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• BASIC RESEARCH •

Expression of sialyl Lewis^a relates to poor prognosis in cholangiocarcinoma

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Abstract

AIM: High levels of serum sialyl Lewis^a (sLe^a) are frequently found in cholangiocarcinoma (CCA) patients and have been suggested to be a serum marker for CCA. However, the significance of this antigen in CCA is unknown. In this study, the clinical significance of sLe^a expression in CCA tissues and the possible role of sLe^a in vascular invasion *in vitro* were elucidated.

METHODS: Expression of sLe^a in tumor tissues of 77 patients with mass-forming CCA and 33 with periductal infiltrating CCA was determined using immunohistochemistry. The *in vitro* assays on adhesion and transmigration of CCA cells to human umbilical vein endothelial cells were compared between CCA cell lines with and without sLe^a expression.

RESULTS: sLe^a was aberrantly expressed in 60% of CCA tumor tissues. A significant relationship was found between the frequency of sLe^a expression and the mass-forming type CCA (P = 0.041), well differentiated histological grading (P = 0.029), and vascular invasion (P = 0.030). Patients with positive sLe^a expression had a significantly poorer prognosis (21.28 wk, 95% CI = 16.75-25.81 wk) than those negative for sLe^a (37.30 wk, 95% CI = 27.03-47.57 wk) (P<0.001). Multivariate analysis with adjustment for all covariates showed that patients positive for sLe^a possessed a 2.3-fold higher risk of death than patients negative for sLe^a (P<0.001). The role of sLe^a in vascular invasion was demonstrated using *in vitro* adhesion and transmigration assays. KKU-M213, a human CCA cell-line with a high expression of sLe^a, adhered and transmigrated to IL-1β-activated endothelial cells of the human umbilical vein more than KKU-100, the line without sLe^a expression (P<0.001). These processes were significantly diminished when the antibodies specific to either sLe^a or E-selectin were added to the assays (P<0.001).

CONCLUSION: This study demonstrates the clinical significance of sLe^a expression in vascular invasion, and an unfavorable outcome in CCA. The role of sLe^a in vascular invasion which may lead to poor prognosis is supported by the *in vitro* adhesion and transmigration studies.

Key words: Cholangiocarcinoma; Sialyl Lewis^a; Poor prognosis

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INTRODUCTION

Metastasis spreads malignant cells from a primary tumor throughout the body resulting in growth of secondary tumors in other tissues or organs. The ability of disseminated cancer cells to re-establish themselves is regulated by a combination of factors, including access to microvasculature and host-tumor cell interaction[1]. Attachment to vascular endothelia is the start of the metastatic cascade and evidence suggests that attachment precedes, and is required for, tumor cell extravasation and subsequent invasion into the target organ parenchyma[2]. Organ-specific receptors have been identified on the luminal surface of microvascular endothelia, specifically recognized by tumor cell ligands, thereby facilitating tumor cell arrest and transmigration into the extravascular space.

Sialyl Lewis^a (sLe^a) antigen, discovered by Koprowski *et al*[3] with the use of monoclonal antibody CA19-9, is a tetrasaccharide epitope (sialylated lacto-N-fucopentaose II) on the tumor cell membrane which may have a role in cancer dissemination[4,5]. There is evidence that sLe^a expressed on tumor cells plays an important role in the adhesion of tumor cells to E-selectin on endothelial cells in the extravasation process[6,7]. Detection of sLe^a, in either tissue or pre- and post-operative serum is a prediction of increased cancer mortality[8]. The association of high levels of serum sLe^a with tumor invasion is common in cancer patients[5,9-11].

Cholangiocarcinoma (CCA), a bile duct cancer, is highly prevalent in Northeast Thailand[12]. Early stage CCA often goes undetected, most patients are diagnosed at an advanced or disseminated stage with a poor prognosis. High levels of serum sLe^a are frequently found in CCA patients and have been suggested to be a serum marker for CCA[13-17]. However, the role of sLe^a in CCA is unclear. We therefore evaluated the association of sLe^a expression in tumor tissues with the clinicopathology and survival of CCA patients. The role of sLe^a antigen in the adhesion and transmigration of human CCA cells to human umbilical vein endothelial cells (HUVEC) *in vitro* was demonstrated.

