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ARTICLE

Lewis x is Highly Expressed in Normal Tissues: a Comparative Immunohistochemical Study and Literature Revision

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An immunohistochemical analysis was employed to determine the expression of carbohydrate antigens associated to mucins in normal epithelia. Tissue samples were obtained as biopsies from normal breast (18), colon (35) and oral cavity mucosa (8). The following carbohydrate epitopes were studied: sialyl-Lewis x, Lewis x, Lewis y, Tn hapten, sialyl-Tn and Thomsen-Friedenreich antigen. Mucins were also studied employing antibodies against MUC1, MUC2, MUC4, MUC5AC, MUC6 and also normal colonic glycolipid. Statistical analysis was performed and Kendall correlations were obtained. Lewis x showed an apical pattern mainly at plasma membrane, although cytoplasmic staining was also found in most samples. TF, Tn and sTn haptens were detected in few specimens, while sLewis x was found in oral mucosa and breast tissue. Also, normal breast expressed MUC1 at a high percentage, whereas MUC4 was observed in a small number of samples. Colon specimens mainly expressed MUC2 and MUC1, while most oral mucosa samples expressed MUC4 and MUC1. A positive correlation between MUC1VNTR and TF epitope (τ=0.396) was found in breast samples, while in colon specimens MUC2 and colonic glycolipid versus Lewis x were statistically significantly correlated (τ=0.28 and τ=0.29, respectively). As a conclusion, a defined carbohydrate epitope expression is not exclusive of normal tissue or a determined localization, and it is possible to assume that different glycoproteins and glycolipids may be carriers of carbohydrate antigens depending on the tissue localization considered. (Pathology Oncology Research Vol 13, No 2, 130–138)

Key words: carbohydrate epitopes, mucins, normal epithelia, immunohistochemistry

Introduction

It is known that mucins are expressed in a tissue-specific manner; studies have been mainly focused on neoplastic tissues which show increased and aberrant expression or, less frequently, loss of expression.[10,58] Increased MUC1 immunoreactivity was observed in malignant tumors of the breast, lung, stomach, pancreas, prostate and ovary.[5,83] In addition, focal aberrant expression of MUC2 and MUC3 epitopes was frequently observed, while other mucins have been related to different malignant tissues.

Mucins are large glycoproteins that can be divided into membrane-bound and secreted types. Nine membrane-bound (MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC16, MUC17 and MUC20) and six secreted mucins (MUC2, MUC5B, MUC5AC, MUC6, MUC7 and MUC19) have been described. The secreted mucins can be subdivided into gel-forming (MUC2, MUC5B, MUC5AC, MUC6 and MUC19) and non-gel-forming mucins (MUC7).[53]

The best characterized of the membrane-associated mucins are MUC1 and sialomucin complex (SMC), the rat homologue of MUC4. These proteins are synthesized as a single polypeptide chain which is cleaved in the ER to generate two subunits that assemble non-covalently as a heterodimer at the cell surface.[45,46] Both MUC1 and SMC can exist either in the membrane-associated or "secreted" forms, with the latter arising by extracellular proteolysis of the heterodimer to release a mucin-like subunit.[57] Whereas the functions of the gel-forming mucins are believed to be primarily protective in nature, the functions of the membrane-associated mucins are less well understood, although recent evidence suggests that they may act as receptors capable of mediating intracellular signaling cascades.[14,52,70] In the case of MUC1, it acts as a docking protein for signaling molecules, while MUC4 acts as a receptor ligand.[33,64]

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MUC5AC and MUC6 are expressed by gastric epithelium; mucus covering the gastric epithelium plays a major role in protection from acidic pH and mechanical aggression. The antral epithelium displays two well-characterized populations of mucous-secreting cells: the superficial epithelium and the deep glands. Cells in the superficial epithelium express MUC5AC in association with type 1 Lewis antigens (Le a and Le b) and produce neutral mucins. It is currently not known if this specific pattern of glycosylation is determined by the primary amino acid sequence of the apomucins or by the fucosyltransferases expressed in each cell type.[49] Mucins contain a unique central, heavily glycosylated serine/threonine-rich domain, comprised of tandem repeating sequences of amino acids and it is this domain that distinguishes one mucin from another.

