Expression of p53, bcl-2, and ki-67 Proteins in the Inflammatory Regenerative and Dysplastic Epithelial Lesions of Flat Colonic Mucosa

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Abstract

The aim of the study was to define the distribution of p53, bcl-2 and Ki-67 proteins in the inflammatory-regenerative and dysplastic lesions of the colon mucosa. The relationship between the presentation of p53, bcl-2 and Ki-67 proteins and the intensity of the inflammatory-regenerative and dysplastic lesions in the colon flat mucosa was investigated as well. Biopsy specimens from 270 patients were examined: 74 were classified as inflammatory-regenerative and 196 as dysplastic lesions (108 mild, 58 moderate, and 30 severe dysplasia). The expression of all three proteins was assessed on the basis of location, quantity, and intensity of immunostaining, by counting antigen positive cells, in comparison with normal mucosa and adenocarcinoma. p53 protein appears only in sporadic cases (6,6%) of severe dysplasia. Bcl-2 expression appears significantly ($p<0,005$) more often in cases of mild dysplasia (61,1%) compared to inflammatory-regenerative mucosa (14,8%). In cases of mild dysplasia, bcl-2 positive cells were spreading from the lower third to the middle third of the crypts. Bcl-2 expression was maintained through the stadiums of moderate and severe dysplasia (75,8%), where antigen positive cells were found all along the crypts. A significant increase ($p<0,005$) in the expression of nuclear protein Ki-67 was noticed in the stadiums of moderate (labelling index =26,3) compared to mild dysplasia (labelling index=16,7), and severe (labelling index=36,7) compared to moderate dysplasia, where the zone of cellular proliferation was widen along the whole crypt length. In the process of the development of epithelial dysplasia in the flat mucosa of colon a degree of the gene p53 alteration is low and appears only in sporadic cases of severe dysplasia. Mutation of the bcl-2 gene is involved in the genesis of the lesion but not in its progression to carcinoma. Increased expression of Ki-67 protein speaks in favour of an increased cellular proliferation which, together with the above mentioned mechanisms, is involved in the process of occurrence and progression of epithelial dysplasia in the flat mucosa of colon.

KEY WORDS: p53, bcl-2, Ki-67, epithelia dysplasia, flat mucosa of the colon

Introduction

Apoptosis is a genetically programmed cell death, regulated by the influence of different genes. De-regulation of responsible genes leads to the inhibition of apoptosis which prolongs the cell survival and supports the development of neoplasms. It is believed that the bcl-2 oncogene and p53 suppressor gene are involved in the process of cell death regulation but not in the process of tumor proliferation. Apoptosis can be identified by different factors, including the damage of DNA, viral infections, mutations of the suppressor gene p53 and activation of the bcl-2 oncogene. Mutations of the p53 gene are, most commonly, genetic changes in cases of carcinomas of different tissues (1-6) but also in the pre-cancer lesions that precede it (6-8). In cases of colon carcinoma, mutations of the p53 gene are registered in 70% of the cases (9-11). Bcl-2 gene located on the chromosome 18 codes a protein that regulates cell proliferation. It is believed that it is also involved in the process of development, mutation and terminal differentiation of cells (12-13). Bcl-2 protein is a great inhibitor of apoptosis and its oncogenic activity is reflected in the prolonged cell survival (14). A mechanism with which bcl-2 protein protects the cell from apoptosis is not known – it is presumed that either there is a change in the mitochondrial function or a change in regulation on the level of the cellular Ca++. A high level of expression or the aberrant protein bcl-2 appears in different tumors (15-17). Numerous studies mention antagonistic action of the p53 and bcl-2 proteins in the development of solid tumors (17,18). Proliferation activity of normal and (pre)malignant tissue is determined by the fraction of cell growth. For evaluation of the cell proliferation an identification of nuclear protein Ki-67, which appears in the G1, S, G2 and M phase of the cell cycle, while it is absent in the G0 phase, is used (19). A loss of the balance between apoptosis and cell proliferation is considered to be a key moment in the process of carcinogenesis. Epithelia dysplasia is a pre-cancerous lesion, in which a cancer develops much more frequently than in normal tissue. Morphology of epithelial dysplasia in the flat mucosa of colon is well studied (20), which is not the case with the biological potential of the lesion, and which is determined by genetic events. Genetic control of the cells is expressed through a mutual interaction of oncogene and tumor suppressor gene. In the development of colon cancer there is no consistent gene expression. Fearon and Vogelstein (21) believe that the full development of cancer is preceded by mutations, happening on about 1 000 different genes. Genetic damages within the frame of pre-malignant lesions have the same complexity. Because most colorectal carcinomas seem to develop from epithelial dysplasia in flat mucosa, studies performed in different stages of colorectal neoplasm may shed some light on the genetic alterations involved in the tumor progression. In this study, the expression of proteins p53, bcl-2 and Ki-67 in inflammatory-regenerative and dysplastic flat mucosa of colon was studied and, afterward, compared with the results from normal mucosa and from the tissue of the de novo (adeno)carcinoma of colon. A relationship between the way of expression of the above mentioned proteins, degree of inflammation and the intensity of epithelial dysplasia has been analyzed as well.

