INTRODUCTION

Colorectal adenocarcinoma represents the fourth most common type of cancer and the second most frequent cause of cancer-associated death in the United States (1). Tumor kinetics studies have suggested that in vivo expansion of neoplastic colonic epithelial cells results not only from increased rates of cell proliferation but also from decreased rates of cell death due to apoptosis (2-4). Furthermore, decreased proportions of apoptotic cells have been documented during progression of these neoplasms from benign adenomatous polyps to invasive cancers (5).

The Bcl-2 family of proteins regulates a distal step in an evolutionarily conserved pathway for programmed cell death and apoptosis (reviewed in Refs. 6-9). Some of the members of this gene family are blockers of cell death, including Bcl-2, Bcl-XL, and Mcl-1; whereas others are promoters of apoptosis, such as Bax, Bak, and Bcl-Xs. Furthermore, in many cases, these proteins can physically interact with each other in a network of homo- and heterodimers in which the relative proportions of the anti-apoptotic and pro-apoptotic members of this family determine the ultimate sensitivity of cells to cell death stimuli (9-19). Of potential relevance to mechanisms of carcinogenesis and resistance to therapy, overexpression of Bcl-2 has been shown to provide protection against a wide variety of apoptotic insults, including growth factor deprivation, loss of cell attachment to extracellular matrix proteins, oncoproteins (c-myc), tumor suppressor genes (p53), cytolytic T cells, radiation, and essentially all currently available chemotherapeutic drugs (reviewed in Refs. 6, 20, and 21).

In this report, we characterized for the first time the expression of several Bcl-2 family proteins (including Bcl-2, Bcl-X, Mcl-1, Bax, and Bak) in a group of 30 colorectal adenocarcinomas and 24 adenomatous polyps.

MATERIALS AND METHODS

All patient materials represented randomly selected cases of colorectal carcinoma or adenoma derived from the St. Luke’s-Roosevelt Center Hospital Department of Surgical Pathology or from snap-frozen colonic tissue obtained at surgical resection. Formalin- or Bouin’s-fixed, paraffin-embedded tumors were stained with H&E and classified histologically by expert pathologists as carcinoma or adenoma derived from the St. Luke’s-Roosevelt Center Hospital. Sections from these cases were immunostained for Bcl-2 family proteins exactly as described previously, using a microwave accentuation technique; antipeptide rabbit antisera specific for Bcl-2, Bcl-X, Mcl-1, Bax, or Bak; and a dianinobenzidine colorimetric detection method (22-26). All of these antipeptide antisera have been described previously in detail (22-26), with the exception of the anti-Bak antibody that was generated in rabbits by using a synthetic peptide corresponding to amino acids 14-36 of the human Bcl protein (NH2-C-E-P-A-L-P-S-A-S-E-E-Q-V-A-Q-D-T-E-E-V-F-R-amide) conjugated to maleimide-activated keyhole limpet hemocyanin and ovalbumin carrier proteins (Pierce, Inc.). The immunostaining specificity was confirmed for all antibodies by preadsorption of antisera with specific peptides that completely abolished immunoreactivity with colorectal tissues and by the failure of irrelevant peptides to block immunostaining (data not shown). In all specimens, residual nonneoplastic epithelial cells, lymphocytes, or peripheral nerves were present that contained specific immunostaining and thus served as...
an internal control and verified adequate fixation and preservation of the relevant epitopes.

Based on comparisons with adjacent normal epithelium to determine relative immunointensity, immunostaining results were scored as <, =, or > normal (−1, 0, +1, respectively). Because the intensity of most Bcl-2 family proteins varies along the crypt-villus axis in normal colonic epithelium, the region of the villi with the strongest immunointensity was used for all comparisons. The approximate percentage of immunopositive epithelial cells was also determined from a minimum of two representative high-power (~×400) fields. Statistical comparisons were performed by unpaired t test, χ² analysis (2 × 2 matrix), or Pearson χ² test (2 × 3 matrix) using a Monte Carlo R × C contingency table test for validation of the P values, using the JMP software package (SAS Institute, Inc.).

In addition to paraffin-embedded tissues, detergent lysates were obtained for five frozen cancers and one adenoma, along with their respective normal resection margins. These lysates were normalized for total protein content (100 μg/lane) and subjected to SDS-PAGE/immunoblot assay as described previously (22–26).

