

MATERIALS AND METHODS

Gastric cancer cases

A total of 846 gastric cancer patients who underwent surgical treatment in the National Cancer Center Hospital, Japan from 2001 to 2004 were studied. Clinical stages and pathological features of primary tumours were defined according to the classification of the International Union Against Cancer (Sobin and Fleming, 1997). There were 567 male and 279 female patients with average age 61.5, and a range of 27–87 years. None of these patients underwent endoscopic mucosal resection or palliative resection. Written informed consent had been obtained from all patients.

Normal controls

For normal negative controls, both peripheral blood and bone marrow were collected from 25 patients having no malignancy from April 2000 to March 2003. This group included 16 cases of gallstone, three cases of common bile duct stone, and six cases of incisional hernia. We extracted BM from the sternum of patients without malignancies but had operations under general anaesthesia.

Bone marrow and blood samples

Aspiration of both BM and PB was conducted immediately prior to operation under general anaesthesia. The aspirated BM was obtained from the sternum using a bone marrow aspiration needle (MDTECH, Gainesville, FL, USA). Peripheral blood was obtained through a venous catheter. The 1 ml of both BM and PB samples was discarded to avoid the contamination of the epidermal cells (Iinuma *et al*, 2006). Each 1 ml sample of BM and PB was immediately mixed with 4 ml of ISOGEN-LS (Nippon Gene, Toyama, Japan) and stored at -80°C until RNA extraction.

Total RNA extraction and first strand cDNA synthesis

Total RNA was according to the ISOGEN-LS manufacturer's protocols. All the clinical samples obtained in National Cancer Center Hospital were sent to our institute. The reverse transcriptase reaction (RT) was performed as described earlier (Mori *et al*, 1995; Masuda *et al*, 2002). The first strand cDNA was synthesized from 2.7 μg of total RNA in a 30 μl reaction mixture containing 5 μl $5\times$ RT reaction buffer (BRL, Gaithersburg, MD, USA), 200 μM dNTP, 100 μM solution of random hexadeoxynucleotide mixture, 50 units of Rnasin (Promega, Madison, WI, USA), 2 μl of 0.1 M dithiothreitol, and 100 U of Molony leukaemia virus RT (BRL). The mixture was incubated at 37°C for 60 min, heated to 95°C for 10 min, and then chilled on ice.

Primers and probes for detecting ITCs and uPAR expression

Quantitative RT-PCR methodology was designed to optimise the specificity and fidelity of the assay. The Kyushu University group had previously investigated the expression of seven representative molecular markers (carcinoembryonic antigen (CEA), CK-7, CK-18, CK-19, CK-20, mammaglobin and mucin (MUC)-1) in 27 cancers and eight non-epithelial cell lines using quantitative RT-PCR. The expression levels of CK-7 and CK-19 showed high sensitivity and specificity for the identification of gastric cancer cells in comparison with the other markers (Masuda *et al*, 2005). Those epithelial cell markers have been widely used as target genes for the detection of ITCs (Mori *et al*, 1996, 1998; Yamaguchi *et al*, 2000; Bessa *et al*, 2003; Sadahiro *et al*, 2005). Thus, CEA, CK-7, CK-19 and GAPDH were studied by quantitative RT-PCR in all 846 patients (Mimori *et al*, 2008). Isolated tumour cells were considered to be 'present' when any single marker was positive.

The reverse transcriptase reaction was performed as described elsewhere (Mimori *et al*, 2008). We performed real-time quantitative RT-PCR using a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) to detect ITCs in peripheral blood and/or bone marrow as the previous study.

Moreover, a uPAR-specific oligonucleotide primer was designed as follows: sense, 5'-TGAATCAATGTCTGGTAGC-3' and antisense, 5'-TGGTTACAGCCACTTTTAGT-3'. The donor and acceptor probe sequences for uPAR identification were 5'-GCTATATGGTAAGAGGCTGTGCAACCGCCT-fluorescein and 5'-LC-Red640-AATGTGCCAACATGCCCCACCTGGG T-phosphorylation. Besides, uPA primers were as follows: forward primer; CTGTGACTGTC TAAATGGAGG; and the reverse primer; GACGATGTAGTCCT CTTTCTT (Nielsen *et al*, 2004).

Preliminary trials were undertaken to assure that results were accurate and reliable. We utilised highly specific hybridisation probes and primers to maintain high specificity for target genes and thereby reduce false positive outcomes.

