Colorectal goblet cell sialomucin heterogeneity: its relation to malignant disease

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SUMMARY Mucus secreted by colorectal cancer differs in three respects from that produced normally: an overall reduction, a loss of O-acetyl substituents in sialic acid, and an increase in neutral mucin. Similar changes have been reported in apparently normal mucosa bordering colorectal cancer. "Normal" left sided colorectal mucosa from 32 patients with rectal cancer was studied. Each case was matched by age and sex to a patient with diverticular disease and a patient with irritable bowel syndrome. Twenty five patients with right sided cancer were matched to patients with Crohn's disease. Sections were stained with mild periodic acid Schiff (mPAS) (selectively stains N-acetyl sialic acid lacking in O-acetyl group) and other closely related techniques. Reactions were graded negative, weak, and intense. An intense reaction was found in 9% of cases; there was no difference between the various matched groups. Phenylhydrazine interposition failed to block the mPAS effect, indicating that a positive result was due to a deficiency of sialic acid with O-acetyl substituents rather than neutral mucin. Different staining patterns in left and right colon were probably due to differing ratios of total sialic acid:fucose. These findings indicate a hitherto unsuspected colorectal goblet cell sialomucin heterogeneity within the general population, but no association with neoplastic disease is apparent.

Colorectal goblet cells are believed to secrete mainly acid mucin but also small amounts of neutral mucin.¹ There are important regional differences in the colon. The right colon secretes more neutral mucin² (higher molar ratio of fucose:sialic acid) and expresses more (fucose rich) blood group antigens. Biochemical differences are found in sialic acid, with greater sensitivity to neuraminidase digestion being evidenced by saponified sialic acid in the left colon.² The structural basis of this change is not known. Most sialic acid residues bear O-acetyl groups in the polyhydroxy side chain or at position C₄, or both.

Colorectal cancer usually secretes less mucus than normal mucosa, which hinders its detailed characterisation. Biochemical and histochemical studies, however, have shown important qualitative changes. These include an increase in the amount of neutral mucin and a reduction in the proportion of side chain and C₄ substituted sialic acid.² Modified periodic acid Schiff techniques, periodate borohydride/potassium

hydroxide/periodic acid Schiff (PB/KOH/PAS)³ and periodate thionin Schiff/potassium hydroxide/periodic acid Schiff (PT/KOH/PAS)⁴, were developed by Culling *et al* to show changes in sialic acid composition—that is, loss of O-acetyl substituents and increased expression of N-acetyl sialic acid. Unfortunately, neither of these methods distinguishes N-acetyl sialic acid from neutral mucin; both are oxidised by periodic acid. This important lack of specificity has now been corrected, but earlier conclusions are open to question.

Several groups have reported "cancer-associated" changes in "normal" colorectal goblet cells adjacent to cancer. A combined biochemical and histochemical study has shown that the mucosa in the proximity of colorectal cancer differs from normal in secreting a reduced proportion of O-acetyl sialic acid.⁵ In 24% of cases no change was observed, but in 42% there was a focal reduction, and in 34% a moderate to severe field reduction was noted. The histochemical observations suggested that the findings were not due to a general loss of sialic acid but due to the deletion of O-acetyl substituents.⁵ Similar changes were reported by Katsuyama *et al*.⁶ Unfortunately, these studies did

Table 1 Interpretation of histochemical staining

	N-acetyl sialic acid lacking in O-acetyl groups	O-acetyl sialic acid	Neutral mucus
mPAS	+	—	—
PAPS	+	—	—
KOH/PAPS	+	+	—
PAS	+	—	+

not include a control group. In contrast, Filipe's studies on sialic acid in "transitional mucosa" showed little change in O-acetyl substitution.^{7,8} Two recent publications using control tissues indicated that sialic acid heterogeneity may be a feature of the general population.^{9,10} This further reinforces the need for caution in accepting the findings of Reid *et al*⁵ and Katsuyama *et al*.⁶

This study was designed to clarify the current position by studying a large number of neoplastic and non-neoplastic colorectal specimens, using a simple battery of mucin histochemical techniques with an acceptable level of specificity.