Mucin *O*-glycosylation is initiated by a transfer of GalNAc (Tn antigen) to threonine residues by pp-GalNAc-Ts41. In the case of MUC2, it is known that it has 14 consecutive and alternating threonine residues in its typical tandem repeat,[41] while MUC1 has a variable number of 20 amino acids (VNTR) which contain five *O*-glycosylation sites (three alternating threonine, and two serine).[32] MUC1 glycosylation has been largely studied; Beum and Cheng[4] pointed out that mucin type glycan biosynthesis begins with the attachment of N-acetylgalactosamine to serine or threonine followed by construction of the remainder of the *O*-glycan unit. Certain sugar residues, such as sialic acid and fucose prevent further elongation of the glycan chain and constitute the peripheral region of the mucin glycan, and they are often found directly attached to one or more reactive sites on the core portion. Furthermore, peripheral structures such as Lewis histo-blood group antigens are attached to the ends of polylactosamine antennae.

Furthermore, aberrant glycosylation of mucins in relation with malignancy has been largely described, and numerous alterations of mucin-associated carbohydrates have been detected in neoplastic epithelial tissues and on circulating mucins in patients with carcinoma.[37] Changes in sialic acid content,[81] fucosylation, branching oligosaccharides[80] and expression of blood group antigens[26,34] have been described. Sialomucins of tumor cells have been shown to express the Galβ1-3GalNAc1 disaccharide (Thomsen-Friedenreich or T antigen oligosaccharide), which in normal cells is usually masked by other carbohydrates.[74] The expression of various sialylated-carbohydrate epitopes may correlate with poor prognosis and enhanced metastatic disease in colorectal and lung carcinomas.[6,75]

Mucin-associated carbohydrate and peptide antigens are currently being investigated for their role in cancer diagnosis, monitoring for progression or metastases, immune suppression and immunotherapy.[9,11,16,23,38,40,41,77] In this sense, knowledge about their expression in normal tissues is crucial, however, systematic correlative studies are scarce. Several groups have examined different carbohydrate epitopes in the context of their studies,[15,16,18,19,38-40,51,56,78] although some reports on studies performed exclusively on normal tissues have also been published.[12,59]

The aim of this study was to evaluate the expression profile of different mucins and carbohydrate-associated antigens of normal mucosa in breast, colon and oral cavity, taking into account whether expression is limited to specific cell types within these tissues.

Table 1. **Antibodies used in this study**

Antigen	*Epitope structure*	*Antibody*	*Isotype and source*	*Producer or reference*
sLewis x	NeuAc2-3Galβ1-4GlcNAcβ1-3Gal-R	KM93	IgM, mouse MAb	Hanai et al[35]
Lewis x	Galβ1-4GlcNAcβ1-3Gal-R	KM380	IgM, mouse MAb	Hanai et al[35]
Lewis y	Gal(α1-2Fuc)β1-4GlcNAc(α1-3Fuc)β1-3Gal-R	C14	IgM, mouse MAb	Brown et al[8]
Tn	GalNAcα-R	HB-Tn1	IgM, mouse MAb	DAKO
sTn	NeuAcα-6 GalNAcα1-R	HB-STn1	IgG1, mouse MAb	DAKO
TF	Galβ1-3GalNAcα1-R	HB-T1	IgM, mouse MAb	DAKO
MUC1VNTR	Arg-Pro-Ala-Pro	C595	IgG3, mouse MAb	Price et al[61]
MUC1CT	SSLSYNTPAVAATSANL (last 17 aa)	CT2	IgG, Armenian hamster MAb	Schroeder et al[70]
MUC1CT	ND	CT33	IgG, rabbit PAb	Croce et al[20]
MUC2	MUC2-GalNAc	PMH1	IgM, mouse MAb	Reis et al[67]
MUC4	ND	anti-MUC4	IgG, rabbit PAb	Ho SB
MUC5AC	VNTR of the MUC5AC apoprotein	CLH2	IgG1, mouse MAb	Reis et al[66]
MUC6	SFQTTTTYPTPSHPQTTLPC	CLH5	IgG1, mouse MAb	Reis et al[68]
CG	ND	C505	IgG, mouse MAb	Price et al[63]
CEA	ND	C363	IgG1, mouse MAb	Price[62]

