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Alkali-catalyzed β-elimination of periodate-oxidized glycans: A novel method of chemical deglycosylation of mucin gene products in paraffin embedded sections

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Altered expression of mucin gene products has been described in many epithelial cancers including colorectal cancer. However, mucins are heavily O-glycosylated making the study of apomucin expression difficult. In this study, we describe a novel method of chemical deglycosylation of mucin gene products on paraffin embedded formalin-fixed tissue sections. In the normal and cancerous colorectum, our results suggest that alkali-catalyzed β-elimination of periodate oxidized glycan method of chemical deglycosylation modifies the structure of carbohydrates sensitive to mild periodate oxidation resulting in less steric hindrance and selectively removes Tn and sialyl-Tn structures, partially exposing the underlying apomucin epitopes. Using this method, we have demonstrated that the MUC1 tandem repeat epitope recognized by MAb 139H2 is masked predominantly due to steric hindrance by carbohydrate structures whereas the MUC2 tandem repeat epitope recognized by MAb CCP58 and pAb MRP and the MUC3 tandem repeat epitope recognized by pAb M3P are masked by the presence of carbohydrate side chains O-linked to Ser/Thr residues within the epitope. Considerable differences in the level and pattern of expression of the epitopes in the tandem repeat region of apomucins of MUC1, MUC2, and MUC3 were observed between normal and cancerous colorectal cancer tissues. We conclude that this novel chemical deglycosylation method that causes selective cleavage of distinct glycans will be useful in unmasking various mucin gene products and glycoproteins containing similar O-glycosidic linkages in the tissue sections of formalin-fixed paraffin embedded normal and pathological tissues.

Keywords: **mucin, periodate oxidation, deglycosylation, immunohistochemistry, MUC1, MUC2, MUC3, colon cancer**

Introduction

Alterations in mucin glycoproteins have been reported to occur in colorectal carcinogenesis and these changes have been associated with differences in the biological properties of cancer cells [1,2]. Because these changes may affect tumor progression, metastasis, and thus prognosis, there is much interest in determining the tissue and cellular expression of mucin gene products in normal and neoplastic epithelium.

The general structural motif of mucins consists of central tandem repeat units which are heavily glycosylated, flanked by sparsely glycosylated "unique" regions. The carbohydrate content of mucin glycoproteins typically range about 50–85% by weight and are attached via O-glycosidic linkages to serine and threonine residues via N-acetyl-Galactosamine (GalNAc) [3]. Because these tandem repeat units consist of distinct amino acid sequences which are specific to each mucin gene product, immunohistochemical studies of apomucins are mainly based on antibodies which react with peptide determinants within the tandem repeat domain [4–8]. A difficulty with this approach is that heavy glycosylation may prevent antibodies from recognizing the cryptic peptide epitope and therefore give false negative results [9].

Two methods have recently been developed to overcome this difficulty. One method is to raise antibodies directed against sparsely glycosylated "unique" regions of apomucins [10]. However, this method is limited by the lack of availability of antibodies and the lack of information about the full-length sequence of mucin gene products and the

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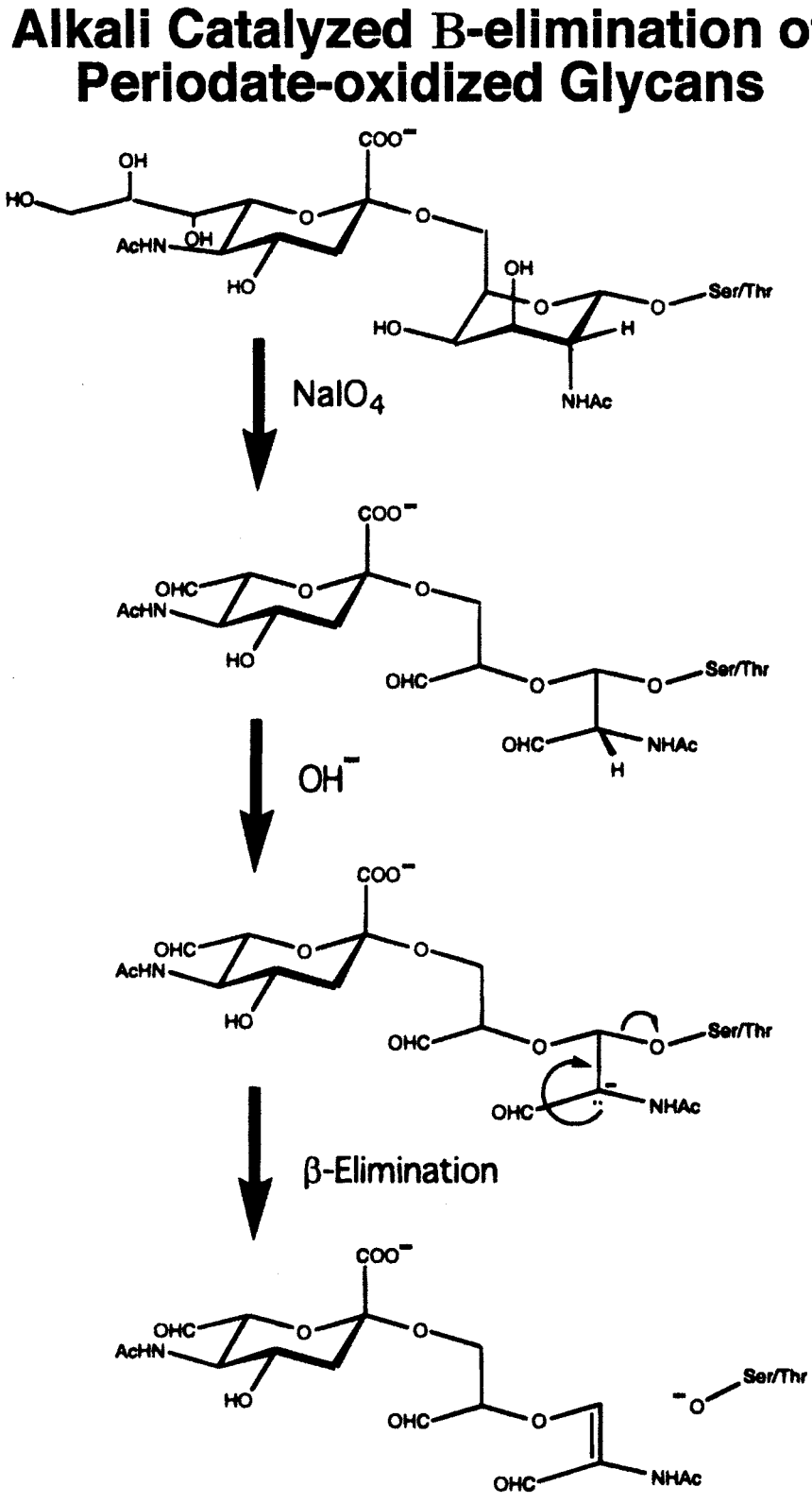

Figure 1. Proposed reaction scheme of alkali-catalyzed β-elimination of periodate-oxidized glycans on sialyl-Tn structure. Periodate oxidation cleaves the carbon-carbon bond between C3 and C4 of GalNAc residue. The resulting moiety is susceptible to β-elimination with mild alkali treatment (modified from Ref. 15).

specificity of the antibodies. These difficulties severely limit the use of this method at the present time. Another method to investigate apomucin expression is to remove the carbohydrate side chains prior to immunostaining. Several investigators have tried both enzymatic deglycosylation as well as chemical deglycosylation methods with limited success. At present, only mild periodate treatment which cleaves carbon-carbon bonds with vicinal hydroxyl groups has been shown to unmask MUC1 but not other apomucin epitopes [11,12]. The carbohydrate side chains of mucin glycoproteins contain numerous carbon-carbon bonds that are prone to oxidative cleavage by periodate [13,14]. Although steric hindrance may be reduced by this treatment, as is the case for MUC1, the cleavage of these bonds results only in the destruction of related carbohydrate epitopes. The resulting carbohydrate moieties are still O-linked to the protein backbone, denying access to antibodies directed against apomucin epitopes.

