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Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line

P. SHAW*†, R. BOVEY*, S. TARDY*, R. SAHLI‡, B. SORDAT§, AND J. COSTA*

*Institute of Pathology, Division of Experimental Oncology, Rue du Bugnon 25, 1011 Lausanne, †CHUV, Institute of Microbiology, Department of Virology, Rue du Bugnon 44, 1011 Lausanne, and §Swiss Institute of Experimental Cancer Research, Ch. des Boveresses, 1066 Epalinges, Switzerland

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ABSTRACT **A wild-type p53 gene under control of the metallothionein MT-1 promoter was stably transfected into human colon tumor-derived cell line EB. Repeated inductions of the metallothionein wild-type p53 gene with zinc chloride results in progressive detachment of wild-type p53 cells grown on culture dishes. Examination at both the light and electron microscopic level revealed that cells expressing wild-type p53 developed morphological features of apoptosis. DNA from both attached and detached cells was degraded into a ladder of nucleosomal-sized fragments. Expression of wild-type p53 inhibited colony formation in soft agar and tumor formation in nude mice. Furthermore, established tumors in nude mice underwent regression if wild-type p53 expression was subsequently induced. Regressing tumors showed histological features of apoptosis. Thus, regression of these tumors was the result of apoptosis occurring *in vivo*. Apoptosis may be a normal part of the terminal differentiation program of colonic epithelial cells. Our results suggest that wild-type p53 could play a critical role in this process.**

p53 was originally discovered as a consequence of its association with simian virus 40 (SV40) large tumor antigen (1, 2). Subsequently, it was shown that mutant p53 could immortalize primary fibroblasts (3, 4) and, in collaboration with mutant ras, could transform them (5, 6). p53 mutations have been found in virtually all types of human tumors examined (7, 8) including tumors of the lung (9, 10), breast (11), liver (12, 13), and colon (14, 15). Homozygosity is observed in most cases of p53 gene mutation, indicating that the wild-type (wt) allele is lost through deletion (16). The conclusion that wt p53 behaves as a recessive oncogene in tumors is supported by cotransfection experiments in which wt p53 suppresses transformation by mutant p53 with a mutant ras (17, 18).

wt p53 stops growth in most transformed cells into which it has been introduced (19, 20). Furthermore, overexpression of wt p53 causes cell cycle arrest near the G_1–S boundary (21–24). *In vitro* replication of SV40 DNA is inhibited by wt p53 (25, 26). Recently, Bargonetti *et al.* (27) have demonstrated that wt p53, but not mutant p53, binds to a region of SV40 DNA adjacent to the origin of replication (27). In addition, it has been reported that a wt p53–gal4 DNA binding domain fusion protein can promote transcription, suggesting that p53 can act as a positive transcription factor (28, 29). Thus, it is possible that p53 regulates a set of genes playing a role in the passage from G_1 to S (16).

We wished to investigate further the function of wt p53 by studying the effects of wt p53 reexpression in human colon tumor cell line EB (30). Induced expression of the transfected wt p53 gene resulted in apoptosis *in vitro*, prevented colony formation in soft agar, and inhibited tumor growth in nude mice. Established tumors in nude mice underwent regression upon induction of the wt p53 gene.

MATERIALS AND METHODS

Cell Culture. EB cells (30) and the stably transfected clones derived from them were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (GIBCO).

Metallothionein (MT) p53 Construction, Transfection, and Selection of Clones. MT p53 was constructed by blunt-end ligating a 212-nucleotide *Sac* I/*Bgl* II fragment of the rat MT-1 promoter (Δ156) (31) to the filled-in *Eco*RI site at the 5′ end of a human wt p53 cDNA clone (32). EB cells were cotransfected with MT p53 and SV-Neo (33) plasmid DNAs by the calcium phosphate procedure. Stable clones were selected for their resistance to G418 (800 μg/ml) and clones were isolated with cloning cylinders. They were then screened for the presence of the wt p53 gene at second passage by PCR (34) using the oligonucleotides IN4AL (5′-AGA GGA ATT CTT CCT CTT CCT GCA GTA C-3′) and IN7AL (5′-TTG AGG ATC CCA AGT GGC TCC TGA CCT G-3′). The amplified DNA fragments are 1100 base pairs (bp) for the endogenous p53 gene and 385 bp for the transfected cDNA construct. Conditions of the 30-cycle PCR were 94°C for 1 min, 63°C for 1.5 min, and 72°C for 2 min in standard PCR buffer conditions (34).

