SPANDIDOS PUBLICATIONS

Sialylation of intracellular proteins of thyroid lesions

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Abstract. Enhanced sialylation represents one of the most frequently occurring alterations of the sugar chain structure in various cancers. However, up to now, sialylation of intracellular proteins of thyroid carcinomas has never been investigated. The aim of this study was comparative analysis of cytoplasmic and nuclear sialoglycoproteins isolated from thyroid benign and malignant tumors as well as non-neoplastic lesions. The sialylation level and types of sialic acid linkages were analysed by lectin blotting and enzyme linked lectino-solid-phase assay (ELLSA) using *Sambucus nigra* (SNA) and *Maakia amurensis* (MAA) agglutinins. The presence of α2,6 and α2,3 linked sialic acid residues was detected in all types of thyroid lesion specimens but there were some differences in the banding and intensity patterns. Analysis of SNA and MAA binding by ELLSA method showed that in the majority of cancer samples the level of sialic acid residues was lower than in non-neoplastic lesions and adenomas. Our present results suggest that decrease in sialylation rather than increase is a characteristic feature of malignant transformation in the thyroid.

Introduction

Glycosylation is a key post-translational modification of cellular proteins that can influence both, the secondary structure and the function of the protein backbone. Carbohydrate portions of glycoproteins undergo neoplastic alterations, and the changes include an increase in branched asparagine-linked and polylactosamine sugar chains as well as sialylation (1-3). Sialic acid residues are generally found at the non-reducing terminal position of both N- and O-linked oligosaccharides of most cellular glycoproteins. These residues are primarily α2,3 or α2,6 linked to galactose residue, or α2,6 linked to N-acetylgalactosaminyl or N-acetylglucosaminyl residue (4,5). A large number of studies have been carried out on sialylation in various cancerous tissues. Increased sialylation has been proposed to be intimately related not only with tumorigenicity but also with invasiveness and metastatic ability (3,6).

Cellular sialic acid levels are mainly controlled metabolically by sialyltransferases and sialidases. Changes in the expression of sialyltransferases have been observed in cancer cells and the regulation of their expression is achieved mainly at the transcriptional level (6,7). ß-galactoside α2,6-sialyltransferase I (ST6Gal I) is one of the most frequently altered glycosylotransferases in human cancers and in some malignancies, such as colon cancer, its increased expression concerns nearly 100% of the patients (8,9). Harduin-Lepers *et al* (6) have found that most breast tumors preferentially expressed ß-galactoside α2,3-sialyltransferase III (ST3Gal III), particularly in large tumors and its high expression was associated with reduced overall survival. mRNA expression of ST6Gal I was enhanced in sqamous cell carcinoma of the cervix but sialyltransferases responsible for α2,3 linkage of sialic acid residues, i.e. ST3Gal I, ST3Gal II and ST3Gal IV, were down-regulated in comparison to normal cervix (10).

There are also several reports on the alterations of endogenous sialidase activity in cancers. Martinez-Zorzano *et al* (11) have reported that sialidase activity toward synthetic substrate 4-methylumbelliferyl-N-acetylneuraminic acid was increased in human colon cancer as compared to normal mucosa. Moreover, increased sialidase activity was observed in breast cancer (12). The results of Kakugawa *et al* (13) showed up-regulation of plasma membrane-associated ganglioside sialidase in human colon cancer as compared to adjacent non-tumor mucosa.

The aim of this study was to check whether alterations in sialylation, as a characteristic feature of malignant transformation, concern also thyroid cancers. To characterize the pattern of sialoglycoproteins of human thyroid neoplasms we examined nuclear and cytoplasmic protein fractions for the ability to bind two sialic acid-specific lectins, *Sambucus nigra* agglutinin (SNA) and *Maakia amurensis* agglutinin (MAA) which preferentially recognize α2,6- and α2,3-linked sialic acid residues, respectively.

