Immunohistochemical staining of colorectal tissues with monoclonal antibodies to ras oncogene p21 product and carbohydrate determinant antigen 19-9

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SUMMARY Two monoclonal antibodies were applied to benign, dysplastic, and malignant human colorectal tissues using immunohistochemical techniques on formalin fixed paraffin embedded material. RAP-5 antibody is directed against a synthetic peptide, reflecting an amino acid sequence of the ras oncogene p21 protein product. Despite using several different techniques and antibody dilutions differential staining between the various epithelial populations was not obtained. RAP-5 also showed other tissue components such as plasma cells, histiocytes, fibroblasts, smooth muscle and vascular endothelium. CA19-9 antibody recognises an epithelial surface carbohydrate antigen originally derived from a human colorectal carcinoma cell line: it did not stain normal colorectal mucosa or adenomatous polyps, but showed focal expression of variable strength in regenerative, dysplastic, and cancerous mucosa in ulcerative colitis, and in non-colic colorectal carcinoma. Neither antibody was found to be a reliable marker of the evolution of malignant mucosal changes, although CA19-9 may be of limited use in confirming adenocarcinoma of gastrointestinal origin.

Recent advances have led to a greater understanding of the role of cellular oncogenes and their products in carcinogenesis in man.^{1,2} Activated cellular ras oncogenes have been found in several solid tumours,^{3,4} and their transforming ability on NIH 3T3 fibroblasts has been shown.⁵ They code for a group of cellular proteins (p21) thought to take part in normal proliferation,⁶ and increased expression of the ras oncogene family has been detected by hybridisation analysis in colorectal tumours.⁷ RAP-5 monoclonal antibody is directed against a synthetic peptide sequence, reflecting positions 10-17 of the human ras (Hu-ras) protein product (p21) derived from the T24 bladder carcinoma.⁸ It has been found to show differential ras gene expression in benign and malignant colonic disease.⁹

A further aid to assessing malignant changes in colorectal mucosa has been the use of epithelial tissue markers by applying antibodies to carcino-embryonic antigen,¹⁰ IgA secretory component,¹¹ and epithelial membrane antigen.¹² Monoclonal antibody CA19-9 reacts with a carbohydrate antigenic determinant (19-9) identified as a sialylated lacto-N-fucopentaose.¹³ It is closely related to epithelial membrane antigen and is found at the epithelial cell

surface. It has been shown immunohistochemically in pancreatic, gastric, and colonic tumours.¹⁴

The purpose of this study was to assess the use of these antibodies as tissue markers in evolving and established cases of colorectal carcinoma.

Material and methods

The material in this study came from the files of this hospital. The tissues had been fixed in 4% aqueous formaldehyde, secondarily fixed in Helly's fluid, and processed to paraffin wax. The sections studied consisted of histologically normal mucosa from the most distant resection limit of non-colic colorectal carcinomas, plus five rectal adenomatous polyps, three villous adenomas, five colorectal carcinomas, and carcinomas from nine patients with ulcerative colitis, also showing regenerative and dysplastic mucosal changes. The colitic mucosa was assessed for dysplasia using a standardised classification.^{15,16} Some of this material also formed the basis of a previous immunoperoxidase study using antibodies to carcino-embryonic antigen and the IgA secretory system.¹⁷

ANTIBODIES AND IMMUNOHISTOCHEMICAL TECHNIQUES

The RAP-5 monoclonal antibody was a generous gift

from Dr J Schlom, NIH, Bethesda, USA. It was applied at varying dilutions between 1/15000 and 1/25000 to trypsinised and non-trypsinised sections. Blocking of non-specific binding of antibody to protein was achieved with ovalbumin 1% and bovine serum albumin. Hydrogen peroxide was used to diminish endogenous peroxidase activity. The NIH protocol, based on a standard avidin-biotin-complex immunoperoxidase procedure, was followed,¹⁸ and colourisation obtained using diaminobenzidine substrate with a light haematoxylin counterstain. Further sections were stained using indirect immunoperoxidase¹⁹ and a streptavidin-biotin peroxidase system.²⁰ The CA19-9 monoclonal antibody (Histocis CA19-9) was obtained by courtesy of CIS

(UK) Ltd, and it was applied to sections undiluted using the manufacturer's recommended avidin-biotin immunoperoxidase kit. In addition, the indirect and streptavidin-biotin techniques were also used. Immunoperoxidase staining was graded qualitatively as light, moderate, or heavy.