MATERIALS AND METHODS

Patients

Surgical specimens of 110 CCAs were obtained from the files of the Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, Thailand. The specimens were classified into 2 types: 77 cases of mass forming CCA and 33 cases of periductal infiltrating CCA. The mean age of patients was 55 years (range, 32 to 75 years). Seventy-two were males, and 31 were females. Informed consent was obtained from each subject and the Human Research Ethics Committee, Khon Kaen University, approved our research protocol. Cancer diagnosis was verified by histology with UICC TNM classification. Clinical follow-up was available for 104 (94.5%) of the patients. Survival of each CCA patient was recorded after surgery until May 15, 2001. Ninety-one patients (82.7%) died by the end of the follow-up period.

Human cholangiocarcinoma cell-lines and HUVECs

The two human cholangiocarcinoma cell-lines used (KKU-M213 and KKU-100) were from the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand. The human umbilical vein endothelial cells (HUVECs) were from the American Type Culture Collection (Manassas, VA).

CCA cells were cultured in a HAM-F12 medium (Life Technologies, Rockville, MD) supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2.5 μg/mL fungizone. sLe^a expression in CCA cell-lines was determined by immunocytochemistry using anti-CA19-9 antibody (Novocastra, Newcastle upon Tyne, UK). sLe^a was highly expressed in KKU-M213, more than 95% of cells had strong, positive staining. In contrast, KKU-100 cells showed no reaction (data not shown).

Immunohistochemical detection of sLe^a

All specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 4-μm-thick serial sections for immunohistochemical staining using avidin-biotin complex technique. Briefly, the paraffin sections were deparaffinized, hydrated and endogenous peroxidase-blocked with hydrogenperoxide. After non-specific staining was blocked with normal horse serum, the sections were incubated with 1:100 anti-sLe^a (anti CA19-9) overnight, followed by biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) and streptavidin-peroxidase (Vector). After washed, the sections were developed in 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co., St Louis, MO), counterstained with hematoxylin, dehydrated, cleared and mounted. When PBS was applied instead of the primary antibody, there was no positive staining.

The intensity of sLe^a expression was semi-quantitatively classified into 4 groups on the basis of the percentage of positive tumor cells: 0%, negative; 1-25%, +1; 26-50%, +2 and >50%, +3.

Adhesion assay

The adhesion assay procedure[18] was as follows. In briefly: HUVECs (2 to 5 passages) were grown in Kaighn's F12K medium, supplemented with endothelial cell growth supplement (Life Technologies, Rockville, MD) and seeded at 4×10^4 cells/well in a 96-well plate, pre-coated with 0.1% gelatin. The plate was then incubated at 37 ℃ in an atmosphere containing 50 mL/L CO_2 for 24 h. After activation of the rIL-1β (100 U/mL) (Life Technologies, Rockville, MD) for 4 h, the medium was removed and the cells were blocked with 1% bovine serum albumin complete media for 1 h.

Cell suspensions of KKU-M213 or KKU-100 (2×10^4 cells), in phosphate-buffered saline (PBS) with 1 mmol/L $CaCl_2$, were added to the HUVEC in each well and incubated for further 45 min. Unbound cells were removed by washing the wells with PBS. Adhered cells were fixed for 15 min with 2.5% glutaraldehyde, then stained with an antibody of 1:400 of pan-cytokeratin (Novocastra Lab, Newcastle upon Tyne, UK) and 1:100 horseradish peroxidase-conjugated goat anti-mouse IgG (Zymed Laboratories, South San Francisco, CA).

The cells that adhered to the HUVECs were counted by microscopy in nine low power fields (×100 magnification). Non-stimulated HUVECs were used as the controls. Triplicate assays were performed and at least two separate experiments were done.

Transmigration assays

A modified transmigration *in vitro* assay was performed as per Yoshida[19]. Approximately 8×10^4 HUVECs were plated on a 0.3-mg pre-coated-Matrigel-culture insert (Becton-Dickinson, San Jose, CA). The monolayer was activated with 100 U/mL IL-1β for 4 h. After blocked with 1% bovine serum albumin complete media for 1 h, 4×10^4 cells of KKU-M213 cells in PBS with 1 mmol/L $CaCl_2$ were added to each insert and incubated for 30 min. Cells on the upper face of the membrane were scraped using a cotton swab and cells on the lower face were fixed with 25% methanol for 15 min and stained with 0.5% crystal violet in 25% methanol. The number of migrated cells on the lower face of the filter was counted under microscopy in nine fields (×100 magnification). KKU-M213 cells incubated in the pre-coated-Matrigel insert without HUVECs were used as control. Assays were done in triplicate and repeated at least twice.