Recently, Gerken and Jentoft developed a novel method of deglycosylating purified samples of mucin glycoproteins which preferentially removes Tn and sialyl-Tn structures [15] (Figure 1). They found that ovine submaxillary mucin, which contains predominantly Tn and sialyl-Tn side chains, can be completely deglycosylated using periodate oxidation followed by β-elimination. However, they also found that mucins containing more complex side chains, such as porcin submaxillary mucin and human tracheobronchial mucin, were not effectively deglycosylated unless pretreated with trifluoromethanesulfonic acid (TFMSA). Treatment with TFMSA causes trimming of mucin oligosaccharide side chains resulting in the reduction of oligosaccharide side chain heterogeneity and increased susceptibility of partially deglycosylated mucin to proteases [15,16]. In their recent study, they were able to remove Tn and sialyl Tn structures selectively from porcine submaxillary mucin by alkali-catalyzed β-elimination of periodate-oxidized glycans after pretreatment with TFMSA [16].

In the present study, we sought to determine if the removal of Tn and sialyl-Tn structures by the combined treatment of periodate oxidation followed by alkali-catalyzed β-elimination is sufficient to unmask previously cryptic apomucin epitopes in tissue sections. Because histological integrity, which is vital to immunohistochemistry, is easily destroyed by extreme pH conditions and by harsh chemical treatment with TFMSA, we modified Gerken and Jentoft's method of chemical deglycosylation of purified mucin samples so that it would be suitable for tissue sections. To access the unmasking of apomucin epitopes after alkali-catalyzed β-elimination of periodate-oxidized glycans, we chose antibodies directed against the heavily glycosylated tandem repeat regions of MUC1, MUC2, and MUC3 which have been demonstrated to be expressed in the normal colonic mucosa from *in situ* hybridization studies [4,5,17] but undetectable immunohistochemically in the goblet vacuoles, and weakly detected in apical secretion, and enterocytes [4,5]. To assess whether certain carbohydrate structures are selectively removed as suggested by Gerken and Jentoft, we treated our specimens with Periodic Acid Schiff reagent and examined their immunoreactivity using antibodies against Tn, sialyl-Tn, sialyl-Lewis^x, and sulfo-Lewis^a. Because the β-elimination step of our modified method involves alkali treatment which saponifies O-acetylated sialic acids resulting in the unmasking of these sialylated antigens [18], all specimens were deacetylated with the same alkali condition prior to treatment. This alkali pretreatment did not affect apomucin expression. In summary, to accomplish our assessment of carbohydrate modification and removal concomitant with apomucin unmasking, the normal and cancerous colorectal tissue sections were either 1) untreated, 2) deacetylated (DA), 3) deacetylated plus periodate oxidized (DA + PO), and 4) deacetylated, periodate oxidized, and β-eliminated (DA + PO + BE) prior to standard immunoperoxidase staining.

Materials and methods

Reagents

Periodic acid was obtained from Fisher Scientific (USA). Biotinylated *vicia villosa* lectin was obtained from Vector Laboratories (USA) [19]. Normal goat serum, biotinylated goat anti-mouse & anti-rabbit IgG, and streptavidin peroxidase conjugate were obtained from Zymed Laboratories (USA).

Antibodies

Table 1 lists the antibodies and lectins used in this study and the antigens they recognize. MAbs TKH2 and SNH4 were generous gifts from Dr. S. Hakomori (The Biomembrane Institute, Seattle, WA) [20,21]. MAb F2 was a generous gift from Dr. E.C.I. Veerman (Vrije Universiteit, Amsterdam, The Netherlands) [22]. MAb 139H2 was a generous gift from Dr. J. Hilkens (The Netherlands Cancer Institute, Amsterdam, The Netherlands) [23]. MAb CCP58 was obtained from Biomeda Corp. (USA) [24]. The antisera to MRP and M3P were raised against synthetic MUC2 and MUC3 tandem repeat sequences respectively as previously described [25,26]. The rabbit polyclonal antisera raised in our laboratory against two synthetic peptides representing an amino terminal and a carboxy terminal domain of non-tandem repeat regions of MUC2 and anti-MRP were used in ELISA of small intestinal mucin as described below.

Alkali-catalyzed β-elimination of periodate-oxidized glycans of mucins in formalin-fixed paraffin embedded tissue sections

After deparafinization, the tissue sections were deacetylated (saponified) with 0.1 M NaOH for 30 min at room temperature prior to overnight periodate oxidation at 4°C with 100 mM $NaIO_4$ in 100 mM acetate buffer, pH 4.5. On the following day, reactive aldehydes were neutralized by incubating with 2% glycine solution for 30 min followed by β-elimination with 0.1 M NaOH for 30 min at room temperature. This procedure

Table 1. Lectins and antibodies used to label apomucins and mucin-associated carbohydrate structures

Carbohydrate/Peptide Epitope	*Antibody/Lectin*	*Antigen*	*Reference*
GalNAcαThr/Ser	VVA	Tn	19
NeuNAcαGalNAcαThr/Ser	TKH2	Sialyl Tn	20
NeuNAcα2,3Galβ1,4(Fucα1,3)GlcNAc-R	SNH4	Sialyl Lewis^x	21
SO_3-3Galβ1,3(Fucα1,4)*GlcNAc-R	F2*	Sulfo Lewis^a	22
PDTRPAPGSTAPPAHGVTSA	139H2	MUC1 Tandem Repeat	23
KYPTTTPISTTTMVTPTPTPTGTQTPTTT	CCP58	MUC2 Tandem Repeat	24
KYPTTTPISTTTMVTPTPTPTGTQT	MRP	MUC2 Tandem Repeat	25
KTTSNSTPSFTSSITTTETTSMS	M3P	MUC3 Tandem Repeat	26
CGNFDHRSNNDFTTRDHMVV	M2AT 1	MUC2 Amino Terminal domain of non-tandem repeat region	**
CVLNDTYYAPGEEVYNGTYG	M2CT 1	MUC2 Carboxy terminal domain of non-tandem repeat region	

*F2 is insensitive to the fucose residue within the sulfated Lewis^a structure and thus recognizes both the native and periodate oxidized conformations.
**Unpublished data.

resulted in consistent and significant increases in the staining of the tissue sections with antibodies directed against mucin tandem repeat peptides.