Materials and methods

Surgical specimens. Surgical specimens were obtained from 116 patients (27 males and 89 females), who underwent surgery for nodular thyroid disease. The mean age of the patients was 50 years (from 11 to 83 years). The studies were performed on 29 specimens of non-neoplastic lesions (nodular goiters),

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30 follicular adenomas, 9 follicular carcinomas and 48 papillary carcinomas (classical type and follicular variant). All cases of follicular carcinoma showed the presence of capsular and vascular invasions but there were no widely invasive cases. The papillary carcinoma cases were classified as stage I and II ($T_{1-2}N_{0-1}M_0$) according to the TNM staging system accepted by International Union Against Cancer (UICC; 2002).

Isolation of nuclear and cytosolic fractions. Nuclei were isolated from thyroid specimens by the sucrose method (14) using 0.5% Triton X-100 to remove membrane ghosts and 1 mM phenylmethylsulfonyl fluoride (PMSF) to inhibit protease activity. The cytosolic fraction was obtained by differential centrifugation of post nuclear supernatant as described previously (15). Supernatant obtained after final centrifugation at 100000 x g for 1 h was considered as the cytosolic fraction.

Western blotting. The samples (50 μg protein/lane) of cytosolic and nuclear proteins of different types of thyroid lesions were resolved by 8 or 11.2% SDS-PAGE (16) and electroblotted onto Immobilon-P transfer membranes (Millipore, Bedford, MA, USA) (17). The detection of lectin-binding glycoproteins immobilized on Immobilon-P sheets was accomplished by the method of Haselbeck *et al* (18). The blots were treated for 1 h with 0.5% blocking reagent (w/v) in Tris-buffered saline (TBS: 50 mM Tris-HCl - 150 mM NaCl, pH 7.5). After washing twice with TBS and once with buffer 1 consisting of TBS, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM $CaCl_2$ at pH 7.5, the membranes were incubated for 1 h with the digoxigenin labeled lectins (SNA, 1 μg/ml; MAA, 5 μg/ml) in buffer 1. After being washed three times with TBS the membranes were incubated for 1 h with polyclonal sheep anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (anti-DIG AP 750 U/ml), which were added to a 1:1000 dilution in TBS. The membranes were again washed three times with TBS and the color reaction was carried out by incubating the membrane with alkaline phosphatase substrates: 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP). Detection was carried out at room temperature using a DIG glycan differentiation kit (Roche Diagnostics GmbH, Mannheim, Germany).

Enzyme linked lectino solid-phase assay (ELLSA). For semi-quantitative analysis of SNA and MAA binding by intracellular proteins, our own modification of McCoy *et al*'s (19) method was used. Samples of cytosolic and nuclear fractions to be assayed for the presence of glycoproteins bearing sialic acid residues were diluted to a final concentration of 2.5 μg/ml in 0.1 M carbonate buffer, pH 9.8. The diluted samples (100 μl) were added to the wells of the 96-well microtiter polystyrene plates (Bio-Rad, Hercules, USA). The attachment of glycoproteins to the wells was effected by overnight incubation at 4˚C and then the plates were washed three times with TBS-buffer containing 0.05% Tween-20 (TTBS). The non-specific binding sites were blocked by the addition of 200 μl of 2% BSA in TTBS-buffer to each well followed by incubation for 1 h at room temperature. Microtiter plates were again washed three times with TTBS and incubated for 1 h at room temperature with 100 μl digoxigenin-labeled lectins (Roche Diagnostics GmbH) per well diluted in TTBS at a concentration of 0.5 μg/ml. After washing the plates three times with TTBS-buffer, the polyclonal sheep anti-digoxigenin Fab fragments conjugated with peroxidase (100 μl per well) were added (dilution of antibodies 1:2500). The plates were incubated for 1 h at room temperature and washed with three changes of TTBS. The color reaction was developed by adding 100 μl per well of peroxidase substrate [0.04% 1,2-phenylenediamine-dihydrochloride/OPD/(Sigma-Aldrich Co., St. Louis, MO, USA) in 0.1 M citric acid phosphate buffer (pH 5.0) and H_2O_2]. After 15 min, at room temperature, the reaction was terminated by addition of 150 μl H_2SO_4 to each well. The absorbance of the wells was measured at 490 nm with an automatic microplate reader (Bio-Rad, model 550).

