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Assessment of cell surface glycoconjugates in normal, benign and malignant human nasal mucosa*

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SUMMARY

Aberrant glycosylation of proteins is a common characteristic of neoplastic changes. No reports exist relating cell surface glycoconjugates to normal, benign and malignant human nasal mucosa. Using lectin affinity histochemistry, glycoconjugate reactivities for peanut agglutinin (PNA), concanavalin A (Con A), Griffonia simplicifolia agglutinin II (GSA-II), soy bean agglutinin (SBA) and Ulex europaeus agglutinin l (UEA-I) were analysed in the following groups: normal, benign (polyp, papilloma, and inverted papilloma) and malignant (squamous cell carcinoma (SCC) alone, SCC arising in inverted papilloma, and adenocarcinoma). The positive rate of lectin staining was evaluated using a quantitative AutoCAD programme. We correlated glycoconjugate expression to clinical features, diagnosis, and malignant transformation. The positive rate of PNA after neuraminidase pre-treatment (NA-PNA) staining was higher in inverted papilloma, while all-negative in polyp and papilloma. NA-PNA staining may be used as a differential diagnostic tool. Both inverted papilloma portions and SCC portions of the SCC synchronized with inverted papilloma subjects showed similar Con A and NA-PNA staining patterns. The biological characteristics define inverted papilloma as a pre-malignant neoplasm. The positive rate of PNA staining was significantly higher in inverted papilloma (inverted papilloma transformed to SCC) compared to inverted papilloma alone. Hence, PNA staining may predict malignant transformation of inverted papilloma. However, further investigations are required to prove this possibly worthwhile prognostic marker.

Key words: glycoconjugates, nasal cancer, inverted papilloma, nasal polyp, nasal papilloma

INTRODUCTION

Aberrant glycosylation of proteins is a common feature during neoplastic change (Hakomori, 1985; Feizi, 1985). The terminal carbohydrates located on cellular surfaces determine many of the final structural and functional properties of proteins conferring essential biological attributes (Yogeeswaran, 1983). Alteration of these cell surface carbohydrates may accompany malignant transformation resulting in the appearance of tumour-associated antigens. Changes in cell surface glycoconjugate expression may result from the absence of or the activation of new tumour-related glycosyltransferases (Freije et al., 1989). These changes in glycoconjugate expression may directly be related to the histological differentiation of the tumour cells (Lalwani et al., 1996). The carbohydrates may be identified by binding of specific glycoconjugates to oligosaccharides, using labels such as biotinylated lectins and avidin-biotin-peroxidase complexes (Hsu and Raine, 1982).

Cancers of the nasal cavity and paranasal sinuses are relatively uncommon, accounting for less than 0.5% of all incident invasive cancer cases (Hayes et al., 1986). The overall incidence of inverted papilloma of the nasal cavity and paranasal sinuses varies from 0.5% to 4% for all primary nasal tumours (Macdonald et al., 1995). Inverted papilloma is regarded as the pre-malignant lesion because of its high rate of recurrence (13-70%) and the tendency for malignant transformation (3-24%; Bielamowicz et al., 1993). Therefore, it provides an ideal system to study the cancer biology of glycoconjugates. However, no data are available regarding cell-surface glycoconjugate expression in human nasal neoplasms.

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The aim of this study has been to investigate the biological characteristics of cell surface glycoconjugates in benign, pre-malignant and malignant nasal mucosa, using lectin affinity histochemistry. We have correlated these findings to clinical features, diagnoses and malignant transformation.

MATERIAL AND METHODS

This study enrolled the following tissue specimens: normal (15 turbinate mucosa), benign (15 nasal polyps, 15 papilloma, 16 inverted papilloma) and malignant (32 squamous cell carcinoma (SCC) and 10 adenocarcinoma) subjects. Of the inverted papilloma subjects, there were 15 males and 1 female, aged from 38-67 years. Six of the inverted papilloma subjects were pre-operatively diagnosed with nasal polyps. However, post-operative specimens were diagnosed as being inverted papilloma synchronized with polyps subjects. Of the malignant subjects, there were 35 males and 7 females with ages ranging from 35 to 72 years. Six of 32 SCC subjects had inverted papilloma alone documented in the surgical specimens (not merely an intranasal biopsy) and upon recurrence obtained SCC (inverted papilloma transformed SCC; *metachronous carcinoma*). The interval between onset of inverted papilloma and onset of SCC ranged from 2 to 8 years. Another 6 of them showed both SCC and inverted papilloma in the same surgical specimens (*synchronous carcinoma*; SCC synchronized inverted papilloma subjects). The SCC arising in inverted papilloma subjects consisted of both metachronous (6) and synchronous carcinoma (6) subjects. The pathological criteria used to establish the diagnosis "inverted papilloma" were those described by Hyams (1971).