Alkali-catalyzed β-elimination of periodate-oxidized glycans of purified human small intestinal mucin

To assess the integrity of the protein core, ELISA studies were performed on purified human small intestinal mucin which was treated with 100 mM $NaIO_4$ (4°C, pH 4.5) followed by alkali-catalyzed β-elimination. The partially deglycosylated mucins were then immobilized on 96-well microtiter plates and immunoreactivity was measured using tetramethylbenzydines as substrate as previously described [27]. Absorbance at 450 nm was measured with a Bio-Rad Microplate Reader. Biotinylated secondary antibodies and horseradish peroxidase conjugated streptavidin were purchased from Zymed (S. San Francisco, CA).

Immunohistochemistry

Standard immunoperoxidase staining method previously described [4,5] using heat induced antigen retrieval in 10 mM citrate pH 6.0 was used to examine 15 pairs of normal human colon and colon cancer specimens. Colon cancer specimens consisted of well to moderately differentiated adenocarcinomas. Aminoethylcarbazole was used as substrate. Sections were counterstained with hematoxylin prior to mounting.

Periodic acid schiff (PAS) staining

Standard PAS technique was used for the chemical estimation of polysaccharides as previously described [27]. Briefly, after deparaffinization, sections were incubated in 0.5% periodic acid solution for 10 min at room temperature followed by 3 rinses with distilled H_2O. The sections were then incubated with Schiff's reagent for 5 min followed again by 3 distilled H_2O rinses. After counterstaining with hematoxylin, the sections were dehydrated and cleared in xylene before mounting.

Scoring of slides

Slides were scored based on the intensity of staining and the prevalence of antigen positive cells in each tissue section. The staining intensity was scored as negative (0), weakly positive (1+), moderately positive (2+), and strongly positive (3+). The prevalence of antigen positive cells was considered as the percentage of each tissue section area with positive staining and was estimated (0 to 100%). The final score was the sum of the

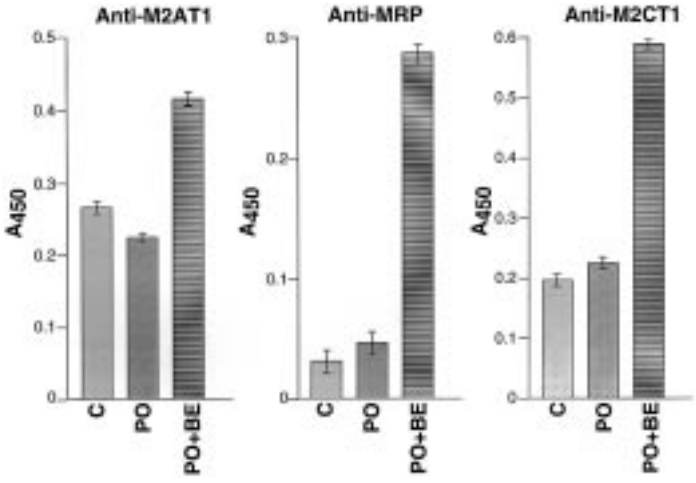

Figure 2. Reactivity of rabbit polyclonal antibodies, anti-M2AT1, anti-MRP and anti-M2CT1 (see Table 1) against purified human small intestinal mucin. Immunoreactivity measured as absorbance at 450 nM. C, control untreated mucin; PO, periodate oxidation; PO + BE, periodate oxidation followed by alkali-catalyzed β-elimination. The number of samples were 6. Mean ± SEM.

staining intensity × percentage weakly positive (1+) product plus staining intensity × percentage moderately positive (2+) product plus staining intensity × percentage strongly positive (3+), as described previously [5].

Results

Optimization of periodate oxidation treatment

Our preliminary study of alkali-catalyzed β-elimination after overnight periodate oxidation at 20 mM $NaIO_4$ (4°C, pH 4.5) did not show an increase in the staining intensity of formalin-fixed tissue sections using antibodies directed against the tandem repeat regions of MUC2 and MUC3. However, when the concentration of $NaIO_4$ was increased to 100 mM and overnight periodate oxidation (4°C, pH 4.5) was carried out, marked and consistent increases in the staining intensity of the tissue sections were observed with these antibodies. However, at concentrations over 100 mM $NaIO_4$, the expression of the same tandem repeat peptide epitopes declined, signifying that the protein core may be compromised by too much $NaIO_4$.

To further assess the integrity of the protein core, ELISA was performed on purified human intestinal mucin which has been treated with 100 mM $NaIO_4$ followed by alkalai catalyzed β-elimination. As shown in Figure 2, ELISA studies showed that after the treatment of human small intestinal mucin with 100 mM $NaIO_4$ followed by alkalai-catalyzed β-elimination, a marked increase in the reactivity of anti-MRP against the tandem repeat peptides of MUC2 mucin was observed as compared to untreated mucin. More importantly, periodate oxidation treatment alone did not cause a decrease in reactivity, suggesting that the peptide core is stable in the presence of 100 mM $NaIO_4$ at 4°C overnight.

A similar but smaller increase in the staining intensity was observed with small intestinal mucin treated with 100 mM $NaIO_4$ (4°C, pH 4.5) overnight followed by alkali-catalyzed β-elimination when ELISA was carried out with antibodies directed against an amino-terminal domain (anti-M2AT1, 1.6X) or a carboxy-terminal domain (anti-M2CT1, 3X) of much less glycosylated non-tandem repeat regions of MUC2. These data indicate that overnight treatment at 4°C with 100 mM NalO4 was not only effective in exposing both the tandem repeat peptide as well as more sparsely glycosylated