DNA, RNA, and Protein Extraction and Analysis. RNA was extracted by the guanidine isothiocyanate/acid phenol method (35). RNA (10 μg) was treated with glyoxal and separated on a 0.8% agarose gel (36). RNA in the gel was transferred to a GeneScreen membrane (NEN) and then hybridized to a p53 cDNA probe (32) labeled by the random primer method (37) in 50% formamide/5× SSC (1× SSC is 150 mM NaCl/15 mM sodium citrate)/50 mM sodium phosphate, pH 6.5/0.5% SDS/denatured salmon sperm DNA (100 μg/ml). DNA and protein were prepared by standard methods (38). DNA was separated on a Tris acetate-buffered gel containing 1.5% agarose. The DNA was then transferred to a GeneScreen membrane (NEN) and hybridized with total human DNA labeled by the random primer method as described above for Northern blot analysis. Western blot analysis was by standard procedures with the pAB240 antibody (39).

MT Promoter Induction. Induction of the wt p53 gene was achieved with either 6 μM cadmium chloride (for screening of stable clones; see Fig. 1*a*) or 100 μM zinc chloride (for all other experiments). Tissue culture medium was not changed during multiple inductions with zinc chloride. When cells were injected into nude mice, the area of injection was marked. For induction of wt p53 in nude mice tumors, zinc chloride [100 μM in phosphate-buffered saline (PBS); 0.4 ml] was injected subcutaneously in the region outside of the marked area, thus avoiding the tumor.

RESULTS

Characterization of MT wt p53 Clones. A wt p53 gene under the control of an inducible promoter was used to avoid

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Abbreviations: wt, wild type; MT, metallothionein; SV40, simian virus 40.

†To whom reprint requests should be addressed.

growth inhibition during establishment of stably transfected cell lines (19, 21–24). A wt p53 cDNA (32) was ligated to a truncated rat MT promoter MT-1 (Δ156), which has been shown to have very low basal activity and yet be strongly inducible with metal ions (31). This construct was cotransfected with SV-Neo (33), as a selectable marker into the EB cell line (30). G418-resistant clones were isolated and tested at second passage for the presence of the wt p53 DNA by PCR. Expression levels of the positive clones were analyzed by Northern blotting. Shown in Fig. 1*a* are the results obtained with clones EB-1, -2, and -5, both uninduced and induced with 6 μM cadmium chloride. All three clones have high levels of a 2-kilobase (kb) p53 mRNA, which is derived from the transfected p53 gene (Fig. 1*a*). This mRNA was undetectable in the uninduced cells, even on long exposures (data not shown). Note also the absence of the 2.4-kb p53 mRNA expected from the endogenous p53 gene (Fig. 1*a*). Southern blot analysis of the EB p53 gene did not reveal any gross alterations in the structure of the gene (data not shown).

Since the MT promoter has been characterized primarily in single induction experiments, we wished to determine whether sustained high levels of wt p53 mRNA could be obtained by daily stimulation of the MT-1 promoter with zinc chloride. This is of obvious importance if a minimal amount of the p53 protein must be maintained throughout the cell cycle to achieve cell cycle arrest (21–24). We examined the amount of p53 mRNA present 4, 8, 24, and 48 h after a single induction with zinc chloride (Fig. 1*b*). Maximal expression was elicited 4 h after induction (Fig. 1*b*) (40). p53 mRNA was still detectable 24 h after a single induction. Subsequent inductions of the introduced wt p53 gene yielded higher levels of wt p53 mRNA than the initial induction (Fig. 1*b*, compare lanes 1× +4h with 2× +4h). The significant increase in mRNA levels upon repeated induction may be the consequence of first induction recruitment of transcription factors that remain in place, resulting in the increased efficiency of subsequent inductions. These results demonstrate that sustained high levels of p53 mRNA could be achieved in our transfected clones by daily inductions with 100 μM zinc chloride.

Western blot analysis of uninduced and induced EB and EB-1 cells is shown in Fig. 2. p53 protein is readily detectable 4 h after a single induction with zinc chloride. Also of interest is that 24 h after a second induction with zinc chloride there is still a significant level of p53 protein. Thus, the mRNA detected by the Northern blot experiments described above is functional. Note the absence of p53 protein in uninduced EB-1 cells and in EB cells with and without zinc chloride induction.