TF: Thomsen-Friedenreich antigen, CG: colonic glycolipid, CEA: carcinoembryonic antigen, MAb: monoclonal antibody, PAb: polyclonal antibody, ND: not determined

Materials and Methods

Tissue samples

Eighteen, 35 and 8 samples of normal epithelia were obtained from breast, colon and oral cavity, respectively. Breast specimens from reductive breast mastectomy were kindly provided by Dr. A. Barbera, Italiano Hospital, while colon and oral cavity samples were obtained from Dr. G. Ramacciotti, Gastroenterology Unit and Dr. A. Pereyra, Head and Neck Surgery Unit of the "General San Martín" Hospital, respectively, all located in La Plata, Argentina. Normal colon and oral specimens were obtained from biopsy samples; all mucosae showed normal histopathological and cytomorphological findings.All procedures fulfilled the World Medical Association Declaration of Helsinki (Helsinki, Finland, 1964). Informed consent was obtained from all subjects included in this study.

Antibodies

The polyclonal and monoclonal antibodies employed are summarized in *Table 1*.

Immunohistochemistry

The technique was performed according to standard procedures.[17] Specimens were fixed in Methacarn (methanol: chloroform: acetic acid 60:30:10) for 2 hours and then transferred into 70% ethanol until processing in paraffin. Tissues were treated with 10 mM sodium citrate buffer at 100°C for 5 minutes for antigen retrieval. Dewaxed sections were placed in methanol with hydrogen peroxide (0.3%) for 15 min to block endogenous peroxidase activity. After three washes in phosphate-buffered saline (PBS, pH 7.2), sections were blocked for non-specific binding with normal horse serum diluted 1:10 in 1% bovine serum albumin (BSA)/PBS for 15 min and rinsed. Then, sections were incubated overnight at 4°C with the first antibodies. After three washes with PBS, samples were incubated with the secondary antibodies, as follows: in the case of mouse MAbs (*Table 1*), peroxidase-conjugated anti-mouse Igs (Sigma, St. Louis, MO, USA) (1:400) was added, incubated for 60 min and washed in PBS. Samples assayed with CT2 MAb were incubated with biotin-SP-conjugated affinity-purified goat anti-Armenian hamster IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) (1:10000) and then with peroxidase-conjugated streptavidin (Jackson ImmunoResearch) (1:500). In the case of polyclonal antibodies (*Table 1*), peroxidase-conjugated goat anti-rabbit Ig (1:150) was employed. Slides were counterstained with hematoxylin and mounted.

Sections were examined by light microscopy and the antibody staining patterns were scored in a semiquantitative manner.[18,25] Staining intensity was graded as negative (–), low (+), moderate (++), or strong (+++). The number of optical fields in a specimen that were positively stained was expressed as a percentage of the total number of optical fields containing tissue. The staining of cytoplasm, plasma membrane and nucleus was evaluated; cells were considered positive when at least one of these components was stained. The pattern of reaction was classified as linear (membrane reaction), cytoplasmic, or mixed (cytoplasmic and membrane),[17,50,69] and the positive reaction in gland lumen content was identified as cellular debris or secretion. Apical and non-apical reaction was also considered. Oral cavity epithelial samples were further evaluated for the presence of stain at different cell layers: basal, spinous, granular and horny.

Statistical analysis

A multiple nonparametric correlation analysis was performed ($p<0.05$). Kendall tau correlation coefficients were calculated including only those variables that showed variance different from zero in their responses. Calculations were performed with STATISTICA for Windows, StatSoft, Inc. (1998, Tulsa, OK, USA).

Results

The antigen expressions in the tissue samples are summarized in *Table 2*.