Material and Methods

SAMPLES

Two to three biopsy specimens of the colon mucosa (at least 30 cm from the anus) were taken by a routine endoscopic examination from the patients with a diagnosis of the inflammatory process of any form. The specimens were taken only from the flat mucosa of the colon. Between 1998 and 2004, biopsy specimens were taken from 270 patients, 208 males and 62 females, all older than 45 years (median age 65 years, range 46-82). During the 2000-2004 period, the specimens of normal colonic mucosa of 40 deceased patients, 26 males and 14 females, between 30 and 70 years old (median age 52), were taken from regular autopsy material regerdless of the main diagnosis and causes of death. The only criterion was the absence of either macroscopic or microscopic evidence of colonic disease. From August 1998 to December 2004, from regular autopsy material the specimens of colon adenocarcinoma from cases with de novo carcinoma have been taken. Carcinoma samples were taken from 40 deceased patients, between 38 and 70 years-of-age (median age 65), 27 males and 13 females.They developed in colonic flat mucosa ranging from 7+/-10 mm in diameter. In 29 cases the surface was slightly sagged, in 8 cases slightly elevated and in 3 cases a discrete swelling in the level of mucosal surface appeared. In the de novo carcinoma tissue on serial sections, the existence of resident adenoma tissue could be established neither by macroscopic or by microscopic method. Other primary mucosal lesion were not found in any of the cases.

METHODS

The specimens were fixed in 10% buffered formalin, embedded in paraffin, cut into 4 micrometre sections and stained by standard haematoxylin-eosin (HE) and analysed immunohistochemically by monoclonal mouse

anti-human p53 protein (Dako, Glostrup, Denmark), mouse anti-human bcl-2 oncoprotein (Dako, Glostrup, Denmark,), and rabbit anti-human Ki-67 antigen (Dako, Glostrup, Denmark). In all specimens stained by HE method, the presence of inflammatory-regenerative and dysplastic changes was searched for. Histological criteria were defined for easier differentiation of inflammatory-regenerative and dysplastic changes and grading of dysplasia intensity. According to those criteria, dysplastic changes are classified into three groups (slight, moderate and severe dysplasia). The classification was based on 19 criteria, graded on a 1- 4 scale with respect to the intensity of changes. The criteria used for classification included size of epithelial cells, shape of nuclei, nucleocytoplasmic ratio, chromasia of nuclei, nuclear stratification, arrangement of chromatin, visibility and number of nucleoli, presence of different cellular types in a crypt, mucus secretion, number of cells in crypt, presence and number of mitotic figures, irregular budding of crypt, crypt branching complex, number of cripts, presence of «back to back» formation, tendency to adopt villous configuration, presence of inflammatory cell infiltrate in the lamina propria, and presence of «crypts abscess». The scores were summed up and the degree of changes was determined mathematically (22). Numerical values of index I for individual categories of changes are: 1.3≤I≤1.8 for inflammatory-regenerative changes; 1,9≤I≤2,3 for mild dysplasia; 2,4≤I≤2,9 for moderate dysplasia; 3≤I≤3,7 for severe dysplasia (22). By semiquantitative method the number of p53 immunoreactive cells was determined within 4 groups: 0=none; 1= <5%; 2=5-25%; 3=25-50%; 4= >50% (23,24); the number of bcl-2 positive epithelial cell nuclei was determined within 4 groups:

0=none; 1=<25%; 2=25-50%;
3=50-75%; 4= >75% (15).

