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MUC1 and Sialoglycan Expression Associated with Cytotoxic T Lymphocyte Infiltration in Eyelid Malignant Tumors

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Purpose: To elucidate the relation between infiltrating cytotoxic T lymphocytes and the expression of MUC1 and sialoglycans in malignant eyelid tumors.

Methods: The distribution of MUC1, *Maackia amurensis* lectin-II (MAL-II)-recognized sialoglycan, and CD8-positive T lymphocytes was examined histochemically in 14 patients with malignant eyelid tumors: three squamous cell carcinomas, six sebaceous gland carcinomas, and five basal cell carcinomas. The density of CD8-positive cells was examined and correlated with MUC1 and sialoglycan expression.

Results: MUC1 was identified in squamous cell carcinoma and sebaceous gland carcinoma, but not in basal cell carcinoma. CD8-positive cells were more densely distributed in those squamous cell and sebaceous gland carcinoma cases that were more intense in MUC1 expression and weaker in MAL-II binding. Tumors with a strong expression of both MUC1 and MAL-II-bound sialoglycans showed few CD8-positive cells.

Conclusions: MUC1 with few sialoglycans is likely to induce an intense infiltration of CD8-positive cytotoxic T lymphocytes in eyelid cancers. **Jpn J Ophthalmol 2002;46:237–243**

Key Words: Cancer, CD8, eyelid, MUC1, sialic acid.

Introduction

Glycoproteins on the cell surface play roles in a variety of cellular functions and intercellular interactions.[1] Mucins are highly O-glycosylated glycoproteins and protect cells from extracellular insults.[2] Among nine human mucins that have been characterized so far, designated MUC1[3] to MUC9[4] in the order of identification, MUC1 glycoprotein is an epithelial cell-related mucin, which was the first of the mucins that were characterized[3] and correlated with the biological and clinical behavior of cancers.[5] In recent years, MUC1 has been shown to be recognized by CD8-positive cytotoxic T lymphocytes in cancers of various organs[6] and myeloma.[7] It is now used not only as a histochemical probe of cancer-associated antigen[5] but also as a target for immunotherapy of MUC1-expressed cancers.[8,9] On the other hand, some other studies reported a contrary finding, that MUC1 inhibits proliferation of active T lymphocytes.[10,11] In the previous studies, the nature of glycans of MUC1 was not examined. Because sialic acid on the terminus of carbohydrate chains in cancer-associated glycoproteins is known to facilitate metastasis of cancer cells by allowing them to escape from the immune system,[12] it can be hypothesized that MUC1 featured in the inhibition of active T lymphocyte proliferation is richly sialylated, whereas MUC1 featured in the induction of cytotoxic T lymphocyte infiltration is poorly sialylated.

Our previous immunohistochemical study of eyelid tumors revealed that MUC1 is expressed in squamous cell carcinomas and sebaceous gland carcinomas, but not in basal cell carcinomas or benign tumors.[13] This finding suggests that MUC1-expressed eyelid malignancies can be a target for immunotherapy. Keeping in mind the potential therapeutic application, it may be justified to test the validity of the above hypothe-

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Vol 46: 237–243, 2002

sis concerning the glycans of MUC1. If MUC1 without sialylation is really recognized by CD8-positive cytotoxic T lymphocytes in eyelid cancers, these lymphocytes possibly infiltrate into the cancer cells with evident MUC1 but not with sialyl residues. On the other hand, if MUC1 with sialyl residues really inhibits proliferation of active T lymphocytes, CD8-positive cells may not be detectable in the cancer cells with expression of both MUC1 and sialyl residues. Thus, we explored whether any relation exists between infiltrated cytotoxic T lymphocytes and the expression of MUC1 and sialoglycans. Herein, we report the immunohistochemical distribution of MUC1 and CD8-positive cytotoxic T lymphocytes and lectin-histochemical distribution of sialoglycans in cases of eyelid cancers.

