Inhibition of Apoptosis in Colon Tumors Induced in the Rat by 2-Amino-3-methylimidazo[4,5-f]quinoline

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Abstract

The cooked meat mutagen 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) produces tumors at multiple sites in the F344 rat, including adenocarcinomas of the colon. In the present study, the development of IQ-induced colorectal tumors was shown to be accompanied by the progressive inhibition of programmed cell death. This was associated with increased expression of the antiapoptosis protein Bcl-2 and decreased expression of bax, a known activator of apoptosis. Carcinomas bearing high levels of bcl-2 expression exhibited low levels of p53, the tumor suppressor protein that in some circumstances has been shown to downregulate bcl-2. Because they lack mutations in the genes commonly associated with increased cell proliferation (APC, Ki-ras, and p53) and show no evidence of microsatellite instability, IQ-induced colon tumors might arise via the deregulation of bcl-2 expression, leading to inhibition of programmed cell death.

Introduction

Colorectal cancer is believed to arise via the accumulation of genetic alterations that activate oncogenes and inactivate tumor suppressor genes (1). However, no such mutations have been detected in the colon tumors induced in experimental animals by heterocyclic amine mutagens from cooked meat. Heterocyclic amines were first identified as potent mutagens in vitro (2). One of the best-studied heterocyclic amines is IQ,3 a compound that produces tumors at multiple sites in the F344 rat, including adenocarcinomas of the colon (3). Molecular analysis has provided evidence that colon tumors induced by IQ lack mutations in genes commonly associated with the control of cell proliferation (e.g., Ki-ras, APC, and p53) and that these tumors show no evidence of microsatellite instability (4–7). Rather than increased cell proliferation or deregulation of DNA repair, the development of IQ-induced colon tumors may occur via a mechanism involving the inhibition of programmed cell death (apoptosis). To test this hypothesis, the colon tumors obtained from a 1-year carcinogenicity bioassy with IQ were examined by TUNEL. Subsequent experiments compared the relative expressions of three apoptosis-related genes in the IQ-induced colon tumors, namely, bcl-2, bax, and p53.

Materials and Methods

Animals and Treatment. Forty male F344 rats were treated with IQ by oral gavage for a total period of 35 weeks, as described previously (8). The average exposure during the time of carcinogen treatment was 4.2 mg IQ/rat per day. The tumors obtained after 1 year were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and examined after staining with H&E. Nine animals had one or more colorectal tumors, and these were examined by TUNEL and immunohistochemical methods as described below.

TUNEL. Apoptotic cells were visualized in situ using a fluorescein ApopTag kit (Oncor). Tissue sections were deparaffinized with xylene, rehydrated with graded alcohols, and incubated with 20 μg/ml proteinase K (Sigma) for 15 min at 37°C. After proteinase treatment, tissue sections were rinsed in PBS (pH 7.2) and incubated for 5 min with 1 × equilibrium buffer (Oncor). After equilibration, the sections were incubated in a humidified chamber with terminal deoxynucleotidyltransferase enzyme for 1 h at 37°C. Sections were soaked in stop-wash buffer (Oncor) for 30 min and then rinsed in three changes of PBS. After rinsing, sections were covered with antidigoxigenin fluorescein (Oncor) and incubated at room temperature in a humidified chamber for 30 min. Sections were rinsed with PBS, counterstained with propidium iodide/antifade (Oncor), and visualized under an Olympus IX70 fluorescence microscope.

Immunohistochemistry. Bcl-2 was stained using an LSAB streptavidin-biotin immunoperoxidase kit (DAKO). Formalin-fixed, paraffin-embedded tissues were deparaffinized and rehydrated as described above, and endogenous peroxidases were blocked by incubating the sections in 3% hydrogen peroxide for 10 min. Sections were rinsed with water and boiled for 14 min in antigen retrieval solution (DAKO). After cooling to room temperature and rinsing in PBS, tissues were blocked with nonspecific swine serum (DAKO) for 10 min. Polyclonal rabbit anti-rat/mouse Bcl-2 antibody (PharMingen) diluted 1000 × in PBS was added to the tissue sections and incubated for 30 min at room temperature in a humidified chamber. The sections were rinsed in PBS and incubated for 10 min at room temperature with linking solution (DAKO), then rinsed in PBS and incubated for 10 min with streptavidin solution (DAKO). After rinsing in PBS, the sections were stained for 30 min with 3,3′-diaminobenzidine chromagen (DAKO), rinsed in water, and counterstained with hematoxylin (Gill No. 3; Sigma). Bax was stained in situ using a procedure that was essentially identical to that used for Bcl-2, except that Bax (N-20) affinity-purified rabbit polyclonal antibody (Santa Cruz Biotechnology) was diluted 500 × in PBS. Similarly, p53 was immunohistochemically localized using the procedure described above, except that purified mouse anti-p53 monoclonal antibody (PharMingen) was diluted 10-fold and incubated overnight at 37°C.

Results and Discussion

In situ TUNEL of colonic mucosa from control rats revealed labeling in the extreme upper region of each crypt (Fig. 1A), whereas the IQ-induced adenomas and carcinomas exhibited a progressive loss of apoptotic cells with stage of tumorigenesis (Fig. 1, B and C). These findings are analogous to the results obtained in endoscopic human mucosal biopsy sections (9), which exhibit a gradual loss of apoptotic cells in the progression from normal tissue through early stages of adenoma to carcinoma.