RT-PCR conditions

PCR amplification was performed using a quantitative fluorescence LightCyclerTM (Roche Diagnostics, Mannheim, Germany) in a 20 μl reaction mixture containing 2 μl of LightCycler FastStart DNA Master Hybridisation Probes (Roche, Diagnostics, Tokyo, Japan), 4.0 μl MgCl_2 , 0.3 μM sense and antisense primers, 0.2 μM fluorescent probe, 0.2 μM LC-Red probe and 5 μl of undiluted template cDNA in LightCycler capillaries (Roche Diagnostics, Tokyo, Japan). The amplification of CEA profile consisted of one cycle at 95°C for 10 min (denaturation) followed by 45 cycles of 95°C for 15 s, 56°C for 15 s and 72°C for 13 s. The amplification of CK-7 profile consisted of one cycle at 95°C for 10 min (denaturation) followed by 50 cycles of 95°C for 10 s, 60°C for 12 s and 72°C for 10 s. The amplification of CK-19 profile consisted of one cycle at 95°C for 10 min (denaturation) followed by 45 cycles of 95°C for 10 s, 60°C for 15 s and 72°C for 16 s. The amplification of uPAR profile consisted of one cycle at 95°C for 10 min (denaturation) followed by 40 cycles of 95°C for 10 s, 62°C for 15 s and 72°C for 8 s. The amplification of GAPDH profile consisted of one cycle at 95°C for 10 min (denaturation) followed by 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 13 s. The amplification of uPA was as follows: 5 min at 94°C , 27 cycles of 1 min at 94°C , 1 min at 56°C , 1 min at 72°C , then 10 min at 72°C (Nielsen *et al*, 2004). Real-time PCR was monitored by measuring fluorescent signals at the end of the annealing phase for each cycle. All primers and probes were synthesized and purified by reverse phase high-performance liquid chromatography and the optimal reagent concentrations and PCR cycling conditions were established and each run of RT-PCR included positive controls synthesized from plasmids by the Nippon Gene Research Laboratories (Sendai, Japan). Real-time RT-PCR assays were repeated in triplicate and adapted the mean value. Quantification data were analysed using the Light-CyclerTM software (Roche Diagnostics, Tokyo, Japan).

Data analysis

A standard curve was prepared with 200–20 000 copies of purified plasmids containing CEA, CK-7, CK-19, uPAR and GAPDH. After proportional baseline adjustment, the fit point method was employed to determine the cycle in which the log-linear signal was first distinguishable from the baseline, and then that cycle number was used as a crossing-point value (Marutsuka *et al*, 2003). The standard curve was produced by measuring the crossing point of each standard value and plotting them against the logarithmic value of concentrations. Those concentrations were calculated by plotting their crossing points against the standard curve.

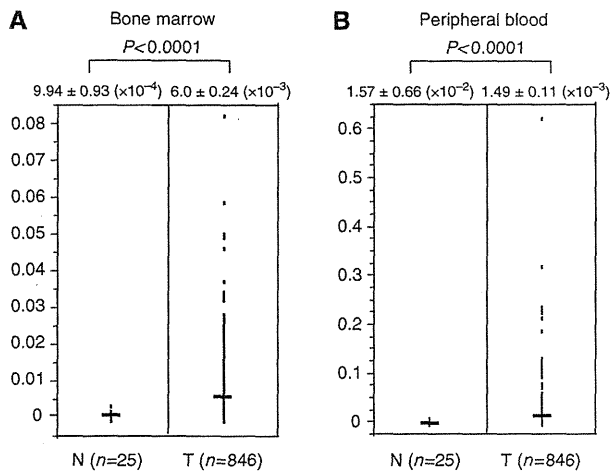


Figure 1 Comparison of *uPAR* mRNA expression between control gastric cancer patients in bone marrow (A) and peripheral blood (B). Horizontal lines indicate the mean expression levels in control and gastric cancer patients. In bone marrow and peripheral blood, the value of the *uPAR* expression of gastric cancer patients was significantly higher ($P < 0.0001$) than those of control cases. The P -value was calculated by a student's t -test.

Expression of CEA, CK-7, CK-19 and *uPAR* was adjusted in each case for GAPDH expression. We set cutoff values for those expression ratios (*CEA*/GAPDH, *CK-7*/GAPDH, *CK-19*/GAPDH, *uPAR*/GAPDH) as the highest value for each marker in 25 normal controls. We distributed high level as 'positive' and low level as 'negative' than the cutoff value. For continuous variables, the data were expressed as the mean \pm s.d. Statistical analysis of group differences was performed using the χ^2 test and the Student's t -test. Logistic regression model was used to identify the independent predictors of distant metastasis. All tests were analysed using JMP software (SAS Institute Inc., Cary, NC, USA). Statistical significance was determined as P -value from two-sided tests of less than 0.05.

RESULTS

Expression of *uPAR* mRNA in BM and PB of gastric cancer patients

In BM, the value of the *uPAR* expression of gastric cancer patients ($6.0 \pm 0.24 (\times 10^{-3})$) was significantly higher ($P < 0.0001$) than those of control cases ($9.94 \pm 0.93 (\times 10^{-4})$), as shown in Figure 1. In PB, the value of the *uPAR* expression of gastric cancer patients ($1.49 \pm 0.11 (\times 10^{-3})$) was also significantly higher ($P < 0.0001$) than those of control cases ($1.57 \pm 0.66 (\times 10^{-3})$).