Table 1. A panel of glycoconjugates including sugar specificities and inhibitory sugars (Gal, galactose; GalNAc, N-acetylgalactosamine).

lectin	abbreviation	major sugar specificities	inhibitory sugar
Canavalia ensiformis	Con A	mannose>glucose	mannose
Griffonia simplicifolia II	GSA-II	N-acetylglucosamine	N-acetyl-glucosamine
peanut agglutinin	PNA	Gal(1,3)GalNAc	D-galactose
soybean agglutinin	SBA	α-D-GalNAc	D-GalNAc
Ulex europaeus I	UEA-I	α-L-fucose	L-fucose

Lectin affinity histochemistry

Formalin-fixed, paraffin-embedded tissue blocks were prepared for this study. Serial sections were cut using a microtome set at 5 μm. These sections were mounted on glass slides pre-treated with acid alcohol. The sections were dried, deparaffinized in xylene and rinsed in 100% ethanol. Endogenous peroxidase activity was blocked by using 0.3% hydrogen peroxide in 0.01 M phosphate-buffered saline (PBS; pH 7.5). They were placed in a moist chamber for 30 min at 37°C. The sections were then treated with 0.2% bovine serum albumin in 0.05 M Tris-buffered saline (TBS; pH 7.5.) for 20 min. After washing with PBS, they were incubated with 50 mg/ml of biotinylated lectins (Sigma, St. Louis, USA; cf. Table 1) for 2 h and washed in PBS for 10 min. Next, the sections were incubated in freshly prepared avidin-biotin-peroxidase complex (ABC; Vector Laboratories) for 30 min. Visualization was achieved by using a developing solution consisting of 0.05% 3',5'-diaminobenzidine (DAB) in TBS (pH 7.5). Lectin binding specificity was verified by blocking with the various specific sugars (at concentrations of 0.2 M) for 20 min prior to incubation (negative controls). The histochemical reactions were defined by microscopical observation of the sections for the presence of oxidized DAB, a brown precipitate.

The penultimate sugar residue to sialic acid was detected by treatment of the sections with neuraminidase (NA; Sigma, St. Louis, USA) followed by PNA incubation (NA-PNA; Bocker et al., 1984). First, the sections were incubated in 0.1 IU/ml neuraminidase prepared in 1% sodium chloride dissolved in 0.05 M sodium acetate buffer (pH 5) for 1 h at 37°C. Then, the sections were washed in PBS for 10 min prior to biotinylated lectin staining.

Evaluation of lectin affinity histochemistry

The positive rate of glycoconjugates staining was evaluated using the quantitative software in AutoCAD programme system (Shin et al., 1994; Urakami et al., 1996). The positive rate of staining was expressed as the mean percentage of epithelial or tumour cells exhibiting positive staining from the total number measured in at least 30 different high-power fields (×200). All numerical data were expressed as mean±SED. We define positive staining for glycoconjugates as distinct staining in >10% of the epithelial cells (Cagle et al., 1994).

Statistical analyses

Comparisons were made by using the Chi-square test along with Yate's correction. The significance level was $p<0.05$.

Table 2. The percentage of positive glycoconjugates staining in specimens from normal, benign and malignant nasal mucosa (number of cases in parentheses).

tissue type	UEA-I	Con A	PNA	NA-PNA	SBA	GSA-II
nasal cancer						
SCC (32)	50	100	72	100	0	0
adenocarcinoma (10)	50	100	80	100	0	0
non-malignant lesions						
IP (16)	75	75	44	88	0	0
papilloma (15)	80	40	0	0	0	0
polyp (15)	100	20	0	0	0	0
normal mucosa						
inferior turbinate (15)	100	0	0	0	0	0

RESULTS

The percentage of positive staining in normal, non-malignant and malignant nasal tissue subjects is illustrated in Table 2. Both SBA and GSA-II failed to stain any of the nasal epithelial tissue. Only 75-50% of the inverted papilloma and SCC subjects showed a positive staining for UEA-I. The epithelium of normal, polyp and papilloma subjects stained weakly for Con A, as compared to malignant subjects. The frequency of positive PNA or NA-PNA staining was higher in inverted papilloma and malignant subjects (Figure 1), whereas they were all-negative in the normal, polyp, and papilloma groups.

