Research In Surgery, Vol 2, Nº 3, November 1990

RESEARCH
IN
SURGERY

Ras Oncogene Protein Product in Ulcerative Colitis

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ABSTRACT
Increased expression of cellular oncogenes has been linked to malignant transformation. To investigate the role of oncogenes in the malignant transformation of colonic epithelium in ulcerative colitis, we compared the levels of *ras* oncogene protein product (p21) in specimens of normal human colonic mucosa (n=16) with levels in specimens of ulcerative colitis with inactive (n=11) and active disease (n=11), low- (n=17) and high-grade dysplasia (n=9) and adenocarcinoma (n=13). p21 content was measured using the RAP-5 monoclonal antibody in a semi-quantitative immunohistochemical assay. Titer was expressed as the highest serial dilution of antibody giving definite staining with the avidin-biotin peroxidase method. There were no statistically significant differences between *ras* p21 levels of low-grade and high-grade dysplasia, of active and inactive ulcerative colitis, and of ulcerative colitis with and without dysplasia. Differences in p21 titer values between normal colonic mucosa, ulcerative colitis without adenocarcinoma and adenocarcinoma in ulcerative colitis were statistically significant using Fisher's exact test (normal colonic mucosa < ulcerative colitis < adenocarcinoma in ulcerative colitis; all $p<0.05$). We conclude that p21 levels in ulcerative colitis are higher than in normal colonic mucosa and that they further increase in adenocarcinoma complicating ulcerative colitis - thus suggesting an important role for the *ras* oncogene in the associated malignant transformation.

Key words: *Ras* oncogene. Ulcerative colitis.

INTRODUCTION
Although adenocarcinoma of the colon is a well known complication of long-standing ulcerative colitis (1), the optimal timing of prophylactic colectomy has not been established. Because colonic cancers have been discovered in about 45% of patients found to have high-grade colonic dysplasia (2-9), a surveillance program of yearly pancolonoscopic biopsies to detect dysplasia is recommended in patients with extensive ulcerative colitis of at least seven years duration (10). Unfortunately, not all colonic cancers are found in association with distant dysplasia; Ransohoff (8) found that high-grade dysplasia was present in only 50% of specimens with colon cancer in ulcerative colitis. Therefore, more sensitive markers or parameters are still needed to identify all patients requiring prophylactic colectomy.

Since increased expression of cellular oncogenes has been linked to malignant transformation (11), with *ras* oncogene being the most commonly implicated in human malignancies, we evaluated the levels of *ras* gene protein product (p21) in histologic sections of patients with long-standing pancolitis and different degrees of non-malignant and malignant colonic changes. By analyzing p21 levels in inactive or active disease, low or high-grade dysplasia,

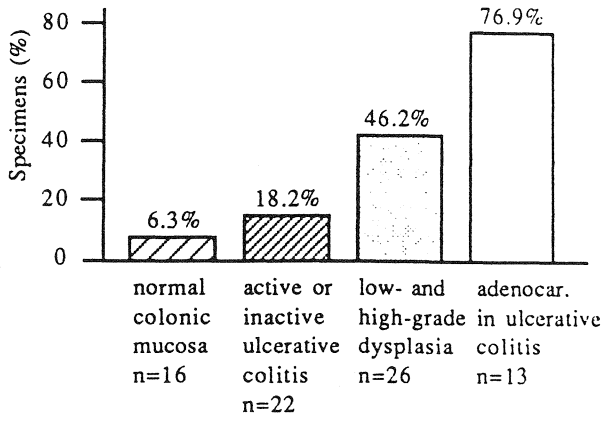

Fig. 1. Percentages of specimens staining at a titer ≥1:40,000 in normal colonic mucosa (n=16), active or inactive ulcerative colitis (n=22), low and high-grade dysplasia (n=26) and adenocarcinoma in ulcerative colitis (n=13).

or adenocarcinoma, we hoped to investigate the potential for p21 determination as an aid in selecting patients requiring colectomy before the development of adenocarcinoma.

MATERIAL AND METHODS

Archival formalin-fixed paraffin embedded specimens were recovered from the Surgical Pathology files of the University of Chicago Medical Center. Specimens examined for levels of *ras* oncogene protein product (p21) included normal colonic mucosa (n=16), active (n=11) and inactive (n=11) ulcerative colitis, low- (n=17) and high-grade (n=9) dysplasia, and adenocarcinoma (n=13) complicating ulcerative colitis. Ulcerative colitis specimens were grouped according to the standardized classification scheme of the Inflammatory Bowel Disease-Dysplasia Morphology Study Group (5). Specimens of normal colonic mucosa were obtained from patients who underwent partial colectomy for trauma. All ulcerative colitis specimens were either from proctocolectomies or from colonoscopic biopsies of patients with pancolitis for seven years or longer. These latter patients were part of a surveillance program conducted by the Section of Gastroenterology at the University of Chicago Medical Center.