Material and methods

Fifty seven specimens of colorectal cancer and adjacent mucosa were studied. Thirty two of the tumours were located in the rectum and 25 in the right colon. These groups were studied separately with their own controls to eliminate the influence of site on the pattern of mucus secretion. The mean age of patients with rectal cancer was 61.9 years (range 37–85). Mucosa immediately adjacent to each cancer was matched with uninflamed mucosa from a specimen resected from diverticular disease (by age and sex). A small fragment of mucosa removed 5 cm proximal to tumour was matched similarly with a normal rectal biopsy specimen from a patient with irritable bowel syndrome. The mean age of patients with right sided cancer was 49.8 years (range 30–70). Mucosa adjacent to each tumour was studied. This was a selected group of patients with a relatively low mean age, because appropriate controls are rarely obtained from middle aged to elderly patients in the absence of malignant disease. The control tissue was derived from right hemicolectomy specimens for ileal Crohn's disease and comprised histologically normal colonic mucosa. An additional 26 total colectomy specimens for chronic constipation were included in this study to increase the number of controls and, more importantly, to permit comparison of left and right sided mucosa from the same subject. In 23 of the cases mucosa was identified as originating from the right (ascending) colon or left (sigmoid) colon.

TECHNIQUES

All specimens were fixed in 10% neutral buffered for-

malin, routinely embedded, serially sectioned at a thickness of 5 µm, and stained by haematoxylin and eosin and the following histochemical methods.

A Mild sodium periodate oxidation and Schiff (mPAS)

This new technique was developed by Veh *et al*¹¹ and is stated to be specific for N-acetyl sialic acid lacking in O-acetyl groups.

- 1 Dewax and bring to distilled water.
- 2 Wash in 0.1 M acetate buffer, pH 5.5, at 2°C for five minutes.
- 3 Treat with 1 mM NaIO₄ in 0.1 M acetate buffer, pH 5.5, at 2°C for 10 minutes.
- 4 Wash in 1% aqueous glycerol for five minutes.
- 5 Wash in distilled water for five minutes.
- 6 Treat with Schiff's reagent at room temperature for 15 minutes.
- 7 Wash three times in 0.5% K₂S₂O₅ in 0.05 M hydrochloric acid for five minutes.
- 8 Wash in running tap water for five minutes.
- 9 Wash in distilled water for five minutes.
- 10 Dehydrate, clear, and mount.

B Periodic acid Schiff¹² (PAS)

Fig 1 Diffuse weak positive staining with mPAS. × 75.

Fig 2 Diffuse intense positive staining with mPAS. $\times 75$.

C Periodic acid phenylhydrazine Schiff (PAPS)

This blocks periodate reactivity due to neutral mucin.¹³

D Potassium hydroxide periodic acid phenylhydrazine Schiff (KOH/PAPS)

Ester on the polyhydroxy side chain is removed by saponification,¹⁴ which renders all types of sialic acid oxidisable by periodic acid, and neutral mucin is blocked with phenylhydrazine interposition.

E Diastase PAS¹² (DPAS)

This excludes the possible contribution of glycogen to periodate activity.

Table 1 summarises the interpretation of these techniques.

CONTROLS

Known positive and negative cases were included in each staining run. Test sections and matched controls were stained at the same time. The specificity of mPAS was tested by phenylhydrazine interposition. This produced little or no change in staining intensity. Statistical evaluation was achieved using the χ^2 test.

Results

Staining by PAS and its various modifications was graded according to the intensity: negative (–); weak (+) (fig 1); and intense (++) (fig 2). This was attempted for both cancerous tissues and normal mucosa.

(All mucosa, including that bordering cancer, are

referred to as normal mucosa in the following.) Two patterns of mPAS staining were observed; a diffuse (graded –, +, or ++) (figs 1 and 2) and a focal, in which small numbers of crypts were lined by intensely positive goblet cells (fig 3), whereas the background mucosa was negative or weakly stained. In any single crypt no differences were seen between goblet cells of crypt base, midcrypt, and surface epithelium.

DIFFUSE mPAS STAINING IN NORMAL MUCOSA FROM CANCER CASES AND CONTROL GROUPS

There were no differences in the distribution of mPAS positive mucus between the three cancer matched control case studies (table 2). There were also no differences in mPAS staining between mucosa immediately adjacent to and 5 cm proximal to cancer of the rectum (table 2).

