Positive correlation between sialyl Lewis X expression and pathologic findings in renal cell carcinoma

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Background. Interaction between tumor cells and endothelium plays a major role in cancer invasion and metastasis. Among various cell adhesion molecules, the cognate interaction between sialyl Lewis antigen expressed in the tumor cell surface and E-selectin expressed on endothelial cells is considered to be crucial for the tumor cell adhesion to the endothelium.

Methods. The sialyl Lewis X (sLX) expression in 45 specimens from renal cell carcinoma patients was examined using immunohistochemistry.

Results. In this study, we demonstrate that the immunoreactivity for sLX in renal cell carcinoma specimens not only correlates with conventional histopathologic parameters but also serves as a useful indicator for the prognosis of renal cell carcinoma.

Conclusion. Since beneficial effect of cimetidine has been reported and ascribed to its inhibitory action on the expression of E-selectin, a ligand molecule of sialyl Lewis antigen, cimetidine may also show inhibitory effect on the tumor recurrence and metastasis of renal cell carcinoma with high level of sLX expression.

In order for tumor cells to metastasize, they first need to interact with endothelial cells. This cell-to-cell interaction requires the cognate interaction of cell adhesion molecules including E-selectin (tethering), and intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (firm adhesion) that are expressed on the endothelial cell surface. Among these cell adhesion events the tethering step and thus the interaction between E-selectin and its ligand molecule sialyl Lewis antigens is considered crucial [1]. The ligands to E-selectin are sialyl Lewis antigens include sialyl Lewis X (sLX) and sialyl Lewis A (sLA). Recent reports have indicated that the beneficial effects of cimetidine for the patients with colorectal cancers are ascribed to its action in inhibiting E-selectin expression on the cell surface [2, 3]. These experimental observations were confirmed by a randomized clinical trial of cimetidine, which clearly showed that the cimetidine treatment dramatically improved the survival of colorectal cancer patients with tumor cells expressing high levels of sialyl Lewis antigens but no such effects were found when sialyl Lewis expression levels were found low or null [3].

The sialyl Lewis antigen has been used as a useful marker for the diagnosis of various cancers in digestive organs, pancreas, gallbladder, liver, lung, and ovary [4–6]. The greater level of the sLX expression was found in the metastasized lesions than the primary tumors in cases of bladder cancer [3]. Although the incidence and significance of expression of sLX antigen in renal cell carcinoma have ever been reported by only Cordon-Cardo et al [7], cimetidine has been shown to have beneficial effects on the survival of patients with renal cell carcinoma. In addition, Kinouchi et al [8] reported that the combined therapy with interferon-α (INF-α) and cimetidine were effective even in advanced cases of renal cell carcinoma. Kobayashi et al [9] found that cimetidine could block the expression of E-selectin on the surface of human umbilical vein endothelial cells (HUVECs), which subsequently reduced the adhesion of tumor cells to the endothelium and prevented liver metastasis in a nude mice model. These findings indicate that the expression and the interaction of these cell adhesion molecules may be a rate-determining step for the initiation of the aggressive expansion of tumor cells such as invasion and metastasis.

In this study, we demonstrate that the level of cell surface expression of sLX antigen in renal cell carcinoma tumors was correlated with the clinical outcome as well as the histopathologic characteristics. The significance of these findings is discussed in terms of the choice of adjuvant cancer therapy.
METHODS

Patients and samples

Forty-five patients who were diagnosed to have renal cell carcinoma and received radical nephrectomy at Nagoya City University Hospital between 1997 and 2002 were enrolled in this study. These patients were subdivided by into pT1 (14 patients), pT2 (25 patients), pT3 (five patients), and pT4 (one patient) groups according to the TNM classification of malignant tumors. Metastases were present at initial diagnosis in 11 patients (M1). No metastatic lesion was found in the other 34 patients (M0). The nuclear grading of cancer was determined based on the General Rules for Clinical and Pathological Studies on Renal Cell Carcinoma proposed by the Fuhrman’s grading system. Histopathologic grading based on the nuclear morphology is as follows: grade 1, nuclei indistinguishable from those of normal tubular cells; grade 2, moderately enlarged, often irregular and slightly pleomorphic nuclei with defined nucleoli and no bizarre forms; and grade 3, numerous bizarre or giant nuclei. The classification of vascular invasion is as follows: pV0, no vascular invasion in specimen; pV1a, microscopic vascular invasion in a renal vein; pV1b, macroscopic vascular invasion in a renal vein; pV2, vascular invasion in vena cava. For the detection of distant metastasis, all the patients were checked at least twice per year for occurrence of metastasis by x-ray studies, computed tomography, and bone scintigraphy during the follow-up period.