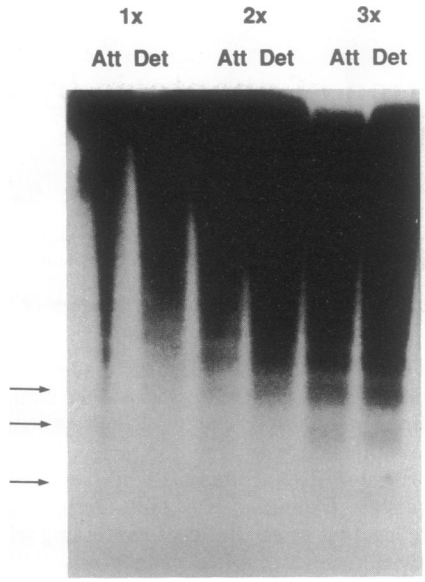

FIG. 2. Time course of DNA degradation in EB-1 after induction of wt p53 with zinc chloride. Attached (lanes Att) and detached (lanes Det) refer to cells that did or did not adhere to the culture dish at the time of cell harvest. 1×, 2×, and 3× refer to the number of inductions by zinc chloride. Cells were harvested 4 h after each induction with zinc chloride. Approximately equivalent amounts of DNA were loaded in each lane. Arrows indicate positions of nucleosomal multimers.

Induction of wt p53 Expression Is Accompanied by Apoptosis. While preparing RNA for the experiments described above, we noticed that the cells of the three wt p53 EB clones detached progressively from the plates over a 4-day period and eventually died. Approximately 10% of the cells detached on day 1, 30% detached on day 2, 50% detached on day 3, and 90% detached on day 4. The parental cell line continued to grow normally in the presence of zinc chloride with a doubling time of ≈45 h (P.S. and R.B., unpublished results). No gross differences in the percentage of cells detaching from the plates were observed for the three wt p53 clones. Cell death can occur by necrosis or apoptosis (41). There are several accepted criteria that can be used to distinguish between these two possibilities—namely, histo-

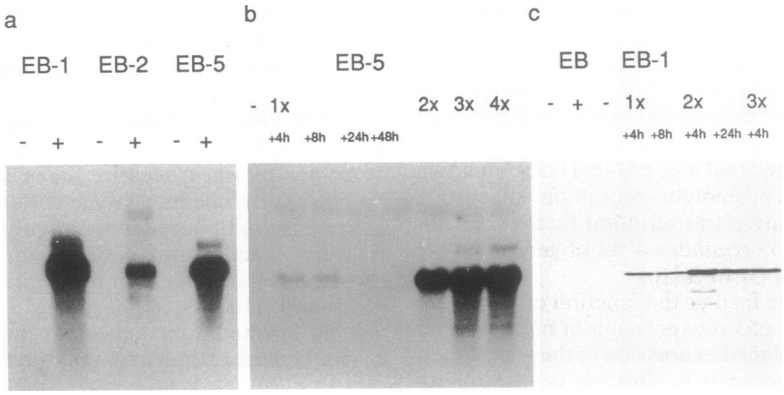

FIG. 1. Analysis of p53 mRNA and protein expression of MT wt p53 clones. (*a*) Ten micrograms of total RNA was analyzed for each clone by Northern blotting. RNA was prepared from cells either uninduced (lanes −) or induced (lanes +) with 6 μM $CdCl_2$ for wt p53 expression. (*b*) EB-5 cells were induced with 100 μM zinc chloride from one to four times. RNA was prepared at the time indicated after induction. 1× +4h indicates that the cells were induced once and that the RNA was prepared 4 h after induction. Subsequent inductions (2×, 3×, and 4×) were at 24-h intervals. (*c*) Western blot analysis of p53 in EB and EB-1. Protein extracts were prepared at the times indicated on the figure and were analyzed on 12% polyacrylamide gels. paB240 was used as the first antibody. Detection was with an alkaline phosphatase-conjugated second antibody.