Clinical magnitude of *uPAR* expression in gastric cancer patients

The correlations between the results for the *uPAR* mRNA level and clinicopathologic factors are summarised in Table 1. Using each cutoff value, 431 (50.9%) and 404 (47.8%) of 846 patients were estimated to be positive for *uPAR* mRNA in BM and PB, respectively.

In BM, the significantly higher population of the *uPAR* mRNA-positive cases belongs to the following clinical subgroups: invasion deeper than the muscularis propria ($P = 0.015$), perioperative overt metastases (including liver and/or lung and/or distant lymph node metastasis, $P = 0.044$) and postoperative recurrence ($P = 0.010$).

In PB, the higher expression was observed significantly in subgroup invasion deeper than the muscularis propria ($P = 0.009$),

perioperative overt metastases ($P = 0.002$), venous invasion ($P = 0.039$), and the clinical stage ($P = 0.008$).

Multivariate analysis for distant metastasis

Univariate and multivariate logistic regression analyses were performed on cases with distant metastasis (Table 2). Univariate regression analysis showed that the following factors were significantly associated with distant metastasis: histological grade, tumour size, lymph node metastasis, lymphatic invasion, venous invasion and *uPAR* mRNA expressions in BM and PB ($P < 0.05$), respectively. Multivariate regression analysis indicated that *uPAR* mRNA high expression group in PB (relative risk (RR); 1.85, 95 % confidence interval (CI); 1.08–3.23, $P = 0.03$) was an independent predictor for distant metastasis next to the incidence lymph node metastasis (RR; 6.50, 95 % CI; 2.99–15.77, $P < 0.0001$) and depth of tumour invasion (RR; 28.2, 95% CI; 14.3–70.0, $P < 0.0001$).

The comparison of *uPA* and *uPAR* expression in representative gastric cancer cases

The potential importance of *uPAR* for the development of minimal residual disease in solid cancer has been focused on for recent two decades; however, several studies revealed that the relevance of a ligand for *uPAR*, *uPA* in mediating tumour-associated proteolysis, invasion and metastasis together with *uPAR* expression (Andreassen *et al*, 2000). We examined *uPA* expression in bone marrow and peripheral blood in representative 83 cases of gastric cancer, including 18 cases of liver and/or lung metastasis, and 12 cases of recurrence of disease among 846 cases of gastric cancer. As we showed in Table 3, we found that *uPA* expression in bone marrow from gastric cancer is correlated with the incidence of lymph node metastasis and recurrence of gastric cancer cases as *uPAR* expression.

The clinical significance of both positive ITCs and positive *uPAR* expression

We identified 66 cases out of 846 (7.8%) that were positive for expression of CEA in bone marrow, whereas 108 (12.7%) were positive in peripheral blood. As for CK-7, 71 patients (8.4%) and 147 cases (20.6%) were positive in bone marrow and in peripheral blood, respectively. Cytokeratin-19 expression was detected in bone marrow in 153 cases (18.1%) and in 251 cases (29.7%) in peripheral blood. Gastric cancer cases with positive expression of any one of the three markers were defined as ITC-positive in bone marrow or peripheral blood. As outlined above, ITCs were detected in 260 (30.7%) cases in bone marrow and 417 (49.3%) cases in peripheral blood.

Moreover, we extracted 126 cases (14.9%) in BM and 200 cases (23.6%) in PB of positive ITCs and positive *uPAR* expression cases. Table 4 shows the clinicopathologic data and positive ITCs and positive *uPAR* cases from the 846 gastric cancer patients. In BM, the incidence of lymph node metastasis was significantly higher ($P = 0.012$) in the both positive group (65 of 126, 51.6%) than in the other group (285 of 720, 39.6%), and the incidence of lymphatic invasion was significantly higher ($P = 0.009$) in the both positive group (67 of 126, 53.2%) than in the other group (293 of 720, 40.7%). Moreover, the clinical stage was significantly higher ($P = 0.024$) in the both positive group (42 of 126, 33.3%) than in the other group (170 of 720, 23.4%). In PB, there was no significance between the both positive group and the other group.