Inhibition of adhesion and transmigration by antibodies to sLe^a and anti-E-selectin

KKU-M213 cells were incubated with 50 μg/mL of anti-sLe^a monoclonal antibody (Chemicon International, Temecula, CA) for 30 min prior to the adhesion or transmigration assays. For HUVEC, a monolayer of activated HUVEC was pre-incubated with 10 μg/mL anti-human E-selectin (Santa Cruz Biotechnology, Santa Cruz, CA) at 37 ℃ for 15 min[7]. The excess anti-E-selectin was washed out with PBS before incubated with tumor cells in the adhesion or transmigration assays. The viability of treated cells determined using trypan blue exclusion dye was 96.86%, which was not significantly different from that of the non-treated sample.

Statistical analysis

Data were presented as mean±SD. The Student *t* test was used for comparisons and $P<0.05$ was considered statistically significant. The association of two categorical variables was analyzed by the χ^2-test or Fisher's exact probability test.

Survival of the patients was compared between the group with positive sLe^a antigen expression and the group with negative sLe^a antigen expression according the Kaplan-Meier method. The significance of the difference in survival between the 2 groups was tested by the log-rank test. Several clinicopathologic factors were subjected to univariate and multivariate analysis using the Cox proportional-hazard regression model. The relative risk of death was compared using the assessment of hazard ratio. Differences were considered statistically significant at $P<0.05$.

RESULTS

Expression of sLe^a and association with clinicopathologic features

By immunohistochemistry, sLe^a was constitutively expressed in normal biliary epithelial cells. It was localized at the apical surface, cytoplasm and/or stroma of CCA tissues (Figure 1).

The expression of sLe[a] was detected in 60% (66/110) of CCA patients. There were 79% (26/33) of periductal infiltrating CCA patients and 52% (40/77) of mass-forming CCA patients who expressed sLe[a] ($P = 0.015$) (Table 1).

The association of sLe[a] expression in CCA patients with clinico-pathologic features was determined. Three variables, mass-forming CCA, well differentiated adenocarcinoma histological grading and vascular invasion were statistically significant and were associated with the expression of tumor sLe[a] (Table 2).

Table 1 Expression of sLe[a] in tumor tissues of CCA patients

Expression of sLe[a]	Tumor type		Total
	Mass-forming	Periductal infiltrating	
0	37	7	44
1+	7	7	14
2+	8	6	14
3+	25	13	38
Total	77	33	110

Table 2 Correlation between expression of tissue sLe[a] and clinicopathologic features

		Expression of sLe[a]				*P*
		0	1+	2+	3+	
CCA type						
Mass-forming		37	7	8	25	0.041
Periductal-infiltrating		7	7	6	13	
Histology type						
Papillary		2	3	1	8	0.029
Well differentiated		8	5	6	14	
Moderately differentiated		8	3	4	5	
Poorly differentiated		19	2	0	9	
Squamous/adenosquamous		7	1	3	2	
Vascular invasion	- No	13	8	9	11	0.030
	- Yes	31	6	5	21	
Neural invasion	- No	27	8	4	14	0.054
	- Yes	17	6	10	24	
Lymphatic invasion	- No	9	5	2	9	0.560
	- Yes	35	9	12	29	

Correlation between sLe[a] expression and cumulative survival rate

Median overall survival in CCA patients with positive and negative sLe[a] expressions was 21.28 wk (95% CI = 16.75-25.81) and 37.30 wk (95% CI = 27.03-47.57), respectively. The survival rate of the patients with positive sLe[a] expression was significantly poorer than that of the patients with negative sLe[a] expression ($P = 0.021$, log-rank test, Figure 2). Well differentiated type CCA ($P = 0.027$) and the expression of sLe[a] ($P = 0.001$) were independently poor prognostic indicators contributing to disease-free survival of CCA (Table 3).