Materials and Methods

Patients and Tissue Preparation

A consecutive series of 14 patients with eyelid cancers underwent surgical treatment in the Kagoshima University Hospital between 1997 and 2001. Their clinical information is summarized in Table 1. Histopathological diagnoses were: three squamous cell carcinomas, six sebaceous gland carcinoma, and five basal cell carcinomas. During a postoperative follow-up of variable periods (range, 5 months to 4 years), a patient with sebaceous gland carcinoma (case 4 in Table 1) had a local recurrence in the bulbar conjunctiva, not in continuity with the edge, 2 years after the initial surgery. The other patients did not have either local recurrence or remote metastasis. A part of the excised tumor in each patient was immersion-fixed in buffered formalin (3.7%) and embedded in paraffin. Serial tissue sections were deparaffinized in xylene, hydrated in a graded ethanol series, reacted with reagents, and examined with a light microscope.

Reagents

A monoclonal antibody to MUC1 core protein with a carbohydrate epitope (mouse IgG, Novocastra Laboratories, Newcastle, UK), a monoclonal antibody to CD8 molecule (specific for cytotoxic T lymphocyte, mouse IgG, Novocastra), a biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA), and a biotinylated MAL-II (Vector) were purchased commercially and used according to the manufacturer's protocols.

Table 1. Clinical and Histopathological Information on Patients with Eyelid Cancers

Case No.	Age	Sex	Histopathological Diagnosis	Staining Pattern*	Density of CD8-positive Cells† Mean (SD)
1	71	Male	Squamous cell carcinoma	II	65.9 (14.7)
2	80	Female	Squamous cell carcinoma	II	91.4 (5.8)
3	80	Female	Squamous cell carcinoma	IV	17.4 (4.7)
4	62	Female	Sebaceous gland carcinoma	I	0.6 (0.7)
5	80	Female	Sebaceous gland carcinoma	I (III)‡	0.5 (0.7) [1.9 (1.3)]
6	71	Female	Sebaceous gland carcinoma	II	64.8 (11.2)
7	91	Male	Sebaceous gland carcinoma	III	1.7 (1.3)
8	72	Female	Sebaceous gland carcinoma	III	1.4 (1.2)
9	71	Female	Sebaceous gland carcinoma	III	3.5 (2.0)
10	71	Female	Basal cell carcinoma	V	1.1 (1.0)
11	68	Female	Basal cell carcinoma	V	3.4 (1.2)
12	79	Female	Basal cell carcinoma	V	3.4 (1.2)
13	62	Female	Basal cell carcinoma	V	1.6 (1.1)
14	87	Female	Basal cell carcinoma	V	1.4 (1.1)

*For explanation, see text.
†CD8-positive cells were counted in 10 randomly selected tissue areas of 300-μm square.
‡A mix of dominant cell type with minor cell type in brackets.

Immunohistochemistry

Serial tissue sections were immersed in 0.01 M sodium citrate buffer, pH 6.0, boiled for 4 minutes in a pressure cooker to unmask antigens, and treated with 0.2% H_2O_2 in methanol for 15 minutes to block endogenous peroxidase activity. After washing in Dulbecco's phosphate-buffered saline (PBS; 0.01 M, pH 7.4), the sections were incubated with 3% horse serum (diluted with PBS) at room temperature for 30 minutes to block nonspecific binding, and then overlaid with primary antibodies (MUC1, 1:100; CD8, 1:40) in PBS with 2% horse serum at room temperature for 1 hour. Serial control sections were incubated with nonimmune C57BL mouse serum (1:100) in PBS with 2% horse serum instead of the incubation with the primary antibodies. The slides were washed in PBS, incubated with biotinylated anti-mouse IgG (1:100 in PBS) at room temperature for 1 hour, washed in PBS, and stained with reagents of the Vectastain Elite ABC kit and diaminobenzidine as the peroxidase substrate following the manufacturer's protocol (Vector).