DISCUSSION

In this study, we examined the clinicopathologic significance of *uPAR* expression in BM and PB in 846 cases of gastric cancer, and found that the depth of tumour invasiveness in the primary

Table 1 Relationship between uPAR expression in bone marrow and peripheral blood and clinicopathologic findings

Total	uPAR expression in bone marrow			P-value	uPAR expression in peripheral blood		
	846	Positive n = 431 (50.9%)	Negative n = 415 (49.1%)		Positive n = 404 (47.8%)	Negative n = 442 (52.2%)	P-value
Age (mean \pm s.d. ^a)		60.4 \pm 0.55	62.7 \pm 0.56	NS	61.5 \pm 0.57	61.6 \pm 0.55	NS
Gender				0.233			0.688
Male	567	297 (69.1)	270 (65.2)		268 (66.5)	299 (67.8)	
Female	279	134 (30.9)	145 (34.8)		136 (33.5)	143 (32.2)	
Histology				0.127			0.174
Differentiated	188	105 (24.4)	83 (20.0)		98 (24.3)	90 (20.4)	
Undifferentiated	658	326 (75.6)	332 (80.0)		306 (75.7)	352 (79.6)	
pT				0.015			0.009
pT1/T2	657	320 (74.3)	337 (81.2)		298 (73.8)	359 (81.2)	
pT3/T4	189	111 (25.7)	78 (18.8)		106 (26.2)	83 (18.8)	
pN				0.707			0.072
pN0	496	250 (58.0)	246 (59.3)		224 (55.5)	272 (61.5)	
pN1	350	181 (42.0)	169 (40.7)		180 (44.5)	170 (38.5)	
pM (Distant metastasis ^a)				0.044			0.002
pM0	743	369 (85.6)	374 (90.1)		340 (84.2)	403 (91.2)	
pM1	103	62 (14.4)	41 (9.9)		64 (15.8)	39 (8.8)	
Postoperative recurrence				0.01			0.115
Non recurrence	833	420 (97.5)	413 (99.5)		395 (97.8)	438 (99.1)	
Recurrence	13	11 (2.5)	2 (0.5)		9 (2.2)	4 (0.9)	
Lymphatic invasion				0.617			0.16
Negative	486	244 (58.3)	242 (58.3)		222 (55.0)	264 (59.7)	
Positive	360	187 (56.6)	173 (41.7)		182 (45.0)	178 (40.3)	
Venous invasion				0.425			0.039
Negative	702	362 (83.9)	340 (81.9)		324 (80.2)	378 (85.5)	
Positive	144	69 (16.1)	75 (18.1)		80 (19.8)	64 (14.5)	
Stage				0.081			0.008
I, II	634	312 (72.4)	322 (77.6)		286 (70.8)	348 (78.7)	
III, IV	212	119 (27.6)	93 (22.4)		118 (29.2)	94 (21.3)	

^aGastric cancer case with liver (H1) and/or lung metastasis (M1), cytology positive of peritoneal washes (CY1), peritoneal dissemination (P), and lymph node metastasis in the distant region (N3).

Table 2 Univariate and multivariate analysis for distant metastasis (logistic regression model)

	Univariate analysis			Multivariate analysis		
	RR	95% CI	P-value	RR	96% CI	P-value
Histological grade (Differentiated/undifferentiated)	2.35	1.31–2.35	<0.0001	0.58	0.24–1.45	0.239
pT (Depth of tumour invasion)	49.91	27.36–99.07	<0.0001	28.17	14.30–60.95	<0.0001
pN (Lymph node metastasis)	22.78	11.56–51.49	<0.0001	6.5	2.99–15.77	<0.0001
Lymphatic invasion	12.12	6.89–23.12	<0.0001	—	—	—
Venous invasion	4.55	2.91–7.10	<0.0001	1.61	0.90–2.88	0.106
uPAR mRNA expression in bone marrow	1.53	1.01–2.35	0.046	—	—	—
uPAR mRNA expression in peripheral blood	1.95	1.28–2.99	0.002	1.85	1.08–3.23	0.0207

tumour was significantly higher in cases of uPAR (+) in BM and/or PB than uPAR(–) cases. In addition, we disclosed an evidence of the concordant relationship between the venous permeation in primary cancer and the incidence of uPAR expression. In spite of the strong association between tumour invasiveness, the presence of uPAR in PB was uncovered to be much more intimate to the incidence of metastasis and could be an independent prognostic indicator for hematogenous metastasis. Therefore, we consider that uPAR might play a consecutive role in cancer cells to invade into vessels and/or invading into the metastatic site. Furthermore, the clinical relevance of uPAR in bone marrow was observed with the incidence of recurrence, not with the synchronous distant metastasis. There was a possible explanation which is as follows, Kook *et al* (1994) reported that uPAR can play a role in tumour cell dormancy. They reported that a uPAR-antisense strategy in a human squamous carcinoma cell line resulting in a significant

reduction of uPAR gene expression, induced tumour cell dormancy in their study. Besides, Laufs *et al* (2006) described this point in the review concerning uPAR. Therefore, the abundant expression of uPAR in bone marrow might indicate the presence of many dormant cells giving rise to recurrence in the future.