Table 3 Significant prognostic factors contributing to disease-free survival by multivariate Cox's proportion-hazard regression model

Variable	Coefficient	SE	Hazard ratio	*P*
Age (55 *vs* >55 yr)	-0.013	0.011	0.987	0.257
Sex (male *vs* female)	0.294	0.250	1.342	0.239
CCA type (mass-forming *vs* periductal-infiltrating)	0.440	0.322	1.552	0.173
Histology				
Papillary	-0.830	0.471	0.436	0.078
Well differentiated	-0.891	0.402	0.410	0.027
Moderately differentiated	-0.736	0.462	0.479	0.111
Poorly differentiated	-0.356	0.405	0.701	0.379
Squamous/adenosquamous	0.264	0.756	1.302	0.727
Vascular invasion (present *vs* absent)	-0.234	0.272	0.791	0.390
Neural invasion (present *vs* absent)	-0.362	0.247	0.696	0.143
Lymphatic invasion (present *vs* absent)	0.053	0.313	1.055	0.865
Tissue sLe[a] (present *vs* absent)	0.834	0.260	2.302	0.001

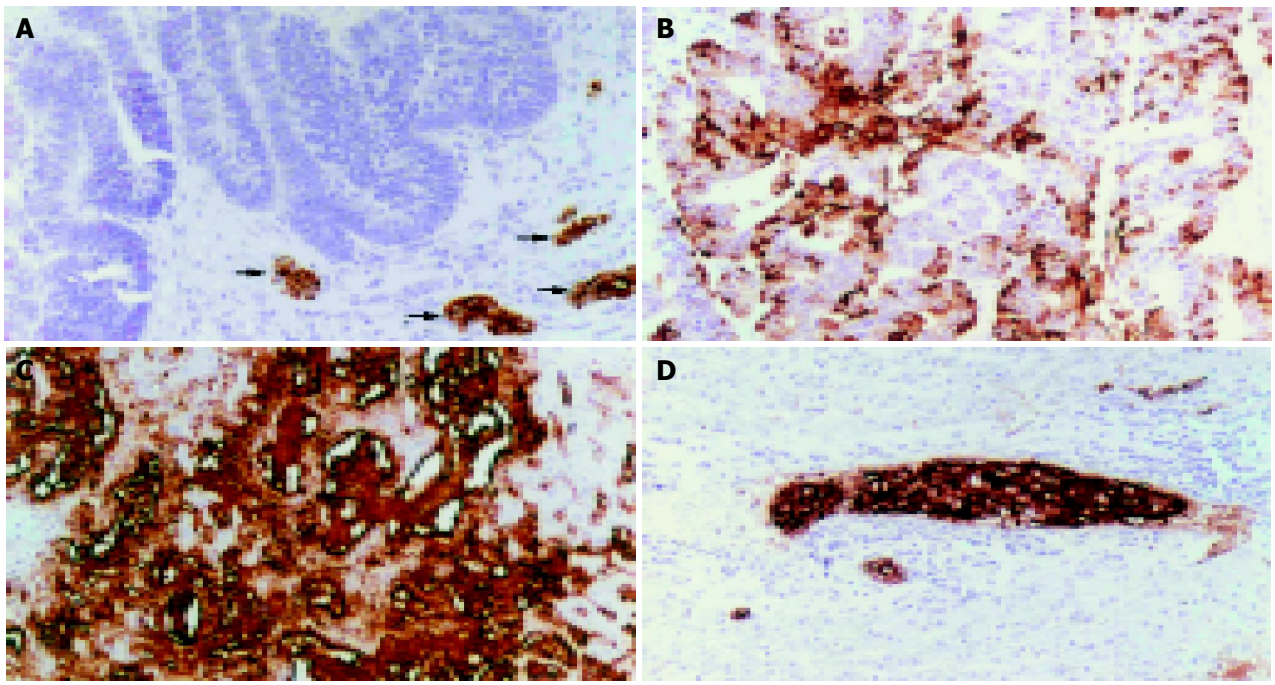

Figure 1 Immunohistochemical detection of sLe[a] in CCA. A: A CCA case with no sLe[a] expression in tumor but with positive staining in the normal bile ducts (arrows); B: and C: CCA cases with positive sLe[a], showing apical and stromal staining, respectively; D: Vascular metastasis of sLe[a] positive CCA cells. (Immunoperoxidase staining, original magnification ×100).

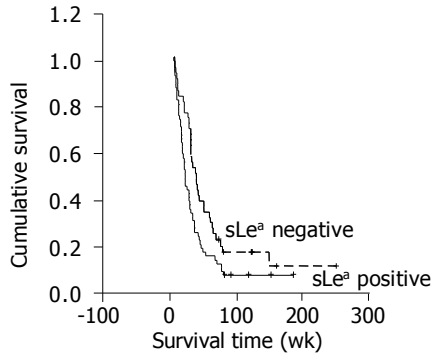

Figure 2 Correlation between expression and cumulative survival rate (Kaplan-Meier method). Patients who were positive for tumor sLe^a had a less favorable prognosis compared to those who were negative (P<0.001).