Immunohistochemistry

Paraffin-embedded tissue sections obtained from 45 patients during the operation were deparaffinized in a cleaning solution (Histochoice) (Amresco, Solon, OH, USA), rehydrated in a graded series of ethanol (100%, 95%, 70%, and 50%), and washed in distilled water. Endogenous peroxidase activity was quenched by 1.5% H2O2 in phosphate-buffered saline (PBS) for 15 minutes followed by washing twice with PBS. Nonspecific protein recognition by the antibody was blocked in casein wash buffer (containing 0.3% casein and 0.5% Tween-20 in PBS) for 30 minutes. Tissue sections were then incubated for 1 hour at room temperature with the primary antibody, monoclonal anti-sLX antibody (Seikagaku Co., Tokyo, Japan), or anti-E-selectin antibody (Dako, Copenhagen, Denmark). After being washed twice in 1:10 casein wash buffer for 5 minutes, and incubated with 1:250 biotinylated antimouse IgG (Vector Laboratories, Burlingame, CA, USA) for 30 minutes, the specific intracellular immunoreactivity was detected by incubation with avidin-biotin/horseradish peroxidase complex (Vector Laboratories) for 45 minutes at room temperature followed by color development in 0.05% diaminobenzidine (DAB)/0.01% H2O2/PBS (pH 7.6) chromogen (Sigma Chemical Co., St. Louis, MO, USA) for 5 minutes. Color development was stopped by washing in distilled water, and sections were lightly counterstained in hematoxylin, dehydrated in a graded series of alcohol, cleared in xylene, and finally mounted in Eukitt.

Semiquantitative analysis of sLX expression

The degree of sLX expression was estimated and classified into one of five grades as described previously [10]. Immunoreactivity of sLX was classified into a scale of 0 to 4 on the basis of staining of tumor cells as follows: 0, no staining; 1, focal, weak staining; 2, strong staining of <25% of cells or moderate staining of 25% to 50%; 3, strong staining of 25% to 50% or moderate staining of >50%; and 4, strong staining of >50%. The immunostained tissue section slides were examined and scored independently by two of the authors blinded to any other pathologic or clinical information. In 60% of cases the decisions were consistent, and the other 40% were reviewed until agreement was achieved.

Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Individual groups (each pathologic grade and TNM classification) were then compared using the nonparametric Mann-Whitney U test, generalized Wilcoxon test and Student t test. For all analyses a probability value of P < 0.05 was considered statistically significant.

RESULTS

Among the 45 renal cell carcinoma patients enrolled in this study, 27 (60%) had grade 1 tumors, 11 (24.4%) had grade 2 tumors, and seven (15.6%) had grade 3 tumors. All the tumor tissues were examined for the expression of sLX and compared with histopathologic findings and clinical characteristics, including the recurrence rate and the incidence of metastasis. The staining of sLX antigen was predominantly detected in the cell membrane of tumor cells or intercellular matrix. In most cases, heterogeneity of sLX staining was noted within individual tumor samples showing either variation in the intensity of staining or patchiness of the DAB staining (Fig. 1).

In Figure 2A, the positive correlation of semiquantitative evaluation of the sLX expression (immunoreactivity) and the tumor staging (pT) are demonstrated. There were significant differences in sLX expression between pT1a and pT3 (P = 0.009 by Mann-Whitney test), and pT2 and pT3 (P = 0.034). Although only single cases were assigned to pT1b and pT4, there was a strong positive correlation between the size of tumor mass and its extension and the extent of sLX antigen expression. Interestingly, the level of sLX expression was positively correlated
with the rate of local recurrence and metastasis of cancer (collectively called “recurrence” in this study) (Fig. 2B). Among 34 patients, the tumor recurrence within 3 years after the radical nephrectomy was noted in 11 cases (32%). In these patients, the level of sL$^X$ expression of the original tumors that were resected was significantly higher than those without recurrence.