In an earlier study by Jauch *et al* (1996) uPAR is a glycosyl-phosphatidylinositol-anchored glycoprotein localised on the outer layer of the plasma membrane of cells, and it binds to its specific ligands such as urokinase-type plasminogen activator (uPA). In this study, we confirmed the concordant relationship between uPAR and uPA in bone marrow and peripheral blood indicating that both proteins have a synergistic role with each other in lymph node metastasis and recurrence of gastric cancer. However, uPAR activation ultimately leads to degradation of the extracellular matrix and fascinates cellular movement for tumour cells, which appears to be necessary for diverse

Table 3 Clinicopathologic significance of uPAR and uPA expressions in bone marrow (BM) and peripheral blood (PB) from gastric cancer cases

		uPAR-BM			uPAR-PB			uPA-BM			uPA-PB			
	<i>n</i>	Mean	s.d.	<i>P</i> -value	Mean	s.d.	<i>P</i> -value	Mean	s.d.	<i>P</i> -value	Mean	s.d.	<i>P</i> -value	
<i>ly</i>	—	58	8.9E-03	6.2E-03	NS	1.4E-02	8.1E-03	NS	2.1E-02	1.3E-02	NS	2.4E-03	2.2E-03	NS
	+	25	1.1E-02	1.1E-02		1.3E-02	9.5E-03		2.2E-02	1.8E-02		3.1E-03	7.3E-03	
<i>v</i>	—	65	9.7E-03	7.8E-03	NS	1.3E-02	8.3E-03	NS	2.1E-02	1.2E-02	NS	2.2E-03	2.1E-03	NS
	+	18	9.3E-03	9.0E-03		1.5E-02	9.2E-03		2.4E-02	2.0E-02		4.0E-03	8.6E-03	
<i>n</i>	—	62	7.9E-03	5.4E-03	0.0006	1.3E-02	8.5E-03	NS	1.8E-02	1.2E-02	0.0002	2.8E-03	5.1E-03	NS
	+	21	1.5E-02	1.2E-02		1.3E-02	8.7E-03		3.1E-02	1.7E-02		2.1E-03	1.3E-03	
<i>cy</i>	—	71	9.2E-03	7.9E-03	NS	1.3E-02	8.4E-03	NS	2.1E-02	1.5E-02	NS	2.5E-03	4.6E-03	NS
	+	16	1.3E-02	8.5E-03		1.5E-02	8.1E-03		2.6E-02	1.5E-02		3.2E-03	2.6E-03	
<i>P</i>	—	80	9.5E-03	7.9E-03	NS	1.3E-02	8.4E-03	NS	2.1E-02	1.5E-02	NS	2.6E-03	4.5E-03	NS
	+	8	1.3E-02	9.4E-03		1.4E-02	7.6E-03		2.6E-02	1.6E-02		2.7E-03	1.9E-03	
<i>met</i>	—	70	9.2E-03	7.9E-03	NS	1.3E-02	8.2E-03	NS	2.0E-02	1.3E-02	NS	2.1E-03	1.9E-03	0.0265
	+	18	1.2E-02	8.7E-03		1.6E-02	8.4E-03		2.8E-02	1.9E-02		4.6E-03	8.7E-03	
<i>rec</i>	—	76	8.9E-03	6.6E-03	0.0072	1.4E-02	8.6E-03	NS	2.0E-02	1.5E-02	0.0409	2.6E-03	4.6E-03	NS
	+	12	1.6E-02	1.3E-02		1.1E-02	5.9E-03		3.0E-02	1.3E-02		2.2E-03	1.5E-03	

cy = cytology of peritoneal washes; ly = lymphatic permeation; met = liver and/or lung metastasis; n = lymph node metastasis; p = peritoneal dissemination; rec = recurrence; s.d. = standard deviation; v = vascular permeation. Significant differences ($P < 0.05$) were described in bold letters.

functions including local invasion and metastasis of tumour cells (Heiss *et al*, 1997).

Then, the second point is what is the origin of cells expressing uPAR gene in BM and especially in PB. As a matter of fact, Heiss *et al* (1995, 1997, 2002) reported that the gastric cancer patients with cells with uPAR protein expression by immunocytology showed significantly poorer prognosis than cases without uPAR expression by Kaplan–Meier analysis in the previous study (Jauch *et al*, 1996; Hardingham *et al*, 2000). They confirmed that uPAR protein expressing cells on the surface cells was a cancer cell by immunocytological study. Their study strongly supported the current study by quantitative RT–PCR assay that the detected uPAR expression by RT–PCR should be originally from gastric cancer cells, and gastric cancer patients with cancer cells with the invasive ability especially in PB. Moreover, we additionally disclosed that cancer patients with simultaneous expression uPAR and epithelial cell markers, CEA, CK19 and CK7 showed a relatively poorer prognosis than ITCs alone. Gastric cancer cell isolated from primary cancer is circulating in the peripheral blood and bone marrow ubiquitously among whole stages (Mimori *et al*, 2008); however, isolated cancer cells with several potential abilities must be required to form metastasis. According to this study, we concluded that uPAR-expressing isolated tumour cells are important in the determination of recurrence through lymph node metastasis.