Role of sLe^a in adhesion and transmigration of CCA cell lines

The adhesion of CCA cells with high positive sLe^a expression (KKU-M213) was compared to that of CCA cells with negative sLe^a expression (KKU-100). The basal adhesion level of these two cell-lines to non-activated endothelial cells was not significantly different. However, in the cytokine-activated HUVECs, the adhesion of KKU-M213 cells was significantly greater than that of KKU-100 cells (P<0.001) (Figure 3). This finding was confirmed by the inhibition assay in which tumor cells were pre-treated with monoclonal antibodies for sLe^a before added to the HUVEC. The number of cancer cells that adhered to the rIL-1β activated HUVECs decreased to base levels, and was significantly less than that without anti-sLe^a (P<0.001) (Figure 3).

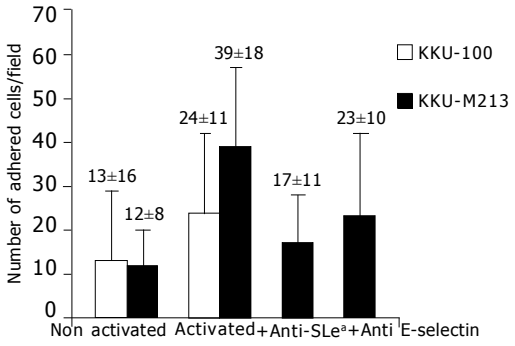

Figure 3 Adhesion of CCA cells to epithelial cells of the human umbilical vein endothelial cells (HUVEC). KKU-M213 or KKU-100 cells were incubated with non-activated or IL-1β activated HUVECs for 45 min at 37 ℃. The basal adhesion levels of KKU-100 and KKU-M213 to non-activated HUVECs were not statistically different, whereas the adhesion levels to activated HUVECs were significantly different (P<0.001). A significant difference occurred between the levels of KKU-100 and KKU-M213 adhesion to non-activated and IL-1β-activated HUVECs (P<0.001). The adhesion level of KKU-M213 significantly declined (P<0.001), when anti-sLe^a or anti E-selectin was added to KKU-M213 or IL-1β-activated HUVECs before the adhesion assay. The results were expressed as mean±SD of triplicate samples of a representative experiment.

To evaluate the contribution of E-selectin to the adhesion of CCA cells, adhesion assays of KKU-M213 to HUVECs were performed with and without rIL-1β-activation. rIL-1β had a clear stimulatory effect on the adhesion of cancer cells to HUVECs (Figure 3). The adhesion of KKU213 to rIL-1β-activated HUVECs was about 4-fold greater than that without activation (P<0.001). The significant contribution of E-selectin was confirmed by showing that incubation of the activated HUVECs with monoclonal antibody to E-selectin, before addition of cancer cells, clearly inhibited cell adhesion (Figure 3) (P<0.001). However, the number of adhered cells was still greater than the basal level (P<0.001).

The contributions of sLe^a and E-selectin to transmigration of KKU-M213 via the HUVECs were evident. The number of cancer cells that transmigrated through HUVEC was significantly reduced, when prior to the transmigration assay. The KKU-M213 cells were treated with anti-sLe^a (P<0.001) or the activated HUVECs were pre-treated with anti-E-selectin (P<0.001) (Figure 4). This observation was contributed mainly via transmigration of KKU-213 through the HUVECs since no KKU-213 cells or HUVECs migrated through the control insert within 30 min of incubation.

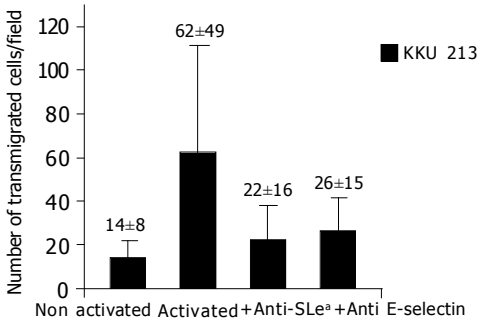

Figure 4 Contributions of sLe^a and E-selectin to transmigration of KKU-M213 to activated HUVECs. Transmigration of KKU-M213 was significantly reduced (P<0.001) when either sLe^a or E-selectin was blocked with specific corresponding antibodies before the transmigration assay. Experiments were carried out in triplicate. The mean±SD of a representative experiment and similar patterns was obtained in the two repeated experiments.