Lectin Histochemistry

Serial tissue sections were treated with 0.2% H_2O_2 in methanol for 15 minutes, washed in PBS, incubated with 3% bovine serum albumin (BSA, diluted with PBS) at room temperature for 30 minutes to block nonspecific binding, and then overlaid with biotinylated MAL-II (1:100 diluted in PBS) with 2% BSA for 1 hour. Serial control sections were incubated with the MAL-II solution mixed with 0.1 M 3′-sialyllactose for 1 hour. The slides were washed in PBS, and then stained following the same method as in immunohistochemistry.

Classification of Staining Patterns

The staining pattern of tumor cells was classified into five types based on the intensities of anti-MUC1 antibody reaction and MAL-II binding as follows.

Type I: MUC1, almost all positive; MAL-II, almost all positive
Type II: MUC1, almost all positive; MAL-II, partially or sparsely positive
Type III: MUC1, partially or sparsely positive; MAL-II, almost all positive
Type IV: MUC1, partially or sparsely positive; MAL-II, partially or sparsely positive
Type V: MUC1, negative

Quantification of CD8-positive cells in relation to the MUC1-distribution and the MAL-II-binding. Photographs of the serial tissue sections, which were incubated with an anti-CD8 antibody, were taken and printed on photopaper. The number of CD8-positive cells was counted at 10 different randomly selected areas, each 300-μm square (corresponding to 4.5-cm square on a 150×-enlarged paper).

Results

Tumor tissues incubated with a nonimmune mouse serum showed virtually no reaction (Figure 1A). The addition of haptenic sugar remarkably inhibited MAL-II binding (Figure 1B). Therefore, the positive staining described below was regarded as specific binding of the antibodies and the lectin.

MUC1 was detected in various degrees in the cell membranes and/or in the cytoplasm of tumor cells from all cases of squamous cell carcinoma and sebaceous gland carcinoma (Figures 2A, 3A, 4A, and 5A), but was scarcely detected in any specimens from the basal cell carcinomas (Figure 6A). The positive reaction was uniformly observed in tumor cells

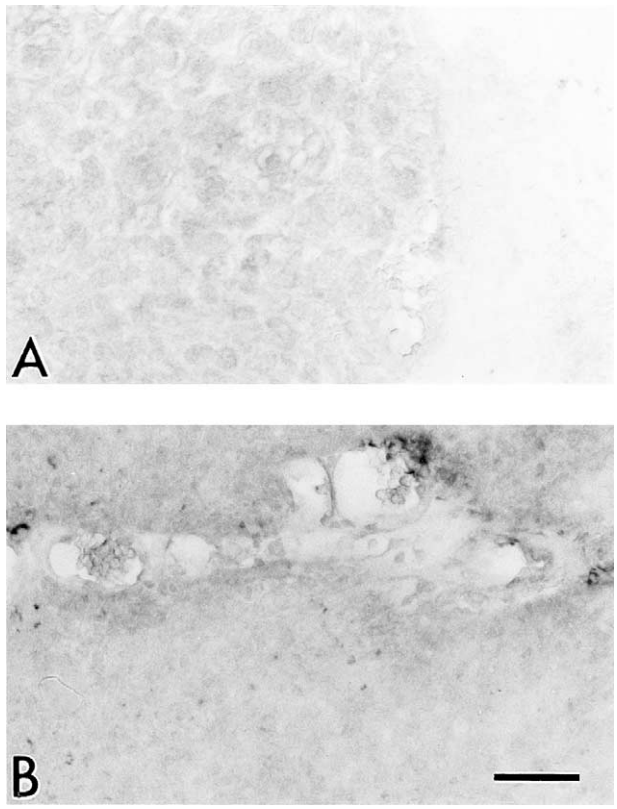

Figure 1. Control stainings [(**A**) sebaceous gland carcinoma, case 7 in Table 1. (**B**) squamous cell carcinoma, case 1 in Table 1]. (**A**) Incubation with a nonimmune mouse serum: no staining. (**B**) Incubation with a mixture of *Maackia amurensis* lectin-II and its haptenic sugar: no staining in the tumor cells except for the punctate staining around the vessel. Bar = 50 μm.