Breast samples

Lewis x was expressed in 13/18 (72%) samples; a high intensity in the apical part of cell ducts was detected (*Fig. 1a*). This pattern was found in all positive samples. sLewis x,

Table 2. **Antigen expression in normal samples by immunohistochemistry**[a]

Antigen	*Normal epithelia*		
	Breast	*Colorectal*	*Oral cavity*
Lewis x	13/18 (72%)	26/35 (74%)	4/8 (50%)
sLewis x	6/18 (33%)	1/35 (3%)	4/8 (50%)
Lewis y	5/18 (28%)	0/35 (0%)	3/8 (38%)
Tn	3/18 (17%)	5/35 (14%)	2/8 (25%)
sTn	0/18 (0%)	0/35 (0%)	2/8 (25%)
TF	4/18 (22%)	0/32 (0%)	0/8 (0%)
MUC1VNTR	7/18 (39%)	23/35 (66%)	5/8 (63%)
MUC1CT	16/18(89%)	21/35 (60%)	6/8 (75%)
MUC2	0/18 (0%)	32/35 (91%)	1/8 (13%)
MUC4	5/18 (28%)	1/35 (3%)	8/8 (100%)
MUC5AC	0/18 (0%)	3/34 (9%)	0/8 (0%)
MUC6	0/18 (0%)	0/32 (0%)	3/8(38%)
CG	ND	32/35 (91%)	3/8 (38%)
CEA	0/18	14/35 (40%)	0/8 (0%)

[a]Results are number of positive samples/total (%).

