Altered Expression of Apoptosis Biomarkers in Human Colorectal Microadenomas

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Abstract

Human colorectal microadenomas are considered the earliest detectable premalignant lesions in the colon. They can be identified as aggregates of enlarged crypts with thicker epithelial linings and elongated luminal openings on the colonic mucosal surface after methylene blue staining and observation under a dissecting microscope. Multiple lines of evidence suggest that a central role in neoplastic development is played by the inhibition of apoptosis, followed by disruption of DNA repair. Understanding the early mechanisms of colorectal carcinogenesis may help develop new approaches of colorectal cancer prevention and treatment. The aim of the present study was to quantify poly-ADP ribose polymerase 1 (PARP-1)–positive cells and to evaluate apoptotic control mechanisms through Caspase-3 active and Bcl-2 protein expression in human microadenomas and in normal colorectal mucosa using immunofluorescence techniques coupled with confocal microscopy and immunoblot experiments. The mean percentage of PARP-1–positive epithelial cells was 3.0 ± 0.37% (SD) and 15.67 ± 0.40% in microadenoma and in normal mucosa, respectively. Proteins involved in programmed cell death were differently expressed in microadenoma and in normal mucosa. Indeed, by semiquantitative immunofluorescence analysis, confirmed by Western blot, microadenoma showed high levels of Caspase-3 active and low levels of Bcl-2 expression, whereas the opposite was true for normal colorectal mucosa. In the stroma of normal colorectal mucosa, fibroblast-like cells and neutrophils were the cells that underwent apoptosis to a greater extent. In conclusion, malfunction of the control mechanisms of programmed cell death seems present in the early stages of colorectal cancer development. Cancer Epidemiol Biomarkers Prev; 19(2): 351–7. ©2010 AACR.

Introduction

Aberrant crypt foci are microscopic epithelial lesions that can be observed on the unsectioned colonic mucosal surface of mice and rats exposed to colon-specific carcinogens (1). Similar lesions have been identified microscopically on the mucosal surface of the human colon after methylene blue staining (2-4). The hypothesis that aberrant crypt foci can be precursors of colorectal adenomas and colorectal cancer resulted from several animal studies (5, 6), in particular, aberrant crypt foci with dysplasia at histology called microadenomas. Indeed, in humans, it has been reported that microadenomas have morphologic and molecular similarities to adenomas, suggesting that they could be precursors of colorectal carcinoma (7-9). It is likely that the inhibition of apoptosis and the deregulated proliferation of colonic epithelium lead to the characteristic buckling crypt epithelium known as serration (10) and may also cause the transition from hyperplastic to dysplastic epithelium. Cells of the normal colonic epithelial surface show some biochemical and occasionally morphologic signs of physiologic apoptosis, required for normal tissue homeostasis (11, 12). Several studies showed a loss of signs of apoptosis during the adenoma-carcinoma sequence, but little is known on the relationship with dysplasia in preneoplastic lesions of the colon (13). It may be speculated that disruption of apoptosis serves a permissive role for the subsequent inactivation of DNA repair mechanisms. Conceivably, loss of DNA repair might drive rapid neoplastic progression (14). Apoptosis is characterized by distinctive morphologic features (15). Caspases, a family of cysteine proteases, are among the essential components of the apoptotic machinery (16). Overexpression of any Caspase family member can induce apoptosis in mammalian cells (17). Molecular ordering of the Caspase activation pathway suggests that Caspase-2, Caspase-8, Caspase-9, and Caspase-10 are initiators, whereas Caspase-3, Caspase-6, and Caspase-7 are the executioner Caspases (18). The cleavage of poly-ADP ribose polymerase-1 (PARP-1) is one of the earliest detectable proteolytic events that occur following high molecular weight fragmentation of chromatin DNA, but before internucleosomal DNA fragmentation (19, 20). PARP-1 (116 kDa), the founding member of Poly-ADP ribose polymerases.
family, can be cleaved into 89- and 24-kDa fragments by almost all Caspases in vitro; however, in vivo it is primarily targeted by Caspase-3 and Caspase-7 (21). The switching on and off of apoptosis is determined by the ratio of proapoptotic and antiapoptotic proteins. Bcl-2 is an example of an antiapoptotic protein that is overexpressed in many cancer cell types, colorectal adenocarcinomas, as well as prostate and breast cancer. Overproduction of the Bcl-2 protein also prevents cell death induced by almost all cytotoxic anticancer drugs and radiation, and contributes to drug resistance in patients with some types of cancer (22, 23).