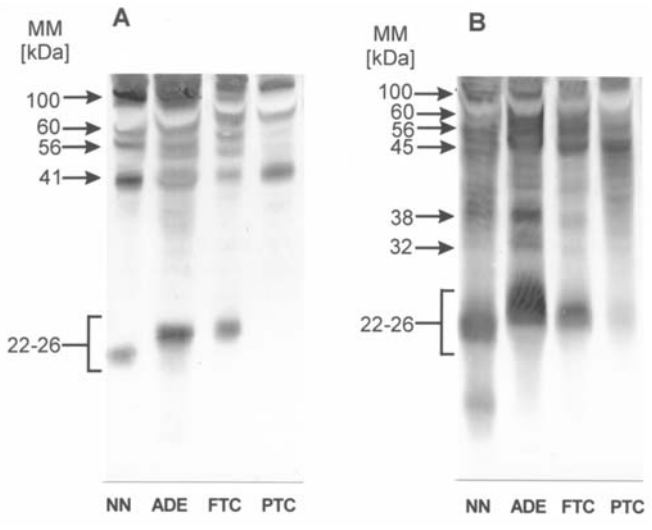

Figure 1. Cytosolic proteins (50 μg) from thyroid pathological specimens. Non-neoplastic lesion (NN), adenoma (ADE), follicular carcinoma (FTC) and papillary carcinoma (PTC) electophoresed on 11.2% acrylamide slab gels were transferred onto Immobilon-P membranes and tested for SNA-(A) and MAA-binding (B).

Measurement of protein concentration. The protein content was assayed using bovine serum albumin as a standard by the method of Lowry *et al* (20).

Statistical analysis. Statistical analysis was performed using the computer program Statistica ver. 5.5 (StatSoft, Inc.). Groups were compared using Mann-Whitney rank sum test. P-value <0.05 was considered statistically significant.

Results

The expression of α2,6-sialylated glycoconjugates was studied using *Sambucus nigra* agglutinin (SNA) as a probe. The specificity of this lectin was reported to include the α2,6-sialylated lactosamine (NeuAcα2,6Gal/GlcNAc) as well as the sialyl-Tn antigen (NeuAcα2,6GalNAc) (21). Representative patterns of cytosolic sialoglycoproteins from thyroid lesions obtained after separation of proteins by electrophoresis in 11.2% slab polyacrylamide gels are shown in Fig. 1. The expression of α2,6-linked sialic acid residues was detected in all types of thyroid specimens; however, there were some differences in

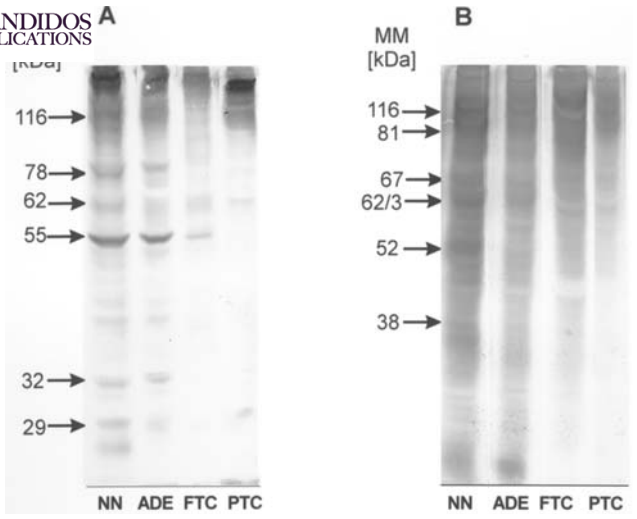

Figure 2. Nuclear proteins (50 μg) from thyroid pathological specimens. Non-neoplastic lesion (NN), adenoma (ADE), follicular carcinoma (FTC) and papillary carcinoma (PTC) electophoresed on 8% acrylamide slab gels were transferred onto Immobilon-P membranes and tested for SNA- (A) and MAA-binding (B).