Jpn J Ophthalmol
Vol 46: 237–243, 2002

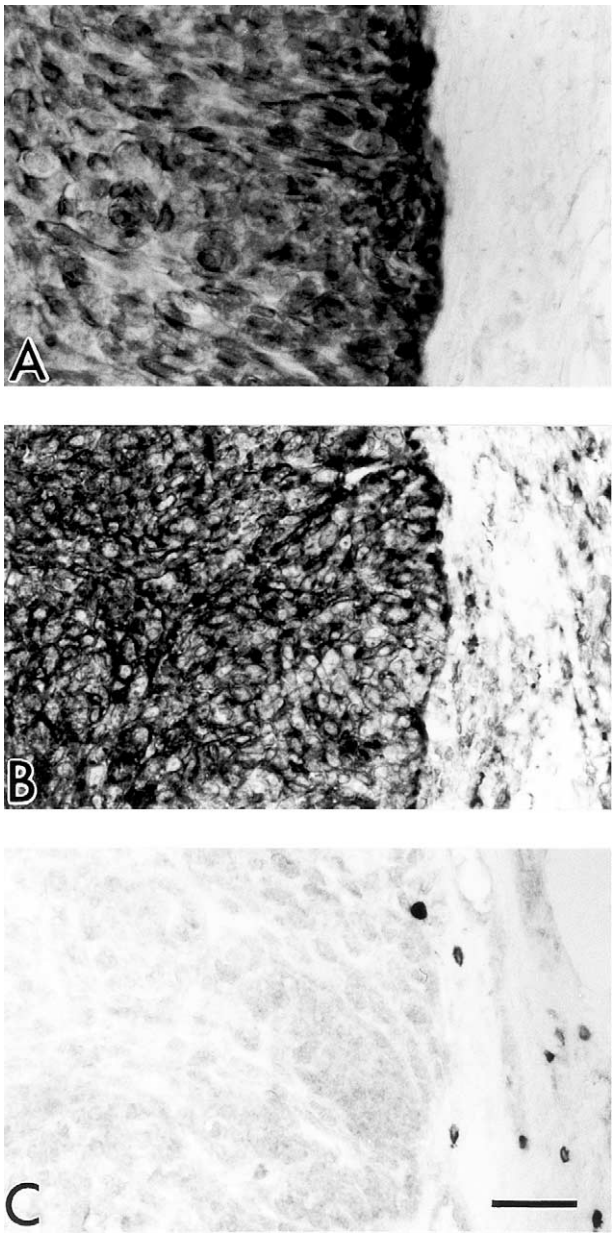

Figure 2. Type I stainings (sebaceous gland carcinoma, case 7 in Table 1). (**A**) Incubation with anti-MUC1 antibody: dense staining in the cell membrane and cytoplasm of tumor cells (left 2/3). (**B**) Incubation with *Maackia amurensis* lectin-II: dense staining in the cell membrane of tumor cells (left 2/3). (**C**) Incubation with anti-CD8 antibody: no positive cell in the tumor mass, but one positive cell on the edge of the tumor mass. Several positive cells in the stroma (right side). Bar = 50 μm.