EFFECT OF PHENYLHYDRAZINE INTERPOSITION UPON mPAS AND PAS STAINING, INCLUDING COMPARISON OF THE LEFT AND RIGHT COLON

The effects of phenylhydrazine interposition were tested in normal mucosa adjacent to all carcinomas of the left and right colon. The effect on mPAS staining was either minimal or resulted in a very slight reduction only in intensity. This confirms the inability of mPAS to stain neutral sugars. On the other hand, PAS staining intensity was reduced by phenylhydrazine interposition (PAPS). In 16 of 32 cases of left sided mucosa there was a moderate reduction, and in 14 and seven of the 25 right sided samples the reduction was judged to be moderate and consid-

Fig 3 Focal mPAS positive crypt against negative background mucosa. $\times 75$.

Table 2 Distribution of diffuse mPAS staining patterns in normal mucosa in three cancer matched control studies and series of colons removed for chronic constipation

Study groups and No of cases		mPAS (-)	mPAS (+)	mPAS (++)
1	Normal mucosa adjacent to rectal cancer (n = 32)*	23	6	3
	Diverticular disease (n = 32)	17	13	2
		χ^2 , p = NS		
2	Normal mucosa 5 cm proximal to rectal cancer (n = 32)*	21	8	3
	Irritable bowel syndrome (n = 32)	22	7	3
		χ^2 , p = NS		
3	Normal mucosa adjacent to cancer of right colon (n = 25)	15	7	3
	Normal mucosa from Crohn's disease (n = 25)	18	6	1
		χ^2 , p = NS		
4	Chronic constipation (left side only) (n = 26)	9	14	3
	Total† = 172	104 (60%)	53 (31%)	15 (9%)

*Derived from same specimens.
†Excludes normal mucosa 5 cm proximal to rectal cancer.

erable, respectively. The differences between level of reduction in the left and right colon were significant ($p < 0.01$). These findings reflect the presence of neutral hexoses within goblet cell mucin of the large intestine and indicate that the right colon contains more neutral mucin than the left colon. This was confirmed in an inpatient study of colectomy specimens removed for chronic constipation (table 3). In 17 of the 23 cases PAS blockade with phenylhydrazine interposition was judged to be greater in the right colon than in the left colon. In five cases no difference was detected, and in one case only the reduction in PAS staining seemed to be greater in the left side of the colon.

EFFECT OF DIASTASE INTERPOSITION ON PAS STAINING

This was performed on all normal mucosa adjacent to cancers of the left and right colon. Diastase interposition did not affect PAS reactivity, indicating a lack of epithelial glycogen stores.

COMPARISON OF mPAS AND PAPS STAINING PATTERNS

A total of 102 specimens were studied by PAPS as well as mPAS (table 4). In theory these techniques should have produced identical results. Both stain

oxidisable sialic acid (lacking O-acetyl substituents) and both fail to stain neutral mucin: with PAPS this is due to phenylhydrazine blockade; with mPAS the low concentration of periodic acid and low temperature combine to inhibit oxidation. For the intense and weak mPAS staining absolute concordance was, in fact, achieved (table 4). Just over one quarter of the mPAS negative cases, however, gave a weak result with PAPS (table 4). This could have been due to incomplete blockade of neutral mucin or increased sensitivity to oxidisable sialic acid.

MILD PAS AND KOH/PAPS STAINING—A COMPARISON OF LEFT AND RIGHT SIDED MUCOSA FROM COLECTOMY SPECIMENS FOR CHRONIC CONSTIPATION

With mPAS staining of the left and right colon, similar patterns were observed, although the intensity of staining was reduced in the right colon. In nine of 13 cases staining weakly in the left colon (table 2) little or no staining could be detected in the right colon. (In the fourteenth case it was not possible to identify a right sided sample.) Saponification with KOH renders all sialic acid oxidisable and therefore capable of giving a positive reaction with PAS. With phenylhydrazine interposition (neutral mucin blockade), this technique specifically shows most, if not all, forms of sialic acid. Although intense staining was achieved in most cases, in about half the intensity of staining was reduced in the right colon. Taken in conjunction with the findings for the effect of phenyl-

Table 3 Inpatient study of colectomy specimens removed for chronic constipation to show reduction of PAS staining following phenylhydrazine interposition in left and right colon