The aim of the present study was to quantify apoptotic cells and to evaluate apoptosis control mechanisms in human microadenomas and in normal colorectal mucosa, to give further support to the hypothesis that microadenomas are precursor lesions in colorectal carcinogenesis. Therefore, we examined samples of microadenomas and of normal colorectal mucosa by indirect immunofluorescence techniques, coupled with confocal microscopy, and immuno blot experiments. Using these techniques, we qualitatively and quantitatively evaluated cleaved PARP-1, Caspase-3, Caspase-9, and Bcl-2 protein expression.

Materials and Methods

Study Population

Twenty-six patients who underwent colonoscopy or surgical resection for colorectal cancer at the University Hospital of Modena were selected for this study. All patients were asked to give an informed written consent to the study protocol, which was approved by the local Ethical Committee. We collected 34 samples of normal colorectal mucosa from 20 patients during colonoscopy, or on surgical specimens immediately after operation, and frozen at −80°C. Of these, 11 subjects had normal colonoscopy (6 men and 5 women; age range, 45-84 y) and 9 patients had colonic or rectal carcinoma (2 patients, women, ages 64 and 73 y), or one or more colorectal adenomas (7 patients, 4 men and 3 women, ages 61-83 y).

Twenty microadenomas were identified in six patients (three had colon and three had rectal cancer, two men and four women, ages 65-85 y) and were removed after an operation on surgical specimens, following staining of colorectal mucosa with 0.1% methylene blue and observation under a dissecting microscope (24). At topology, the average size of these lesions ranged from 80 to 180 crypts per microadenomas. In all 20 microadenomas, the luminal shape of the crypts was very irregular and elongated, suggesting the presence of dysplasia (24). Indeed, all these lesions at histology showed unequivocal dysplasia when examined by a trained pathologist (loss of mucosal polarity with elliptic crypts and varying orientation of the lumens in the same lesion, increased size and number, elongation, and hyperchromatism of nuclei with focal or extensive stratification; ref. 9). Thus, they could appropriately be called microadenomas.

Immunofluorescence Confocal Microscopy

Immunofluorescence analysis was carried out to evaluate the percentage of apoptotic epithelial cells, quantifying PARP-1–positive cells, and to analyze the expression of Caspase-3 active and Bcl-2 proteins. Moreover, we identified the type of stromal cells undergoing apoptosis using a panel of antibodies (see below). Samples of normal colorectal mucosa and microadenomas were fixed in 4% paraformaldehyde in PBS, cryoprotected in 15% sucrose in PBS, and frozen in iso-pentane cooled in liquid nitrogen. Horizontal cryosections of the samples were cut at 10 μm, and H&E staining was done on sections to control tissue integrity. After a treatment with 3% bovine serum albumin (BSA) in PBS for 30 min at room temperature, the cryostatic sections were incubated with the primary antibodies (mouse anti–Cleaved PARP-1 from Cell Signaling, mouse anti–Bcl-2 from BD Transduction Laboratories, rabbit anti–Caspase-3 active from Sigma; mouse anti-mast cell Tryptase and rat anti–Pro-Collagen I from Chemicon; rabbit anti–Myeloperoxidase from DakoCytomation, mouse anti-CD3 from DAKO, and mouse anti-CD20 from Immunomarkers) diluted at 1:25 dilution in PBS containing 3% BSA for 1 h at room temperature. After washing in PBS, the samples were incubated for 1 h at room temperature with the secondary antibodies diluted at 1:20 in PBS containing 3% BSA (sheep anti-mouse FITC conjugated, goat anti-rabbit Cy3 conjugated; SIGMA rabbit anti-rat TRITC conjugated; SIGMA).