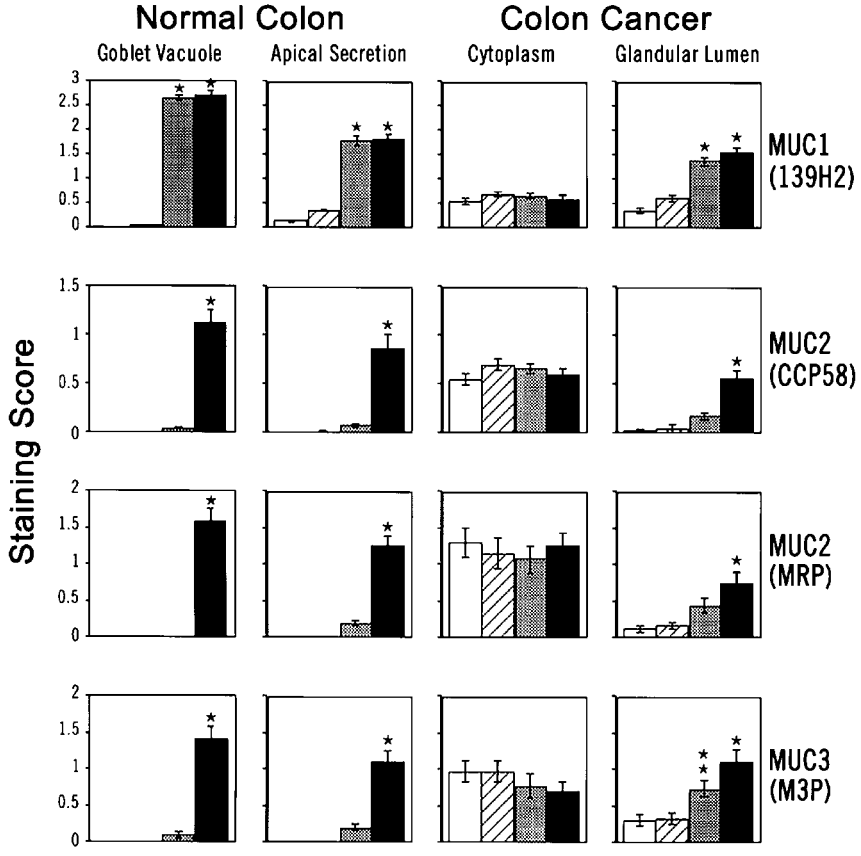

Figure 3. Apomucin expression in normal colon and colon cancer. The specimens were: untreated (open bar), DA treated (striped bar), DA + PO treated (shaded bar), or DA + PO + BE treated (solid bar). Statistical significance versus untreated specimens. The number of specimens were 15 for both normal colon and colon cancer. Mean ± S.E.M. (* = $p < 0.01$, ** = $p < 0.05$).

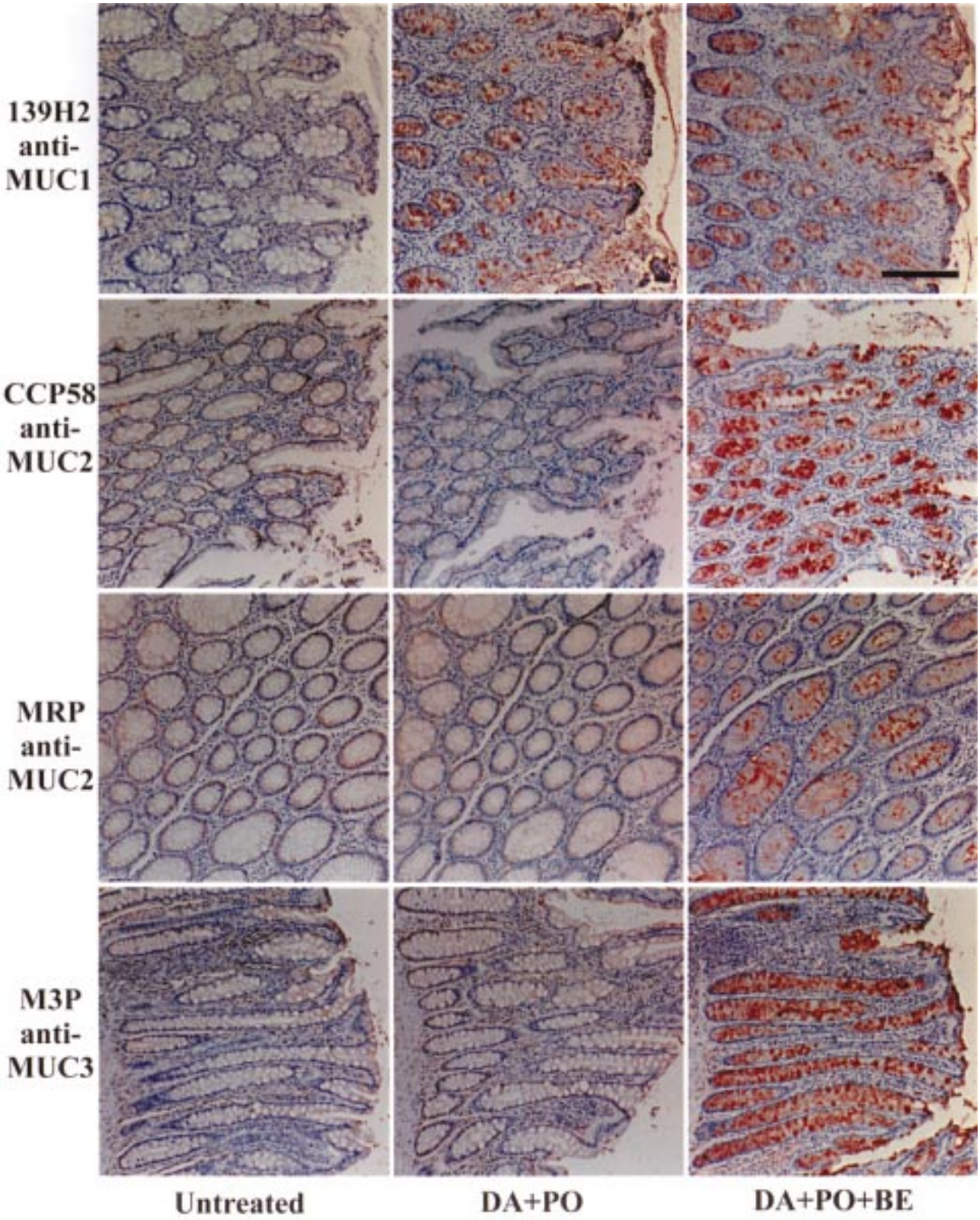

Figure 4. Immunostaining of apomucin in the normal colonic mucosa. mAb 139H2, anti-MUC1, shows goblet and lumenal staining after DA + PO treatment. β-elimination did not further increase staining with 139H2. Both mAb CCP58 and pAb MRP, directed against MUC2 apomucin, show perinuclear staining in untreated and DA + PO treated specimens. Unmasking of MUC2 apomucin in goblet vacuoles and lumenal secretion occurred only after DA + PO + BE treatment. Results of pAb M3P directed against MUC3 apomucin were similar to antibodies against MUC2 apomucin. Bar represents 100 μm.