Results

RAP-5 MONOCLONAL ANTIBODY

Epithelial staining using this antibody was of a diffuse distribution throughout the cell cytoplasm. There was no accentuation of positivity in the supranuclear Golgi zone, the cell apex, or along the luminal border. Global uniformity of staining was seen in the various

Fig 1 (a) RAP-5 monoclonal antibody. Normal epithelial cells of mucosa, muscularis mucosae and tumour epithelium (left) all show diffuse staining. Goblet cell mucin and tumour stroma is negative. (Avidin-biotin immunoperoxidase.) $\times 230$. (b) RAP-5 monoclonal antibody. Positive staining in ganglion cells (G), lymphatic tumour embolus (T), and vascular muscle coat and endothelium (M). (Avidin-biotin immunoperoxidase.) $\times 270$.

epithelial populations represented as follows: histologically normal mucosa; mucosa with transitional features; regenerative and dysplastic mucosa in ulcerative colitis; dysplasia in adenomatous polyps and both colitic and non-colitic adenocarcinoma. Non-epithelial components also stained. These included plasma cell cytoplasm, occasionally lymphocyte and histiocyte cytoplasm and fibroblasts, ganglion cells, and nerve branches. The smooth muscle in the muscularis mucosae, the muscle coat, and submucosal vessels also displayed staining product along with the endothelial cells of submucosal vessels and granulation tissue capillaries. Goblet cell mucin and tumour collagenous stroma were negative (fig 1). Differential epithelial staining was not improved by antibody dilution, blocking, and digestion, or by using the alternative immunohistochemical methods. Further colorectal blocks of tissue processed without secondary Helly's fluid fixation showed similar results but with decreased muscle coat reaction.

CA19-9 MONOCLONAL ANTIBODY

This antibody failed to stain histologically normal and transitional like mucosae. The table summarises the staining of diseased colorectal tissue. Regenerative epithelium in ulcerative colitis showed focal positivity varying from light to heavy. The staining pattern was one of product deposition in the immediate supranuclear and cell apex regions and as a fine luminal rim over the surface epithelium (fig 2). Dysplasia in adenomatous polyps and villous adenomas was negative, but was positive in a focal manner, and of variable strength in ulcerative colitis. This showed cell apex staining, but the surface luminal border was particularly prominent and was sometimes associated with cryptal and surface debris (fig 3). Two adenocarcinomas were negative, 12 showed positivity, albeit focal and light in eight, and moderately diffuse or heavy in only four cases of the 13 cancers. The staining pattern was similar to that of dysplasia with luminal rim accentuation. Central debris in tumour acini and diffuse cytoplasmic staining along with single tumour cells surrounded by a halo effect were also seen (fig 4). Using indirect immunoperoxidase

Fig2 CA19-9 monoclonal antibody. Regenerative epithelium with staining occurring in supranuclear and cell apex regions, and as a luminal surface rim. Two glands (bottom) are negative. (Avidin-biotin immunoperoxidase.) $\times 370$.

Table Staining reaction of monoclonal antibody CA19-9 with colorectal tissues

Histological diagnosis	No of cases studied	Staining intensity			
		0	Light	Moderate	Heavy
Regeneration in ulcerative colitis	14	6	5	1	2
Dysplasia in ulcerative colitis	11	5	4	1	1
Adenocarcinoma in ulcerative colitis	9	1	6	1	1
Adenomatous polyp, villous adenoma	8	8			
Adenocarcinoma	5	1	2	2

Fig 3 CA19-9 monoclonal antibody. Ulcerative colitis with high grade dysplasia. Staining occurs in cell apex and as a strong luminal surface rim. (Avidin-biotin immunoperoxidase.) $\times 370$.

and streptavidin-biotin peroxidase strengthened the product reaction but did not alter the staining distribution within a given tissue. Goblet cells and non-epithelial components were consistently negative.

Discussion

Eukaryotic cells contain proto-oncogenes, which code for a number of cell products concerned with normal growth and differentiation.² The Hu-ras gene family elaborate several proteins, with binding and