MORPHOLOGICAL CHANGES	Index*	No. of patients
Inflamatory-regenerative	1,3	7
	1,4	10
	1,5	16
	1,6	9
	1,7	10
	1,8	12
TOTAL		74
Mild dysplasia	1,9	
	2,0	21
	2,1	10
	2,2	29
	2,3	30
		18
TOTAL		108
Moderate dysplasia	2,4	14
	2,5	15
	2,6	8
	2,7	6
	2,8	11
	2,9	4
TOTAL		58
Severe dysplasia	3,0	5
	3,1	9
	3,2	4
	3,3	6
	3,4	2
	3,5	2
	3,6	2
	3,7	0
TOTAL		30

*Index (I) is a numerically calculated range of the fluctuation of morphological changes (22).

TABLE 1. Classification of morphological changes in the colon mucosa in 270 patients with inflammatory-regenerative and dysplastic epithelial lesions

Labelling Index (LI) of Ki-67 antibodies represents a number of antigen positive cells which is divided with a total number of crypt cells and is expressed in percentages (25). Using a 400x magnification, almost 200 cells were examined in 10-15 microscopic fields. Each crypt was divided in 3 parts: basal, middle, and upper third. Statistics analysis we have used a Chi-square test, standard deviation and "t" test. A probability value $p<0,005$ for bcl-2, and $p<0,01$ for Ki-67 was taken to indicate statistical significance. Statistical program used was SPSS Version 9,0 for Windows Operating System.

Results

Among microscopically examined biopsy samples of the colon mucosa of 270 patients, chronic ulcerative colitis was found in 205 cases, lymphocytic colitis in 40 cases and eosinophilic colitis in 25 cases. In 74 cases, changes were defined as inflammatory-regenerative and in 196 cases as dysplastic. Mild dysplasia was found in 108 cases, moderate in 58 cases and severe in 30 cases (Table 1). Table 1. Classification of morphological changes in the colon mucosa in 270 patients with inflammatory-regenerative and dysplastic epithelial lesions In normal, inflammatory-regenerative, mild and moderately dysplastic mucosa of the colon no expression of p53 nuclear protein has been found. In 2 cases (6,6%) of severe dysplasia, whose index value is I=3.6, a small number of cells showed expression of the p53 protein (group 1). Antigen stained light yellow and there was no zonal arrangement among antigen positive cells. In 23 cases (76, 6%) of adenocarcinoma of the colon, expression of p 53 oncogene was found in 25-50% of

		MORPHOLOGIC CHANGES				
			EPITHELIAL DYSPLASIA			
Score	Normal mucosa N=40	Inflammatory-regenerative N=74	Mild dysplasia N=108	Moderate dysplasia N=58	Severe dysplasia N=30	Adenocarcinoma of colon N=40
0	5 (12,5%)	3 (4,1%)	0	0	0	7 (17,5%)
1	35 (87,5%)	60 (78,1%)	30 (27,8%)	10 (17,3%)	3 (10,0%)	25 (62,5%)
2	0	11 (14,8%)	67 (61,1%)	44 (75,8%)	22 (73,3%)	5 (12,5%)
3	0	0	11 (10,1%)	4 (6,9%)	5 (16,6%)	3 (7,5%)

Score: 0=none; 1=<25%; 2=25-50%; 3=50-75%; 4=>75% (23,24).