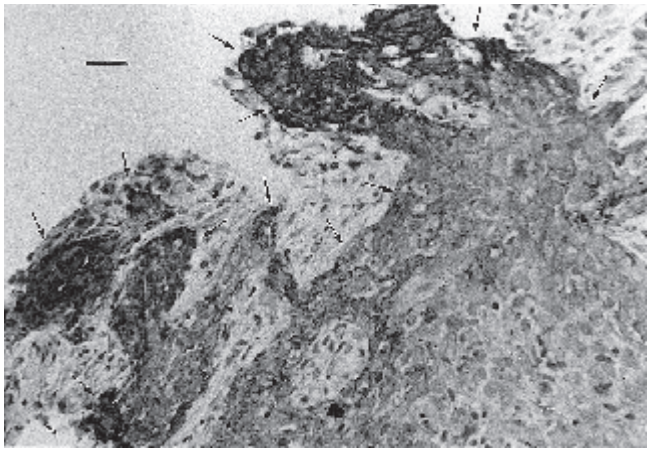

Figure 1. SCC subject exhibiting positive NA-PNA staining of the neoplastic cells (scale bar: 45 μm).

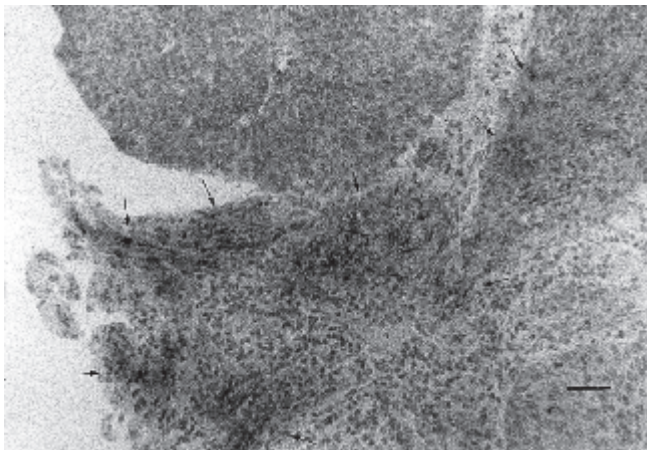

Figure 2. Positive PNA staining (arrows) is noted in this inverted papilloma (inverted papilloma transformed to SCC) subject. This patient was diagnosed with SCC 7 years later (scale bar: 25 μm).

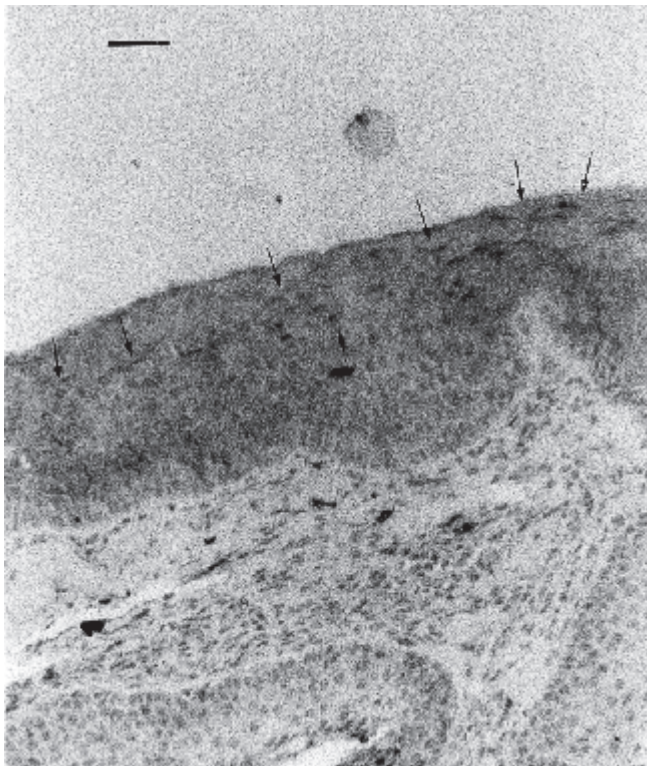

Figure 3. Positive NA-PNA staining (arrows) is observed in the epithelium of this inverted papilloma subject (scale bar: 25 μm).