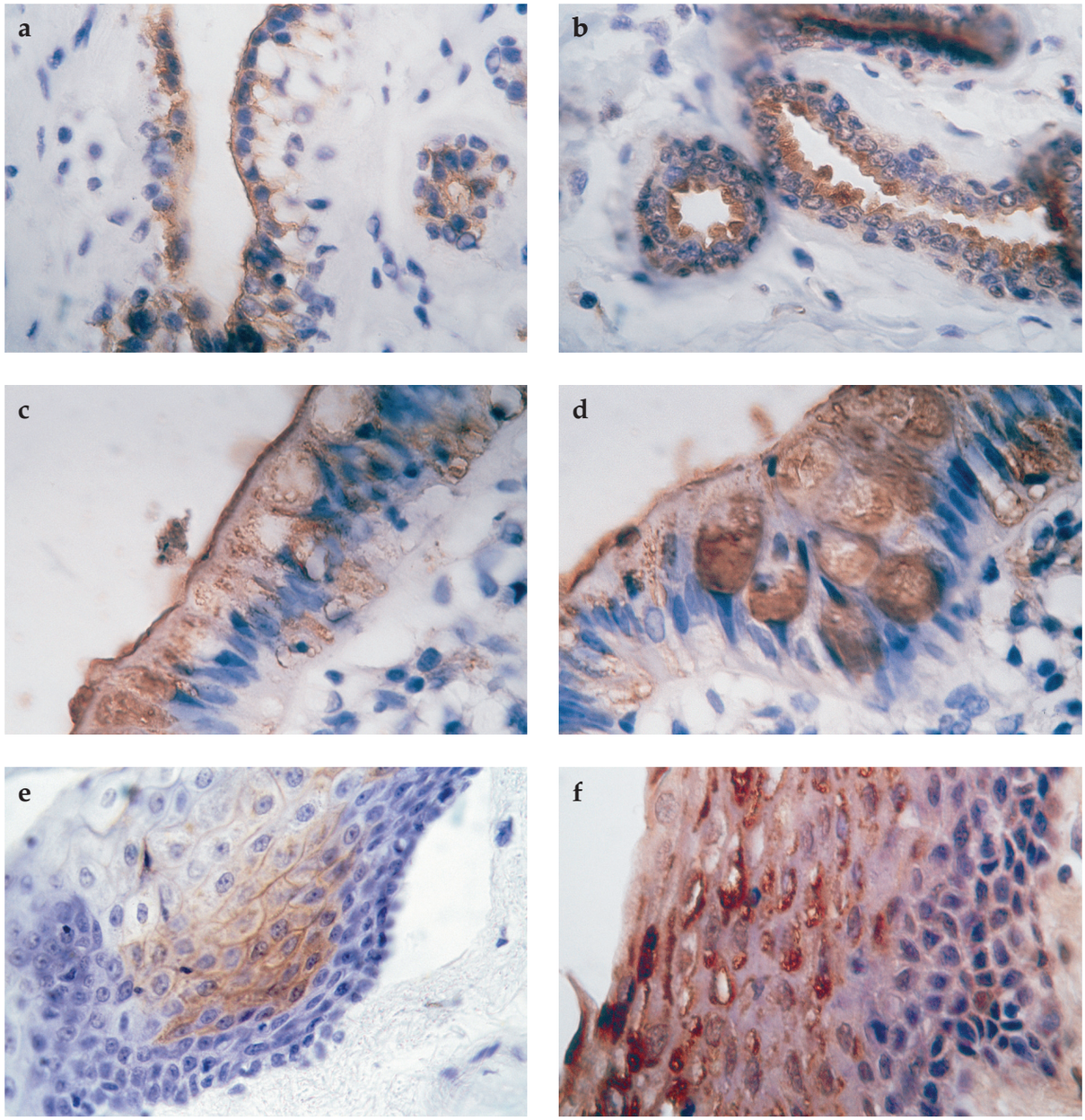

Figure 1. *Immunohistochemical results. The left column shows tissue sections incubated with anti-Lewis x MAb, while the right column shows the reaction with anti-mucin Abs. Expression of Lewis x* ***(a)*** *and MUC1CT* ***(b)*** *in breast sections; positive reaction is observed mainly at the apical membrane and cytoplasm.* ***(c)*** *and* ***(d):*** *colonic sections.* ***(c)*** *Strong positive reaction with anti-Lewis x MAb at the apical membrane and cytoplasm.* ***(d)*** *Positive reaction with anti-MUC2 MAb; goblet and columnar cells show an apical staining at cytoplasm and membrane as well.* ***(e)*** *and* ***(f):*** *oral mucosa samples.* ***(e)*** *Positive reaction with anti-Lewis x MAb; spinous and granular layers are reactive.* ***(f)*** *Strong reaction for MUC4 throughout the mucosa.*

Lewis y, Tn and TF epitopes were detected in some cells belonging to a few samples with a cytoplasmic pattern and a low intensity.

MUC1 detected with both MAbs employed showed a high expression with an apical pattern mainly restricted to the plasma membrane (*Fig. 1b*); the lumen content was also stained. On the contrary, MUC4 was expressed in a few samples with a cytoplasmic pattern with a moderate intensity. The other mucins studied did not show any reaction.

A statistically significant positive correlation between TF and MUC1VNTR staining was found (τ=0.396), with 3 cases positive and 10 cases negative for both parameters. Although not statistically significant, a positive tendency was observed when Lewis x was compared with MUC1CT and MUC1VNTR; 11 samples were positive for MUC1CT and Lewis x and 1 negative for both parameters and, finally, 6 samples were positive and 4 negative for both MUC1VNTR and Lewis x.

Colon samples

Most cells from 26/35 (74%) tissue sections showed a strong reaction with anti-Lewis x MAb; generally, the cytoplasm and the plasma membrane were reactive showing a mixed, apical pattern (*Fig. 1c*). On the other hand, Tn hapten was expressed in a few cases (5/35) with low intensity, restricted to the apical cytoplasm. Only one specimen showed a positive reaction sLewis x, while Lewis y and sTn did not show any reactivity.

As it was expected, MUC2 showed a positive reaction in most samples (32/35, 91%); goblet cells as well as columnar cells were positive in some specimens (*Fig. 1d*). A varied pattern of staining was found: some specimens showed a strong vesicular cytoplasmic reaction, while in several samples the luminal content was also stained. Frequently, cytoplasmic reaction was observed together with a membrane staining in a mixed pattern (*Fig. 1d*). Employing an anti-MUC1VNTR MAb (C595), 23/35 (66%) specimens showed a positive staining with a mixed pattern and a moderate intensity, although several samples stained only at the apical plasma membranes (linear pattern). MUC1CT reacted in 21/35 (60%) samples with a predominant expression at the apical membrane, while in some other cases a mixed pattern was observed. Normal colonic glycolipid (CG) showed a high percentage of reaction (32/35, 91%); most specimens displayed a non-apical mixed reactivity with a strong intensity and a granular pattern. In 20/32 (62%) cases, reaction comprised the entire specimen, and in some of them staining of the luminal content was also observed. Finally, carcinoembryonic antigen (CEA) was detected in 40% (14/35) samples with a non-apical mixed pattern and low intensity.

