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 sL^X antigen in RCC tumors was correlated with the clinical outcome as well as the histopathological characteristics. The significance of these findings is discussed in terms of the choice of adjuvant cancer therapy.

Materials and Methods

Patients and Samples.

Forty-five patients who were diagnosed to have RCC 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 (5 patients), and pT4 (1 patient) groups according to the TNM classification of malignant tumors. Metastases were present at initial diagnosis in 11 patients (M1). No metastatic legion was found in the other 34 patients (M0). The nuclear grading of cancer was determined based on the Fuhrman's grading system and Japanese general rules for clinical and pathological studies . Histopathological 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; 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, computed tomography and bone scintigraphy during the follow-up period.

Immunohistochemistry

Paraffin-embedded tissue sections obtained from 45 patients during the operation were deparafinized in a cleaning solution (Histochoice, Amresco, 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% H_2O_2 in PBS for 15 min followed by washing twice with PBS. Non-specific protein recognition by the antibody was blocked in casein wash buffer (containing 0.3% casein and 0.5% Tween 20 in PBS) for 30 min. Tissue sections were then incubated for 1 h at room temperature with the primary antibody, monoclonal anti-sialyl Lewis X 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 min, and incubated with 1:250 biotinylated anti-mouse IgG (Vector Laboratories, CA) for 30 min. The specific intracellular immunoreactivity was detected by incubation with avidin-biotin/horse radish peroxidase complex (Vector Laboratories, CA) for 45 min at room temperature followed by color development in 0.05% diamino-benzidine/0.01% H_2O_2/PBS (pH7.6) chromogen (Sigma, MO) for 5 min. 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 sL^X expression

The degree of sL^X expression was estimated and classified into one of five grades as described previously[10]. Immunoreactivity (IR) of sL^X 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 $<80\%$; 3, strong staining of 25 - 50% or moderate staining of $>80\%$; 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 pathological grade and TNM classification) were then compared using the nonparametric Mann-Whitney U-test, generalized Wilcoxon test and Student's *t*-test. For all analyses a probability value of $p < 0.05$ was considered statistically significant.

Results

Among the 45 RCC patients enrolled in this study, 27 (60.0%) had grade-1 tumors, 11 (24.4%) had grade-2 tumors, and 7 (15.6%) had grade-3 tumors. All the tumor tissues were examined for the expression of sL^X and compared with histopathological findings and clinical characteristics including the recurrence rate and the incidence of metastasis. The staining of sL^X antigen was predominantly detected in the cell membrane of tumor cells or intercellular matrix. In most cases, heterogeneity of sL^X staining was noted within individual tumor samples showing either variation in the intensity of staining or patchiness of the DAB staining (Fig. 1).

In Fig. 2A, the positive correlation of semiquantitative evaluation of the sL^X expression (IR) and the tumor staging (pT) is demonstrated. There were significant differences in sL^X 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 sL^X antigen expression. Interestingly, the level of sL^X 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 (IR) are depicted in Fig. 2C. These observations clearly illustrates that the tumor with high expression of sL^X antigen had a higher level of vascular infiltration ($p<0.001$, over all). 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 pathological subtypes of RCC.

Among 45 cases studied 36 (80%) and 9 (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 IR 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 Fig. 2D, we examined the relationship between the pathological 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 7 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 pathological 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); 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 Fig. 3, we found a significant difference in the rate of tumor-free survival between cases with IR$\leqq$2 (low sL^x expression) and those with IR$>$2 (high sL^x expression) (p=0.0047 by generalized Wilcoxon test). The cumulative 3-year tumor-free survival rate of the IR$\leqq$2 group of RCC patients (n=16) was 90.0%, whereas that of the IR$>$2 group (n=11) was only 38.5%. Any greater differences were found when we classified the RCC cases by tumor grading, tumor cell types, or pT staging (data not shown).