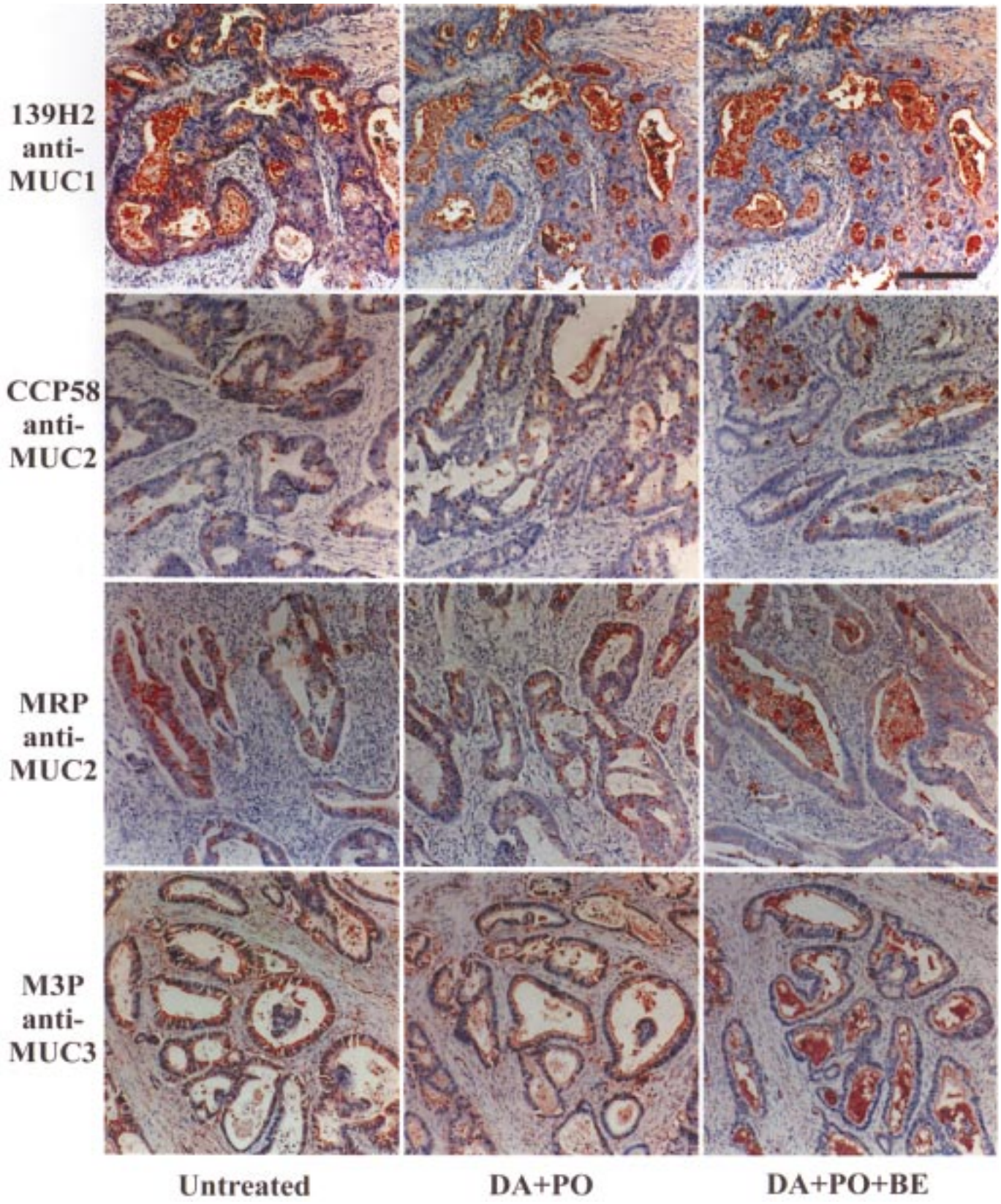

Figure 5. Immunostaining of apomucin in colon cancer. MUC1, MUC2, & MUC3 all exhibited weak to moderate cytoplasmic staining with little to no enhancement after DA + PO + BE treatment. Lumenal staining of MUC2 & MUC3 was markedly enhanced only after DA + PO + BE treatment whereas lumenal staining of MUC1 increased after just DA + PO treatment. Bar represents 100 μm.

non-tandem repeat peptides, but also mild enough to preserve the integrity of the protein core.

Apomucin expression after alkali-catalyzed β-elimination of periodate-oxidized glycans

In normal colonic mucosa there was no immunostaining in the luminal secretion, goblet vacuoles, and enterocytes of untreated specimens in MUC1 and MUC2 tandem repeat peptide epitopes examined due to the heavily glycosylated nature of mature mucins (Figures 3 and 4). MUC2 apomucin epitope was detected only in the supranuclear region of goblet cells. MUC3 apomucin epitopes were expressed weakly in the upper colonic crypt cells. After DA, all apomucin epitopes still remained cryptic. However, after DA + PO, the MUC1 apomucin epitope recognized by MAb 139H2 became exposed, in contrast to apomucin epitopes of MUC2 and MUC3 that remained cryptic. Only after DA + PO + BE treatment did the MUC2 and MUC3 apomucin epitopes become unmasked in the goblet vacuoles and the luminal secretion. Interestingly, there was no difference in the MUC1 staining scores between DA + PO treated specimens and DA + PO + BE treated specimens.

In colon cancer, untreated specimens stained with 139H2 (MUC1) exhibited weak diffuse cytoplasmic staining while specimens labeled with CCP58, MRP, and M3P showed moderate diffuse cytoplasmic staining (Figures 3 and 5). Even after AD + PO + BE, there was no further unmasking of these epitopes in the cytoplasm of cancer cells. With regard to the immunostaining of glandular luminal content, while no unmasking occurred with DA treatment, glandular lumen staining scores after DA + PO treatment increased nearly 4-fold for MUC1 and MUC2. For MUC3, there was roughly a 2-fold increase. The epitopes of MUC2 and MUC3 apomucins were further unmasked another 2 to 3-fold after DA + PO + BE treatment. However, there was no difference in the staining scores of MUC1 between DA + PO and DA + PO + BE treated specimens.

Carbohydrate antigen expression after alkali-catalyzed β-elimination of periodate-oxidized glycans

In the normal colonic mucosa, O-acetylation of sialic acids (NeuNAc) masks the expression of sialyl-Tn and sialyl-Lewis^x structures [18]. This is evident by the strong immunostaining of goblet vacuoles and apical secretions after DA treatment as shown in Figures 6 and 7. After DA + PO treatment, expression of Tn, sialyl-Tn, and sialyl-Lewis^x were significantly reduced. Sulfo-Lewis^a expression was not reduced by DA + PO treatment because the epitope recognized by the MAb F2 does not include the periodate sensitive fucose residue [22]. Since the oxidation (DA + PO) of GalNAc in Tn and sialyl-Tn results in the destruction of carbohydrate structures critical to these antigens and is also a prerequisite for β-elimination, DA + PO + BE treatment did not differ from DA + PO treatment alone.

In colon cancer, where sialic acids are rarely O-acetylated, both untreated and DA treated specimens exhibited moderate to strong staining of the glandular lumen but weak to moderate staining in the cytoplasm of cancer cells in all 4 carbohydrate antigens examined (Figures 6 and 8). After DA + PO treatment, immunostaining was reduced to almost negligible levels except for sulfo-Lewis^a which showed no change. DA + PO + BE treatment did not further reduce the staining scores.

Quantification of carbohydrate content by PAS staining

In the goblet vacuoles, apical secretion, and enterocytes of normal colonic mucosa, DA treated specimens had a PAS staining score 3 to 4-fold higher than untreated specimens because O-acetylated sialic acids are not periodate sensitive (Figures 6 and 7). There was no difference in PAS staining of DA treated versus DA + PO treated specimens because the PAS staining method involves a redundant periodic acid cleavage of carbon-carbon bonds with vicinal hydroxyl groups into aldehydes which is then localized by Schiff's reagent [28]. After DA + PO + BE, PAS staining decreased but was not abolished, suggesting that only a portion but not all of the carbohydrate side chains of mucin glycoprotein was removed by alkali-catalyzed β-elimination of periodate-oxidized glycans.