RESULTS

Adenocarcinomas. The intensity of Bcl-2, Bcl-X, Mcl-1, Bak, and Bak immunostaining in neoplastic epithelium was scored relative to that in adjacent normal colonic epithelium in 19 of 24 adenomatous polyps and in all 30 adenocarcinomas, all of which contained residual nonneoplastic epithelium for direct comparisons. Although the results presented represent the average intensity, the immunointensity among immunopositive tumor cells was generally homogeneous within each particular case; thus, tumor heterogeneity was not a significant concern in terms of scoring for intensity. Fig. 1 summarizes the results. Compared to that of normal epithelium, the intensity of Bcl-2 was significantly reduced in 25 of 30 (83%) carcinomas (P = 0.0001). In contrast to Bcl-2 and the other Bcl-2 family proteins studied here, Mcl-1 immunoreactivity in normal and malignant colonic tissue was generally weak. Nevertheless, Mcl-1 immunointensity was clearly reduced compared to normal adjacent mucosal epithelium in 20 of 30 (67%) primary cancers (P = 0.0001), similar to that of Bcl-2. In contrast, Bcl-X immunointensity was increased relative to adjacent normal epithelium in 18 of 30 (60%) carcinomas (P = 0.0001). Although decreased expression of the pro-apoptotic protein Bak might have been expected, Bak immunointensity relative to that of normal epithelium was more often increased (13 of 30; 43%) than decreased (7 of 30; 23%). In contrast, the immunointensity of the pro-apoptotic protein Bak was clearly reduced compared to that of normal mucosal epithelial cells in 27 of 30 (90%) colorectal adenocarcinomas (P = 0.0001). Fig. 2 shows representative examples of these immunostaining results for Bcl-2, Bcl-X, Mcl-1, Bak, and Bak in colorectal adenocarcinomas, contrasting tumor cells with normal colonic mucosa.

Adenomas. The intensity of immunostaining for Bcl-2, Bcl-X, Mcl-1, Bak, and Bak was also compared with that of normal epithelium for the 24 adenomas analyzed here, providing insights as to how early in the tumor progression process alterations in the levels of these apoptosis-regulating proteins may occur. Bcl-2 immunointensity was diminished relative to that of normal epithelium in 9 of 24 (38%) benign tumors (Fig. 1) and was increased compared to that of normal mucosa in only 1 of 24 adenomas. Conversely, Bcl-X immunointensity was increased relative to that of normal epithelium in 12 of 24 (50%) cases and was not decreased relative to that of normal epithelium in any of the 24 adenomas. Mcl-1 immunointensity was also higher in some adenomas (7 of 24; 29%) than in normal tissue. Immunostaining for the pro-apoptotic protein Bak was decreased compared to that for normal tissue in only 3 of 24 (13%) adenomas. Surprisingly, Bak immunointensity was actually higher than that of adjacent normal epithelium in 15 of 24 (63%) benign tumors. In contrast, Bak immunointensity was markedly reduced in 22 of 24 (92%) adenomas. Thus, reductions in Bcl-2 and Bak as well as elevations in Bcl-X and Mcl-1 can occur as relatively early events in colorectal tumor progression.

Comparisons of Cancers and Adenomas. Comparisons of the immunointensity data for adenomas and carcinomas revealed a statistically significant decline in Bcl-2 immunointensity in association with tumor progression (P = 0.0002; Fig. 1), suggesting that although some adenomas may contain reduced Bcl-2 levels, decreases in Bcl-2 immunointensity are significantly more frequent in carcinomas. Similarly, significant differences were noted when comparing Mcl-1 immunointensity data for adenomas and carcinomas (P = 0.0001), reflecting the observations that Mcl-1 immunointensity was sometimes higher in adenomas than in normal epithelium but was frequently lower in carcinomas than in normal colonic mucosa. No significant differences were noted for immunointensity of Bax, Bcl-X, or Bak when adenomas were compared with carcinomas.