On the contrary to the hypothesis of the origin of uPAR expression in cancer cells, several studies have uncovered findings of the uPAR from host side cells in BM or PB from cancer patients. Pyke *et al* (1993) reported the abundant expression of uPAR in macrophage (Dubuisson *et al*, 2000), and Sugai *et al* (2004) disclosed that advances in gastric cancer cases indicated the activation of inflammatory cytokines, such as IL-10 and IL-12 from macrophages. Furthermore, Hildenbrand *et al* (1998) mentioned

that the abundant expression of uPAR was observed in endothelial cells, which has been recently really focused on as the key player for the initial development of metastasis. Mancuso *et al* (2001) reported that the number of circulating endothelial cells (CECs) in PB from cancer patients are more than that in healthy volunteers (Beerepoot *et al*, 2004). Asahara *et al* (1997) reported that bone marrow-derived endothelial cell progenitor cells were disseminated to the neovascularisation of the cellular surface of malignant cells (Peters *et al*, 2005). In addition, EPC-specific gene, Id-1, was reported to be identified and its consecutive role for metastasis has been reported in the recent study. Therefore, we considered that the presence of CEC or EPC in PB should be important to form metastasis, and our current study elucidated the role of uPAR especially in PB as the independent marker for metastasis.

In this study, we concluded that the RT–PCR assay for uPAR expression in PB can be one of the favourite tumour markers to predict DFS in gastric cancer outpatients. Then, we disclosed the abundant expression of uPAR in gastric cancer cases with invasion and with venous invasion abilities. Earlier Heiss *et al* distinctively disclosed that uPAR expression in BM and PB in gastric cancer is originally from cancer cells themselves (Heiss *et al*, 1995). However, as the clinicopathologic significance and the predictive role for metastasis is much more consecutive in uPAR in PB than in BM, uPAR might be originally expressed in endothelial (progenitor) cells as the host side reaction in gastric cancer patients. Further study will be required to address this controversial issue.

ACKNOWLEDGEMENTS

This work was supported by the following grant sponsors: CREST, Japan Science and Technology Agency (JST); Japan Society for the

Table 4 Relationship between epithelial marker and uPAR expression in bone marrow and peripheral blood and clinicopathologic findings

		Epithelial marker and uPAR expression in BM			Epithelial marker and uPAR expression in PB		
Total (n = 846)		Both positive n = 126	Others n = 720	P-value	Both positive n = 200	Others n = 646	P-value
Age (mean ± s.d.*)		60.9 ± 0.97	61.6 ± 0.43	NS	60.8 ± 0.81	61.7 ± 0.45	NS
Gender				0.403			
Male	567	88 (69.8)	479 (66.5)		130 (65.0)	437 (67.6)	0.526
Female	279	38 (30.2)	241 (33.5)		70 (35.0)	209 (32.4)	
Histology				0.645			
Differentiated	188	30 (23.8)	158 (21.9)		45 (22.5)	143 (22.1)	0.914
Undifferentiated	658	96 (76.2)	562 (78.1)		155 (77.5)	503 (77.9)	
pT				0.183			
pT1/T2	657	92 (73.0)	565 (78.5)		147 (73.5)	510 (79.0)	0.11
pT3/T4	189	34 (27.0)	155 (21.5)		53 (26.5)	136 (21.0)	
pN					0.012		
pN0	496	61 (48.4)	435 (60.4)		112 (56.0)	384 (59.4)	0.389
pN1	350	65 (51.6)	285 (39.6)		88 (44.0)	262 (40.6)	
pM (Distant metastasis)				0.108			
pM0	743	105 (83.3)	638 (88.6)		170 (85.0)	573 (88.7)	0.171
pM1	103	21 (16.7)	82 (11.4)		30 (15.0)	73 (11.3)	
Postoperative recurrence				0.145			
Non recurrence	833	122 (96.8)	711 (98.8)		197 (98.5)	636 (98.5)	0.961
Recurrence	13	4 (3.2)	9 (1.2)		3 (1.5)	10 (1.5)	
Lymphatic invasion				0.009			
Negative	486	59 (46.8)	427 (59.3)		117 (58.5)	369 (57.1)	0.73
Positive	360	67 (53.2)	293 (40.7)		83 (41.5)	277 (42.9)	
Venous invasion				0.102			
Negative	702	98 (77.8)	604 (83.9)		162 (81.0)	540 (83.6)	0.399
Positive	144	28 (22.2)	116 (16.1)		38 (19.0)	106 (16.4)	
Stage				0.024			
I, II	634	84 (66.7)	550 (76.4)		141 (70.5)	493 (76.3)	0.101
III, IV	212	42 (33.3)	170 (23.4)		59 (29.5)	153 (23.7)	

*s.d. = standard deviation.