Discussion

Currently, urological surgeons do not have powerful measures to assess the aggressiveness of advanced RCC and predict future prognosis of the patients besides pathological 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 RCC tumor cells showed strong positive correlation with both macroscopic and microscopic pathological 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 RCC [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 sL 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 human umbilical endothelial cells (HUVEC) and that the cimetidine administration in nude mouse model also inhibited the trans-splenic liver metastasis [9]. Other possible effects of cimetidine include: (i) inhibition of the activity of suppressor T lymphocytes bearing a histamine type 2 receptor in cancer patients [11,13]; (ii) cimetidine, acting as an antioxidant, inhibits tumor growth [14], (iii) prevention of postoperative alterations of lymphocyte subpopulations [15], and 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 RCC involves primarily sL^X in at least three RCC cell lines in cell culture experiments[19]. Furthermore, Steinbach et al. [19] concluded that cytokines significantly affect the adhesion of RCC 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 IL-1β. The various inhibitors of the 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 nothing that a possible involvement of other regulatory molecules such as p38 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 expressionmost likely at the level of posttranscription [20-25]. In this context, the issue of whether cimetidine but not other H2R antagonists could interfere with such signaling cascade should also be explored.

The design of our study took into account the similarity between RCC and colorectal cancer in that cimetidine has beneficial effects on both cancers. As shown in this study, the immunoreactivity to sL^X on RCC specimens was remarkably correlated with T stage and tumor- free survival (Figs. 2, 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 RCC 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 RCC 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 RCC with a high level of sL^X expression.

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Figure legends

Fig. 1. Histopathological findings of the tissue expression of sL^X in RCC. (x 400)

sL^x staining of three representative RCC cases are presented. A,C, E: immunostaining of sL^x antigen. B, D, F: hematoxylin-eosin staining of RCC. The tissue sections of the same patients (A and B; C and D; E and F) were stained. The level of sL^x expression is expressed as the immunoreactivity (IR) to the mouse monoclonal antibody to human sL^x antigen (see Materials and Methods for the details). The pathological diagnosis, based on the General Rules for Clinical and Pathological Studies on RCC proposed by the Japanese Urological and Pathological Association (11), of each patient is also indicated (G: nuclear morphology; pT: tumor size; pV: vascular infiltration).

Fig. 2. Positive correlation of the level of sL^x antigen expression and the tumor staging (pT), the cancer recurrence, the degree of vascular infiltration of tumor, and the nuclear grading of tumor cells. (A) sL^X expression of tumor tissues and T stages. The level of sL^X expression is determined according to the results of immunostaining of each tumor tissue ans expressed as immunoreactivity (IR) score. The tumor tissues are classified according to the tumor staging (pT) based on the size of primary tumor. The average sL^X expression levels were compared among these groups and the differences were assessed by Mann-Whitney test. n, number of RCC cases in each category. (B) sL^X expression and the cancer recurrence. The average sL^X expression levels were compared between groups with and without cancer

recurrence (local recurrence and detection of distant metastasis). The statistical differences were found between these two groups ($p<0.05$). (C) Vascular infiltration of tumor cells and sL^X expression. The extents of vascular invasion of tumor cells were determined by microscopic examination of the resected pathological specimen obtained during surgical operation. Note that significant statistical differences ($p< 0.05$) were found between pV0 and pV1a, and pV0 and pV2. No significant differences were found between pV1a and pV2. (D) Positive correlation between the pathological grading and the level of sL^x expression. The pathological grading was determined for each case based on the nuclear morphology of tumor cells (11). Significant differences in the level of sL^X expression were found between pathological grades 1 and 3 ($p<0.001$), and grades 1 and 2 ($p<0.05$).

Fig. 3. The level of sL^x expression and the metastasis-free period.

All the RCC cases were classified into two groups: low sLx expression (IR$\leqq$2) and high sL^x expression (IR$>$2). Twenty-seven cases without metastasis upon the initial diagnosis were followed up for 3 years. All patients received medical check-up including X-ray, computed tomography, and whole body bone schintigraphy at least twice a year during the follow-up period. The level of sL^x expression was assessed by immunohistochemistry using the resected tumor tissue upon radical nephrectomy (year=0). Statistically significant difference in the metastasis-free period was observed between low sLx expression (IR$\leqq$2) and high sLx expression (IR$>$2) (p=0.0047).