enzyme activities influencing cell metabolism.⁶ Proto-oncogenes can exert an effect on mucosal proliferation and carcinogenesis, either quantitatively by an increased expression of the normal gene or qualitatively by mutagenesis of the gene sequence. The activated cellular oncogene produces either increased concentrations of normal cellular protein or an abnormal peptide sequence, events favourable to cell transformation. This process may be brought about by encountering a cofactor event such as a chemical carcinogen, viruses, or radiation. Heightened ras gene expression has been described in hybridisation studies in colonic polyps and tumours.⁷ On tissue sections using a different monoclonal to ras p21 (Y13 259 Hu-ras Harvey) than the one presently studied, two groups of workers found variable staining in normal colorectal mucosa and carcinoma,^{21 22} with an increased intensity in adenomatous polyps.²² RAP-5 antibody has been described as showing preferential staining in human mammary and colonic carcinomas over benign inflammatory and dysplastic lesions in these tissues.^{8 9} Its expression has been postulated to correlate with the depth of colonic wall tumour invasion and in areas of severe epithelial dysplasia, indicating a relatively late event in malignant transformation.⁹ Our present study did not show differential staining of the various epithelial cell populations or between different levels of tissue in the same lesion. In contrast to the findings of other studies,^{8 9} we also noted strong staining of non-epithelial tissue components as seen previously in the colon²¹ and breast.²³ Ghosh *et al*²³ recently reported similar findings to ours in a range of breast lesions. We must agree with their interpretation that either RAP-5 detects a normal cell product not enhanced in carcinogenesis, or that it does not show the abnormal p21 product, derived from mutagenesis and oncogene activation, which is necessary for neoplasia. An explanation for the discrepancy in colorectal studies using the two p21 antibodies may be that RAP-5 reacts on solid phase radioimmunoassay with both T24 Hu-ras and Hu-ras Harvey peptides, which show an amino acid difference at position 12 of the p21 molecule.^{8 9} This indicates RAP-5 reactivity with a group of p21 proteins, and therefore the detection of a specific mutant p21 product relevant to tumour formation is made less likely.²³ This in no way excludes the role of ras genes in colonic carcinogenesis but does seem to indicate a limited use in our hands for monoclonal antibody RAP-5 in diagnostic colorectal pathology.

Neoplasia tissue markers include the expression of oncofetal antigens (carcinoembryonic antigen) and the loss of normal cell function with the evolution of malignancy (IgA secretory system). An allied approach has been to raise antibodies against the

Fig 4 (a) CA19-9 monoclonal antibody. Negative mucosa (left) contrasts with strong epithelial surface staining in adenocarcinoma (right). (Avidin-biotin immunoperoxidase.) $\times 130$. (b) CA19-9 monoclonal antibody. High power view of tumour epithelial surface staining. (Avidin-biotin immunoperoxidase.) $\times 370$.

immunodominant antigen on tumour cell surfaces. MC2 and MC4²⁴ and antibody to epithelial membrane antigen achieve this by showing cell surface carbohydrate oligosaccharides. Monoclonal antibody CA19-9 recognises a closely related fucosylated pentasaccharide, sialylated lacto-N-fucopentaose, developed by immunisation of BALB/c mice with a human cell line SW1116, derived from a colorectal carcinoma.^{25 26} Its main use has been as a serum tumour marker in upper gastrointestinal disease,^{27 28} and it has been shown in adenocarcinoma tissue sections of stomach, pancreas, and colon.¹⁴ The findings in this study confirmed positive CA19-9 staining in 12 of 14 colonic carcinomas, although it was focal and light in eight of these 12. Epithelial dysplasia showed variable staining, being totally negative in nine pre-

malignant polyps but giving patchy, light deposition in just over half of the regenerative and dysplastic cases of ulcerative colitis. It is possible that CA19-9, like carcinoembryonic antigen and epithelial mucins (personal observations DCA), may reflect not only dysplasia but also regeneration in ulcerative colitis,^{17 29} where cell turnover and proliferation is known to be increased.³⁰ The supranuclear and cell surface staining of CA19-9 also corresponds to the Golgi apparatus and cell apex localisation of carcinoembryonic antigen seen on ultrastructural study of a human colorectal adenocarcinoma cell line.³¹ The explanation for the spectrum of staining found in similar histological changes within the mucosa remains unanswered. Thus the results for differential staining of these epithelial subtypes were less reliable than the

previously used carcinoembryonic antigen and IgA secretory system markers.¹⁷ Therefore, CA19-9 cannot be recommended for diagnostic use in tracing the evolution of malignant changes in the colorectal mucosa, particularly as a basis for discrimination between regeneration and dysplasia in ulcerative colitis. The disparity in staining between non-colitic premalignant polyps and carcinoma is of interest, and further work needs to be done to assess its ability to indicate carcinomatous change within mucosal polyps. At present, CA19-9 may be of limited use to the histopathologist in established malignancy when used in combination with other markers (eg CEA) in confirming metastatic adenocarcinoma of gastrointestinal origin.

In the absence of reliable immunohistochemical markers of colonic regeneration, dysplasia, and carcinoma one can only recommend that the pathologist gains sufficient experience of the spectrum of possible histological changes in colorectal epithelium and that his judgment be refined by application of standardised classifications.^{15 32}

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