Promotion of Science (JSPS) Grant-in-Aid for Scientific Research, Grant numbers 17109013, 17591411, 17591413, 18390367, 18590333, 18659384 and 18790964; The Ministry of Education, Culture, Sports,

Science and Technology (MEXT) Grant-in-Aid for Scientific Research on Priority Areas, Grant number 18015039; Third Term Comprehensive 10-year Strategy for Cancer Control, Grant number 16271201.

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Original Communications

Diameter of splenic vein is a risk factor for portal or splenic vein thrombosis after laparoscopic splenectomy

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Background. Splenomegaly is a risk factor for post-splenectomy portal or splenic vein thrombosis (PSVT) due to large splenic vein stump. The relationship between splenic vein diameter (SVD) and PSVT has not been established.

Objectives. To investigate whether SVD is a risk factor for PSVT.

Methods. Forty patients who underwent laparoscopic splenectomy were analyzed. Preoperative and postoperative enhanced helical computed tomographic scans were obtained in all patients, and subsequent follow-up was performed in patients with PSVT during anticoagulant therapy. SVDs at the junction of portal vein (PV) 2, 4, and 6 cm from the junction of PV were measured preoperatively and postoperatively. Multivariate analysis was performed using logistic regression model.

Results. PSVT was diagnosed in 52.5% (21/40) patients. Preoperative SVD was significantly larger in patients with PSVT than in those without PSVT. Seventy-two percent of patients (16/22) with PSVT in splenic veins with a diameter of >8 mm developed PSVT. Multivariate analysis identified preoperative SVD as a significant and independent determinant of PSVT. At a cutoff value of 8 mm, receiver operator characteristic analysis for prediction of PSVT provided an area under the curve of 0.8552 (95% CI 0.821–1.000).

Conclusion. Preoperative SVD is a risk factor for post-splenectomy PSVT. We recommend measurement of SVD preoperatively in patients elected to undergo splenectomy, and a close follow-up of patients with SVD greater than 8 mm. (*Surgery* 2009;145:457–64.)

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PORTAL OR SPLENIC VEIN THROMBOSIS (PSVT) is a serious but rare complication of elective splenectomy.^{1,2} However, with the improvement in diagnostic modalities and increased interest in this disease entity, it is becoming apparent that the incidence of PSVT is greater than clinically appreciated.^{3–6} Since the clinical manifestations of PSVT are unspecific and most of the patients with radiologically detected PSVT are asymptomatic, diagnostic work-up, prophylaxis, and treatment for PSVT remain to be established.⁷

Accepted for publication June 23, 2008.

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0039-6060/\$ - see front matter

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doi:10.1016/j.surg.2008.06.030

PSVT occurs as a result of one or several prothrombotic disorders. These factors can be grouped into systemic and local factors.⁸ After splenectomy, the systemic factors include postoperative hypercoagulable state and thrombocytosis, while local factors such as venous flow congestion at the stump of the splenic vein may promote thrombus generation.

From a clinical point of view, splenomegaly, thrombocytosis and myelodysplastic disease are recognized risk factors for PSVT.⁹ Some authors recommended imaging surveillance for patients with these risk factors.^{3,4,10} Splenomegaly is considered a risk factor, because a large stump of splenic vein causes blood turbulence, which in turn can result in a local increase in coagulability and enhanced thrombus formation.^{2,4} Postoperative thrombocytosis may also have a direct impact on thrombus formation.¹¹ Some groups recommended ligation of the splenic vein at the junction of the inferior

mesenteric vein (IMV) to prevent blood stasis.¹² Therefore, the size of splenic vein stump after splenectomy may play a role as a local factor, however, the relationship between the size of splenic vein stump and PSVT has not been established.

Because splenomegaly is caused by a variety of conditions, eg, portal hypertension, hypersplenism, inflammatory reaction, and tumor growth, the weight of the spleen cannot simply represent the size of splenic vein stump. Prediction of spleen weight before operation is time consuming. We hypothesized that the diameter of the splenic vein is directly related to the hemodynamic changes in the portal venous system, excluding patients with portal hypertension, and thus can be a more easily measurable predictor for PSVT than spleen weight. Accordingly, postoperative splenic vein diameter (SVD) may represent the flow dynamics of portal venous system after splenectomy, which could provide information regarding treatment strategy.

The aims of this study were to evaluate the impact of preoperative SVD on PSVT after splenectomy, and to determine the relationship between postoperative SVD changes and treatment outcome using prospectively collected data.