Fig. 1 Tozawa et al.

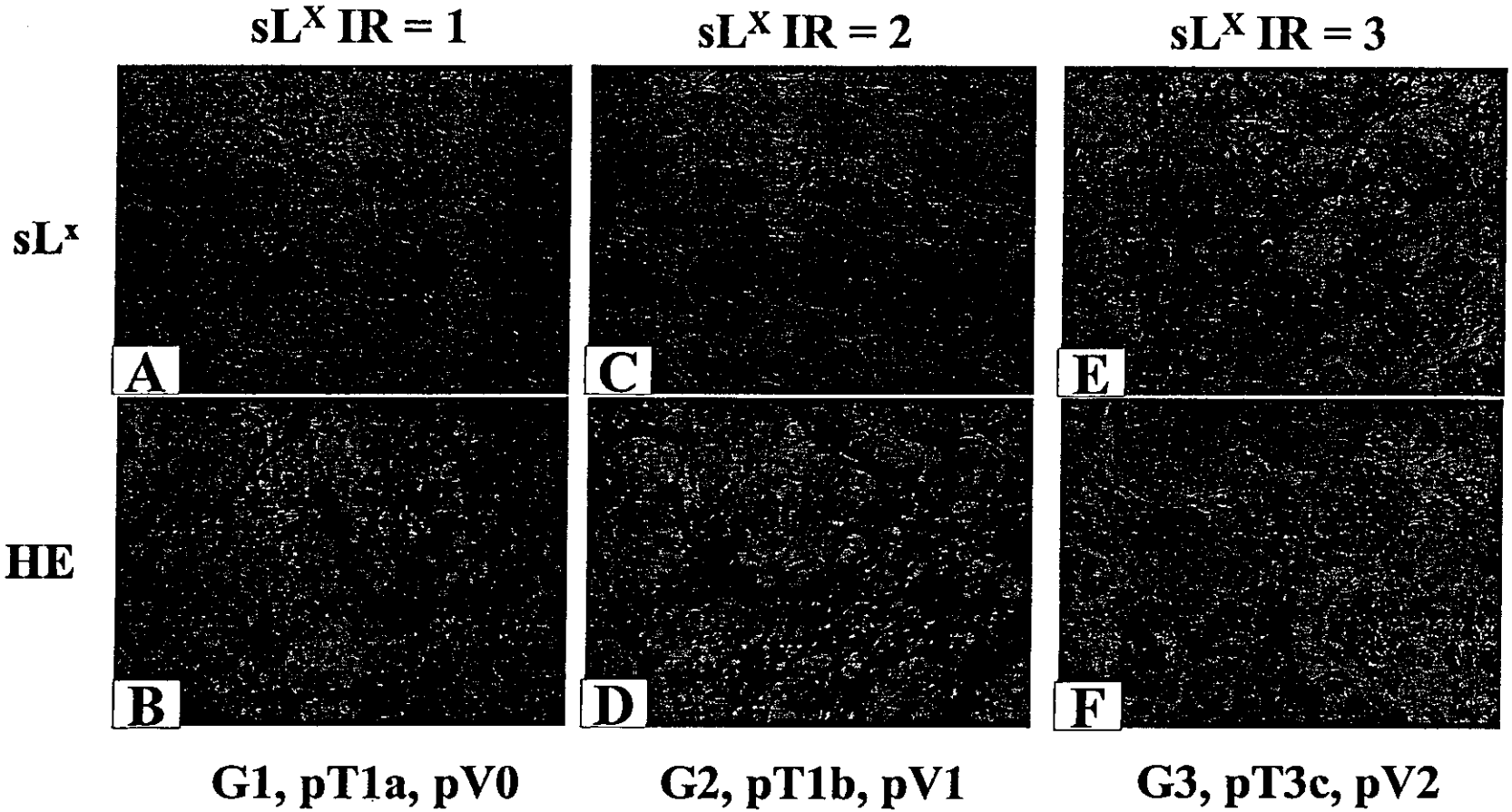

Fig. 2A Tozawa et al.