Histological Subtypes of Adenomas and Carcinomas. In an effort to pinpoint more precisely the stages in tumor progression where changes in the levels of immunointensity for Bcl-2 family proteins occur, the immunostaining results were also compared among different histological subcategories of neoplasms (e.g., adenomas with versus without severe dysplasia; well-differentiated carcinomas versus moderately/poorly differentiated carcinomas; Table 1). Significant decreases in Bcl-2 immunointensity were noted for 7 of 14 (50%) adenomas with severe dysplasia (P = 0.0002) but not for 2 of 8 (25%) less histologically advanced adenomas without severe dysplasia (P = 0.16). Conversely, the intensity of Bcl-X immunostaining was significantly higher than that of normal mucosa in 9 of 14 (64%) adenomas with severe dysplasia (P = 0.0003) compared to only 3 of 8 (38%) adenomas without severe dysplasia (P = 0.06). Thus, decreases in Bcl-2 immunostaining and increases in Bcl-X immunostaining tended to be associated with histological progression of adenomas, at least among the specimens evaluated in this study. The transition from adenoma without severe dysplasia to adenoma with severe dysplasia was not associated with statistical differences in the intensity of Mcl-1 or Bak immunostaining (Table 1). However, comparisons of Bax immunointensity in adenomas without severe dysplasia and those with severe dysplasia indicated significantly more cases...

_Bcl-2 FAMILY GENE EXPRESSION IN COLORECTAL CANCER_
Fig. 2. Representative photomicrographs of immunostaining results. Tissue sections were immunostained for Bcl-2 (A and B), Bcl-X (C and D), McI-1 (E and F), Bax (G and H), and Bak (I and J) using specific antipeptide antisera. Antibodies were detected by a 3,3'-diaminobenzidine colorimetric method (brown), and nuclei were counterstained with hematoxylin (blue). In the top row (A, C, E, G, and I; ×100), normal epithelial crypts are uppermost with carcinoma below. The bottom row (B, D, F, H; ×400) shows higher power views of carcinoma, or carcinoma with normal colonic crypts containing immunopositive cells (J, ×400). For Bcl-2, McI-1, and Bak, note the reduced intensity of immunostaining compared to normal adjacent epithelium. Conversely, immunointensity is elevated for Bcl-X compared to that of normal tissue. K, the results obtained when the anti-Bak antiserum was preadsorbed with Bak peptide before performing the immunostaining procedure, thus confirming the specificity of the Bak immunoreactivity seen in I and J.
of elevated Bax immunointensity among the more histologically advanced adenomas ($P = 0.008$; Pearson $\chi^2$, $2 \times 3$ matrix).

Comparisons of well-differentiated carcinomas with moderately/poorly differentiated tumors were hampered by the small number of less histologically advanced tumors available for this study. Nevertheless, the data suggest that elevations in Bcl-X and diminutions in Bak immunointensity were more frequent in moderately/poorly differentiated tumors than in well-differentiated carcinomas. For Mcl-1, the differences in immunointensity between well-differentiated and moderately/poorly differentiated tumors reached statistical significance ($P = 0.02$). In contrast, Bak immunointensity was decreased regardless of differentiated phenotype, whereas Bax expression in carcinomas, regardless of their degree of histological differentiation, was not reproducibly different from that in normal epithelial cells (Table 1).

### Scoring for Percentage Immunopositivity

In addition to immunointensity, the percentage of immunopositive tumor cells was estimated for all specimens. As shown in Fig. 3, the percentages of Bcl-2 immunopositive cells in carcinomas were significantly lower than in adenomas (mean $\pm$ SE, 44 $\pm$ 6% versus 73 $\pm$ 5% respectively; $P = 0.001$). Moreover, the percentage of Bcl-2 immunopositive cells was lower in moderately/poorly differentiated tumors than in well-differentiated cancers (39 $\pm$ 6% versus 70 $\pm$ 11%, respectively; $P = 0.05$; data not shown). In contrast, the percentages of Bcl-X, Mcl-1, Bax, and Bak immunopositive cells were not significantly different when comparing adenomas versus carcinomas (Fig. 3) and well-differentiated tumors versus less-differentiated tumors (data not shown). The average number of Mcl-1 immunopositive cells was lower for moderately/poorly differentiated cancers (50 $\pm$ 6%) than for well-differentiated tumors (75 $\pm$ 5%).