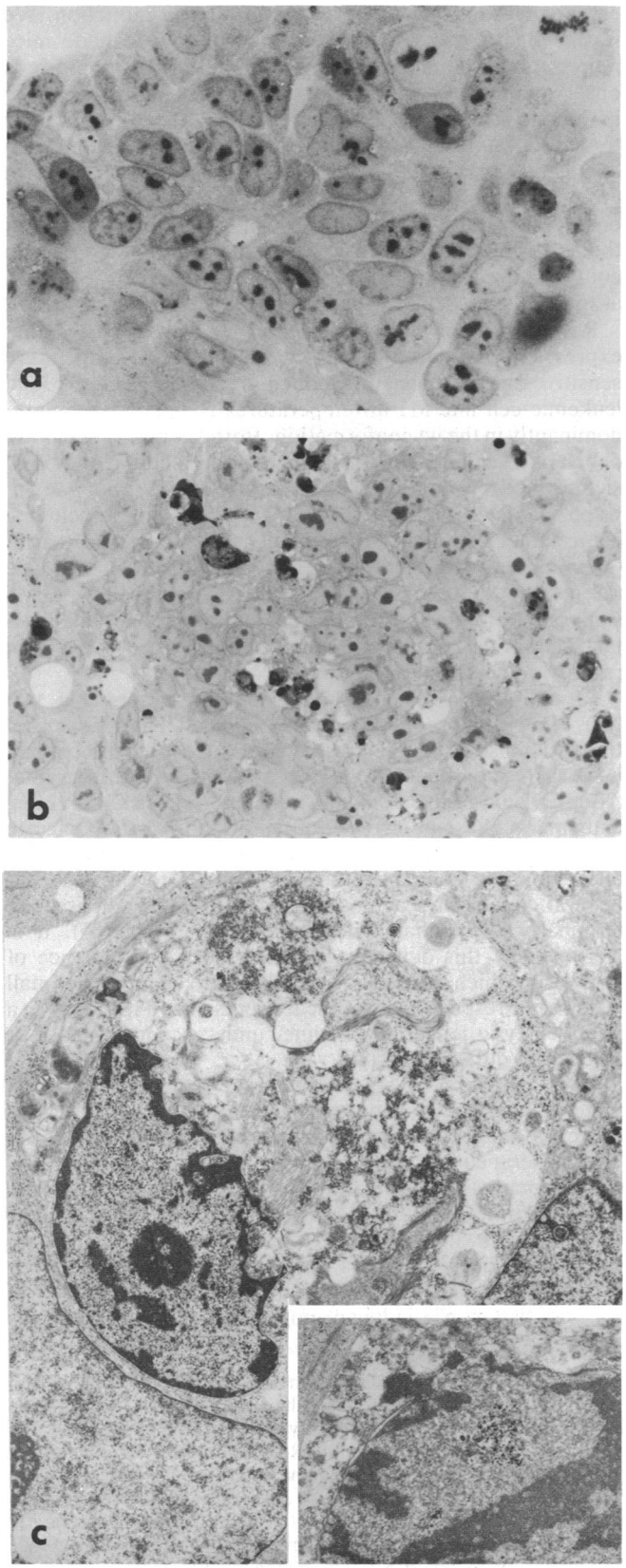

FIG. 3. Histology of induced and uninduced EB cell clones. (*a*) Histology of uninduced EB-1 cells grown *in vitro*. (*b* and *c*) Histology and electron micrographs of EB-1 cells after two inductions with 100 μM zinc chloride. (*Inset*) Leakage of chromatin from the nucleus is shown. (*a*, ×2400; *b*, ×2850; *c*, ×5900; *Inset*, ×8100.)

logical morphology and pattern of DNA degradation. We thus analyzed the state of the DNA in the parental cell line EB and in the transfected cell line EB-1 growing in the presence of zinc chloride. Parental EB cells contained only high molecular weight DNA (data not shown). On the other hand, DNA from cells expressing wt p53 underwent progressive degradation yielding a ladder of DNA fragments corresponding to multimers of nucleosomal-sized DNA (Fig. 2). This pattern of degradation is typical of apoptosis (41). It was striking that after only 4 h of metal ion stimulation, DNA degradation was already evident in the detached cell fraction. Thus, a small proportion of the cells underwent apoptosis very rapidly. After three consecutive inductions, the DNA was heavily degraded in both the attached and detached fractions. Morphological examination of EB-1 cells expressing wt p53 at the light and electron microscopic levels demonstrated typical features of cells undergoing apoptosis: condensation of chromatin in crescentic caps adjacent to the nuclear membrane, incomplete nuclear membranes, and translucent cytoplasmic vacuoles (Fig. 3 *a–c*).