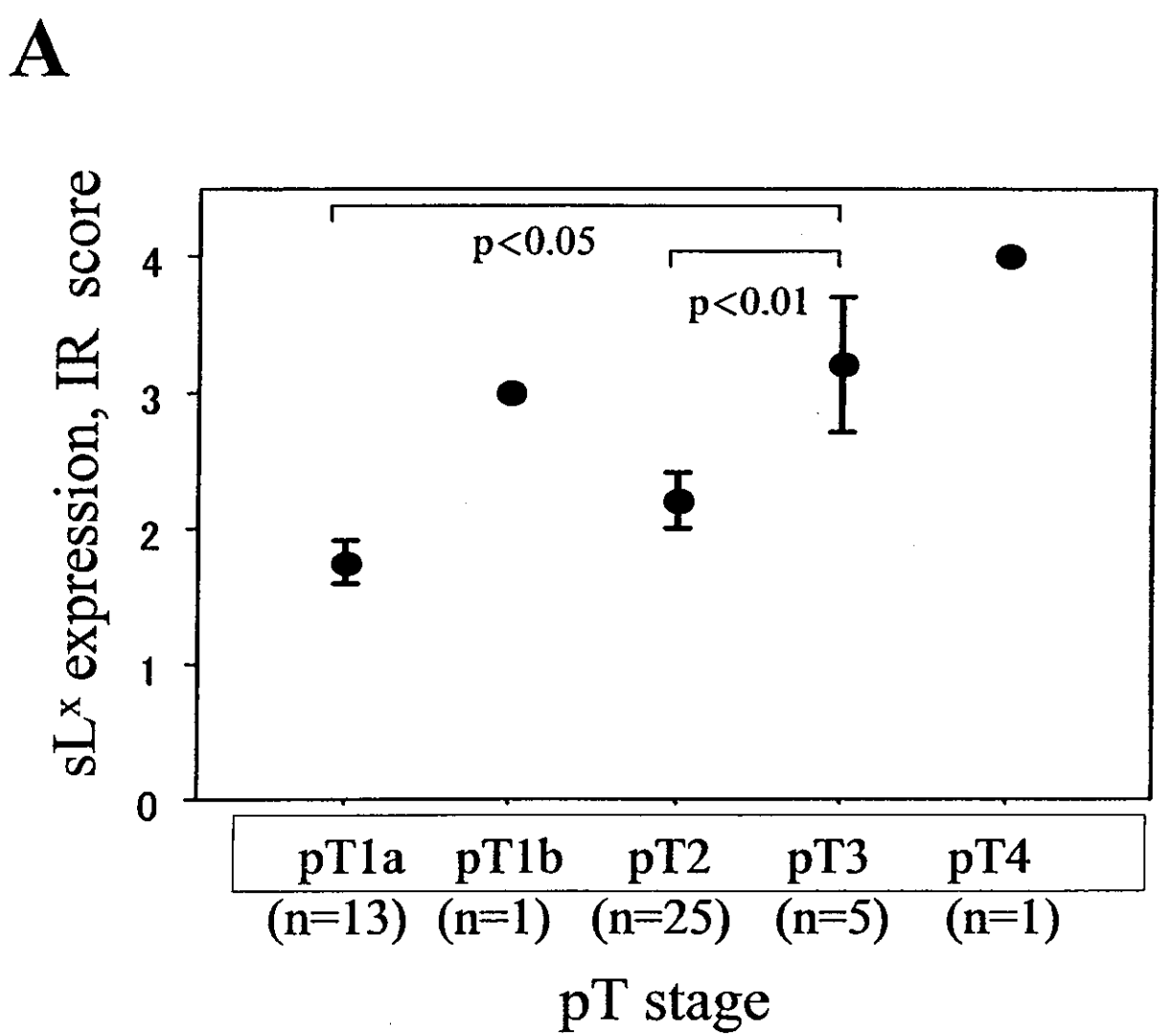

Fig. 2B Tozawa et al.