### Immunoblot Analysis of Bcl-2 Family Proteins in Colorectal Neoplasms

Frozen tissue was available for immunoblot analysis from five colorectal carcinomas and one adenoma, along with adjacent normal colon mucosa. Compared to those in normal tissue, the relative levels of Bcl-X$_L$ protein were elevated in all five cancers but not in the adenoma (Fig. 4). The Bcl-X$_L$ protein migrated as a doublet band at $M_r \sim$ 28,000–30,000, consistent with previous investigations of this protein (23). A $M_r$ 19,000 band compatible with the Bcl-X$_S$ protein was detected in 4 of 5 carcinomas and the adenoma, but relative levels of Bcl-X$_S$ were not appreciably different in neoplastic and normal tissue paired-samples.

Compared to the levels found in adjacent normal mucosa, Bcl-2 protein levels were elevated in 2 of 5 carcinomas, equivalent in 1 of 5 carcinomas, and lower in 2 of 5 carcinomas. Bcl-2 protein levels were not altered in the adenoma specimen, compared to those in normal adjacent tissue (Fig. 4). In addition to the expected $M_r$ 26,000 Bcl-2 protein band, an additional band with $M_r$ $\sim$ 30,000 was detected in some samples. This additional protein band corresponds to a phosphorylated form of Bcl-2, based on experiments where protein phosphorylation studies of colon cancer lines, and peptide-mapping studies (39). Previous studies have suggested that phosphorylation of Bcl-2 inactivates this protein (27).

### Table 1

#### Intensity of Bcl-2 Family Protein Immunostaining Compared to Normal Colonic Epithelium

<table>
<thead>
<tr>
<th>Protein</th>
<th>Adenomas w/o SD</th>
<th>Adenomas w/SD</th>
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<tbody>
<tr>
<td>Bcl-X</td>
<td>3/8 (↑)</td>
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Fig. 3: Comparisons of the percentage of immunopositive cells for Bcl-2, Bax, Mcl-1, Bcl-x, and Bak in adenomas and carcinomas. The approximate percentages of immunopositive malignant cells were determined for 22 cases of adenoma and two hyperplastic polyyps (Ad) and for 30 adenocarcinomas (Ca). Bars, mean percentage-positivity. Statistical significance was determined by unpaired t test, comparing Ad to Ca.

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2425
or absence of the p30 form of Bcl-2 was noted between normal versus tumor tissue. Furthermore, in all specimens, unphosphorylated p26-Bcl-2 was the predominant form of Bcl-2 protein present (Fig. 4).

The Bax antiserum detected a single Mr ~21,000 band, typical of the Bax-α protein. The relative levels of Bax protein were higher than those in adjacent normal tissue in 4 of 5 adenocarcinomas and the adenoma. Thus, Bax was similar to Bcl-X in that its relative levels were typically higher in neoplasms than in normal colonic tissue. Immunoblot analysis of Mcl-1 revealed only faint bands (data not shown), consistent with the generally weak immunoreactivity seen for Mcl-1 in normal and malignant colonic tissue.

Finally, the anti-Bak peptide antiserum (described here for the first time) reacted with a Mr ~25,000 single band (Fig. 4), demonstrating the monospecificity of this antiserum and showing that the Bak protein migrates in this gel system at its expected molecular weight based on cDNA cloning results (15–17). This antiserum also specifically reacted with recombinant Bak protein produced in yeast and with in vitro-translated Bak (data not shown). Despite the clearly evident reduced intensity of Bak immunostaining seen in most adenomas and carcinomas, we were unable to detect decreases in Bak protein by Western blotting, perhaps due to the presence of contaminating nonneoplastic cells in these samples.

**DISCUSSION**

In this report, we demonstrate for the first time the overexpression of the Bcl-X protein in colorectal adenocarcinomas, particularly in those tumors with less-differentiated histological features. Some adenomas, especially those with severe dysplasia, also had elevated Bcl-X immunointensity, suggesting that up-regulation of Bcl-X expression can occur as a relatively early event in the progression of colorectal cancers. Immunoblotting showed that Bcl-X was the predominant form of Bcl-X protein in colorectal cancers. Like Bcl-2, the Bcl-X protein has been shown to inhibit apoptosis induced by growth factor withdrawal, antimitabolites, and many anticancer drugs (28). The up-regulation of Bcl-X in approximately two-thirds of the colorectal cancers evaluated here, may contribute to the origins and the progression of these colorectal tumors, as well as to the general resistance of colorectal cancer to chemotherapy (29).