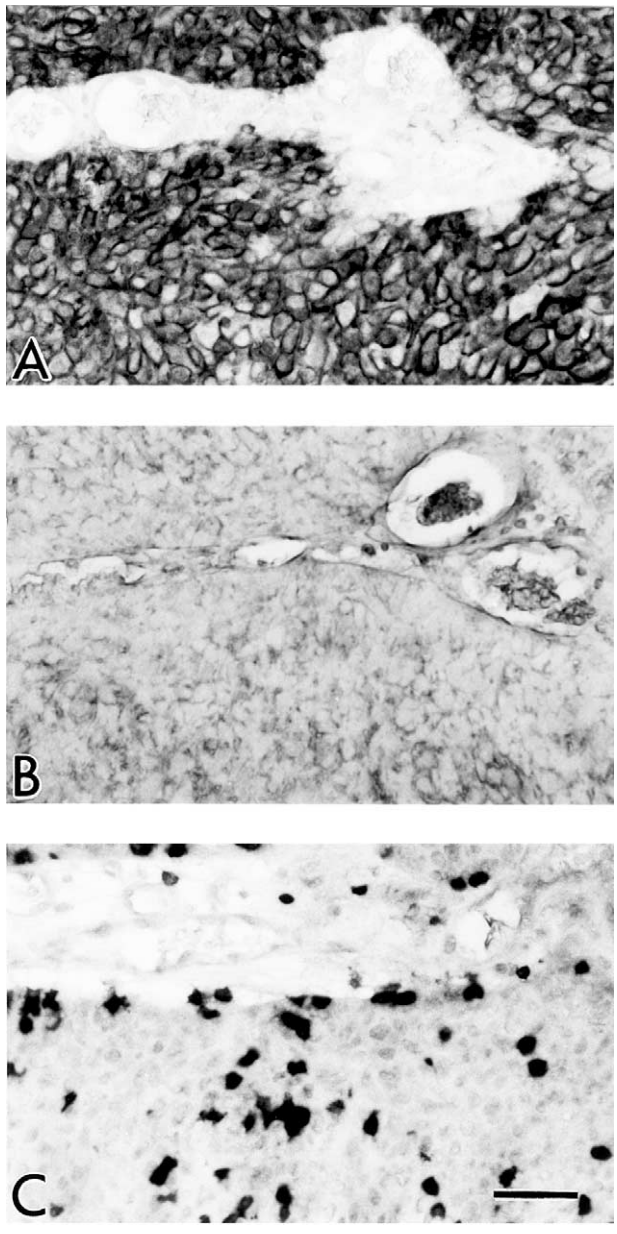

Figure 3. Type II stainings (squamous cell carcinoma, case 1 in Table 1). (**A**) Incubation with anti-MUC1 antibody: dense staining in the cell membrane of tumor cells. (**B**) Incubation with *Maackia amurensis* lectin-II: sparse staining in the cell membrane of tumor cells. (**C**) Incubation with anti-CD8 antibody: a number of positive cells in the tumor mass. Bar = 50 μm.

of cases 1, 2, 4, and 6 in Table 1 (Figure 3A, case 1), while it was partially or sparsely detected in those of cases 3, 5, 7, 8, and 9 in Table 1 (Figure 5A, case 3).] MUC1 staining was variable with the area examined in case 5 in Table 1 (Figures 2A, 4A).

MAL-II bound to the cell membranes of tumor cells from all cases irrespective of their pathological diagnosis although the staining intensity varied among the cases (Figures 2B, 3B, 4B, 5B, 6B).

Figures 2, 3, 4, 5, and 6 show the staining pattern of type I, II, III, IV, and V, respectively, defined by