After washing in PBS and in H2O, the samples were counterstained with 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) in H2O and then mounted with antifading medium [0.21 mol/L DABCO and 90% glycerol in 0.02 mol/L Tris (pH 8.0)]. Negative control samples were not incubated with the primary antibody. The confocal imaging was done on a Leica TCS SP2 AOBS confocal laser scanning microscope. For DAPI and Cy3 double detection, samples were sequentially excited with the 405 nm/25 mW line of a blue diode laser and with the 543 nm/1.2 mW line of a HeNe laser. The emission signals from DAPI and Cy3 were detected by two photomultiplier tubes. The samples, processed for triple fluorescence (DAPI, FITC, and Cy3), were sequentially excited with the 405 nm/25 mW line of a blue diode laser, the 488-nm/20 mW lines of the Argon laser, and the 543 nm/12 mW line of a HeNe laser.

Excitation and detection of the samples were carried out in sequential mode to avoid overlapping of signals.

Sections were scanned with laser intensity, confocal aperture, and gain. Black-level setting was kept constant for all samples. Optical sections were obtained at increments of 0.3 μm in the Z-axis and were digitized with a scanning mode format of 512 × 512 or 1,024 × 1,024 pixels and 256 gray levels. The confocal serial sections were processed with the Leica LCS software to obtain three-dimensional projections. Image rendering was done by Adobe Photoshop software.

Evaluation of Cleaved PARP-1 Immunofluorescence

The original green fluorescent confocal images were converted to gray scale and median filtering was done.
An intensity value ranging from 0 (black) to 255 (white) was assigned to each pixel. Background fluorescence was subtracted and immunofluorescence intensity was calculated as the average for each selected area. To quantify PARP-1–positive cells, all blocks were sectioned exhaustively and three to four slides were examined at \( \times40 \) magnification for each patient to count ~1,000 epithelial cells of the colonic crypts, corresponding to 16 to 23 crypts for each sample. Starting randomly, every third field of vision on one section was used for sampling all crypt cells within the unbiased sampling frame. This absolute sample size is near-optimal independent of the
dimensions of the object. To each sample was assigned a code number and the score was determined by an observer who was blind to tissue groups during analysis.

**Evaluation of Caspase-3 Active and Bcl-2 Immunofluorescence**

The evaluation was carried out in a subset of samples; six for normal colorectal mucosa and six for microadenomas were randomly selected. The original green fluorescent confocal images were converted to gray scale and median filtering was done. An intensity value ranging from 0 (black) to 255 (white) was assigned to each pixel. Background fluorescence was subtracted and an immunofluorescence intensity was calculated as the average for each selected area. To quantify Caspase-3 active and Bcl-2 expression, all blocks were sectioned exhaustively and three to four slides were examined at ×40 magnification for each patient to consider ~60 crypts. Starting randomly, every third field of vision on one section was used for sampling all crypts within the unbiased sampling frame. The fluorescence intensity at the selected areas, linearly correlated with the number of pixels, was quantitatively analyzed using the standard imaging analysis software of an NIS-Elements system. To each sample was assigned a code number and the score, called immunofluorescence intensity score (IFIS), was determined by an observer who was blind to tissue groups during analysis (25).

**Western Blot Analysis**

Whole-cell lysates were obtained from the samples of normal colorectal mucosa and microadenomas extracted with hypotonic buffer [50 mmol/L Tris-Cl (pH 7.8), containing 1% Nonidet P40, 140 mmol/L NaCl, 0.1% SDS, 0.1% Na deoxycholate, 1 mmol/L Na3VO4, and freshly added protease inhibitor cocktail]. Lysates were then cleared by centrifugation for 15 min in a refrigerated centrifuge, max speed, and immediately boiled in SDS sample buffer. Forty micrograms of protein extract from each sample (microadenomas and normal mucosa) were electrophoresed on SDS-PAGE and were transferred to nitrocellulose membrane. The protocols of the Western blot were done as described in ref. (26). The membrane was blocked with 3% dry milk and 2% BSA in PBS-T and was incubated with the following antibodies diluted at 1:1,000 overnight at 4°C under agitation: anti-mouse cleaved PARP-1 (Cell Signaling), anti-rabbit Caspase-9 (Cell Signaling), anti-rabbit active Caspase-3 active (SIGMA), and anti-mouse Bcl-2 (BD Transduction Laboratories). After washing, the membrane was incubated with secondary Horseradish Peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (1:10,000) or HRP-conjugated sheep anti-mouse IgG antibody (1:3,000) for 30 min at room temperature. Immunoreactive proteins were detected with enhanced chemiluminescence (Amersham). Furthermore, the membranes were stripped and incubated with anti-mouse β-tubulin (SIGMA) to control and correct for loading error.