PAS staining of untreated versus DA treated colon cancer specimens showed no differences in the staining score due to the predominantly non-O-acetylated nature of sialomucin in colorectal neoplasm (Figures 6 and 8). After DA + PO + BE treatment, PAS staining of the glandular lumen was only about a third that of untreated, DA treated, and DA + PO treated specimens. Again, this suggests that carbohydrates were not only modified but also actually removed by our chemical deglycosylation method.

Discussion

Periodate oxidation cleaves the carbon-carbon bond with vicinal hydroxyl groups of carbohydrates without altering the underlying peptide structure [13,14]. Because of its ability to discriminate, periodate oxidation has been widely used to characterize the carbohydrate or peptidic nature of epitopes recognized by newly developed monoclonal antibodies [29,30]. However, this technique is applied mainly to ELISA method using purified antigens. Recently, investigators have applied periodate oxidation on gastric and colonic tissue sections resulting in the unmasking of MUC1 apomucin in the normal epithelium [11,12]. In neoplastic colorectum as well as normal and carcinomas of breast and pancreas where MUC1 is detectable, immunostaining is enhanced by pretreatment with periodate [12]. Although periodate oxidation does not cause deglycosylation, the modification of carbohydrate structure is thought to reduce steric hindrance and allow antibodies access to the underlying apomucin epitope. However, the unmasking of apomucins by periodate oxidation is limited to MUC1. This

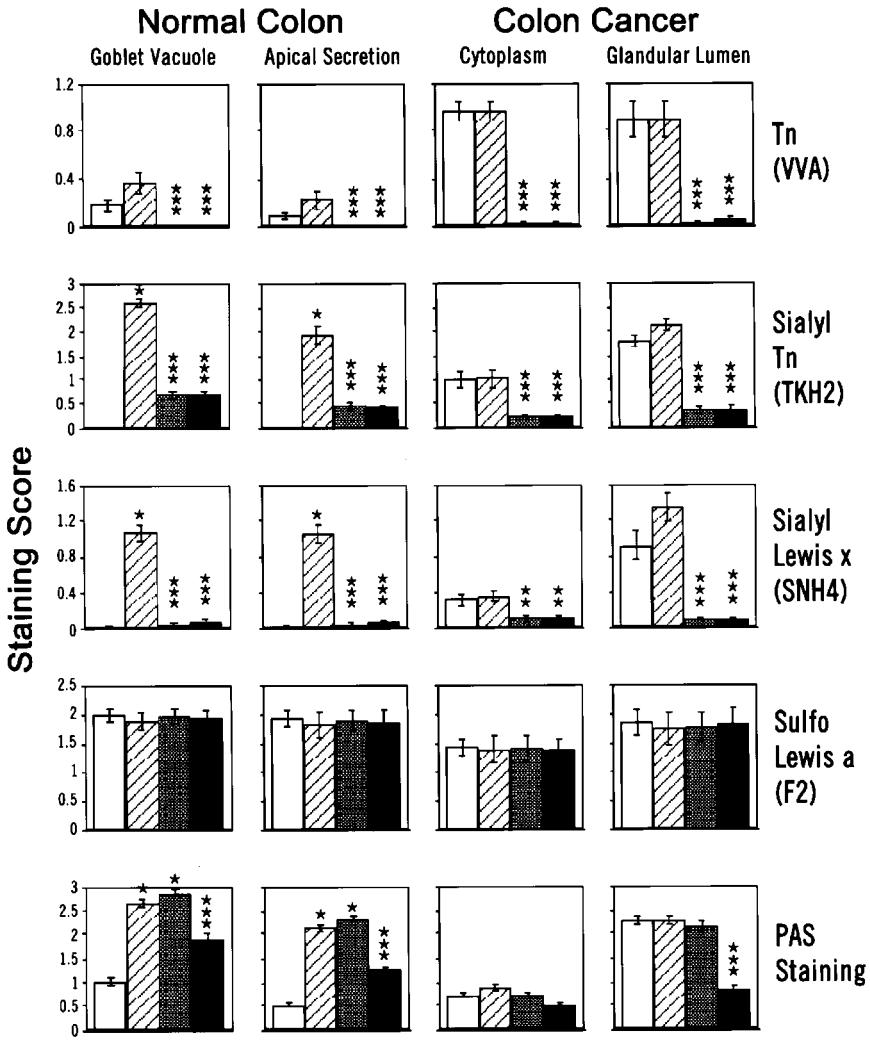

Figure 6. Carbohydrate antigen expression in normal colon and colon cancer. The specimens were: untreated (open bar), DA treated (stippled bar), DA + PO treated (shaded bar), or DA + PO + BE treated (solid bar). Statistical significance versus untreated specimens. The number of specimens were 15 for both normal colon and colon cancer. Mean ± S.E.M. (* = $p < 0.01$) Statistical significance versus DA treated specimens. (** = $p < 0.05$, *** = $p < 0.01$).

can in part be explained by the relatively low percentage of hydroxy-amino acids, which are potential O-glycosylation sites, in the tandem repeat region of MUC1 (25%) compared to the tandem repeat region of other apomucins (60–70%) [1,2].

Our novel method of deglycosylating mucin glycoproteins in paraffin embedded sections takes advantage of the oxidative cleavage of carbon-carbon bonds between unsubstituted C3 and C4 of GalNAc residues by periodic acid (Figure 1) [15,16]. The chemistry of the resulting aldehyde functional group causes the hydrogen on C2 to become acidic, making the entire carbohydrate moiety susceptible to β-elimination with mild base treatment [15,16]. Studies on the composition and type of oligosaccharide side chains present on mucin glycoproteins [30–33] have shown that Tn and sialyl-Tn are the only structures present in detectable amounts anchored to the polypeptide via unsubstituted GalNAc at C3 and C4. These studies have also shown that sialylated and sulfated Lewis structures tend to reside at the terminal end of side chains anchored to the protein backbone via GalNAc with substituents at C3 and/or C4. The recent study of porcine submaxillary mucin also indicates that O-glycan structures in the tandem repeat domain of apomucin are not uniformly distributed and that their length can be modulated by peptide sequences [34]. They further showed that Tn and sialyl Tn structures were selectively removed by periodate and β-elimination. Thus, it could appear from these studies that while Tn and sialyl Tn structures may be preferentially removed by alkali-catalyzed β-elimination of periodate-oxidized glycans, the Lewis structures anchored via substituted GalNAc should remain attached. Four important observations from our data on the immunostaining pattern of carbohydrate antigens after each step of our deglycosylation method tends to support this conclusion. 1) Immunostaining of