In contrast to Bcl-X, expression of Bcl-2 seemed to decline during progression of adenomas to carcinomas and in moderately/poorly differentiated tumors compared to well-differentiated adenocarcinomas. Indeed, the immunointensity data for Bcl-2 and Bcl-X displayed a strong inverse correlation (P = 0.0001), suggesting that these proteins are reciprocally regulated in colorectal cancers. Reciprocal regulation of Bcl-2 and Bcl-X has been observed previously in thymocytes in which the immature cortical thymocytes immunostain for Bcl-X but not Bcl-2 and, conversely, the mature medullary thymocytes express Bcl-2 but not Bcl-X (23). Analogous to thymic differentiation, the expression of Bcl-2 and Bcl-X may be inversely regulated to some extent during differentiation and dedifferentiation of colorectal cancers. Given that Bcl-2 expression is associated with more differentiated histology in adenocarcinomas of the breast, squamous non-small cell lung cancers, neuroblastomas, and thyroid cancers (26, 30–34), it may be of particular interest to contrast the expression of Bcl-2 with Bcl-X in these other types of cancer.

The decreases in Bcl-2 immunostaining seen here in most tumors differ from previous reports that have suggested that Bcl-2 immunoreactivity is elevated in colorectal adenocarcinomas and benign adenomas compared to adjacent normal colonic mucosal epithelium (5, 35, 36). One potential explanation for this difference may be the relative bias of our study toward less-differentiated cancers and of previous studies toward well-differentiated tumors.

Similar to Bcl-2, immunostaining for Mcl-1 tended to decline during progression from adenoma to carcinoma and was particularly low in less-differentiated tumors. In normal colonic epithelium, as well as in other complex epithelia in the skin, upper respiratory tract, and esophagus, immunostaining for Mcl-1 is known to be greater in the more-differentiated cells near the epithelial surface than in the less-differentiated cells located in the base of the crypts in the colon or along the basement membrane in the epithelia of the skin, nasopharynx, and esophagus (24). Thus, the relative decline in Mcl-1 immunostaining may be attributable to the less-differentiated phenotype of the preponderance of the tumors evaluated in this study.

In contrast to the frequent reductions in Bax immunostaining reported recently in breast adenocarcinomas (25, 37), Bax expression was more often elevated than decreased in colorectal cancers. How-
ever, the context in which Bax is expressed may determine whether it promotes or inhibits apoptosis. For example, although loss of Bax in knockout mice has been associated with hyperplasias in several organs (consistent with a pro-apoptotic function for the Bax protein), spermatogenesis in male mice is defective due to excessive cell death (38). Therefore, under some circumstances, Bax may be necessary for cell survival. Nevertheless, reduced Bax immunointensity was observed in 7 of 30 (23%) adenocarcinomas evaluated here; and 3 of 30 (10%) tumors were comprised of ≤30% of Bax immunopositive cells. Thus, reductions in Bax expression can occur, albeit infrequently, in colorectal carcinomas. Therefore, it may be of interest to determine whether reduced Bax immunostaining defines a subgroup of colorectal cancers that displays poorer responses to chemotherapy or radiation, given the recent report of poorer responses to chemotherapy of colorectal cancers that displays poorer responses to chemotherapy or radiation, given the recent report of poorer responses to chemotherapy and shorter survival in some subgroups of patients with breast cancer whose tumors contained reduced Bax immunostaining (25).

Similar to Bax, the Bak protein seems to function predominantly as a pro-apoptotic protein when overexpressed in most cell types but can paradoxically promote cell survival in some cell lines (17). The reduced Bax immunostaining seen in nearly all of the adenomas and carcinomas evaluated here suggests that Bak probably functions as a pro-apoptotic protein within the context of colonic epithelium. Moreover, the high incidence of reduced Bak immunostaining suggests that decreases in the expression of this gene define an early event in the pathogenesis of colorectal cancers. However, future studies are required to gain greater insights into the biological repercussions and the clinical significance of the alterations in Bcl-2, Bcl-X, Mcl-1, Bax, and Bak expression described here in colorectal adenocarcinomas.

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Elevated Expression of Bcl-X and Reduced Bak in Primary Colorectal Adenocarcinomas

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