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Advances in Brief

The Role of *p53* in Spontaneous and Radiation-induced Apoptosis in the Gastrointestinal Tract of Normal and p53-deficient Mice[1]

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Abstract

Three h after whole-body irradiation (8 Gy) of C57BL × DBA/2 F_1 mice, p53 protein was expressed strongly in the stem cell compartment of the small intestine but at lower levels in the colon. At this time, apoptotic cells were also observed in the stem cell position of the small intestine, with fewer in the colon. In mice without copies of the p53 gene (nulls), the levels of spontaneous apoptosis, in both the small intestine and the colon, were not different from wild-type. Irradiation of the nulls with 8 Gy of γ-rays failed to induce any further apoptosis: the loss of p53 essentially rendered the epithelial cells, from both the small intestine and the colon, radioresistant. The response of the epithelial stem cells of the small intestine suggests that p53 may play a role in the deletion of damaged cells with carcinogenic potential, whereas this process is limited in the colon.

Introduction

Despite the similarities in structure and proliferation of the small intestine and colon, small bowel cancers in humans are rare whereas those in colon are relatively common (1). The crypts of the murine and human small intestine and colon are highly polarized, are rapidly proliferating, and have a cell hierarchy which can be related to the position of individual cells along the crypt axis seen in longitudinal crypt sections (reviewed in Ref. 2). In the mouse it is evident that both tissues have only a few stem cells (2) located at the crypt base in the colon and at about cell position 4 from the base in the small intestine, immediately above or among the Paneth cells. Previous studies have established that damage-induced apoptosis had a greater incidence in the small intestine compared to the colon (3–7). Moreover, these dead cells occurred specifically at the stem cell position (about cell position 4) in the small intestine, whereas in the large bowel apoptosis was not associated specifically with the stem cell position but with cells higher up the crypt, *i.e.*, later in the hierarchy. This led to the conclusion that apoptosis in the small intestine is a protective mechanism, efficiently removing cells with damage from the tissue (2). For some reason, this mechanism is defective, attenuated, or inactive in stem cells of the large bowel, thus increasing the risk of damage perpetuation and hence carcinogenic risk. The protein product of the p53 gene has been suggested to act as "the guardian of the genome" (8). Evidence suggests that DNA damage is, in some way, "sensed," resulting in a posttranslational increase in p53 protein. p53 has been proposed to inhibit progression through G_1 into S phase via transactivation of WAF1/Cip1 which inhibits G1 cyclin-dependent kinases (9, 10). Presumably this delay allows time for DNA repair, a process somewhat similar to the idea that G_0 was important for genetic "housekeeping" purposes (11). In other circumstances, which may be related to cell phenotype or the level of DNA damage, p53 stabilization may be involved in the initiation of apoptosis (12–16). In this way a damaged cell can be either repaired or deleted from the tissue. Data presented here, using normal and p53-null mice, link p53 with this process in intestinal epithelial stem cells *in vivo*, and suggest an explanation for the greater incidence of cancers of the colon.

Materials and Methods

Measurements of Apoptosis. Male C57BL × DBA/2 F_1 (hereafter called $BD2F_1$) mice 10–12 weeks old or p53-null (17) heterozygous and wild-type mice, generated by the appropriate crosses and genotyped as described (18), were used. Groups of mice were irradiated (8 Gy) with a ^{137}Cs γ source (dose rate, 3.8 Gy/min) and sacrificed at various times later. Small intestine and colon were fixed in Carnoy's fluid and transverse 3-μm sections of the intestine were prepared and stained with hematoxylin and eosin (4–7). The distribution of apoptotic cells along the length of 50 half crypts from each of 4 mice was scored and the data were smoothed over 3 consecutive cell positions as described previously (6, 7).