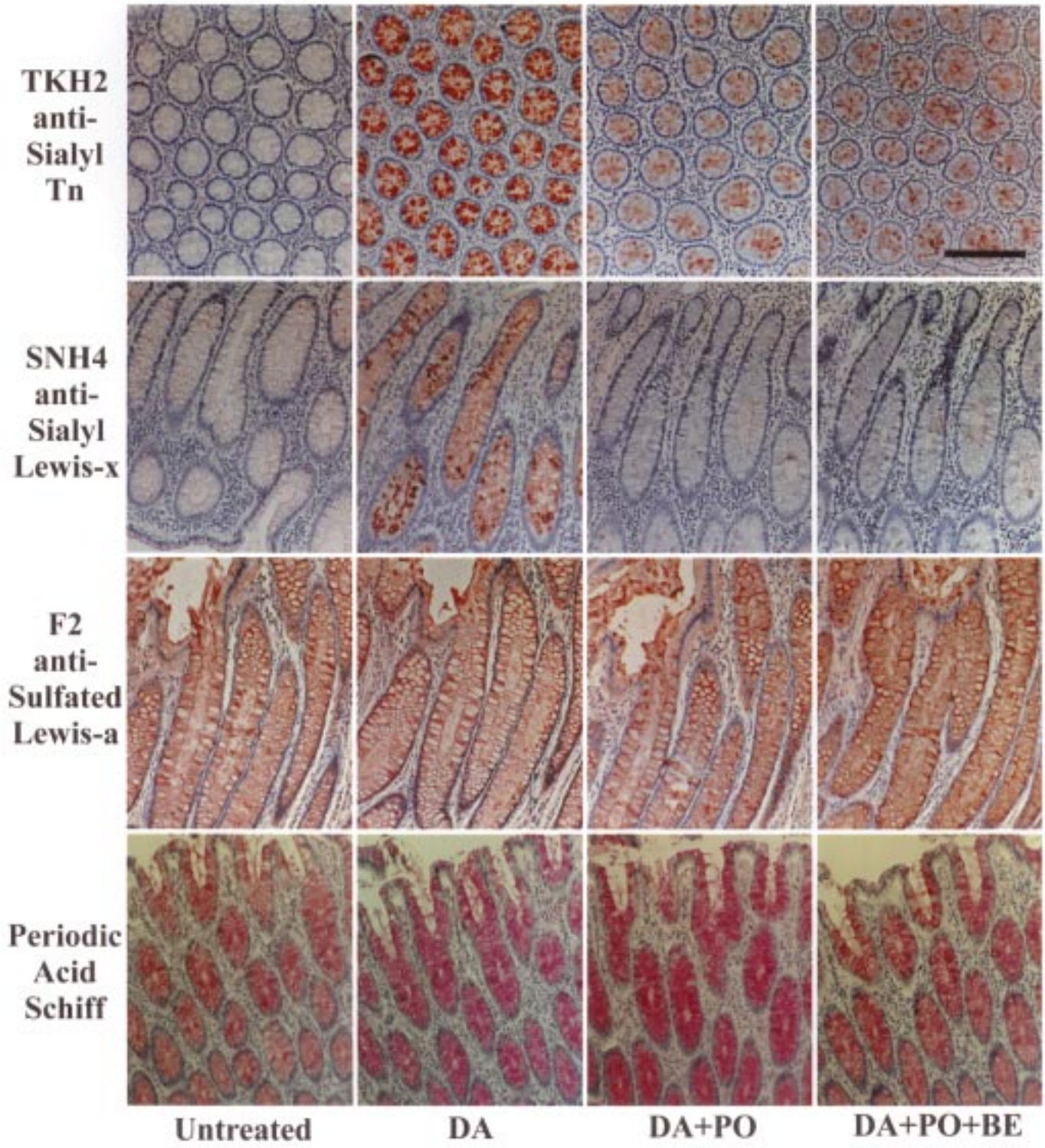

Figure 7. Immunostaining of carbohydrate epitopes in normal colonic mucosa. Short and long chain carbohydrate antigens sialyl-Tn and sialyl-Le^x were unmasked after saponification with DA treatment. These antigens were then destroyed with DA + PO treatment. Immunostaining with mAb F2, which recognizes both sulfo-Le^a and its periodate oxidized product, was not reduced even after DA + PO + BE treatment suggesting that long carbohydrate side chains were not removed by our method. PAS staining was markedly enhanced after DA treatment as a result of increased periodate sensitive bonds due to saponification. After DA + PO + BE, PAS staining decreased but was not abolished, suggesting that a portion but not all of the carbohydrate side chains was removed by our method. Bar represents 100 μm.