Reduction in left colon	No of cases	Reduction in right colon	No of cases
None to slight	14	None to slight	2
		Moderate	8
		Pronounced	4
Moderate	9	None to slight	1
		Moderate	3
		Pronounced	5

Table 4 Comparison of mPAS and PAPS staining patterns

mPAS	No of cases	PAPS	No of cases
Negative	62	Negative	45
Weak	30	Weak	17
Intense	10	Weak	30
		Intense	10

Fig 4 Schematic drawing to illustrate variation in amount of sialic acid and neutral mucin in normal colorectal mucosa. Staining intensities for N-acetyl sialic acid lacking O-acetyl groups (mPAS) and total sialic acid (KOH/PAPS) increase from right to left. There is no evidence from this study to suggest any intrapatient site related variation in ratio of O-acetyl:N-acetyl sialic acid.

hydrazine interposition it seems that an inverse relation exists between sialic acid and neutral sugars, with a higher total sialic acid:neutral sugar ratio existing in the left colon (fig 4). It also seems that the total amount of sialic acid is similar within a particular site of the colon in a series of subjects.

MUCIN PROFILES IN CANCER v ADJACENT NORMAL MUCOSA

Difficulties were encountered because of considerable differences in the amount of mucus secreted by the cancers. Whereas six of 32 normal mucosal samples from the left colon gave a weak reaction with mPAS (table 2), 22 left sided cancers gave a weak reaction. This probably indicates an increased expression of N-acetyl sialic acid lacking in O-acetyl groups within tumour mucus (presumably at the expense of O-acetyl sialic acid). Three cancers gave an intense reaction with mPAS, but these were not the three cases that showed an intense positive reaction within normal adjacent mucosa.

In samples of cancer from the right colon six cases gave an intense reaction with mPAS. The adjacent normal mucosa was intensely positive in two, weakly positive in two, and negative in the remaining two cases. In the third case with an intensely mPAS positive normal mucosa the cancer mucus was negative. Cancers also showed increased PAS reactivity compared with that of adjacent normal mucosa. This effect was reduced by phenylhydrazine blockade in half the cases, showing that the increase was, in part, due to neutral mucin.

FOCAL STAINING PATTERNS IN NORMAL MUCOSA

In samples of normal mucosa giving a negative or weak diffuse result with mPAS one crypt, or sometimes small numbers of crypts (up to five), were observed, in which the entire goblet cell population gave an intense positive result with mPAS. The reverse effect (focal negative or weak staining in an intensely positive background) was never seen. Focal positive crypts were also stained intensely with PAPS and PAS. These foci occurred more often in cancer cases than in controls when background staining was negative, but not when weak positive staining featured in the background (table 5).

Discussion

Mucosa adjacent to colorectal cancer differs from normal large bowel mucosa in giving a blue staining reaction with the high iron diamine alcian blue sequence.¹⁵ This method is based on differential ion binding and is not specific.¹⁶ A blue colour does not exclude the presence of sulphate, nor does a brown

Table 5 Focal positive mPAS staining against negative and weak positive background mucosa

Background mucosa	Carcinoma	Control
Negative	11/38 (28.9%)*	3/35 (8.6%)*
Weak positive	4/13 (30.8%)	4/19 (21.2%)

No of focal positive cases/total.
* χ^2 test, $p < 0.05$.

reaction product necessarily signify the absence of sialomucin. Culling *et al* introduced a range of modified PAS techniques^{3,4} that are specific for O-acetyl sialic acid. They showed that a loss of O-acetyl substituents is found not only in benign and malignant neoplasms¹⁷ but is also evident in the mucosa bordering colorectal cancer.⁵ Their early studies suggested that a switch was taking place from O-acetyl sialic acid to N-acetyl sialic acid, but their original methods failed to distinguish between neutral mucin and N-acetyl sialic acid. Although blockade of neutral mucin may be achieved by the interposition of phenylhydrazine,^{13,18} the complexity of their original methods has deterred many workers from investigating their utility as research or diagnostic tools.