Table 2. Detection of the bcl-2 protein in 270 cases of inflammatory-regenerative and dysplastic flat mucosa of the colon, 40 cases of normal mucosa and 40 cases of the tissue of colon adenocarcinoma

Morphologic groups in which the significance is tested	Number of freedom degrees	Value of the chi-square test	The conclusion about the significance of differences between morphologic groups and the level of significance
1. Normal mucosa – Inflammatory-regenerative changes	1	4,499	significant, p<0,005
2. Normal mucosa –mild dysplasia 2		53,949	significant, p<0,005
3. Normal mucosa – moderate dysplasia	1	53,190	significant, p<0,005
4. Normal mucosa – severe dysplasia	1	44,915	significant, p<0,005
5. Normal mucosa – adenocarcinoma	2	9,999	significant, p<0,01
6. Inflammatory-regenerative changes-slight dysplasia	2	57,247	significant, p<0,005
7. Mild dysplasia-moderate dysplasia	2	3,046	not significant
8. Moderate dysplasia – severe dysplasia	2	2,362	not significant
9. Severe dysplasia – adenocarcinoma	2	26,875	significant, p<0,005

TABLE 2a. Results of the testing of Table 2

the cells (group 2), in which antigen stained more intensively than the one in the cells of severe dysplasia. The way of expression of bcl-2 protein in inflammatory-regenerative and dysplastic mucosa of the colon, in comparison to normal mucosa and the tissue of adenocarcinoma of the colon, are presented in Table 2.

In majority of the cases with normal mucosa of the colon, bcl-2 protein was present in less than 25% of the cells (group 1) of the crypt base. In inflammatory-regenerative mucosa, a significant increase (p<0,005) in the number of bcl-2 positive cells (score 2), which were still mainly located in the crypt base, was noticed. A signifi-

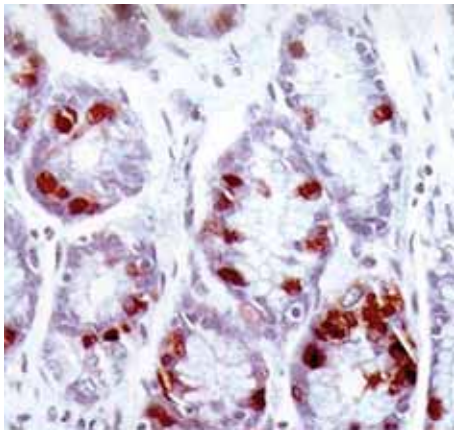

FIGURE 1. Moderate epithelial dysplasia in colonic mucosa. Immunostaining for bcl-2 showing positive nuclear reaction (X 250 magnification).

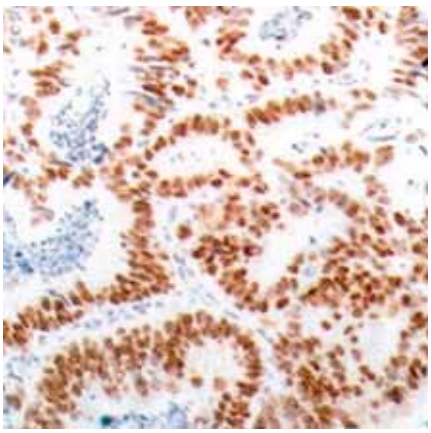

FIGURE 2. Adenocarcinoma of the colon. Immunostaining for Ki67. Note that almost all tumour cell nuclei are stained (X 250 magnification).

		MORPHOLOGIC CHANGES			
			DYSPLASIA		
Location of antigen in the crypts	Normal mucosa N=40	Inflammatory-regenerative N=74	Slight N=108	Moderate N=58	Severe N=30
Upper third	2,1	3,3	3,1	8,2	21,2
Middle third	12,1	12,0	13,8	29,5	32,4
Lower third	27,9	29,2	33,1	41,2	56,4
Mean value	14,03	14,83	16,7	26,3	36,7
Standard deviation	13,01	13,18	15,20	16,73	17,98

* LI (labeling index) – the number of Ki-67 positive cells divided by the total number of cells in the crypt, expressed in percentages (25).