Table 1. Suppression of tumor formation in nude mice

Cell line	Uninduced	Induced*
EB†	4/4	2/2
EB-1	4/4	0/2
EB-2	4/4	0/2
EB-5	4/4	0/2

Each nude mouse was injected with 5×10^6 cells.
*Zinc chloride (100 μM) was administered daily for ≈3 weeks starting 3 days after subcutaneous injection of cells into mice.
†Parental cell line.

Inhibition of Tumor Formation in Nude Mice by wt p53. Colony formation in soft agar and tumor formation in nude mice are criteria for tumorigenicity. All wt p53 clones, as well as the parental cell line, yielded colonies in soft agar at frequencies of 1–1.5%. Induction of the wt p53 gene completely abolished colony formation in all three cell lines—EB-1, -2, and -5 (data not shown). Colony formation in the parental cell line EB was unaffected by the presence of zinc chloride in soft agar.

Tumor growth in mice injected with the parental cell line proceeded normally, even in mice injected daily with zinc chloride. Tumors were obtained in nude mice injected with the three wt p53 clones only in the absence of zinc induction (4/4 in all cases; Table 1). When zinc chloride was administered daily, starting 3 days after injection of the cells, no tumors developed (0/2 mice for each clone). Thus, wt p53 expression prevented tumor formation in nude mice.

Regression of Established Tumors with wt p53 Expression. We further analyzed the effect of wt p53 induction on several established EB-1 tumors. Six weeks after injecting 5×10^6 cells, palpable tumors were present. We then induced wt p53 expression for a period of 3 weeks. This resulted in tumor regression, which was accompanied by morphological evidence of apoptosis (compare Fig. 4*a* and Fig. 4 *b* and *c*). The presence of scattered single necrotic cells is characteristic of apoptosis (41). The residual tumor mass comprised mainly neutrophils and scavenging macrophages (41). Induction of wt p53 for 2 months in established tumors resulted in total elimination of histologically detectable tumor cells at the site of cell injection (data not shown). Daily zinc chloride injections had no effect on established EB tumors (data not shown). Thus, expression of wt p53 in EB cells results in apoptosis both *in vitro* and *in vivo*.

DISCUSSION

The present experiments show that expression of wt p53 in the colon tumor-derived cell line EB induces apoptosis in cells grown *in vitro*, prevents colony formation in soft agar, prevents tumor growth in nude mice, and induces regression

