and resuspended in SDS/PAGE buffer [63 mM Tris-HCl, pH 6.8/10% (vol/vol) glycerol/5% 2-mercaptoethanol/2% SDS/0.025% bromophenol blue] and then boiled for 5 min. Total cell lysate was loaded and separated by SDS/12% PAGE in a minigel apparatus (Bio-Rad). Proteins were transferred to a poly(vinylidene difluoride) membrane as described (28). The membranes were blocked and incubated with p21 or p53 monoclonal antibodies (Oncogene Science) and then incubated with horseradish-conjugated goat anti-mouse antibody and signals were detected by ECL (Amersham).

Northern Blot Analysis. Total RNA was isolated from $\approx 70\%$ confluent cell monolayers after MMC treatment with RNazol B (Biotecx Laboratories, Houston). RNA ($10 \mu\text{g}$ per lane) was denatured and electrophoresed through a 1.0–1.2% agarose-formaldehyde gel and transferred to a nylon membrane (Zeta-Probe; Bio-Rad). Northern blot hybridization was carried out as described (28).

RESULTS

Apoptotic Responses of Normal and Tumor-Derived Cells to SSP and MMC. The cellular response to two apoptosis-inducing agents, SSP and MMC, was examined by microscopic examination of cells stained with the fluorescent DNA binding dye Hoechst 33258. In these experiments, cells were treated for various times with SSP ($0.2 \mu\text{M}$) or with MMC ($30 \mu\text{M}$). The morphological effects on five cell types described above are shown in Fig. 1.

SSP- and MMC-treated 70N cells underwent morphological changes typical of apoptosis (1, 3) (Fig. 1 A–C). The nuclei shrank and chromatin became very dense, collapsing into dense spheres (arrows indicate apoptotic nuclei). Breast carcinoma cells were more resistant to both agents than 70N. MCF-7 tumor cells expressing wild-type p53 (Fig. 1 D–F) became smaller and the chromatin condensed, but it did not fragment as seen with 70N cells until 30 h or more after treatment. 21NT tumor cells (Fig. 1 G–I) and 21PT (data not shown), both p53 null, responded to SSP at a low level, but not at all to MMC, as predicted by the absence of p53. MDA-MB-231 (Fig. 1 J–L) and MDA-MB-435 (data not shown) tumor lines with mutant p53 responded to both drugs at intermediate levels. Unexpectedly, the HPV immortalized lines lacking p53, 76N/16E6 (Fig. 1 M–P) and 76N/16E6E7 (data not shown), underwent total apoptosis in response to both drugs.

Flow Cytometric and Microscopic Analysis of Apoptosis. A flow cytometric program using PI as the DNA-binding dye (30)