Table 3. Comparison of positive lectin staining in SCC alone and SCC arising in inverted papilloma subjects (number of cases in brackets; percentage in parentheses).

	Con A	PNA	NA-PNA
SCC alone [20]	20 (100%)	14 (70%)	20 (100%)
SCC arising in IP [12]			
SCC (from IP) [6]	6 (100%)	5 (83%)	6 (100%)
SCC synch. IP [6]			
SCC portion	6 (100%)	4 (67%)	6 (100%)
IP portion	6 (100%)	2 (33%)	6 (100%)

Table 4. Comparison of positive PNA and NA-PNA staining in human nasal polyps and inverted papilloma subjects (number of cases in parentheses. Positive rate of staining is expressed as mean ±SED *: significant difference ($p<0.05$) as compared to inverted papilloma alone. †: significant difference ($p<0.01$) as compared to polyp).

	PNA	positive rate	NA-PNA	positive rate
polyp (15)	0	6.78 [2.43]	0	5.83 [1.75]
IP synch. polyp (6):				
polyp portion	0	5.62 [1.32]	5 (83%)	47.5 [8.45]†
IP portion	2 (33%)	25.6 [8.96]	5 (83%)	53.9 [7.49]†
IP alone (10)	4 (40%)	34.5 [8.36]	9 (90%)	51.6 [10.3]†
IP (IP trans. SCC) (6)	5 (83%)	69.3 [10.8]*	5 (83%)	62.7 [9.63]†

The characteristics of positive glycoconjugate staining for SCC alone and SCC arising in inverted papilloma subjects are represented in Table 3. Both SCC portion and inverted papilloma portion of SCC synchronized with inverted papilloma subjects showed similar Con A and NA-PNA staining patterns. There were no significant differences in lectin reactivity between SCC alone and SCC arising in inverted papilloma subjects.

The characteristics of positive PNA and NA-PNA staining in human nasal papilloma, polyp, inverted papilloma synchronized with polyps, inverted papilloma and inverted papilloma (inverted papilloma transformed into SCC) subjects are illustrated in Table 4. There was a significant increase in PNA reactivity in inverted papilloma (inverted papilloma transformed into SCC) subjects (Figure 2), as compared to inverted papilloma alone or inverted papilloma synchronized with polyps subjects. NA-PNA reactivity was significantly higher in all inverted papilloma subjects (Figure 3), as compared to polyps and papilloma subjects.

DISCUSSION

Glycoconjugates have been used extensively to study alterations in cell surface carbohydrates associated with malignant transformation (Cummings, 1994). Cell surface glycoproteins with heterogeneous glycosyl residues play an important role in the regulation of cell proliferation and epithelial growth. They are often altered in neoplastic cells and aberrantly proliferating cells (Hakomori, 1985). They are also ideal molecular probes for studying tumour histogenesis (Miettinen et al. 1983), heterogeneity of tumour cell populations (Bresalier et al., 1985) and the relationship of tumours to inflammatory and pre-neoplastic conditions (Cooper et al., 1987). In this study, histochemical staining methods were applied to formalin fixed, paraffin-embedded tissues in order to provide us with the advantage of

higher sensitivity (Hsu and Raine, 1982) and more detailed morphology (Kuhlmann et al., 1983; Wirbel et al., 1984).

There were no positive staining controls in the study. Thus, negative staining caused by technical error should be considered. We repeated these specimens with different positive specimens to avoid a false-negative result. Glycoconjugate reactivity of nasal mucosa epithelial cells showed a wide reactive range. The staining characteristics of glycoconjugates were similar to p53 immunostaining (Fang et al., 1997). It is important for analysing these histochemical data to define a positivity of staining using the positive rate of staining. Thus, positivity was defined as positive rate over 10% according to the definition of Cagle et al. (1994). Both SBA and GSA-II reactivity were confined to the cell surfaces of the epidermis cells.