Staining of p53 Protein. Animals were sacrificed, intestines were fixed in 4% formal saline, and routine 3-μm paraffin sections were prepared. Deparaffinized sections were heated to 95°C for 10 min in 0.01 M citrate buffer, pH 6, in a microwave oven. The sections were then immunostained using the ABC peroxidase method followed by a weak hematoxylin counterstain. The primary antibody used was CM5 raised in a rabbit to recombinant mouse p53 and purified essentially as described earlier for human p53 (19) by affinity chromatography on purified murine p53 coupled to agarose beads.

Results and Discussion

In order to test the idea that p53 may be involved in the damage recognition-apoptosis induction process in the intestine and to establish a molecular basis for the preferential carcinogenesis of the colon, sections from normal and irradiated wild-type animals were stained with an affinity-purified rabbit polyclonal αp53 antibody and analyzed for apoptosis, in parallel experiments. Fig. 1 shows that in the small intestine and colon from control unirradiated animals very few cells were found which expressed nuclear, wild-type p53. However, 3 h after irradiation (8 Gy) there was an increase in p53-positive nuclei in the small intestine which were often near the base of the crypt, specifically around cell position 4 or 5, *i.e.*, in the putative stem cell region (Fig. 1*B*). Immunoreactive cells were also seen at higher positions in the crypt and among the intercalated cells (the cells scattered among the Paneth cells near the crypt base). The intercalated cells may also be part of the stem cell compartment (2). In the crypts of irradiated colon there were p53-positive nuclei (Fig. 1*D*) but they were fewer in number, were more broadly distributed throughout the crypt,

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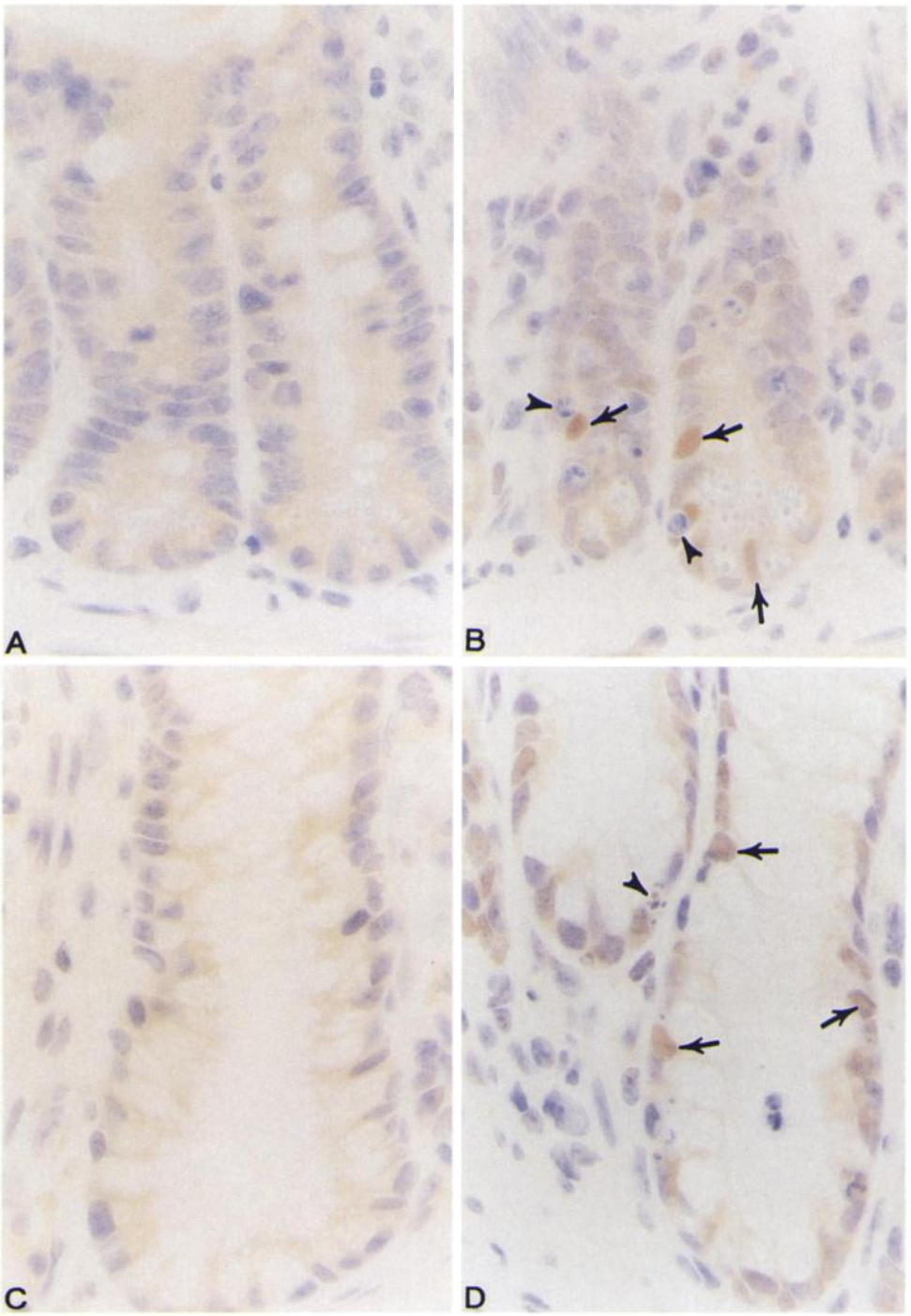