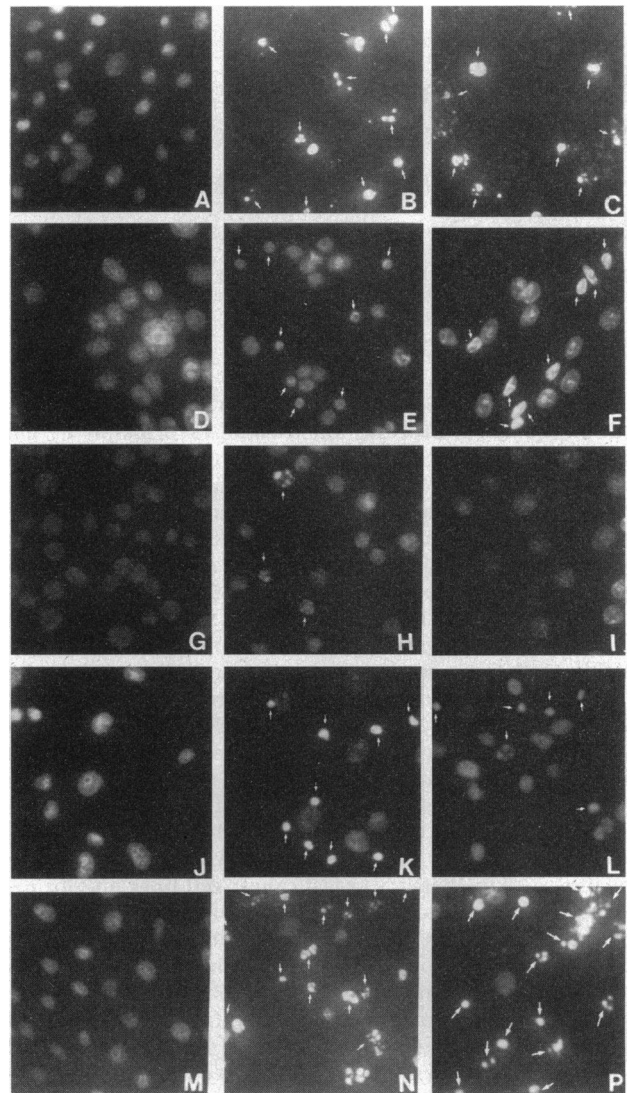


Fig. 1. Morphological response of breast cells to SSP and MMC. Cells were fixed *in situ* with methanol and stained with Hoechst 33258. Arrows indicate apoptotic nuclei. (A–C) 70N; (D–F) MCF-7; (G–I) 21NT; (J–L) MDA-MB-231; (M–P) 76N/16E6. (A, D, G, J, and M) Untreated; (B, E, H, and K) $0.2 \mu\text{M}$ SSP for 24 h; (N) $0.2 \mu\text{M}$ SSP for 12 h; (C) $30 \mu\text{M}$ MMC for 48 h; (F, I, L, and P) $30 \mu\text{M}$ MMC for 32 h. ($\times 180$).

was applied to four cell types—70N, MCF-7, 21PT, and 16E6—as shown in Fig. 2. In these and subsequent experiments, adherent and nonadherent cells were combined. In each cell type, the control panels show a typical exponential growth pattern, with cells distributed in G_1 , S, and G_2/M . Essentially no cells were present with DNA values below diploid, which is indicative of apoptosis.

The differences in response of the four cell types to MMC treatment for 24 h are dramatic. Whereas 21PT showed little if any response, the 16E6 cells displayed extensive apoptosis. Similarly, MCF-7 cells showed a very abnormal DNA pattern. Although the 70N cells showed little response to MMC within 24 h (Figs. 2 and 3A), they were highly responsive after 32–48 h (Figs. 1 and 3A). In contrast, after treatment for 24 h with SSP, the 70N, 21PT, and 16E6 cells lost their exponential growth pattern and sub- G_1 apoptotic peaks were seen.

To clarify the significance of these cytofluorometric patterns, we determined the frequency of apoptotic figures by fluorescence microscopy. The results are summarized in Fig. 3A (MMC) and Fig. 3B (SSP). The most rapid and complete