TABLE 3. Ki-67 LI* in 270 cases of inflammatory-regenerative and dysplastic mucosa of the colon, compared to normal mucosa (40 cases)

Morphologic groups tested for the difference significance	"t" test value	Conclusions about significance The difference and level of significance
1. Normal mucosa-inflammatory-regenerative changes	t=0,306	not significant
2. Normal mucosa-mild dysplasia	t=0,881	not significant
3. Normal mucosa-moderate dysplasia	t=3,534	significant, p<0,01
4. Normal mucosa-severe dysplasia	t=5,842	significant, p<0,01
5. Inflammatory-regenerative changes-mild dysplasia	t=0,868	not significant
6. Mild dysplasia-moderate dysplasia	t=3,648	significant, p<0,01
7. Moderate dysplasia-severe dysplasia	t=3,283	significant, p<0,01

TABLE 3a. Results of the testing of Table 3.

cant increase of the bcl-2 protein expression (p<0,005) was noticed in the phase of mild dysplasia. In mild dysplasia (Figure 1), antigen positive cells involved the basal and the middle third of crypts. There was no significant increase in the number of bcl-2 positive cells in cases of moderate and severe dysplasia, where antigen positive cells were found all along the crypts. In the tissue of colon adenocarcinoma, the way antigens were organized and the arrangement of antigen positive cells within the glands was identical as in the cases of severe dysplasia. Majority of Ki-67 positive cells, in the normal mucosa of colon, are located in the basal third of the crypt, while small number of them were seen in the middle and upper third of the crypt. In the inflammatory-regenerative mucosa, no significant increase in the number of Ki-67 positive cells has been noticed. In cases of mild dysplasia, an increase in the number of Ki-67 positive cells was not significant. In the inflammatory-regenerative and mild dysplastic mucosa Ki-67 positive cells have maintained an identical arrangement along the crypts. A significant increase (p<0,005) of Ki-67 positive cells has been noticed in moderate and severe dysplasia, with their movement along the whole crypt length. In the tissue of adenocarcinoma, a significant increase (p<0,001) in the number of Ki- 67 positive cells continued, without outstanding zonal arrangement (Figure 2).

Discussion

We have investigated the expression of the p53, bcl-2 and Ki-67 proteins in the inflammatory-regenerative and different phases of dysplastically changed flat mucosa of colon. We have also analyzed the relationship between the presence of these proteins and the intensity of the inflammatory process and the intensity of epithelial dysplasia, and these results were compared with the status of these proteins in normal mucosa and in the tissue of the de novo colon adenocarcinoma. In this study of epithelial dysplasia in the flat mucosa of colon, the occurrence of a nuclear p53 protein has been noticed in a small number of cells in sporadic cases of severe dysplasia and in the tissue of de novo (adeno)carcinoma of the colon mucosa. Occurrence of p53 was associated with epithelia dysplasia of high index value I. In the tissue of carcinoma it was not noticed in all cancer cells, which is in agreement with the results of some other researchers (25,26). Late occurrence of p53 in epithelial dysplasia could be a sign of the lesion's progression from benign to malignant. This protein can not be taken as a marker in the process of initial screening, but can be of prognostic value in the evaluation of biological behaviour of severe epithelial dysplasia. We have noticed an increase in the expression of bcl-2 nuclear protein in cases of inflammatory-regenerative mucosa of the colon, which