Fig. 1. p53 protein expression identified by the affinity-purified CM5 polyclonal antibody in murine small intestine (*A* and *B*) and colon (*C* and *D*). *B* and *D*, crypts 3 h after 8.0 Gy of γ-irradiation, *A* and *C*, unirradiated controls. *Arrows*, p53-positive cells near the base of the crypts in the small intestine and scattered throughout the crypt in the colon; *arrowheads*, apoptotic cells. × 900.

and did not show a particular preference for the putative stem cell region (*i.e.*, cell positions 1–2).

When, after irradiation, mice were analyzed for p53 expression at each cell position in the small intestine, peak levels of p53-positive nuclei were seen at positions 4–5, *i.e.*, the stem cell positions (Figure 2). Preliminary observations (data not presented) suggest that the levels of p53 expression decline progressively at later times after irradiation. When the spatial distribution of apoptosis was analyzed in the same mice, the frequency plot of apoptotic fragments almost exactly followed that of p53 staining (Fig. 2). The temporal and spatial correlation between apoptosis and p53 expression in the small intestine strongly suggests that they may be linked. In contrast, in the colon, apoptosis and p53 expression were not as closely related as in the small intestine, specifically in the lower regions of the crypt which contain putative stem cells.

Recently, homologous recombination in embryonic stem cells has allowed the production of mice constitutively lacking functional p53 (15–17). The concept of p53 as "guardian of the genome" (8) has been supported recently by reports which showed that thymocytes from p53-null mice failed to undergo apoptosis after irradiation *in vitro* (15, 16). To test further the hypothesis that p53 is involved in DNA damage recognition and apoptosis induction in intestinal epithelial lineages *in vivo*, we compared the levels of spontaneous and radiation-induced apoptosis *in vivo* in the crypts of the small intestine and colon of mice homozygously lacking (null) (−/−), heterozygous (−/+) or wild-type (+/+) p53 (17). Background levels of spontaneous apoptosis in the

crypts of p53-null mice were not significantly different from those of the heterozygotes, homozygote, wild-type, or conventional, wild-type, $BD2F_1$ animals (Fig. 3; Table 1) showing that p53 is not involved in spontaneous apoptosis. It is possible that the spontaneous apoptosis observed in intestinal epithelia is not the consequence of infidelity of normal DNA replication or due to the occasional damage induced by background radiation or other potentially cytotoxic damage which imposed p53-responsive DNA double strand breaks. We suggest that the incidence of spontaneous apoptosis in adults and apoptosis observed during development involves other mechanisms, possibly removing genomically intact cells which may be in excess to requirements. For example cells produced in a crypt by an occasional symmetrical stem cell division, in a system that normally divides asymmetrically (20), would need to be deleted to prevent hyperplasia.