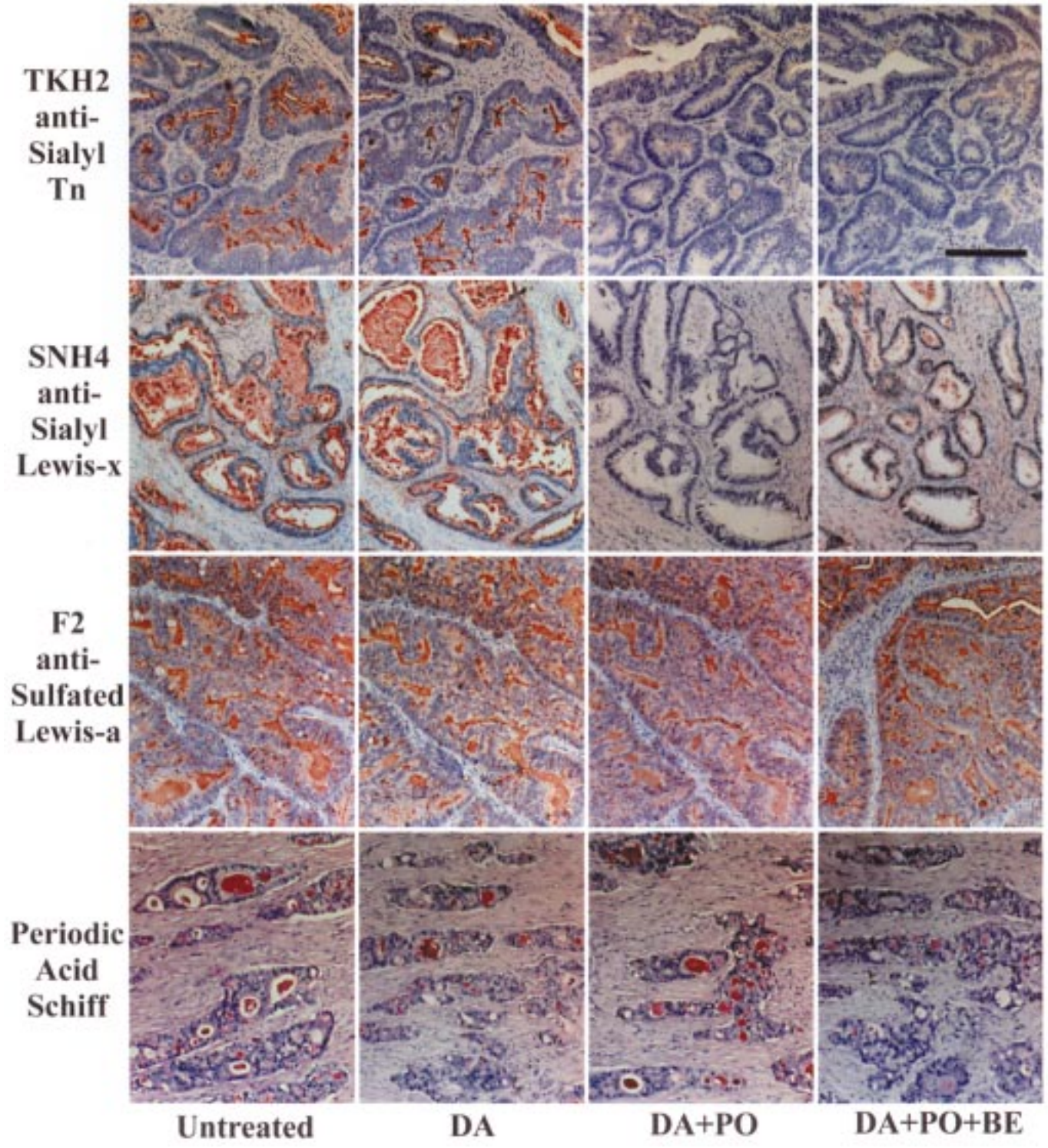

Figure 8. Immunostaining of carbohydrate epitopes in colon cancer. Staining of carbohydrate antigens sialyl-Tn and sialyl-Le^x were markedly decreased by DA + PO treatment. Again there was no change in the staining of sulfo-Le^a with mAb F2 after DA + PO + BE treatment suggesting that long chains were not removed. PAS staining after DA + PO + BE treatment decreased, again suggesting that some side chains were removed by our method. Bar represents 100 μm.

Tn and sialyl Tn antigens were nearly abolished after DA + PO treatment confirming that they were oxidized by periodate, resulting in a product susceptible to *β*-elimination. 2) In both normal and colon cancer, there was a marked

decrease in the PAS staining score between DA + PO treated and DA + PO + BE treated specimens. This suggests that removal of some carbohydrates took place after β-elimination. 3) The immunostaining pattern with MAb F2, which is insensitive to the fucose residue within the sulfated-Lewis[a] structure and thus recognizes both the native and periodate oxidized product [22], was unchanged after DA + PO + BE treatment. This suggests that oligosaccharide side chains anchored via C3 and/or C4 substituted GalNAc are not removed. 4) It is well documented that differential glycosylation of mucins exists in colorectal cancer resulting in increased Tn and sialyl Tn expression [35–38]. Similarly, PAS staining score of colon cancer specimens after DA + PO + BE treatment decreased by 65% compared to DA + PO treatment alone. In the normal colon, the decrease was only 35%, suggesting that Tn and sialyl Tn may be the preferential structure removed by our method.

Using alkali-catalyzed β-elimination of periodate-oxidized glycans, we were able to enhance the expression of all tandem repeat peptide epitopes of apomucins we examined. Our results for MUC1 showed significant unmasking of the apomucin tandem repeat peptide epitope after DA + PO treatment alone as observed by other investigators. More importantly, DA + PO + BE treatment did not further enhance MUC1 staining in both normal and colon cancer. This suggests that steric hindrance plays a significant role in the masking of the MUC1 epitope recognized by MAb 139H2. Recently it has been reported that some MUC1 apomucin antibodies show an increased reactivity towards Tn and sialyl Tn glycosylated MUC1 tandem repeat [34]. It is therefore possible that increased reactivity of the MAb 139H2 observed after PO alone may reflect some sort of unique recognition of the antibody to oxidized MUC1 tandem repeat. Immunostaining using antibodies directed against the tandem repeat sequence of MUC2 and MUC3 exhibited a supranuclear staining pattern of goblet cells in the normal colonic mucosa [4]. After post-translational modifications in the Golgi stacks involving heavy glycosylation, these epitopes became cryptic as evident in the lack of staining in the goblet vacuoles and apical secretion. Although DA + PO treatment did not expose these epitopes, DA+ + PO + BE treatment significantly unmasked these epitopes in MUC2 and MUC3, resulting in intense staining of goblet vacuoles and apical secretion. This strongly suggests that in MUC1 apomucin, steric hindrance by carbohydrate side chains is mainly responsible for masking the apomucin epitope whereas in MUC2 and MUC3, removal of carbohydrates may be required to expose the apomucin epitope. However, the possible role of steric hindrance by carbohydrate side chains of MUC2 and MUC3 cannot be ruled out.

In the glandular lumen of colon cancer specimens, we noticed a subtle yet modest increase in the staining of MUC2 and MUC3 apomucins after DA + PO treatment alone. These results are consistent with our current understanding of cancer associated alterations in the carbohydrate moieties of mucins. As mentioned, differential glycosylation of mucins exists in colon cancer, arising in part from incomplete synthesis of oligosaccharide side chains and decreased glycosyltransferase activity [39,40]. This results in increased apomucin exposure, shorter oligosaccharide side chains, and the appearance of core region carbohydrates Tn and sialyl Tn which are masked in normal tissue [37,38]. Although DA + PO + BE treatment results in significant unmasking of MUC2 and MUC3 apomucins, periodate oxidation alone can cause limited unmasking due to the fewer and shorter oligosaccharide side chains in colonic cancer mucin. Interestingly, in the cytoplasm of colon cancer cells where there is already a moderate staining of MUC1, MUC2, and MUC3 apomucins, β-elimination did not further unmask these epitopes. We speculate that the apomucins in the cytoplasm are sparsely glycosylated and that periodate mediated β-elimination cannot or can only minimally further enhance staining. While intracellular mucin in cancer cells did not become unmasked by DA + PO and DA + PO + BE, extracellular secreted apomucin did become unmasked, indicating that even in cancer cells, additional glycosylation may act as a signal for exocytosis.

To summarize, alkali-catalyzed β-elimination of periodate-oxidized glycans significantly unmasks apomucin epitopes by removing carbohydrate side chains linked via unsubstituted GalNAc at C3 and C4. Using this novel method, previously cryptic MUC2 and MUC3 epitopes became exposed concomitant with the loss of carbohydrates with minimal peptide core degradation. Our data on carbohydrate antigens suggest that shorter carbohydrate side chains such as Tn and sialyl Tn may be the structures preferentially removed by this method. Our results also indicate that alkali-catalyzed β-elimination of periodate-oxidized glycans is an excellent tool for examining changes in glycosylation, detecting cryptic antigens, and determining the tissue and cellular expression of mucin gene products in normal and neoplastic epithelium in paraffin embedded formalin-fixed tissue sections. Because the family of mucin gene products all contain similar O-glycosidic linkages [2], we expect this chemical deglycosylation method will be useful in unmasking other products of the mucin gene family in normal and pathological tissues. Although we focused our study only on colon cancer, cancers involving other organs and various other disease states such as inflammatory bowel disease, chronic bronchitis, cystic fibrosis and cholelithiasis have also been suggested to have altered expression of mucin genes [1,2,7,8]. While *in situ* hybridization has helped to elucidate quantitative and qualitative alterations in the mucin gene expression in the tissues of these disease states, it has been difficult to demonstrate the level of expression of mucin gene products using immunohistochemical methods due to heavy glycosylation. Since there are limited studies available on the correlation between mucin mRNA expression and mucin glycoprotein expression, we feel this novel method, which can be used in formalin-fixed paraffin embedded archival tissues, will be important in resolving these issues.

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