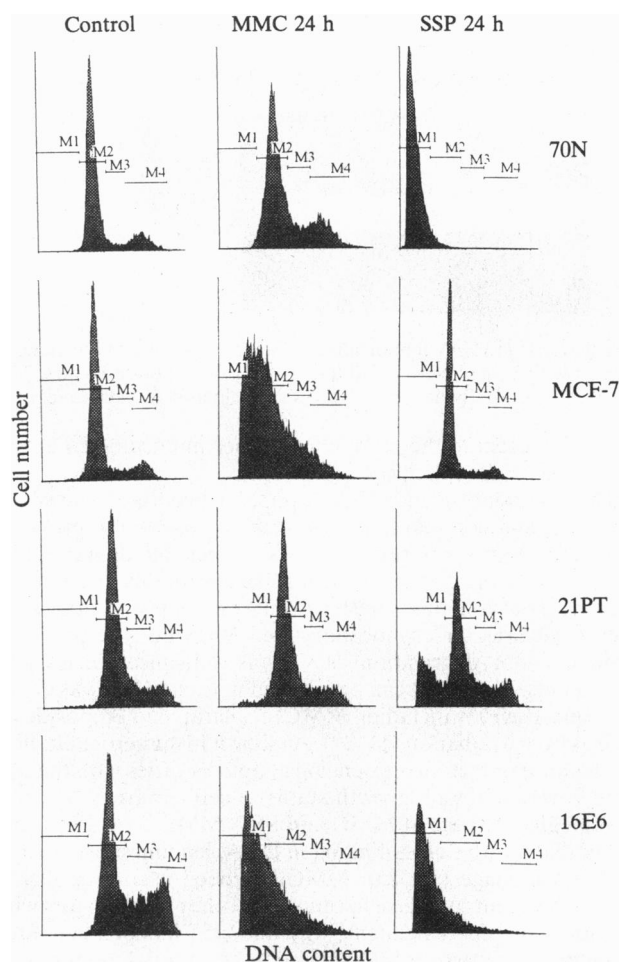


FIG. 2. Flow cytometric analysis of DNA content in SSP- and MMC-treated breast cells. Cells were plated in 60-mm dishes and treated with SSP and MMC. After treatment, supernatant was collected and the attached cells were treated with trypsin. Nonadherent and adherent cells were combined and the pellet was resuspended in PI solution (50 $\mu\text{g/ml}$) with 0.1% Nonidet P-40 and 0.1% sodium citrate. Fluorescence of cells stained with PI was measured by flow cytometry. Bars: M1, apoptotic cells; M2, G_0/G_1 -phase cells; M3, S-phase cells; M4, G_2/M -phase cells.

apoptosis induced by MMC was in the HPV cell lines, which contain no p53 protein (Fig. 4). 70N and MCF-7 cells also showed extensive apoptosis, although the time course was unusual for 70N cells as noted above; only after 32 h did the 70N cells undergo apoptosis. The mammary tumor lines MDA-231 and MDA-435 (both expressing mutant p53) showed intermediate values, whereas 21NT and 21PT, which totally lack p53 protein, showed no response to MMC.

SSP induced extensive apoptosis in 70N and in the HPV lines, but lower levels were seen in the tumor lines. The results are largely consistent with those in Fig. 2. The experiments reported in Fig. 3 were done 2–4 times with similar results. Overall, the maximum values reached were somewhat lower by microscopy than by flow cytometry.

Effect of MMC on the Expression of p53 and p21. To determine whether MMC treatment could override p53 degradation by HPV E6-activated ubiquitinylation, the expression of p53 protein was examined by immunoblotting after MMC treatment (Fig. 4). Normal 70N cells expressed abundant p53 protein (27), which was further upregulated by MMC within 4 h. However, p53 protein was not detectable in HPV immortalized cells with or without MMC treatment. In MCF-7 cells, p53 protein was undetectable until induced by MMC treatment. Despite the different resting levels of p53 in 70N and