**Statistical Analysis**

All quantitative data in normal colorectal mucosa and in microadenomas are reported as means ± SD. The PARP-1–positive cells, expressed as mean percentage, and the difference between Caspase-3 active or Bcl-2 mean expression, determined as IFIS, were tested for statistical significance by the Student’s t test. A P < 0.05 was chosen to indicate a statistically significant difference.

**Results**

**Apoptotic Cells in Normal Mucosa and Microadenomas**

Some representative staining patterns in normal colorectal mucosa and microadenomas are shown in Fig. 1 (A-I) where nuclear labeling of apoptotic cells is evident, as revealed by DAPI staining. The presence of apoptotic stromal cells is markedly evident in normal colorectal mucosa especially adjacent to crypts with lower levels of apoptotic cells (Fig. 1G-I). On the other hand, microadenomas do not show an appreciable PARP-1 staining of stromal cells (Fig. 1D-F).

To clarify these features, we tried to identify the type of stromal cells mostly involved in the apoptotic process. The several antibodies specific for different stromal cells that were used, coupled with PARP-1 staining, were as follows: antiamast cell tryptase that shows reactivity also to basophils although to a lesser degree, anti–procollagen I specific for fibroblast-like cells, antitymelyoperoxidase specific for neutrophils and macrophages, and anti-CD3 and anti-CD20 that recognize T and B lymphocytes, respectively. Interestingly, the staining profiles observed showed a greater amount of fibroblast-like cells and neutrophils undergoing cell death, as revealed by coimmunostaining with PARP-1 and the antibodies specific for these cell types (Fig. 1I).

To quantitatively evaluate differences in the rates of apoptosis between normal colorectal mucosa and microadenomas, we performed immunofluorescence experiments on 20 microadenomas and 20 samples of normal colorectal mucosa by means of confocal microscopy, which allows a good resolution of subcellular structures in very thick samples. The percentage of apoptotic cells was determined using cleaved PARP-1 antibody, which detects endogenous levels of the large fragment (89 kDa) of the human protein resulting from the cleavage of the native protein and does not recognize the full-length PARP 1 or other isoforms. In all the specimen of normal colorectal mucosa, many PARP-1–positive epithelial cells were detected, whereas in microadenomas, the number of positive cells was steadily lower (Fig. 1A-F). The percentage of labeled cells was 15.67 ± 0.40% (SD) in normal colorectal mucosa and 3.0% ± 0.37% (SD) in microadenomas (Fig. 2).

**Immunofluorescence Analysis of Caspase-3 Active and Bcl-2 Proteins**

Confocal analysis of a subset of six samples randomly selected using Caspase-3 active and Bcl-2 antibodies
allowed the definition of the distribution pattern and the evaluation of the quantitative consistence of these proteins in normal colorectal mucosa and microadenomas.

Caspase-3 protein aggregated in small clumps distributed in the cytoplasm at the lateral and basal portion in epithelial cells, both in normal colorectal mucosa and microadenomas, and their presence was particularly evident in the surface epithelium (Fig. 1J-O). The samples of normal colorectal mucosa were strongly stained, whereas microadenomas showed lower staining; these features were confirmed by the measurement of the expression levels, with an IFIS of 78.97 (±1.29, SD) for normal colorectal mucosa and of 35.37 (±1.14, SD) for microadenomas (Fig. 3).

Bcl-2 protein accumulated predominantly at the apical region of epithelial cells toward the lumen of colonic crypts either in normal colorectal mucosa and in microadenomas. The Bcl-2 expression level trend was the opposite of Caspase-3: microadenomas showed an IFIS of 85.68 (±1.79, SD), whereas normal colorectal mucosa had an IFIS of 42.72 (±1.02, SD; Fig. 3).