Both SBA and GSA-II did not stain any of the nasal epithelial tissue subjects. This suggests that the N-acetylgalactosamine and N-acetylglucosamine moieties are not expressed on the cellular surface of either epithelial or malignant cells (Leathem and Atkins, 1983). UEA-I is known to stain the respiratory epithelium in normal and polyp subjects. In the papilloma, inverted papilloma and malignant groups even less UEA-I staining was present.

An increasing trend for positive Con A staining was observed for benign subjects (polyp, papilloma, and inverted papilloma) followed by malignant subjects. This trend is due to an increase expression of Con-A-reactive cell-surface D-mannose units or a change in the distribution of ligand-binding sites, which may follow neoplastic transformation of nasal epithelial cells (Reano et al., 1982). This is similar to data presented by Huang et al. (1994). Seventy-five per cent of the inverted papilloma subjects showed positive Con A staining. The characteristics of tumour recurrence and progression may be associated with Con A staining (Campo et al., 1988; Freije et al., 1989); however, the prediction of recurrence for inverted papilloma cannot be made by microscopical examination (Hyams, 1971; Batsakis, 1979).

The staining patterns between the inverted papilloma portions and SCC portions of SCC synchronized with inverted papilloma subjects are similar for Con A and NA-PNA. These biological characteristics support the hypothesis that inverted papilloma is a pre-malignant lesion of SCC (Suh et al., 1977; Feinmesser et al., 1985; Nielsen et al., 1991). A better prognosis is described for SCC arising in inverted papilloma compared to SCC alone (Lavertu et al., 1989; Benninger et al., 1990). However, no characteristic differences in lectin staining existed between these two subject groups.

PNA staining is predominantly intracellular and probably reflects a change in the synthesis of mucus by the Golgi apparatus. An increased expression of galactose-related sugars has also been observed following malignant transformation (Cooper et al., 1987; Bocker et al., 1984). This is shown by the increased staining of PNA prior to NA treatment. Neuraminidase pretreatment may permit detection of the Thomsen-Friedenreich antigen (T-antigen) by removing the terminal sialic acid exposing Galß1→3GalNAc or Galß1→4GalNAc residues (Bocker et al., 1984). A significant increase in positive PNA staining was noted in malignant subjects. This is similar to previous data showing that laryngeal carcinoma, oral carcinoma, bladder carcinoma, colon carcinoma and breast cancer stain intensely, while non-neoplastic tissue stains weakly or not at all using PNA (Bocker et al., 1984). In inverted papilloma and SCC tissue, positive PNA staining is indicative of the T-antigen being detected. This reflects the loss of sialic acid during neoplastic change. According to their biological characteristics polyps and papilloma are benign lesions, while inverted papilloma possesses tumorigenic qualities (Newman et al., 1979; Feinmesser et al., 1985). The inverted papilloma synchronized with polyp subjects showed a lower PNA reactivity, compared to inverted papilloma alone or inverted papilloma (inverted papilloma transformed into SCC) subjects. The biological characteristics may support the suggestion that inverted papilloma synchronized with polyp is more stable and has a lower tendency for malignant change (Nielsen et al., 1991). The positive rate of PNA staining in inverted papilloma (inverted papilloma transformed into SCC) was significantly higher than in the inverted papilloma alone group. A strongly positive PNA staining may characterize inverted papilloma subjects who possess the potential for transformation into SCC. This may indicate that the cells, although demonstrating a normal histology, may already have undergone some neoplastic changes. Previous data has shown that strong PNA staining may be associated with an early stage of head-and-neck carcinoma (Lalwani et al., 1996). Hence, PNA staining may be used as a worthwhile prognostic marker. However, further investigations are required to prove its prognostic relevance.

NA-PNA staining showed similar patterns between the polyp portion and inverted papilloma portions of inverted papilloma synchronized with polyp subjects. This may partially support the theory that inverted papilloma originates from ordinary nasal polyps via epithelial proliferation (Ringertz, 1938). However, most scientists regard inverted papilloma as a true neoplasm, distinct from ordinary nasal polyps (Osborn, 1970; Norris, 1963; Suh et al., 1977). A significantly higher positive rate of NA-PNA staining was noted in inverted papilloma, compared to polyp and papilloma subjects. Thus, NA-PNA staining could assist in solving the clinical dilemma of differential diagnosis regarding polyp, papilloma or inverted papilloma. This may result in an earlier detection of tissue presenting neoplastic characteristics and an early treatment.

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