the banding and intensity patterns between different thyroid lesions (Fig. 1A). Several different glycoproteins with molecular masses in the range of 41-100 and 22-26 kDa reacted with SNA in non-neoplastic thyroid lesions and benign tumors. In the majority of papillary carcinoma specimens SNA binding was remarkably decreased in comparison to non-neoplastic lesions and adenomas. In the group of follicular carcinomas the expression of sialic acid residues was rather high. The qualitative differences between cytosolic sialoglycoproteins of different types of thyroid lesions were seen especially among these with molecular masses of 22-26 kDa. There was no SNA binding by these cytosolic glycoproteins in papillary carcinoma samples. *Maakia amurensis* agglutinin (MAA) was utilized as the best lectin specific to α2,3-linked sialic acid in N-glycans. MAA binds with low affinity to the O-glycans possessing sialic acid α2,3 linked to galactose (21). Also in the case of cytoplasmic glycoproteins recognized by MAA the lectin binding was usually lower in papillary carcinoma samples than in the other lesions (Fig. 1B). However, contrary to the results with SNA, in cancer samples there were some sialoglycoproteins with molecular masses of 22-26 kDa bearing α2,3-linked sialic acid residues, but the intensity of the bands corresponding to them was lower than in the rest of the thyroid lesions.

The profiles of SNA and MAA binding nuclear glycoproteins from non-neoplastic thyroid lesions and adenoma samples were very similar (Fig. 2). In both cases nuclear glycoproteins bearing α2,3 and α2,6 bound sialic acid residues were distributed from ~29 to >180 kDa. Both non-neoplastic and adenoma samples from the majority of the analyzed patients showed a similar banding and intensity patterns. Nuclear fractions of papillary and follicular carcinoma samples were less abundant in sialoglycoproteins. In general, staining was poorer and the detected glycoprotein bands were restricted to the upper half of the transfer membrane (region where glycoproteins with molecular masses of 55-200 kDa were located) (Fig. 2).

The results obtained from the ELLSA method showed significant differences in lectin binding by cytosolic glycoproteins of benign and malignant thyroid lesions (Fig. 3). The majority of samples of non-neoplastic lesions and adenoma indicated stronger SNA and MAA binding in comparison with papillary thyroid carcinomas ($p=0.00005$ and 0.0002 for SNA; $p=0.004$ and 0.003 for MAA). There were no statistically significant differences between adenomas and non-neoplastic lesions ($p>0.05$).

The results obtained from the ELLSA method indicate also some differences in SNA and MAA binding by nuclear glycoproteins of the examined specimens (Fig. 4). Also in

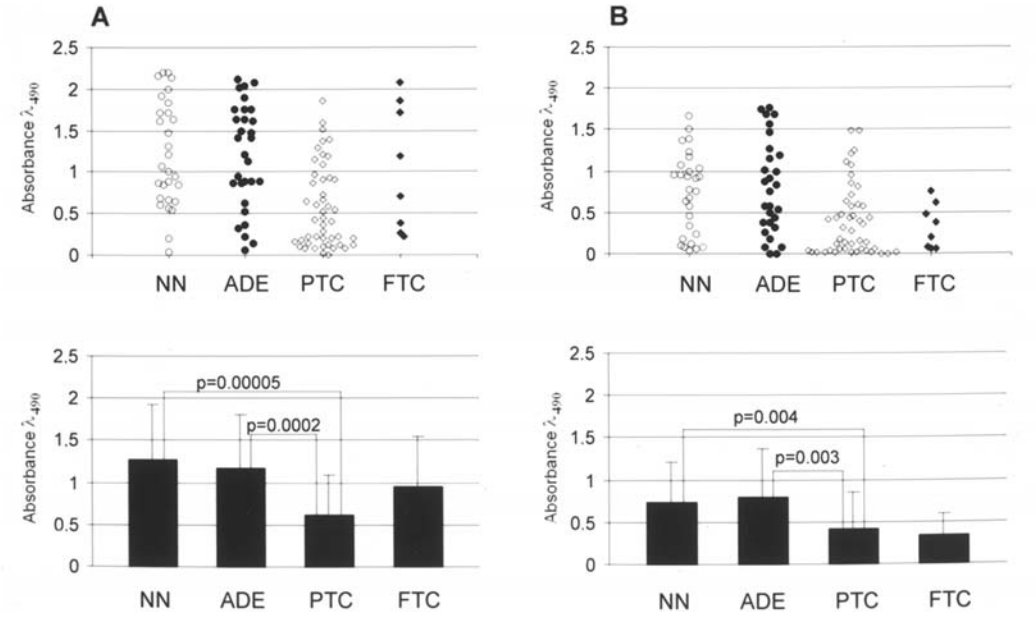

Figure 3. Analysis of SNA (A) and MAA (B) binding to cytosolic proteins of thyroid pathological specimens by ELLSA method. NN, non-neoplastic specimens; ADE, adenoma; PTC, papillary carcinoma; FTC, follicular carcinoma.