The RAP-5 mouse IgG2a anti-p21 monoclonal antibody was obtained from the laboratory of Dr. J. Schlom (NIH, Bethesda, Maryland). A semi-quantitative immunohistochemical assay was performed using a previously described avidin-biotin method (12). Briefly, 4 µm thick paraffin sections were deparaffinized in xylene and rinsed in absolute ethanol. Endogenous peroxidase activity was blocked by placing the tissue in 0.3% hydrogen peroxide-methanol for ten minutes. After rinsing in phosphate buffered saline (PBS) adjusted to pH 7.4, nonspecific binding to charged proteins was blocked by incubating tissue sections with 10% normal horse serum (Vector Laboratories, Burlingame, CA) for 30 minutes. The normal horse serum was then blotted off the slide and the primary antibody was applied. Primary incubation (30 min. at 25°C) was carried out with serial dilutions of RAP-5 antibody from 1:5,000 to 1:160,000 in PBS, 0.1% BSA, 0.05% sodium azide in Tris buffer. After washing with PBS, biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) was employed as the secondary antibody (30 min. at 25°C). Following PBS wash, the avidin-biotin complex (Vector Laboratories) was applied to sections for 30 minutes. The peroxidase reaction was then initiated by the addition of 0.06% diaminobenzidine (Sigma Laboratories, St. Louis, MO) and 0.01% hydrogen peroxide for five minutes. Counterstaining was with Gills Hematoxylin #3. One slide per case was employed as a negative control using leukocyte common antigen as the primary antibody (Dako Corporation, Santa Barbara, CA). A specimen of human colon carcinoma with known high concentrations of p21 served as a positive control for p21 immunoreactivity.

A semiquantitative estimate of p21 expression was obtained by determining the highest dilution of RAP-5 which elicited definitive cytoplasmic staining. Subtle uniform brown blushes were considered negative. The slides were examined blindly and independently by two investigators, with any disagreements moderated by a third blind independent reader. Statistical comparisons were based on Fisher's exact test, global Chi-square and Chi-square test for linear trend (13,14).

RESULTS

The immunoperoxidase reaction for p21 protein appeared as a diffuse brown cytoplasmic stain. At low dilutions all epithelial and stromal elements displayed some degree of staining. However, at higher dilutions normal stromal fibroblasts and smooth muscle cells did not stain, leaving the colonic epithelium to exhibit different degrees of stain. No mucosal reaction was obtained when RAP-5 was replaced by leukocyte common antigen.

Specimens in each group exhibited a wide range of p21 expression. Based on our previous work (15) demonstrating that titers of *ras* oncogene protein product in normal colonic mucosa were always less than 1:40,000, the results of this study were analyzed calculating the percentage of specimens staining at a titer equal to or greater than 1:40,000. Since there were no statistically significant differences between the *ras* p21 levels of low-grade and high-grade dysplasia, and between active and inactive ulcerative colitis (Table I), we divided the specimens into four groups: normal mucosa, active or inactive ulcerative colitis, dysplasia, and adenocarcinoma. Only 6.3% of specimens from normal colonic mucosa stained at titers ≥ 1:40,000 compared to 18.2% of specimens with ulcerative colitis, 46.2% of dysplastic specimens and 76.9% of adenocarcinomas (Fig. 1). The global Chi-square value (global Chi-square = 19.996, 3 degrees of freedom, $p<0.001$) can be totally explained by linear trend (trend Chi-square = 19.219, 1 degree of freedom, $p<0.001$). The observed increase in the proportion of specimens with titers ≥ 1:40,000 with increasing histologic severity is statistically significant.