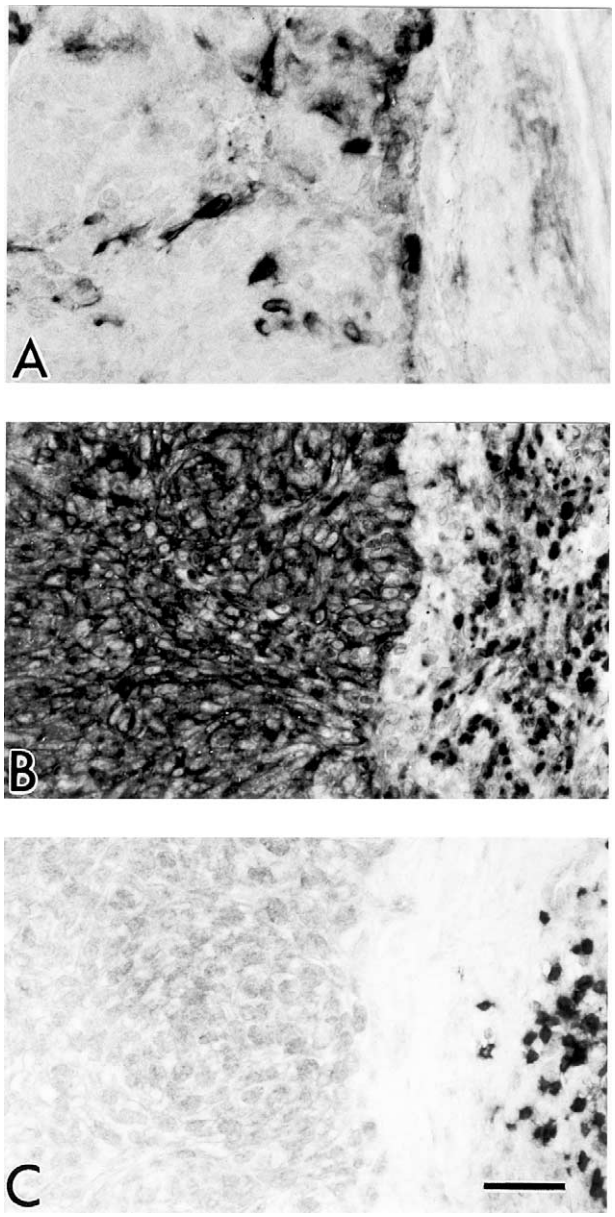

Figure 4. Type III stainings (sebaceous gland carcinoma, case 7 in Table 1). (**A**) Incubation with anti-MUC1 antibody: sparse staining in the cell membrane and cytoplasm of tumor cells. (**B**) Incubation with *Maackia amurensis* lectin-II: dense staining in the cell membrane of tumor cells. (**C**) Incubation with anti-CD8 antibody: no positive cell in the tumor mass. A number of positive cells in the stroma (right side). Bar = 50 μm.

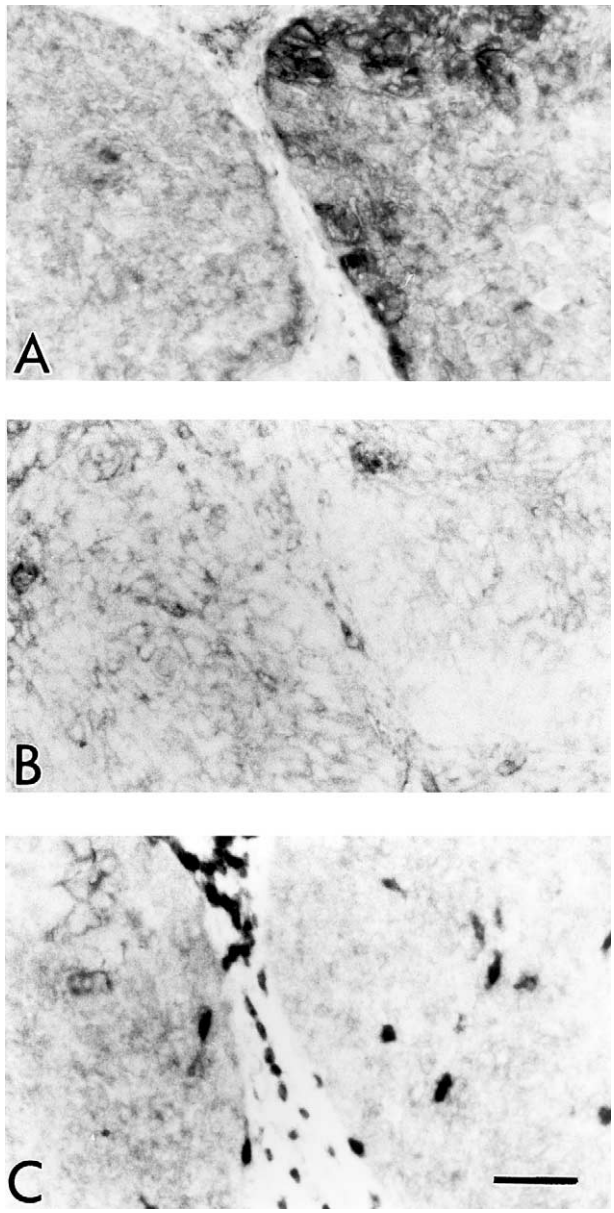

Figure 5. Type IV stainings (squamous cell carcinoma, case 3 in Table 1). (**A**) Incubation with anti-MUC1 antibody: sparse staining in the cell membrane and cytoplasm of tumor cells. (**B**) Incubation with *Maackia amurensis* lectin-II: sparse staining in the cell membrane of tumor cells. (**C**) Incubation with anti-CD8 antibody: some positive cells in the tumor mass. A number of positive cells in the stroma between the tumor masses. Bar = 50 μm.