DISCUSSION

The present study demonstrated that 60% of CCA tumor tissues were aberrantly expressed sLe^a. The univariate analysis revealed mass-forming type CCA, well-differentiated histological type and presence of vascular invasion tumor associated with the expression of tumor sLe^a. Tsuji *et al*[15] and Minato *et al*[20] supported our immunohistology results that sLe^a was expressed in 60% of intrahepatic CCAs and the expression of sLe^a antigen was more frequent in well-differentiated adenocarcinoma cells. In addition, the high level of serum sLe^a (CA19-9) in CCA patients was recently demonstrated to be related to venous invasion, perineural invasion and lymph node metastases[14].

The multivariate analysis indicated the expression of tumor sLe^a as an independent prognostic factor affecting disease-free survival and overall survival. From our literature search, this appears to be the first report on the association of sLe^a expression with poor prognosis in CCA. Patients with positive sLe^a in tumor tissues had significantly shorter survival than those with negative sLe^a. Therefore, the presence of tumor sLe^a can be used as a prognostic risk factor related to survival of CCA patients and may help select patients with poor prognoses that can then be offered adjuvant therapy.

A key event in cancer metastasis is the transendothelial migration of tumor cells. This process involves multiple adhesive interactions between tumor cells and the endothelium. After adhering to the surface of endothelial cells, tumor cells must penetrate the endothelial junction. The contribution of sLe^a to the adhesion of tumor cells to endothelial cells via E-selectin has been observed in various cancer cell-lines[21-23]. In the present

study, the contribution of sLe[a] to vascular invasion was demonstrated not only by a statistical association analysis but also in the *in vitro* adhesion and transmigration assays of CCA cells to E-selectin-mediated human endothelial cells.

The role of sLe[a] in endothelial cell adhesion was assessed by comparing the adhesion levels of two CCA cell lines: one with a high expression of sLe[a] (KKUM213) and one with undetectable sLe[a] (KKU-100). These two cell lines had comparable basal adhesion to non-activated HUVECs. However, upon rIL-1β-activation, the number of cells adhering to the activated-HUVECs of KKU-M213 was significantly greater than that of KKU-100.

Our *in vitro* studies with KKU-M213 and KKU-100 had a low, but measurable basal adhesion to non-activated endothelial cells. Blocking-activated HUVECs with antibody to E-selectin did not completely keep adhesion at a basal level, suggesting the involvement of other, as yet unknown, carbohydrate ligands on CCA cells, and/or receptors on activated HUVECs, in the adhesion of these cell-lines.

The importance of sLe[a] to allow or enable attachment of CCA cells to endothelial cells has therefore been confirmed by the selective blocking of sLe[a] (marked reduction of adhesion of KKU-M213) by specific antibodies. Moreover, the treatment inhibited the binding of KKU-M213 to activated HUVECs close to the basal level obtained from KKU-M213, with non-activated HUVECs. The observation indicates that the KKU-M213 cells adhering to the activated HUVECs was mainly via sLe[a]. The same conclusion is drawn from the transmigration study.

Blocking either sLe[a] or E-selectin, with a specific neutralizing antibody, can inhibit adhesion and transmigration of CCA cells to endothelial cells, confirming the involvement of sLe[a] and E-selectin in these processes.

The sLe[a] antigen is expressed at trace levels in normal biliary cells but is expressed in a high level in tumor cells and can be detected as a tumor marker in serum. The increased serum level of sLe[a] in CCA has been reported ranging from 57 to 100%[13-16]. The discrepancies between various investigators might be due to the differences in etiology and incidence[24-29].

Several lines of evidence and our results point to the cancer-associated carbohydrate antigen, sLe[a], in the vascular invasion via the adhesion and transmigration of cancer cells to vascular endothelial cells. It may also contribute to the hematogenous metastasis of cancer and unfavorable outcome. SLe[a] is immunogenic and potentially a target for passive-and active-specific immunotherapy for human cancers in which the sLe[a] antigen could be expressed as a tumor-differentiation antigen[30]. CCA is a highly metastatic cancer with a poor prognosis. The association of sLe[a] expression with a poor prognosis in CCA and the contribution of sLe[a] to CCA-cell adhesion and transmigration via E-selectin-mediated HUVECs demonstrated in this study suggest the possible use of this ligand as a target for specific immunotherapy of CCA in the prevention of metastases, especially in patients with aberrant expression of sLe[a].

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