At 4.5 h after animals had received 8 Gy of irradiation, high levels of apoptosis were present in the colon and small intestine of both heterozygous and wild-type mice but apoptotic events were not observed at levels above the background in the p53-null mice, in both tissues (Fig. 3, and Table 1). Neither were apoptotic cells seen at later times (6, 9, 12 h) (data not shown) in the irradiated p53-null mice, suggesting that the process is prevented and not delayed. The virtual absence in p53-null mice of radiation-induced apoptosis in the stem cell regions of intestinal crypts provides very strong evidence for a direct link between radiation-induced DNA damage, p53 expression, and apoptosis in stem cells of this surface epithelium and shows that the loss of p53 renders these cells radioresistant. This is the first time this relationship has been shown for epithelial cells *in vivo*.

With respect to the observation that small intestinal cancers are rare, the observations of a differential expression of p53 in the stem cells of the small and large bowel (Fig. 2) are consistent with the hypothesis that the small bowel stem cell pool is efficiently protected against genetic damage, initiating apoptosis to remove cells with these defects (2, 6, 7). This stem cell-specific process is attenuated in the large bowel (6, 7), providing a possible partial explanation for the differential cancer incidence in these two sites. In other experiments, we have observed that the protooncogene *bcl*-2, which prevents or delays

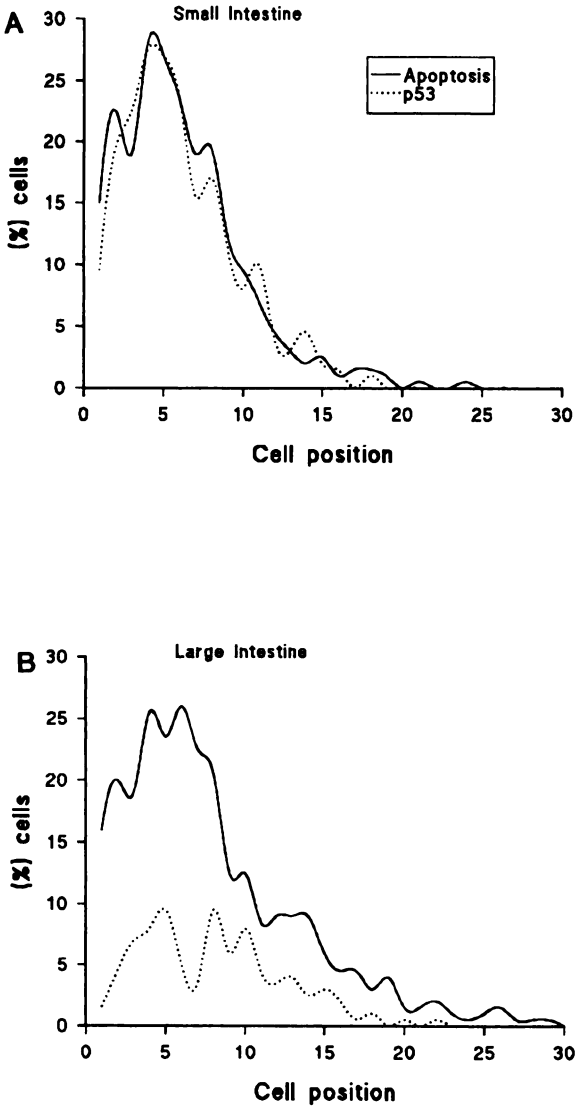

Fig. 2. Relationship between the distribution of apoptotic fragments (——) and p53-positive nuclei (.....) along the length of the crypt of the murine small or large intestine 3 h after 8.0 Gy ^{137}Cs γ-rays.