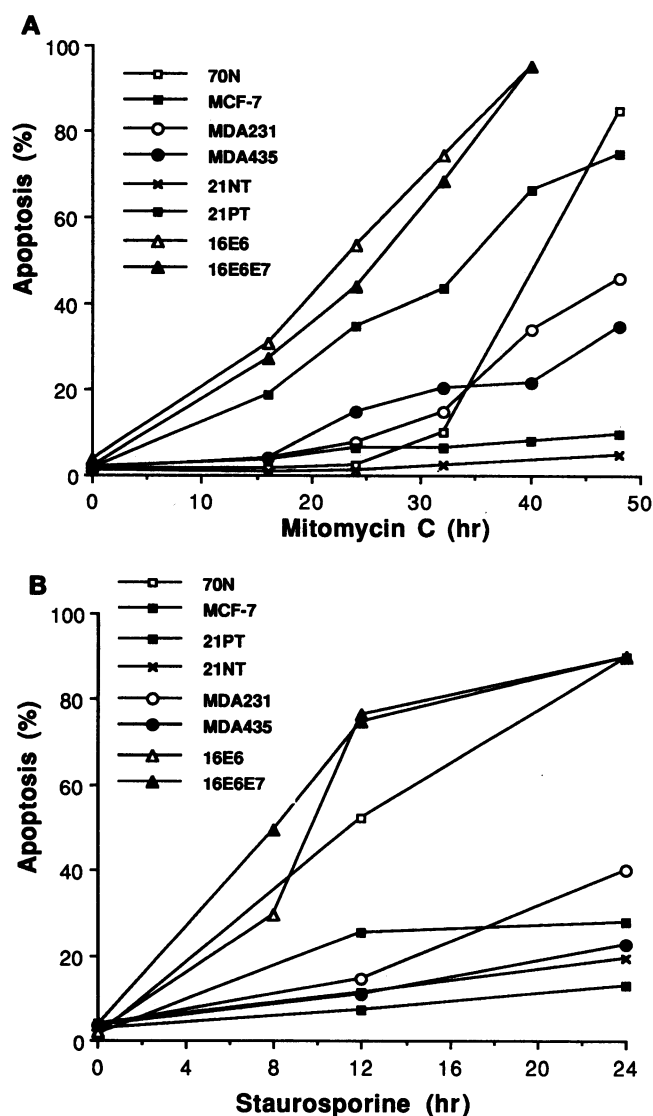


FIG. 3. Differential induction of apoptosis in HMECs, carcinoma cells, and HPV immortalized cells by MMC (A) and by SSP (B). Cells were plated in D medium at $3\text{--}5 \times 10^4$ cells per 35-mm dish. Monolayer cultures were treated with 30 μM MMC or 0.2 μM SSP at the indicated times. Cells were washed, fixed with 100% methanol, and stained with Hoechst 33258. Apoptotic nuclei were recognized by their condensed, sometimes fragmented chromatin by fluorescence microscopy. At least 400 cells were counted from each sample. Experiments were done 2–4 times with similar results.

MCF-7 cells, both underwent extensive apoptosis after MMC treatment (Fig. 3A). In fact, MCF-7 cells initiated apoptosis much more rapidly than 70N cells.

The same Western blot (Fig. 4) was stripped and retested with anti-p21 antibody. The protein was present at all time points in both 70N and MCF-7 cells but not at all in the HPV lines. Thus, the rapid and extensive induction of apoptosis by MMC in HPV cells utilizes a mechanism that is both p53 and p21 independent. Furthermore, the presence of p21 in the untreated MCF-7 cells prior to induction of p53 and the stable levels of p21 after p53 induction demonstrate that p21 is not under p53 control in these cells.

Since no p21 protein could be detected in the HPV cells, we wondered whether p21 mRNA had been induced. We found to our surprise that p21 mRNA was indeed induced by treatment of 16E6 cells (Fig. 5) and 16E6E7 cells (data not shown) with MMC. The high expression levels were similar for 70N and for the HPV lines. Thus, transcription of p21 is induced by MMC