The pairwise comparisons of the fractions of the four groups staining at titers of at least 1:40,000 using

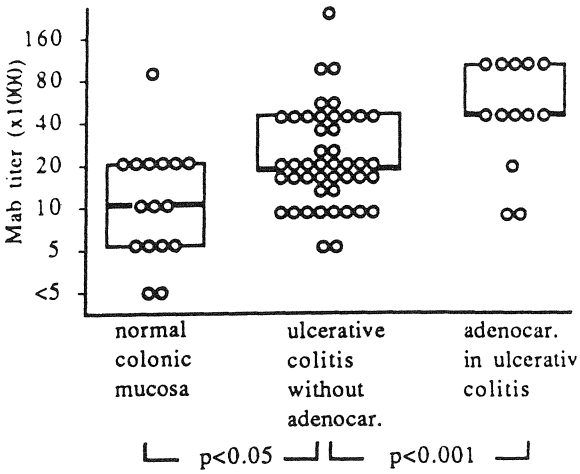

Fig. 2. Highest titers of monoclonal antibody RAP-5 staining ras oncogene protein product. The vertical scale is the logarithm of the highest dilution of monoclonal antibody RAP-5 staining ras oncogene protein product in normal colonic mucosa (16), ulcerative colitis without adenocarcinoma (47) (active and inactive disease, low- and high-grade dysplasia) and adenocarcinoma in ulcerative colitis (13). Boxes represent the middle 50% of the distribution of values obtained for each class of specimens. Each individual observation is marked with a dot. The thickened line represents the median. The pairwise comparisons are based on the fractions of specimens in the three groups, staining at titers of at least 1:40,000.

Fisher's exact test demonstrate that the fraction of specimens with ulcerative colitis that had p21 levels of at least 1:40,000 did not differ significantly from the fraction of normal specimens. On the other hand, that percentage was significantly greater among specimens with dysplasia ($p<0.008$) and adenocarcinoma ($p<0.003$) compared with normal specimens. Compared to specimens with ulcerative colitis, the percentage was significantly greater in specimens with adenocarcinoma ($p=0.001$) and approached a significant difference in specimens with dysplasia ($p=0.065$). Finally, the fraction of specimens with dysplasia and p21 titers of at least 1:40,000 approached a significant difference compared to the percentage of adenocarcinoma with similar P21 titers ($p=0.09$).

The groups of specimens were then organized into three statistically significant distinct groups based on *ras* p21 expression: normal colonic mucosa exhibited the lowest p21 expression, adenocarcinoma in ulcerative colitis exhibited the highest expression and ulcerative colitis without adenocarcinoma exhibited intermediate expression (Fig. 2). There was a statistically significant increase in the percentage of specimens with titers ≥1:40,000 with increasingly severe histology (global Chi-square = 33.341, 8 degrees of freedom, $p<0.001$).

DISCUSSION

The oncogene most commonly implicated in a wide variety of human malignancies is the *ras* gene. This gene codes for a 21,000 dalton protein commonly known as p21, which is localized to the inner side of the cell's plasma membrane. p21 has GTP-binding capability and GTPase enzyme activity (16,17) and has amino acid homology with signal transducing G proteins (18). Accordingly, it has been proposed that *ras* p21 acts like a G protein, conveying messages from growth factor receptors to intracellular targets (19) and possibly playing an important role in the control of cellular proliferation.

Malignant transformation has been associated both with point mutations of the *ras* gene, affecting the structure of the protein product and with enhanced expression of the *ras* gene affecting the quantity of the protein product. Point mutations have been found that result in an alteration of the amino acid sequence at either position 12, 13 or 61 of the p21 protein. When transfected to NIH 3T3 cells, these mutated *ras* genes are able to induce transformation (21). Similarly, injection of high levels of purified *ras* p21 protein into NIH 3T3 cells has been demonstrated to be sufficient to induce transformation (21).

Investigation of *ras* oncogene expression in premalignant and neoplastic tissues has been greatly facilitated by the monoclonal antibody RAP-5. RAP-5 (Ra, ras; P, peptide) antibody was produced in mice against a synthetic peptide corresponding to positions 10-17 of the Hu-*ras* protein product from the T24 bladder carcinoma (22), and its specificity for the *ras* p21 protein was established using Western immunoblots and solid-phase radioimmunoassay (22,23). Using RAP-5 in immunohistochemical studies, enhanced expression of the *ras* oncogene has been demonstrated in prostate (24), bladder (25), thyroid (26), stomach (26,27), breast (22,29,30), and colorectal carcinomas (2,15,31,32). These data have been complemented by further studies using spot hybridization (27,32-35), Western immunoblotting (4) and quantitative liquid competition radioimmuno-assay (23,27,30) demonstrating enhanced *ras* expression in stomach, breast, colon and head and neck carcinomas. Thus, the results of these studies suggest that increased transcription and translation of the normal cellular *ras* p21 protein play a role in human carcinogenesis.