Expression of Apoptotic Regulatory Proteins in Normal Mucosa and Microadenomas

To confirm the previously described immunofluorescence observations and investigate the expression profile of some regulatory proteins involved in apoptotic process, cell lysates of normal colorectal mucosa and microadenomas specimens were analyzed by Western blotting. We evaluated a panel of antibodies to investigate the apoptotic process to avoid the pitfalls of relying on a single antibody. The results clearly showed a single intense band at the expected molecular weight (89 kDa) for cleaved PARP-1 in normal colorectal mucosa, whereas a very low band was slightly detectable in microadenomas samples (Fig. 4). In some microadenomas, the band corresponding to cleaved PARP-1 was not detectable.

The expression profile of full-length and cleaved Caspase-9 showed in Fig. 4 seemed to be different in normal colorectal mucosa and in microadenomas. Full-length Caspase-9, stand for the 47-kDa band, was more evident in microadenomas, whereas the band of cleaved Caspase-9 at 37 kDa was well detectable in normal colorectal mucosa but was very slight in microadenomas. This fact was also confirmed by the evaluation of Caspase-3 active: the corresponding band at 17 kDa, well recognizable in normal colorectal mucosa, is hardly detectable in microadenomas.

Moreover, we further assessed the Bcl-2 expression profile. This antiapoptotic protein gave, at the expected molecular weight (25 kDa), a strong band in microadenomas and a slightly appreciable band in normal colorectal mucosa.

Discussion

In this study, the percentage of epithelial apoptotic cells in microadenomas, the earliest microscopically detectable premalignant lesions in the colon, was quantitatively evaluated and compared with that obtained in normal colorectal mucosa. The results showed for the first time the reduction in the rate of apoptosis in human microadenoma compared with normal mucosa. It is established that apoptosis is vital for normal crypt homeostasis and its impairment may be an early event in...
colorectal tumorigenesis. The apoptotic index is related to the existence of colorectal neoplastic lesions, despite of differences between carcinomas and adenomas. Indeed, some articles found a decreased apoptosis ratio (27) and others an increase (28). Moreover, several works strongly support the hypothesis that microadenomas are the earliest identifiable lesions in the adenoma-carcinoma sequence in humans because of their resistance to apoptosis, which can lead to a selective growth advantage and a transformed morphology (28, 29). Turning the apoptotic machinery on and off is determined by the ratio of proapoptotic and antiapoptotic proteins. The data of the present study show a dramatic downregulation of apoptosis in human colorectal carcinogenesis. The data of the present study suggest that a deregulated expression of proapoptotic proteins, that is PARP-1 and Caspase-3 and Caspase-9, and of anti-apoptotic proteins as Bcl-2, in epithelial cells of microadenomas confer a resistance to signals leading to apoptosis. These resistant cells could then undergo subsequent genetic alterations, which in turn would lead to an overt malignant phenotype.

Another finding of this study is the presence of many apoptotic cells in the stroma of the normal mucosa, which are not detectable in the stroma of microadenomas. Our results show that fibroblast-like cells and neutrophils are the cells that most frequently undergo cell death. However, we could not obtain quantitative data and this observation needs further confirmation by “ad hoc” studies.

In summary, the results of the present study clearly show a dramatic downregulation of apoptosis in human microadenomas, compared with normal mucosa. Therefore, the evaluation of apoptotic biomarkers may be useful in colorectal cancer prevention and may offer additional perspectives for the therapeutic use of activators or inhibitors of the apoptotic pathway.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
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References

Correction: Altered Expression of Apoptosis Biomarkers in Human Colorectal Microadenomas

In this article (1), which was published in the February 2010 issue of Cancer Epidemiology, Biomarkers & Prevention, there is an error in lines 13 and 14 of the abstract (p. 351): “high levels of Caspase-3 active and low levels of Bcl-2 expression.”

The correct sentence follows. “Indeed, by semiquantitative immunofluorescence analysis, confirmed by Western blot, microadenoma showed low levels of Caspase-3 active and high levels of Bcl-2 expression, whereas the opposite was true for normal colorectal mucosa.”

Reference


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