We then looked at the extent of vascular infiltration of the original tumor tissue. Whereas no vascular infiltration of tumor cells was noted in 23 out of 24 patients without recurrence, significant vascular infiltration was evident in all the patients with tumor recurrence (11 patients). Only one patient had a sign of mild local vascular infiltration but no recurrence was detected. These findings of vascular infiltration with regard to the level of sL$^X$ expression (immunoreactivity) are depicted in Figure 2C. These observations clearly illustrate that the tumor with high expression of sL$^X$ antigen had a higher level of vascular infiltration ($P < 0.001$, overall). The patients with the tumor with high expression of sL$^X$ antigen showed a significantly higher rate of tumor recurrence ($P < 0.05$). There was no significant difference between the expression of sL$^X$ and pathologic subtypes of renal cell carcinoma.

Among 45 cases, studied 36 (80%) and nine (20%) cases were pathologically diagnosed as the clear cell type and the chromophobe cell type, respectively. However, there was no statistical significance in the level of sL$^X$ expression with regard to the cell types. No statistical significance was found between the tumor cell type and the rate of tumor occurrence. In either cell types, the tumor of low sL$^X$ immunoreactivity showed less probability of local recurrence. Moreover, the level of sL$^X$ expression in patients with distant metastasis was significantly higher than that without distant metastasis ($P < 0.0001$). Thus, the expression levels of sL$^X$ antigen appear to be a significant predictor for the development of metastases and tumor-free survival rate. In Figure 2D, we examined the relationship between the pathologic grading based on the nuclear morphology and the level of sL$^X$ expression. Whereas most of the cases (27 patients) were classified into grade 1, tumors from 11 and seven patients were classified into grades 2 and 3, respectively. Interestingly, there was a strong difference in the levels of cell surface sL$^X$ expression and this pathologic classification. Lower grade tumor showed significantly lower levels of sL$^X$ expression: between grades 1 and 2 ($P = 0.04$ by Mann-Whitney test); between grades 1 and 3 ($P = 0.0002$ by Mann-Whitney test); and no statistical significance between grades 2 and 3.

Finally, we have examined the metastasis-free period by the classification based on the level of sL$^X$ expression. As shown in Figure 3, we found a significant difference in the rate of tumor-free survival between cases with immunoreactivity $\leq 2$ (low sL$^X$ expression) and those
with immunoreactivity >2 (high sL$^X$ expression) ($P = 0.0047$ by generalized Wilcoxon test). The cumulative 3-year tumor-free survival rate of the immunoreactivity $\leq 2$ group of renal cell carcinoma patients ($N = 16$) was 90%, whereas that of the immunoreactivity $> 2$ group ($N = 11$) was only 38.5%. Any greater differences were found when we classified the renal cell carcinoma cases by tumor grading, tumor cell types, or pT staging (data not shown).

**DISCUSSION**

Currently, urologic surgeons do not have powerful measures to assess the aggressiveness of advanced renal cell carcinoma and predict future prognosis of the patients besides pathologic diagnosis. Neither do we have any better adjuvant therapeutic options to construct effective therapeutic strategies according to the individual characteristics of tumor other than radical nephrectomy. In this study, we demonstrate that expression of sL$^X$
antigen on renal cell carcinoma tumor cells showed strong positive correlation with both macroscopic and microscopic pathologic findings, and clinical outcomes such as metastasis and tumor-free survival.