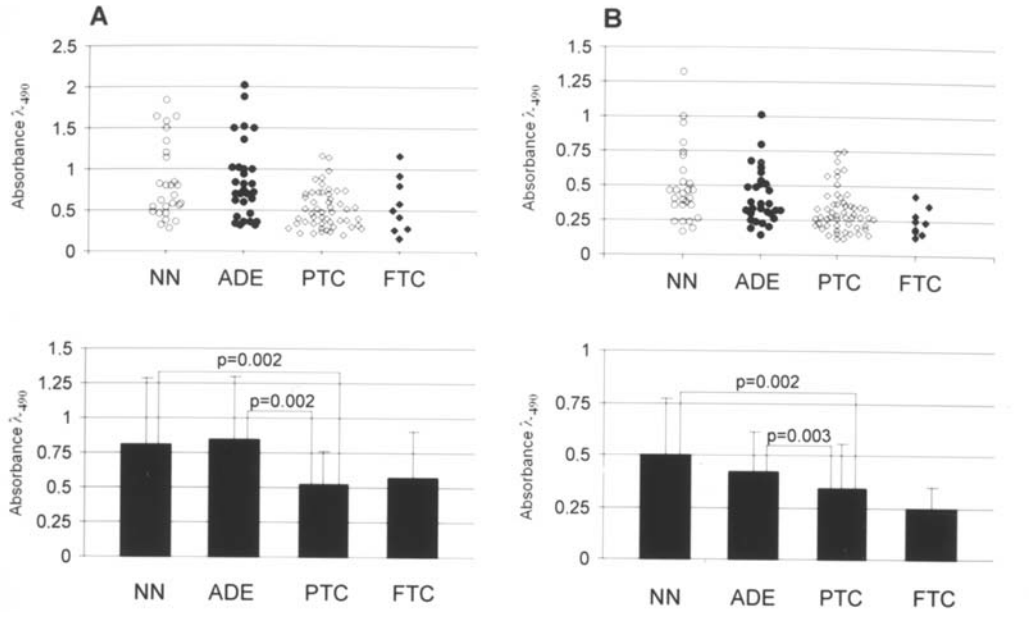

Figure 4. Analysis of SNA (A) and MAA (B) binding to nuclear proteins of thyroid pathological specimens by ELLSA method. NN, non-neoplastic specimens; ADE, adenoma; PTC, papillary carcinoma; FTC, follicular carcinoma.

the case of nuclear fraction most carcinoma samples showed lower expression of sialic acid residues than non-neoplastic lesions and adenomas.

Discussion

Aberrant glycosylation occurs in all types of experimental and human cancers and has been implicated as an essential mechanism in defining stage, direction and fate of tumor progression (reviewed in refs. 22-24). Despite the growing interest in the changes of glycosylation during oncogenic transformation there is sill little information available about the glycoprotein patterns in thyroid lesions. Our previous studies concerning glycosylation of intracellular proteins in benign and malignant thyroid neoplasms showed significant quantitative and qualitative differences in binding of *Erythrina cristagalli* (ECA) and *Ricinus communis* (RCA-120) agglutinins that recognized N-acetyllactosamine or galactose residues (15). Lower level of lectin binding in the case of carcinomas versus adenomas and non-neoplastic specimens could have been attributed to a lower level of general glycosylation of some cytosolic proteins in cancer. Alternatively, weaker binding of ECA and RCA-120 could have resulted from sialylation of terminal galactose residues of glycoproteins, as introduction of sialic acid either to C3 or to C6 galactose position inhibits or completely abolishes lectin's affinity to galactose (25). Taking into consideration the growing amount of evidence of increased sialylation during cancer progression the second possibility seemed to be more reliable.