continued throughout all studies of epithelial dysplasia but with a significant decrease in the tissue of de novo carcinoma. High levels of bcl-2 protect the cell from induced apoptosis promoted by the normal type of p53 protein. Reduced quantities of the bcl-2 protein in the tissue of carcinoma can be a sign that this protein is not responsible for further progression of the lesion, from severe dysplasia to carcinoma, as well. Some researchers believe that, in some tumors, the expression of this protein can be an epiphenomenon with no biological importance (27). The presence of increased levels of bcl-2 with a lack of p53 protein can be a sign that apoptosis induced by p53 protein could be blocked by increased values of bcl-2. In our research, a significant increase in the number of dividing Ki-67 positive cells was noticed in cases of moderate and severe dysplasia in the flat mucosa of colon, and especially in the tissue of de novo carcinoma. This increase in the number of dividing cells in epithelial dysplasia was followed by a simultaneous redistribution of the cells with an occurrence of the movement of antigen positive cells toward the surface of the crypts. An expansion of the proliferating zone, which spreads toward middle and upper third of the crypts, is a typical occurrence in the phase of moderate and severe dysplasia. The occurrence and development of epithelial dysplasia in the flat mucosa of colon are supported by the extended life span of cells and increased volume of cellular proliferation, which are expressed through an increased expression of bcl-2 and Ki-67 proteins. Kricha and colleagues (28) have found that the p53 protein is not detectable in normal and regenerative mucosa unlike actively inflamed mucosa of the colon in cases of chronic ulcerous colitis. They believe that in those cases deal with reversible accumulation of the mutated form of the p53 protein. It has been noticed that the intensive staining of the p53 protein in cases of chronic ulcerous colitis is a sign of the development of epithelial dysplasia (8,29), although O'Neill (30) believes that the mutation of the p53 protein can have different effects, depending on the type of cells. Mutation of the p53 gene is common in all types of neoplasms and are often associated with poor prognostic parameters and poor clinical course (18,31). An increase of the number of p53 positive cells in the tissue of colon adenocarcinoma is considered to be a sign of an increased aggressiveness of tumor (29). Differences in the p53 expression, depending on the location of carcinoma in colon, are noticed; in carcinomas located more distally, p53 expression is more pronounced (32). Results of some researchers (32,25) show that the p53 expression occurs late in the tissue of adenocarcinomas which develop from adenomatous formations. In some carcinomas of other tissues, which showed tendency toward invasion, expression of the p53 protein has been registered (10). This is specially registered in breast cancer, laryngeal cancer and oral cavity cancer (33). There are much more studies about the behaviour of this protein in the tissue of cancer in different tissues than about the pre-dysplastic lesions. Beside p53 protein, a bcl-2 protein is involved in the regulation of apoptosis. It has opposite effect of p53 gene, i.e. it acts inhibitory on apoptosis. It is believed that its duty is to maintain the maturation and terminal differentiation of cells since, in the organized epithelia, it is located within stem cells and in the proliferative zone (basal part of the mucosa gland in the small intestine and colon, basal layer of epidermis) (14). Expression of this gene, through inhibition of apoptosis, leads to the accumulation of poorly differentiated cells, i.e. to occurrence of pre-malignant lesions. Although the initial stimulus of malignant transformation of the colon mucosa epithelium is not known, the maintained expression of bcl-2 alleviates the survival of the clone of tumor cells, which have the capability to inhibit apoptosis and to extend the cells' life (34). Bcl-2 does not promote the cell proliferation and, in the absence of additional genetic alterations, bcl-2 positive tumors have tendency to be relatively less aggressive. Visualization of the Ki-67 protein showed the fraction of proliferating cells within the tissue. The amount of nuclear protein Ki-67 in different cancer tissues is always bigger compared to normal tissue (34). In stomach cancer, expression of this protein is in correlation with metastatic potential of the tumor (35). In breast cancer, expression of the Ki-67 is in the correlation with clinical-pathological parameters (36). In colon cancer, no correlation between the level of this protein and clinical-pathological parameters, such as the depth of the tumor invasion, involvement of pericolic lymph nodes and occurrence of distant metastases, has been found (37). Abnormalities of cell proliferation can be expressed as a simple increase in the number of dividing cells, increased DNA synthesis and/or as a change in the distribution of those cells in the tissue. Increased cellular proliferation is a well known finding in the zones of healing and active colitis (38). Maintenance of the Ki-67 positive cells in the lower third of the colon mucosa crypts in cases of chronic ulcerous colitis excludes the existence of dysplasia (8). As a conclusion, this study suggests that the degree of the p53 genetic alteration in dysplastic lesions of the flat mucosa of colon is very low, i.e. the activation of the p53 suppressor gene is not involved in the process of epithelial dysplasia genesis. It has noticed an increase in the expression of bcl-2

nuclear protein in cases of inflammatory-regenerative mucosa, and in the phase of mild dysplasia, and is maintained trought other phases of epithelial dysplasia. Since the mutation of bcl-2 gene in a small number of cases occurs in the tissue of the de novo (adeno)carcinoma, it can be concluded that this gene is not involved in the process of further progression of the tumor, i.e. that the expression of the bcl-2 is an early step in the evolution of tumor and the mechanism of apoptosis does not participate in tumor's progression. Increased cellular proliferation, expressed through an increased number of Ki-67 positive cells distributed along the crypts is also a part of the development and progression of epithelial dysplasia in the flat mucosa of colon.

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