The major benefit of these findings in terms of a proposal of novel therapy comes from previous reports with colorectal cancers and the dramatic beneficial effect of cimetidine [2, 9, 11, 12]. For example, Matsumoto et al [2, 12] reported that the treatment with cimetidine markedly reduced the frequency of metastasis and significantly increased the survival rate in the patients whose tumor cells expressed higher levels of the sL^X and the sL^A epitopes. However, cimetidine was not effective in the patients with lower levels (or none) of these epitopes, although such cancers are considered to be less aggressive. It was demonstrated that a 1-year course of cimetidine produced a 10-year survival rate of 96% in patients whose tumor had high sL^X expression with cimetidine, compared to only 35% in control cases without cimetidine treatment [11]. Similar observations with cimetidine were reported with renal cell carcinoma [8].

Although the mechanism of cimetidine to endow cancer patients of high sL^X antigen expression in tumors with the beneficial effects, Kobayashi et al [9] clearly showed that this effect of cimetidine is ascribed to the down-regulation of E-selectin, a ligand molecule for sialyl Lewis antigens, that is expressed on the endothelium. They demonstrated that cimetidine could block the expression of E-selectin and thus inhibited the adhesion of tumor cells to the HUVECs and that the cimetidine administration in nude mouse model also inhibited the transsplenic liver metastasis [9]. Other possible effects of cimetidine include (1) inhibition of the activity of suppressor T lymphocytes bearing a histamine type 2 receptor in cancer patients [11, 13]; (2) cimetidine, acting as an antioxidant, inhibits tumor growth [14]; (3) prevention of postoperative alterations of lymphocyte subpopulations [15]; and (4) maintenance of natural killer cell activity [16].

Metastasis is the hallmark of malignant phenotype of cancer. The involvement of either sL^A or sL^X in adhesion to the endothelium is still controversial and may depend on the tissue types of cancers [17, 18]. The hematogenous metastasis of colorectal cancer and pancreatic cancer is mainly mediated by sL^A/E-selectin interaction [18] whereas that of renal cell carcinoma involves primarily sL^X in at least three renal cell carcinoma cell lines in cell culture experiments [19]. Furthermore, Steinbach et al [19] concluded that cytokines significantly affect the adhesion of renal cell carcinoma to the endothelium, and that cytokine-induced increases in tumor endothelial binding are mediated at least in part by the E-selectin/sL^X interaction.

In one of our previous studies [1], we reported that adhesion of tumor cell line QG90 derived from lung cancer to HUVEC was dependent on E-selectin expression on the cell surface of HUVEC. The adhesion of cancer cell to HUVEC and E-selectin expression was induced by interleukin (IL)-1β. The various inhibitors of the nuclear factor-kappaB (NF-κB) activation cascade could block the cell adhesion mediated by E-selectin and sL^X as E-selectin gene expression is under the transcriptional control of NF-κB. However, the action of cimetidine in blocking E-selectin does not appear to be at the level of transcription but rather at a step after transcription [9]. In this regard, it may be worth noting that a possible involvement of other regulatory molecules such as p38 mitogen-activated protein kinase (MAPK), in addition to NF-κB, also participating in the IL-1β signaling [20, 21] should be examined as p38 MAPK is required for the E-selectin expression most likely at the level of post-transcription [20–25]. In this context, the issue of whether cimetidine but not other histamine receptor (H2R) antagonists could interfere with such signaling cascade should also be explored.

The design of our study took into account the similarity between renal cell carcinoma and colorectal cancer in that cimetidine has beneficial effects on both cancers. As shown in this study, the immunoreactivity to sL^X on renal cell carcinoma specimens was remarkably correlated with T stage and tumor-free survival (Figs. 2 and 3). Semiquantitative analysis revealed that elevated expression of the sL^X epitope was associated with the potential for metastasis, suggesting the importance of this epitope as a ligand for E-selectin. If the cimetidine given to the patients can efficiently block the expression of E-selectin on vascular endothelial cells, even malignant renal cell carcinoma cells expressing higher levels of sL^X would not be able to
adhere to the endothelium and the frequency of metastasis in the patients would be reduced, resulting in the beneficial effects for the patient survival. Taken together, these results suggested a beneficial effect of cimetidine on renal cell carcinoma patients, presumably by blocking the expression of E-selectin on vascular endothelial cells and inhibiting the adhesion of cancer cells. Future studies should clarify the effect of cimetidine on renal cell carcinoma with a high level of sL\textsuperscript{X} expression.

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