To reveal whether sialylation is increased in thyroid malignant carcinomas, in the present study we examined this modification using plant lectins SNA and MAA, which preferentially bind to α2,6- and α2,3-linked sialic acid residues, respectively. Our results have shown that both types of sialylation occur in cytosolic and nuclear fraction of all thyroid lesions. However, there are significant differences in lectin binding by intracellular glycoproteins of benign and malignant thyroid neoplasms. Contrary to our expectations, in the majority of cancer samples especially in papillary carcinoma, lectin binding was rather weaker than stronger when compared with adenomas and non-neoplastic specimens. There were not many differences in sialylation level between non-neoplastic lesions and adenomas. In these types of thyroid lesions the lectin binding was variable but in most cases it was high. In Western blot analysis the most striking difference was lack of SNA binding and very weak MAA binding with cytoplasmic sialoglycoproteins with molecular masses in the range of 22-26 kDa in papillary carcinoma samples.

Increased sialylation has been reported in various kinds of cancers (26-29), but so far, sialylation of thyroid carcinoma proteins has never been evaluated. Our present results suggest that the decrease in sialylation rather than increase can be a characteristic feature of malignant transformation in thyroid. However in the follicular carcinomas group it was not so evident as in the papillary group. There is some difficulty in distinguishing follicular carcinoma from adenoma and it is still obscure whether this kind of cancer can arise from follicular adenoma. So it would be very valuable to find any specific marker that could be useful in diagnosis. Unfortunately the small number of cases did not enable us to draw any conclusions.

The cause of the decreased level of sialic acid in thyroid cancer cells remains obscure to us. One of the possible explanations could be the increased expression of specific cellular sialidases which was often observed in cancer cells. However, taking into consideration the results concerning ECA and RCA-120 binding, we can risk a suggestion that not only sialylation but overall glycosylation in thyroid cancers was affected.

Carbohydrate moieties of glycoconjugates are constructed by complex interactions involving a sequential action of glycosylotransferases. Sialic acid is a sugar terminating the oligo-

chains of glycoproteins and glycolipids. Therefore, differences in the levels of carbohydrates detected by SNA and MAA may result from quantitative differences in the activities of sialyltransferases or glycosylotransferases responsible for core structure formation. There is no information concerning activity of sialyltransferases in thyroid cancers but increased mRNA expression of ST6Gal I was observed in some other thyroid lesions, i.e., autonomously functioning thyroid nodules (AFTNs) and Graves' disease (30-32). Carcinoma of the thyroid arising in autonomously functioning nodules is uncommon. The majority of thyroid carcinomas present as a cold nodule on radionuclide scintigraphy. Interestingly, Frenzel *et al* (31) found that mRNA expression of ST6Gal I in AFTNs is increased but in cold thyroid nodules it is decreased or shows no differences with surrounding tissue. According to these findings it is very probable that the decreased sialylation level of intracellular proteins in thyroid carcinomas, especially papillary ones, observed by us was due to decreased expression of sialyltransferases. However, further study is needed to elucidate this problem.

As carbohydrate structure can affect the biological activity or metabolic half-life of many proteins it is possible that the sialylation defect can influence a broad spectrum of cellular functions. Recent reports suggest that sialic acid metabolism and thyroid function can be interdependent (31-32). Increased mRNA expression of ST6Gal I in primary thyrocyte cultures after thyrotropin stimulation as well as increased cell-surface expression of the thyrotropin receptor (TSHR) after ST6Gal I cotransfection have been shown (31). The sialylation of the TSHR can improve and prolong its cell-surface expression, thereby regulating the availability of the transmembrane receptor for ligand signaling (31).

Alterations in the sugar chain structures of glycoproteins, that have been found in thyroid tumors can be expected to be the basis of their abnormal behavior. So irrespective of the cause of changes in glycan structure, comparative studies of oligosaccharide chains of glycoproteins, as produced by malignant cells and non-neoplastic lesions, can provide useful information for the diagnosis, prognosis and therapy of tumors.

Acknowledgements

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