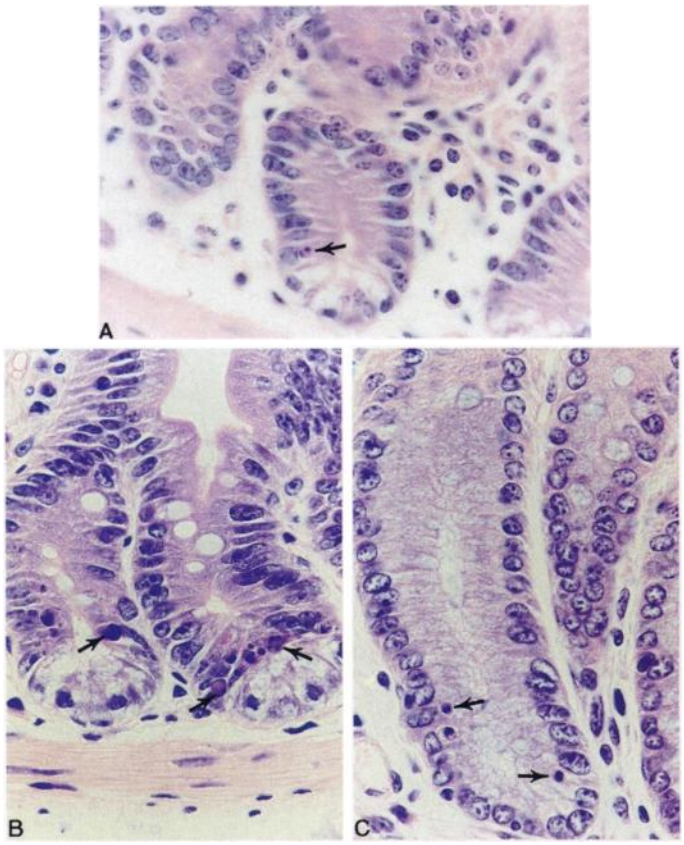

Fig. 3. Examples of (*A*) apoptotic figures (*arrows*), in sections from *A*, untreated p53-null mice (−/−), *i.e.*, spontaneous apoptosis and in irradiated (*B*, *C*, 4.5 h, 8 Gy) wild-type mice (+/+) (*B*, small intestine; *C*, colon). Staining of 3-μm paraffin sections. H & E, × 900.

Table 1 *Apoptotic frequency following irradiation of p53 null (−/−), heterozygous (+/−), homozygous (+/+), and wild-type $BD2F_1$ mice*

Apoptotic fragments were scored in the lower third of the crypts (cell positions 1–7, encompassing the stem cell region). Numbers are representative of a typical experiment. In heterozygotes and wild-type animals between 300 and 450 fragments were seen in the lower third of 200 half-crypt sections from 4 mice. In complete contrast, in the p53-null mice only 10 fragments were observed in the small intestine and 1 in the large intestine, numbers consistent with spontaneous levels of apoptosis.

Total apoptotic fragments at cell positions 1–7 in 200 half-crypt sections				
	Small intestine		Large intestine	
Allelotype	Control	4.5 h, 8 Gy	Control	4.5 h, 8 Gy
−/−	30	10	9	1
−/+	18	360	5	441
+/+	19	397	2	297
$BD2F_1$	28	308[a]	1	305[a]

[a] Observed at 3 h.

apoptosis (21), is expressed in the stem cell region of the colon but not in the small intestine.[5] Thus, determinants of an advantageous cell survival in colonic stem cells, including *bcl*-2 expression and an attenuated p53 response, may promote the critical first step of carcinogenesis, the survival of a cell with a damaged genome. Thereafter, mutated cells arising from this background may continue to be characterized by this survival advantage so as to be able to accumulate further genetic changes (22) and to be inherently resistant to chemotherapy and radiotherapy.

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