Bcl-2 expression in normal rat colon was localized immunohistochemically to the proliferative zone at the base and lower half of the...
Fig. 1. Inhibition of apoptosis in IQ-induced colon tumors in the rat and associated changes in the expression of bcl-2. A-C, fragmentation of DNA in cells undergoing apoptosis was identified in situ using TUNEL. A, section of normal colonic mucosa from a control rat (original magnification, ×10). B, sparse distribution of apoptotic nuclei in an IQ-induced adenoma (×10). C, almost complete loss of apoptotic nuclei in a late-stage carcinoma (×10). The viable cells in each section display PI⁻ and FITC⁺ nuclei (red), whereas the cells undergoing apoptosis have FITC⁺ nuclei (yellow fluorescence). D-G, altered expression of Bcl-2 protein as determined by immunohistochemical staining. D, bcl-2 expression in normal colonic mucosa from a control rat...
INHIBITION OF APOPTOSIS IN IQ-INDUCED RAT COLON TUMORS

Fig. 2. Changes in (A-C) bax and (D-F) p53 expression in IQ-induced colon tumors in the rat. A. Immunohistochemical localization of bax protein (brown) to the epithelial cells present in the upper third of each crypt (control rat colon, ×10). B. Expression of bax in an IQ-induced adenoma, with low level immunoreactivity dispersed throughout the epithelium (×20). C. Loss of bax expression in an IQ-induced carcinoma shown to express high levels of bcl-2. Immunohistochemical localization of p53 expression in normal rat colon mucosa (D), an IQ-induced adenoma (E), and an IQ-induced adenocarcinoma (F) shown to express high levels of bcl-2.

The Bcl-2 protein most commonly seen was identified by a granular appearance throughout the cytosol and staining of the nuclear membrane. Compared with adjacent normal-looking tissue, the colorectal tumors induced by IQ exhibited a marked increase in the intensity of staining for Bcl-2 (Fig. 1, E-G). In the carcinomas, bcl-2 expression was detected throughout the malignant epithelium, with no obvious topographic distribution (Fig. 1G). Similar increases in bcl-2 expression have been reported in human tissues (9–11), although one recent study suggests that an alternative inhibitor of apoptosis, the protein Bcl-XL, may be elevated in less-differentiated cancers (12).

Increased bcl-2 or Bcl-XL expression could conceivably occur in conjunction with changes in the expression of other members of the bcl-2 family, including those that counteract the antiapoptotic effects of Bcl-2. One candidate in this regard is Bax, a dominant repressor of Bcl-2 that has been shown to form Bcl-2/Bax heterodimers (13). In accordance with previous work using mice (14), we found bax expression in control rat colonic mucosa to be localized to the region

4309
undergoing apoptosis, namely, the upper third of each crypt (Fig. 2A). Bax protein was sporadic in the IQ-induced adenomas, interspersed at low levels throughout the entire tumor with no discernible topographic localization (Fig. 2B). Most striking, however, was the finding that carcinomas with high levels of Bcl-2 expressed almost complete loss of bax expression (Fig. 2C). These results support a reciprocal relationship between bcl-2 and bax expression during the development of IQ-induced colon tumors.

Reciprocal changes in the levels of Bcl-2 and Bax proteins have been linked to the tumor suppressor p53 in several studies (14–18): (a) p53-deficient mice display increased Bcl-2 protein levels and decreased bax gene expression; (b) a temperature-sensitive p53 caused decreases in Bcl-2 and concomitant increases in bax expression that were temperature-dependent; and (c) wild-type p53 can down-regulate Bcl-2 levels via a negative response element on the bcl-2 gene. Based on these observations, we studied whether changes in p53 expression might account for the increased Bcl-2 and decreased Bax levels in IQ-induced colon tumors. p53 was detected in both normal tissue and IQ-induced adenomas (Fig. 2, D and E), but not in carcinomas bearing high levels of bcl-2 expression (Fig. 2F). Because the increases in bcl-2 expression preceded any changes in p53, the latter would seem to be an unlikely upstream regulator of bcl-2 in IQ-induced colon tumors.

The colon tumors induced by IQ lack mutations in such genes as p53, APC, and Ki-ras, but we have detected Ki-ras mutations in colon aberrant crypt foci induced by IQ in the rat (19). This suggests that, in some cases, early ras mutations can be lost in the progression to carcinoma. What might be the mechanism for this loss of genetic alterations? One hypothesis discussed recently (20) is that under some circumstances, cells bearing ras mutations might fail to proliferate because the surrounding region is too healthy to permit invasion. A second hypothesis (19) proposes that treatment with certain carcinogens might cause an overall increase in the rate of cell proliferation throughout the tissue, thereby removing the selective advantage for clonal expansion of cells bearing mutant p21\(^{\text{ras}}\). However, rats treated chronically with IQ or a related heterocyclic amine colon carcinogen, PhIP, exhibit only marginal increases in the basal proliferation rate of the colonic mucosa\(^4\) (21). Furthermore, IQ-induced colon tumors completely lack the microsatellite instability that has been detected in the colon tumors of rats given PhIP (7).

Rather than increased cell proliferation or deregulation of DNA repair, the present study suggests that IQ-induced colon tumors might arise via the deregulation of bcl-2, leading to inhibition of apoptosis. To our knowledge, this is the first study to examine the inhibition of apoptosis in an animal model given a dietary colon carcinogen. This investigation compared normal tissue to IQ-induced colon tumors, but the results do not formally distinguish between IQ-specific and colon-specific events. Therefore, it will be important for future studies to examine the specificity of the inhibition of apoptosis using various heterocyclic amines, as well as other classes of colon carcinogen. For example, the question of specificity might be addressed by comparing the activities of IQ and PhIP with other heterocyclic amine carcinogens that do not produce tumors of the colon, such as 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline.

\(^{4}\) R. H. Dashwood, unpublished observations.

**References**


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