In this study we used a battery of simple techniques to test the possibility that modifications in sialic acid might precede neoplastic transformation in the large intestine. The mPAS technique usually gave a negative result throughout the length of the colon. This supports the assertion by Veh *et al*¹¹ that the method stains neither O-acetyl sialic acid (except, possibly, 7 O-acetyl sialic acid) nor neutral mucin; these are normally well represented within the colorectum. In the cases that were positive with mPAS phenylhydrazine interposition (for neutral mucin blockade) did not produce any appreciable reduction in the staining intensity. On the other hand, alcianophilia was reduced by prior digestion with neuraminidase (data not given). We are therefore confident that N-acetyl sialic acid was detected by mPAS. Although weak and, less commonly, intense mPAS staining was

observed in the mucosa bordering left and right sided cancers, these findings occurred with a similar incidence in control tissues from age and sex matched subjects. We therefore believe that constitutional factors may be responsible for goblet cell sialic acid heterogeneity within the general population.

Deficiency of O-acetyl transferase activity, determined by homozygosity for a recessive gene, could account for the 9% of subjects secreting N-acetyl sialic acid as opposed to O-acetyl sialic acid. This would be analogous to the slow acetylator phenotype, which is caused by an N-acetyl transferase defect. Most histopathologists must be familiar with the occasional rectal biopsy specimen showing an unusually intense result with routine PAS staining. Alternatively, some may have wondered at the weak staining of most rectal biopsy specimens. Differences in the PAS staining of rectal biopsy specimens do not reflect inconsistent laboratory practice but are determined by sialic acid heterogeneity within the general population. This heterogeneity is not associated with either blood group secretor state or acetylator phenotype (unpublished observations), nor is it related to neoplastic disease. This conclusion contradicts the findings of Reid *et al*,⁵ but receives support from Lev *et al*.⁹ It has been reinforced by our failure to show a concordance in staining patterns for cancer and adjacent mucosa.

Fig 5 shows the usual staining patterns in the left colon. mPAS and PAPS generally gave concordant results (table 4). PAPS, however, takes longer and entails use of the highly toxic chemical phenylhydrazine. When a weak or intense mPAS reaction

Fig 5 Usual goblet cell staining patterns in left colon. (black = intense, dots = weak, white = negative).

Fig 6 Schematic drawings to illustrate three main types of sialic acid expression based on patterns shown in fig 5. □ = neutral mucus; ▨ = O-acetyl sialic acid; ▩ = N-acetyl sialic acid lacking in O-acetyl groups.

occurred in the left colon of patients with chronic constipation, this was mirrored in the right colon, though the intensity was usually slightly diminished. KOH/PAPS stains total sialic acid. In most of the cases tested the left and right colon again gave concordant results, though the intensity was relatively less strong in the right colon. On the other hand, the PAS method (which stains neutral sugars as well as some sialic acid moieties), resulted in more intense staining of the right colon. The principal terminal sugars in goblet cell mucin are probably fucose and sialic acid.¹⁹ Biochemical and histochemical studies^{2,6} show that the fucose:total sialic acid ratio is higher in the right than the left colon. Our staining patterns support this view and have been illustrated schematically (fig 4). It is also relevant that Ulex europaeus lectin (specific for fucose) binds selectively to the goblet cell mucin of the right colon²⁰ and that right sided colonic mucus is known to express fucose rich blood group substances.²¹ We therefore suggest that the total amount of sialic acid depends on the region

of colon under study, whereas the ratio of O-acetyl sialic acid: N-acetyl sialic acid is influenced by constitutional factors (fig 4). The ratio of O-acetyl sialic acid and N-acetyl sialic acid may be the same throughout the colon in any subject (fig 4). The relatively simple models depicted in fig 6 will be slightly complicated by the superimposition of focal changes shown by us and other workers.^{5,9}

We found that focal mPAS positive crypts were more numerous in specimens harbouring cancer, but we interpret the change as a functional metaplasia rather than as a sign of early neoplastic change⁵ as identical changes are featured in metaplastic polyps of the colorectum.²² The existence of heterogeneity in large bowel sialic acid in the general population may have important implications. We have shown that mPAS positive sialic acid is susceptible to neuraminidase digestion (unpublished observations) and are now studying possible associations between the secretion of this variant of sialic acid and susceptibility to ulcerative colitis. Furthermore, it is now essential to establish the inpatient sialic acid state when investigating changes associated with disease by histochemical or biochemical means.

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