A number of past studies have investigated *ras* p21 content in ulcerative colitis. However, most of these studies either examined a single specimen (2,23) or never distinguished between the histological grades of the ulcerative colitis specimens examined (15,32,36). In the only comprehensive study of *ras* p21 content in ulcerative colitis the authors (37) used highly concentrated RAP-5 monoclonal antibody and, therefore, were not able to demonstrate differential staining among regenerating mucosa, dysplasia and adenocarcinoma. On the other hand, using the RAP-5 monoclonal antibody in a semiquantitative immunohistochemical assay, we found *ras* p21 expression increased in a stepwise fashion from specimens of normal colonic epithelium to specimens of premalignant ulcerative colitis and finally to specimens of adenocarcinoma in ulcerative colitis.

These results demonstrate that *ras* p21 content parallels the degree of malignant transformation in ulcerative colitis and are in agreement with several other studies dealing with premalignant and malignant lesions which have found a correlation between *ras* p21 levels and the malignant potential of the particular lesion under examination (15 24, 25, 29).

	<1:5	1:5	1:10	1:20	1:40	1:80	1:160
Normal colonic epithelium (n=16)	2	4	3	6	-	1	-
Inactive ulcerative colitis (n=11)	-	1	3	5	2	-	-
Active ulcerative colitis (n=11)	-	-	1	8	1	1	-
Low-grade dysplasia (n=17)	-	1	3	5	7	-	1
High-grade dysplasia (n=9)	-	-	1	4	3	1	-
Adenocarcinoma in ulcerat. colitis (n=13)	-	-	2	1	5	5	-

Table I. Cellular ras oncogene protein content in specimens of normal colonic mucosa and ulcerative colitis (titers x1000).

Although p21 is a membrane-bound protein, the immunoperoxidase reaction appeared as a diffuse brown cytoplasmic stain. Although we believe that this is secondary to RAP-5 reacting with cytoplasmic peptide precursors of the final p21 protein, this issue was not addressed by the present study. Additionally, since RAP-5 cannot distinguish the normal p21 product gene from the p21 of the point mutated gene (22), this study does not directly address whether the elevated levels of p21 detected are products of normal or mutated *ras* genes.

Recently, several authors have suggested that RAP-5 is not a specific monoclonal antibody for p21 and that it shows cross-reactivity with other proteins (38-40). Furthermore, the immunohistochemical assay has come under criticism because its interpretation is subjective.

In support of the specificity of the RAP-5 antibody, experiments on mammary (23,30), colonic (23) and gastric (27) normal and neoplastic mucosa have demonstrated a correlation between quantitative evaluation of *ras* p21 by direct binding liquid competition radioimmunoassay and the degree of staining observed by RAP-5 immunohistochemically. Specificity of RAP-5 for p21 expression was also confirmed in these studies by parallel immunohistochemical assays with Y13-259, another anti-p21 antibody. Staining patterns and anatomical location of immunoreactivity were almost identical for both RAP-5 and Y13-259.

In a study using an antipeptide antibody similar to RAP-5, Nigg (41) demonstrated that at low concentration the antibody reacted specifically with its protein and that cross-reactivity occurs mainly at high concentrations of a monoclonal antibody. Accordingly, by considering only the highest dilution of antibody giving definite staining, as in this study, cross-reactivity is eliminated as a potential problem. Furthermore, by having each specimen read blindly by as many as three investigators, subjectivity of interpretation is minimized. Thus, although truly quantitative analysis of *ras* oncogene protein products may be obtained only with direct binding liquid competition radioimmunoassays, relative levels may be detected with the immunohistochemical assay employed in this study. The relatively greater ease by which the immunohistochemical assay may be performed allows for a more rapid exploration of the clinical importance of the *ras* oncogene family and its p21 product.

In conclusion, this study suggests that enhanced expression of *ras* oncogene is associated with malignant transformation in ulcerative colitis. Nevertheless, further studies are necessary to clearly define the role of *ras* in the process of malignant transformation in ulcerative colitis. Perhaps then high risk patients could be monitored via oncogene expression and treated surgically before actual malignant transformation occurs.

Acknowledgements

This study was partially supported by award 87-100 of the American Cancer Society, and by grant RO1-CA45259-02 of the National Cancer Institute.

We thank Dr. J. Schlom, NIH, Bethesda, Maryland for the generous gift of RAP-5 monoclonal antibody. Mrs. Roberta Carden for her expert secretarial help.

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