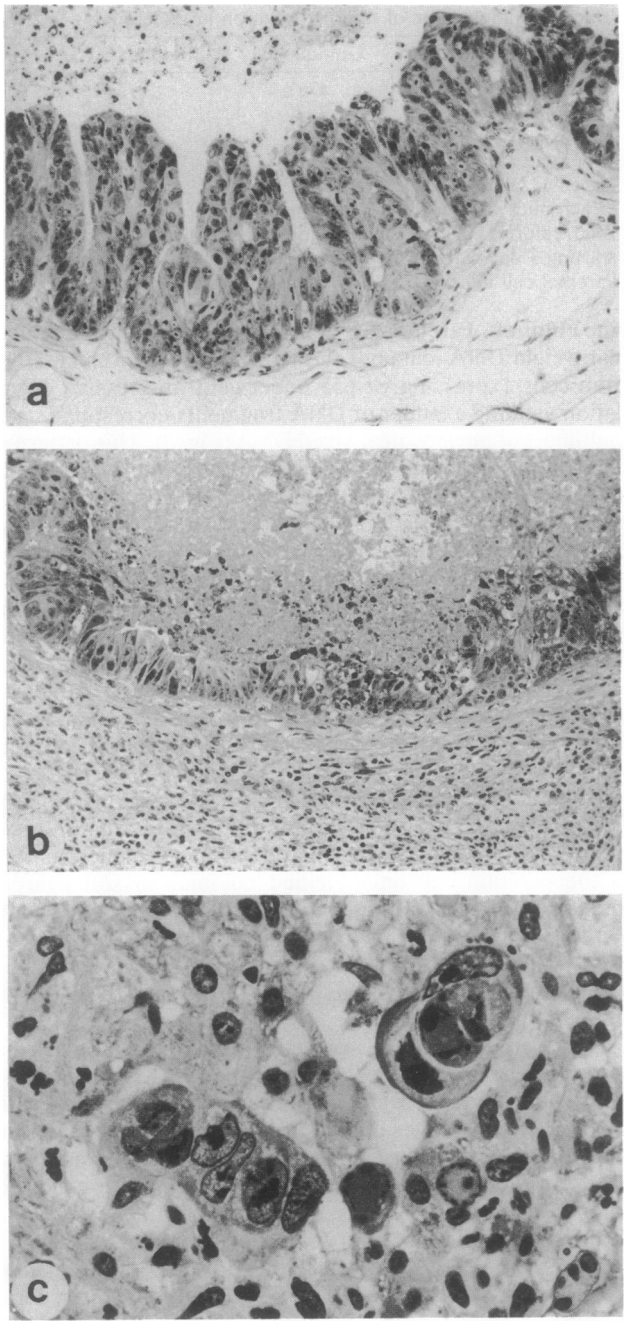

FIG. 4. Histology of regressing and nonregressing tumors from nude mice injected with EB-1 cells. (*a*) Control uninduced EB-1 tumors. (*b* and *c*) EB-1 tumors in which wt p53 was induced with zinc chloride. Tumors were allowed to form for ≈6 weeks. Induction of wt p53 by subcutaneous injections of 100 μM zinc chloride was initiated and continued daily for 3 weeks. Animals were sacrificed and dissected, and tumors were prepared for histological examination. Note the presence in *c* of several apoptotic cells. (*a* and *b*, ×750; *c*, ×2850.)

of established nude mice tumors *in vivo* by induction of apoptosis.

The generally accepted criteria for apoptosis—namely, nucleosomal DNA ladders and morphological features—are both observed when wt p53 expression is induced in EB cells. Zinc chloride, the metal inducer used in the present experiments, does not induce apoptosis in the parental cell line. In fact, the endonuclease responsible for the nucleosomal ladder observed in apoptosis is known to be inhibited by zinc chloride, although at considerably higher concentrations (42). Apoptosis of cells in culture was quite rapid, being essentially complete after 4 days of wt p53 induction. We further demonstrate that established tumors in nude mice regress upon induction of wt p53 expression. Apoptosis is observed in this situation as well. There is a large difference in the rate at which apoptosis proceeds in the tumor relative to cells in culture. Established tumors take ≈2 months to complete apoptosis. This may be due to a difference in growth rate of the cells in the tumor relative to cells in culture or to accessibility to the inducer zinc chloride. We feel that the first is more likely, since we have observed that cells in culture undergo apoptosis more rapidly when they are grown at low density (R.B. and P.S., unpublished observations).

Recently, Oren and co-workers (43) have reported that expression of a murine p53 mutant that is temperature sensitive for transformation induces apoptosis in the myeloid leukemic cell line M1 at temperatures at which p53 is predominantly in the wt conformation. Both EB and M1 cells are deficient in endogenous p53 expression. Whether this is of significance to induction of apoptosis by wt p53 is not known. wt p53 has been expressed in a variety of cell types, including colon tumor cell lines (19); yet only this report and that of Oren and co-workers describe induction of apoptosis. The rareness of the observation suggests that the cell type and state of differentiation before transformation may be important factors determining whether or not wt p53 expression results in apoptosis.

EB cells give rise to well-differentiated tumors (Fig. 4*a*) (44). They form pseudo-crypts and tight cell junctions, structures reminiscent of the normal colon. Cells of the colonic mucosa derive from stem cells located at the base of crypts. After division of the stem cells, daughter cells stop dividing and enter a phase of terminal differentiation as they migrate to the surface of the mucosa, such that there is a gradient of differentiation from the base of the crypt to the mucosa. Several days after reaching the surface of the mucosa, the terminally differentiated cells are sloughed off (45). It is possible that this detachment occurs as a consequence of apoptosis, which has been demonstrated to occur in the small intestine in response to chemical agents (46). HL-60 cells, a promyelocytic leukemic cell line, undergo apoptosis when induced to differentiate (47).

Apoptosis has been studied in a large variety of biological systems. Information concerning the relationship between cell cycle stage and apoptosis is, however, quite meager. Chemotherapeutic drugs, such as cisplatin and etoposide, induce apoptosis after arrest in G_2 (48). Mouse embryo cells, upon epidermal growth factor deprivation (49), and immature T cells treated with anti-T-cell receptor antibody (50) undergo apoptosis. The latter cases are associated with G_1 arrest. Although the examples are limited, it is provocative to consider that wt p53-induced apoptosis in our colon cell lines also occurs after an arrest in G_1.

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