A statistically significant positive correlation was found between MUC2 and Lewis x (τ=0.28) with 24 cases positive and 2 cases negative for both antigens (n=35). When expression of Lewis x was compared with that of CG, a positive correlation was also found (τ=0.29) with 25 samples positive and 2 negative for both antigens (n=35).

Oral cavity

Anti-Lewis x MAb was reactive in 4/8 (50%) samples and showed variable intensity with a predominant linear pattern. Spinous and granular layers were always reactive (*Fig. 1e*), but some specimens displayed a positive reaction at the stratum corneum. sLewis x epitope was detected in 4/8 (50%) samples, with a frequent reaction of low to moderate intensity, located at the spinous and granular layers; a linear pattern of reaction was always observed. Three out of eight samples were reactive with anti-Lewis y MAb with a low intensity and a predominant linear pattern which was mainly detected at granular and/or corneum layers. Tn and sTn reaction was observed in a few cells of two samples with a cytoplasmic pattern and a low intensity of the basal layer.

An interesting feature is that all samples reacted with anti-MUC4 MAb with a strong staining and a mixed pattern comprising all epithelial layers (*Fig. 1f*). MUC1VNTR expression showed a low intensity and a mixed pattern at the basal layer, while a linear pattern was detected at the stratum corneum. MUC1CT was observed in most samples; in three out of eight, location and pattern of reaction was similar to those of MUC1VNTR. On the other hand, three other samples showed a very intense reaction extended throughout the mucosa.

Finally, other mucins such as MUC6 and MUC2 and also CG were detected in some cells belonging to a few samples; MUC2 showed an intense reaction with a mixed pattern comprising the whole sample while the other MAbs showed a very low intensity staining restricted to spinous and granular layers.

Discussion

The present study showed that the expression of Lewis x is widely distributed in normal breast, colon and oral mucosa with a mixed and apical pattern, co-expressed with different mucins according to the localization. It also revealed a statistically significant correlation between the expression of Lewis x and MUC2 or normal colonic glycolipid in colon specimens, while in breast samples between MUC1VNTR and the TF epitope.

In fact, Lewis x was strongly expressed with the highest percentage of reactivity in normal colonic and breast mucosa. In this sense, Nakagoe et al[54] found similar results; they detected 95.2% (40/42) positive normal colorectal mucosa samples, which was maintained in colorectal carcinomas (90.8%, 79/87). Taking into account the relationship between the expression of Lewis x and MUC2 and also normal colonic glycolipid, it may be possible to speculate that these molecules and also MUC1 could be Lewis x carriers.

Since first reports,[28,79] it is well recognized that Lewis x determinants can be expressed on both cellular glycoprotein and glycolipid molecules. Furthermore, it is possible that interaction between carbohydrates may be due to Lewis x residues.[34]

In accordance with findings of other authors,[13] we detected MUC1 in normal colorectal epithelia; we found

MUC1 and MUC2 staining in both goblet and columnar cells, although other reports[1] described MUC2 as a goblet cell mucin while MUC1 has been found associated to columnar cells. As it has been stated, MUC1 and MUC2 may be carriers for a variety of carbohydrate epitopes on their threonine residues. Accordingly, we have detected some breast samples which were reactive with anti-sLewis x, -Lewis y and -Tn MAbs and a few colorectal specimens stained with anti-sLewis x and Tn.

Controversial findings have been reported regarding the expression of the Thomsen-Friedenreich (TF) antigen in normal specimens when different MAbs were used. Orntoff et al[56] described that normal colon *O*-linked mucin-type glycoproteins express TF antigen which lacks in carcinomas with the accumulation of Tn and sTn antigens. These findings were corroborated by Cao et al,[12] although in an earlier report,[11] they did not find TF expression in normal colorectal mucosae. Related to these antigens, we did not find TF and sTn expression, although a few normal colon specimens were reactive with anti-Tn MAb. In contrast, MUC1VNTR and TF have shown a statistically significant correlation in normal breast samples, although the number of positive TF specimens was only four out of 18. In 5 samples, Cao et al[12] did not find positive staining with anti-TF G/A7 and HH8 MAbs.