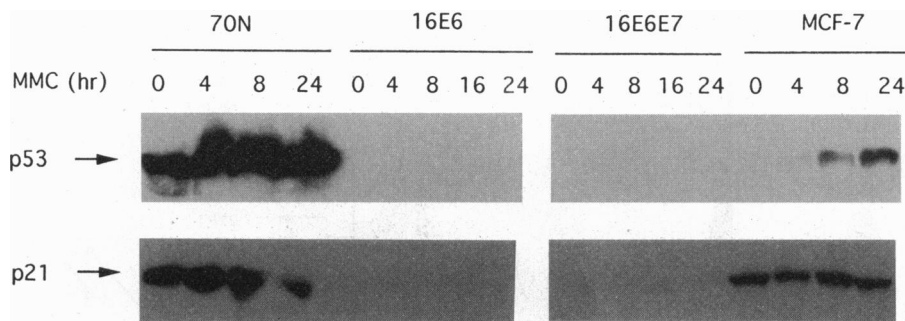


FIG. 4. Induction of p53 and p21 proteins in MMC-treated normal, carcinoma-derived, and HPV immortalized HMECs by Western blot analysis. The cells (70N, 16E6, 16E6E7, and MCF-7) were grown in 100-mm dishes, treated with 30 μ M MMC, and harvested at the indicated times. The same blot was probed with p53 (DO-1) and p21 (Ab-1) antibodies (Oncogene Science). The lysate from 10^6 cells was loaded in each lane.

in the absence of p53 but no protein is found in the HPV lines. These results indicate that p21 mRNA is induced by DNA damage in the absence of detectable p53 and, perhaps more importantly, that p21 protein levels can be controlled by posttranslational mechanisms. We need now to establish whether p21 is destroyed through the E6-mediated ubiquitination pathway.

DISCUSSION

This work was initiated to investigate the apoptotic responses of normal and tumor-derived HMECs, with the hope of identifying conditions or agents that might offer therapeutic opportunities in the treatment of breast cancer. We found that (i) apoptosis was induced both by MMC, a DNA-cross linking and damaging agent, and by SSP, a protein kinase inhibitor with no known effects on DNA; and (ii) that the extent of MMC-induced apoptosis correlated with p53 expression in the normal and tumor-derived cell types. Unexpectedly, however, we found that HMECs, immortalized by transfection with the E6 gene of HPV 16, were extremely sensitive to SSP- and to MMC-induced apoptosis despite the absence of p53 protein. Thus, the HPV lines and the 21T lines, both lacking p53 protein, behaved very differently in response to MMC and SSP, probably because of the presence of E6 in the HPV lines. The effect of MMC in inducing expression of p21 mRNA but

not p21 protein in the HPV lines further implicates E6 as the apoptosis-mediating agent.

How is apoptosis induced? A popular hypothesis envisions opposing and competing forces—e.g., “grow/do not grow”—inducing apoptosis in response to irreconcilable demands (4). Factors promoting growth, such as oncoproteins and growth factors, would in this “clash” hypothesis oppose antigrowth factors such as underphosphorylated Rb, wild-type p53, and growth factor deprivation as well as anticancer drugs and treatments. Apoptosis can be induced in tumors by a variety of anticancer agents including MMC, cisplatin, and etoposide as well as by γ -irradiation (11, 29). As shown in numerous studies, the balance of factors influencing apoptosis varies with the cell type *in vivo* and with growth status in cell culture.

Biological Effects of MMC and SSP. MMC is an alkylating agent that induces cross-linking in DNA, leading subsequently to DNA damage (24, 25). MMC has been used as a DNA-damaging agent to induce apoptosis by other investigators who report both p53-dependent (12) and p53-independent (10) induction. However, MMC can also be activated—for example, by xanthine oxidase or xanthine dehydrogenase—to produce reactive oxygen (24). Whether both modes of action are involved in inducing apoptosis is not known. SSP is a nonspecific serine/threonine protein kinase inhibitor that disrupts cell cycle progression (23) and triggers apoptosis, presumably by activating cyclin-dependent kinases involved in cell cycle regulation (29).

We found that the presence, absence, or mutated state of p53 in tumor-derived cells governed their apoptotic response to MMC much as described for other anticancer agents acting on other cell types. (e.g., see refs. 8–11). Normal cells (70N) and MCF-7 tumor cells, expressing wild-type p53, were susceptible to apoptosis induced by MMC, whereas cells lacking p53 (21NT and 21PT) or expressing mutant p53 (MDA-MB-231 and -435) were quite insensitive to MMC. The tumor cells were all relatively unresponsive to SSP, consistent with other reports that tumor cells are less responsive to apoptosis-inducing agents than normal cells (4). In contrast to these results, HPV-immortalized HMECs that express E6 were highly sensitive to apoptosis induced either by MMC or by SSP.