the behavior to these two probes (see explanation in the section of Materials and Methods).

As regards the infiltration of CD8-positve cells, the type I and type III tumors showed only a few cells (Figures 2C and 4C). The type II and type IV tumors showed numerous cells (Figures 3C and 5C).The type V tumor had much less infiltration (Figure 6C). The results of infiltrated cell counting in each case are shown in Table 1. The statistical analysis revealed that all cases showing type II tumor (cases 1, 2, and 6) were more dense in CD8-positive-cell density than the other cases showing either type

Jpn J Ophthalmol
Vol 46: 237–243, 2002

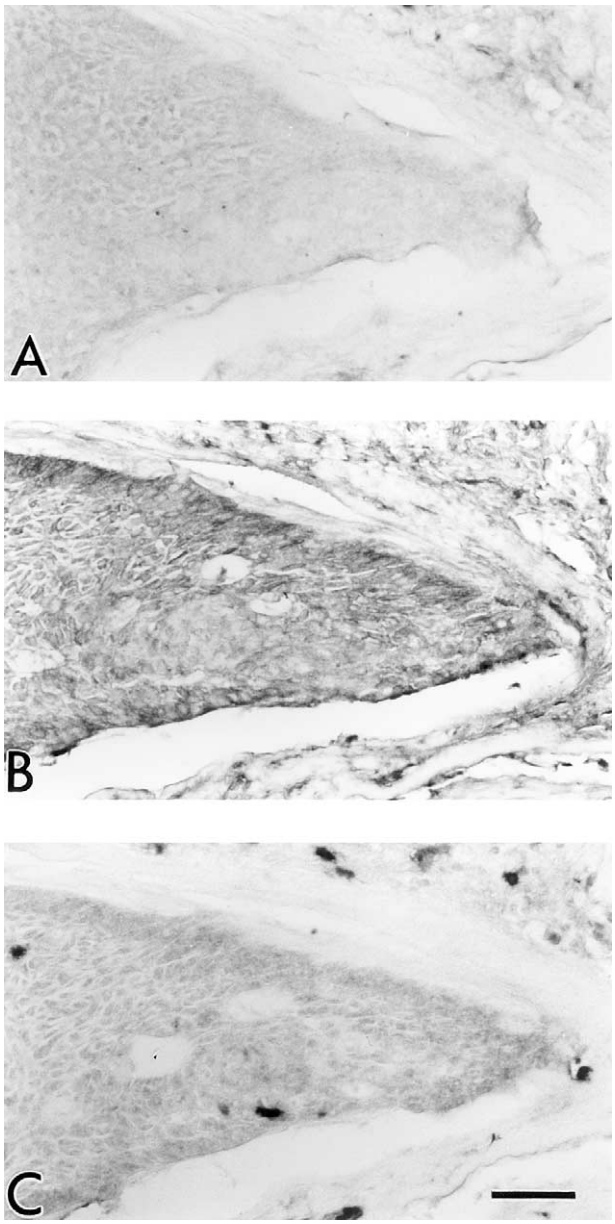

Figure 6. Type V stainings (basal cell carcinoma, case 13 in Table 1). (**A**) Incubation with anti-MUC1 antibody: no staining in the tumor cells. (**B**) Incubation with *Maackia amurensis* lectin-II: relatively dense staining in the cell membrane of tumor cells. (**C**) Incubation with anti-CD8 antibody: some positive cells in the tumor mass and the stroma. Bar = 50 μm.