Finally, in breast specimens, it has been observed that MUC1CT showed a higher expression compared to MUC1VNTR. This observation was expected since MUC1VNTR may be shed into the extracellular space,[31,44,3,72] and we have found similar results in an earlier report.[20]

The immunocytolocalization pattern in colon and breast specimens was mainly linear, but some cells showed a diffuse cytoplasmic staining which, according with Reis et al,[68] may be due to the variability of the type/rate of glycosylation in different cells.

Diverse reports have been published in relation to MUC1VNTR epitope expression in oral normal epithelia. Fernandez et al[27] and Tatemoto et al[76] have reported a very low expression of MUC1VNTR in superficial layers, while Jeannon et al[43] found high levels (85%) of MUC1, employing an antibody reactive with a carbohydrate epitope associated to mucin (Ma695). Other authors,[42,55] employing anti-MUC1 DF3 MAb, did not find MUC1VNTR expression, while Sengupta et al[71] found MUC1 associated to minor salivary gland ducts and also to the surface part of normal oral epithelia. We present here that MUC1 protein core and cytoplasmic tail are expressed at basal and horny layers.

Liu et al[47] demonstrated the presence of transcripts for MUC1 and MUC4 in both parotid and submandibular glands and *in situ* hybridization localized these transcripts in epithelial cells lining ducts and in some serous acinar cells. They also detected soluble forms of both mucins in parotid secretion after immunoprecipitation with mucin-specific antibodies. Weed et al[82] identified MUC4 by immunocytochemical staining throughout the normal upper aerodigestive mucosa. In this localization, it is possible that both MUC4 and MUC1 may be Lewis x carriers; Spielman et al[73] reported that in rat mammary ascites tumor cell sublines derived from a carcinogen-induced solid tumor, the extracellular domain of MUC4 is mainly formed by carbohydrates (70%) the main components of which are core 2 structures and sialylated derivatives.

In this report, we have also detected other Lewis antigens; sLewis x[29] was expressed in normal oral cavity, in some breast samples and in one colon specimen while Lewis y was detected in breast and oral tissues. sLewis x has been largely associated to metastatic behavior in lung, colorectal and breast carcinoma [6,69,75] and, for colon carcinoma, a switch from core 3-based polylactosamine-*O*-glycans to shorter core 2-based oligosaccharides has been reported.[7,60] The degree of sialylation increased in these carcinomas, resulting in the formation of sTn antigen, sLewis x and sLewis a.[2] In breast carcinomas there is a switch from preferential core 2-based polylactosamino-glycans to shorter sialylated core 1-based oligosaccharides.[36,48]

In oral mucosa, other authors[22] have found Lewis y present on parabasal cells, whereas in epithelial dysplasias the expression of Lewis y is seen in cell surfaces of the superficial spinous cells, possibly reflecting a lack of normal epithelial differentiation. Some of the aberrant expression patterns of carbohydrate antigens were seen in premalignant lesions without epithelial dysplasia,[9,21] suggesting that histo-blood group antigen changes appear early in the development of malignancy.[65] Studies showed that premalignant lesions that developed later into cancer exhibited a loss of histo-blood group antigen A years before malignant transformation. This fact could be due to allelic loss of the ABO glycosyltransferase-encoding genes and, also, to post-transcriptional down-regulation of the gene transcript may be involved.[30] Some of the changes found in premalignant and malignant lesions are also seen in non-malignant circumstances such as in wound healing.[24] The prognostic value of aberrant histo-blood group antigen expression in oral premalignant lesions is largely unknown.[65] Our results showed a predominant expression of MUC4 and MUC1 mucins in several epithelial layers of the oral cavity. Carbohydrate epitope expression was in relation with cell differentiation through the epithelia. A simple carbohydrate-like Tn (GalNAc-R) was located at the basal layer, while Lewis x, sLewis x and Lewis y stained upper epithelial layers, in particular Lewis y, found at the corneal layer.

Contradictory results have been reported in relation to the carbohydrate and mucin expression in normal samples depending on the MAbs employed. A defined carbo-

hydrate epitope expression is not exclusive of normal tissue or a determined localization, and it is possible that individual mucin genes have distinct patterns of expression within mucin-producing tissues, suggesting that the various mucin gene products may play distinct functional roles. On the other hand, it is possible to assume that different glycoproteins and glycolipids may be carriers of carbohydrate antigens depending on the tissue localization considered.

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