A Role for E6 in Apoptosis? Might E6 be triggering the destruction of p21 and of other proteins in addition to p53? Recent studies have shown that E6 acts through a cascade of E6-associated proteins, leading into the ubiquitin pathway. Ubiquitinylation in turn leads to the programmed destruction of a number of regulatory proteins, including cyclins (31, 32), through the proteasome mechanism (33).

It seems likely, therefore, that the sensitization of HPV lines to apoptosis might involve degradation of proteins that have a survival function in these cells. p21 protein is an obvious candidate, since it is transcriptionally activated by MMC in the HPV cells, but no p21 protein is present despite the high mRNA level. This result is very suggestive of rapid protein

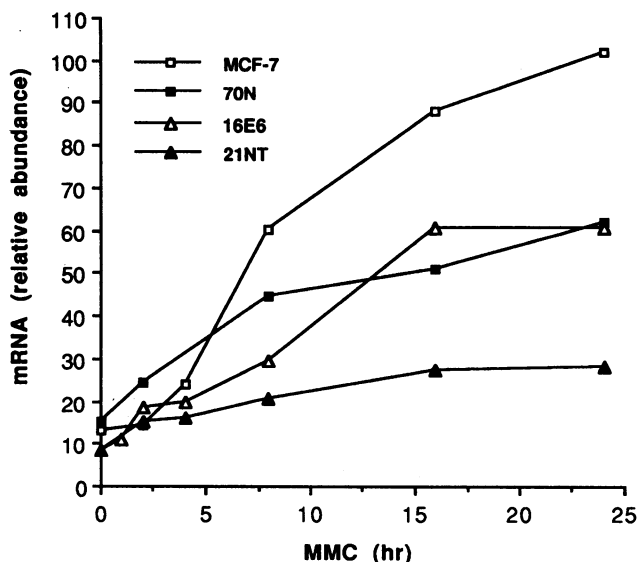


FIG. 5. Induction of p21 mRNA by exposure to MMC. Exponentially growing cells were treated with MMC and harvested at the indicated times. Relative densities of bands on x-ray film were quantitated and normalized with 36B4 loading control by image densitometer (Bio-Rad).

degradation triggered by E6 through the ubiquitin pathway. Since the HPV cells are similarly triggered for apoptosis by SSP and MMC, they may share some common E6-sensitive precursors.

p21 and Apoptosis. Several lines of evidence in addition to ours support the proposal that p21 may act as a survival factor in p53^{-/-} cells in which it is induced by a p53-independent pathway. (i) High levels of p21 mRNA are present in quiescent and senescent fibroblasts in which p53 levels are low and in cells lacking p53 (20). p21 might act to block apoptosis and maintain the integrity of this nondividing but metabolically active cell compartment. A similar discordance between p53 and p21 expression levels was seen in a series of breast carcinoma cell lines (34). In our cells (Fig. 4), p21 protein expression levels are not temporally related to p53 levels. (ii) In HL-60 cells, which lack p53, p21 mRNA (35) and protein (21) are elevated in response to the induction of differentiation in the absence of apoptosis. (iii) p21 mRNA can be induced by serum or serum growth factors in fibroblasts from p53^{-/-} mice. Whether or not apoptosis will be induced in each instance depends on other factors—for example, bcl-2 acting as a survival factor for p53⁻ lymphoblasts (10).

In summary, we have found that mammary epithelial cells immortalized with HPV 16E6 rapidly undergo apoptosis after treatment with the DNA-damaging agent MMC or with SSP. The presence of E6 may promote apoptosis through the ubiquitin-mediated destruction of p21 and perhaps other proteins involved in the balance between survival and apoptosis. If so, E6 or a cellular protein with similar activity may have therapeutic potential in the destruction of tumor cells by apoptosis.

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