I, III, IV, or V tumors ($P < .05$). Case 3 showing type IV tumor was more dense in CD8-positive-cell density than the cases showing either type I, III, or V tumors ($P < .05$). Among the cases showing either type I, III, or V, there was no statistical difference in cell density ($P > .05$). With respect to the relationship between cell density and histopathological diagnosis, the density was higher in squamous cell carcinoma than in the other malignant tumors except for case 6 ($P < .05$).

Discussion

These results confirm the previous study that MUC1 is expressed in squamous cell carcinoma and sebaceous gland carcinoma, but not in basal cell carcinoma.[13] An extension of the study with a combined histochemical study of MUC1-expression and MAL-II-binding and quantification of CD8-positive cells provides a further understanding of the relationship between MUC1-expression and the immune system in malignant eyelid tumors.[14]

The CD8-positive cells were numerous in incomparable density in the type II and IV tumors (squamous cell carcinoma: 3/3 cases; sebaceous carcinoma: 1/6 cases), in such a way that the combination of the dense expression of MUC1 with the sparse binding of MAL-II is closely related to the infiltration of cytotoxic T lymphocytes. This finding correlates with our hypothesis and suggests that MUC1 with only a few sialoglycans or asialoglycans allows infiltration of numerous cytotoxic T lymphocytes. Most of these cells may infiltrate through the endogenous immune system for tumor rejection, because recent studies confirmed that MUC1-specific CD8-positive cells exhibit antitumor activity against MUC1-positive metastasis[15] and prevent MUC1-expressing tumor formation.[16] The reason why all cases of squamous cell carcinoma showed tumor types with few sialoglycans, and most cases of sebaceous carcinoma showed tumors with rich sialoglycans should be clarified in future studies. On the other hand, it is difficult to reconcile with an alternative hypothesis that densely sialylated MUC1 inhibits the proliferation of active T lymphocytes, in view of no significant difference in its expression between the type I tumor and either the type III or the type V tumors.

As regards clinical prognosis, only one case (case 4 in Table 1) with the type I tumor had a local recurrence 2 years after surgery, whereas the other cases remained favorable. It is difficult to discuss whether any relation exists between the clinical outcome and the tumor type because of a short period of follow-up (range, 5 months to 4 years). Therefore, we retrospectively examined the prognosis of three type I cases previously reported.[17] Two of the three cases already had metastasis to neck lymph nodes at the time of the report,[17] and the other case of low differentiated carcinoma had metastasis later. The edges of the

excised tumor in all three cases were free of malignant cells by histopathological examination at the time of surgery. Thus, MUC1 expression with highly sialylated glycans may relate to the biological properties of invasion and metastasis of cancer cells. Because highly sialylated MUC1 is presumably not recognized by cytotoxic T lymphocytes, the residual tumor cells may have proliferated without rejection by the T cells in the invaded and/or metastasized regions. This has prompted us to follow carefully a case of mixed type I and III tumor cells (case 5 in Table 1).

In a quantitative assessment of CD8-positive cells, basal cell carcinoma (type V) with low risk of metastasis or recurrence showed a negligibly low density of CD8-positve cells, which was comparable with other carcinomas with high risk of metastasis. This finding suggests that the density of CD8-positive cells alone is not crucially related to the biological property of metastasis. Instead, in view of the anti-adhesive function of MUC1,[18] the presence of MUC1 on the tumor cell surfaces is likely to be relevant to their metastatic properties. As regards basal cell carcinoma, the diffuse homogeneous distribution of cytokeratin 14 may play an additional role in the defense against metastasis.[19,20]

The present study indicates the direction of future immunotherapy for malignant eyelid tumors. To achieve successful immunotherapy with MUC1-immunization, selective inhibition of sialoglycan formation of cancer-associated glycoconjugates is mandatory. In view of reduced sialic acid on the surfaces of apoptotic bodies of malignant eyelid tumors,[21] it is justified to further extend a glycobiological study concerning the selective inhibition of sialyltransferase expression.

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