PATIENTS AND METHODS

Patient population. Fifty-three consecutive elective splenectomies, including laparoscopic splenectomy (LS) ($n = 41$) and hand-assisted LS (HALS, $n = 12$), were performed between April 2001 and June 2007 at the Department of Surgery, Osaka University Medical Hospital. Before entry into the study, informed consent was obtained from all patients. The operative technique was performed as described previously.¹³ Age of the patients, sex, indication for surgery, operating time, blood loss, platelet count before the operation and on a post-operative day (POD 7 ± 1), spleen weight, occurrence of PSVT, and postoperative complications were recorded prospectively. Of the 53 patients, 17 patients overlapped with patients reported in our previous study.¹³ Five patients (LS:4, HALS:1) were excluded from this analysis because of hypersensitivity to the intravenous contrast media used in helical computed tomography (CT), renal dysfunction, or anomalies of the portal venous system. Another group of 8 patients (LS:0, HALS:8) with portal hypertension due to liver cirrhosis was excluded from this analysis because the high portal vein pressure could influence SVD. Thus, the study population consisted of 40 patients (LS:37, HALS:3). These consisted of 26 patients with idiopathic thrombocytopenic purpura, 7 with malignant

lymphoma, 2 with splenic lymphangioma/hemangioma, 2 with hereditary spherocytosis, 1 with autoimmune hemolytic anemia, 1 with Evans syndrome, and 1 with splenic cyst (Table I).

Detection and diagnosis of PSVT. All 40 patients underwent preoperative and postoperative helical CT with intravenous contrast media. Imaging analysis was performed prospectively before operation, and after splenectomy between POD 3 and POD 11 (median 6.0 POD). Detection of PSVT was based on the criteria defined previously.^{3,13} In brief, PSVT was diagnosed when an unenhanced region was detected in a dilated splenoportal system, which was otherwise free of any abnormality in the preoperative CT. PSVT was classified into 5 types according to the location of the thrombus.¹³ Distal splenic vein thrombosis (dSVT) was defined as thrombosis located in the splenic vein distal to the junction of IMV. Thrombi between the portal vein and IMV were classified as proximal SVT (pSVT). Total splenic vein thrombosis (tSVT) (pSVT+dSVT) was defined as thrombosis involving the entire splenic vein. When IMV was directed towards the superior mesenteric vein (SMV), splenic vein thrombosis was defined as dSVT. Thrombosis in SMV, intra- and extra-hepatic portal vein was classified as SMVT, iPVT, and ePVT, respectively.

Intermittent pneumatic foot pump was used for perioperative prophylaxis of deep venous thrombosis until full ambulation, but no anticoagulant was used for all patients.

Measurement of SVD. SVD was measured on helical CT scan transaxial images using Aquarius NET server (TeraRecon Inc., San Mateo, CA) at 4 different locations; at the junction of splenic vein and PV (Fig 1, A, location *a*), 2 cm from the junction (Fig 1, A, location *b*), 4 cm from the junction (Fig 1, A, location *c*), and 6 cm from the junction (Fig 1, A, location *d*). SVD was measured on at least two or three sequential enlarged CT images ($\times 2$ – $\times 3$) at all 4 locations, and the largest diameter was selected for analysis.

Statistical analysis. Continuous data are expressed as median and range, unless otherwise specified. Statistical analysis was performed using the Chi-square test or the Fisher exact test for categorical data and the Mann-Whitney *U* test for nonparametric continuous data. The Wilcoxon signed rank test was used to compare preoperative and postoperative or post-treatment SVD. The relationships between clinical features and the incidence of PSVT were examined using logistic regression models in the single-variate and multivariate analyses. The above statistical analyses were completed using StatView 5.0J software

Table I. Comparison of clinical features of patients with or without portal or splenic vein thrombosis

	No PSVT (n = 19)	PSVT (n = 21)	P value†
Age (years)*	56, 17–74	37, 18–73	.233
Sex (M:F)	7:12	5:16	.369
BMI (kg/m ²)*	22.6, 17.9–30.3	22.1, 18.2–24.3	.364
Operating time (min)*	135, 65–200	115, 66–358	.291
Blood loss (ml)*	40, 6–180	20, 10–160	.156
Platelet count: preoperative ($\times 10^4/\mu\text{L}$)*	8.8, 1.4–21.9	9.7, 2.6–27.4	.218
Platelet count: POD 7 \pm 1 ($\times 10^4/\mu\text{L}$)*	19.5, 0.7–52.4	28.8, 2.9–75.7	.06
Spleen weight (g)*	122, 11–608	220.5, 61–2315 (n = 20)	.028
Surgical procedure (LS:HALS)	19:0	3:18	.087
Indication for surgery			
Benign:Malignant	16:3	17:4	.787
Idiopathic thrombocytopenic purpura	15	11	
Splenic lymphangioma/hemangioma	0	2	
Hereditary spherocytosis	0	2	
Autoimmune hemolytic anemia	0	1	
Evans syndrome	0	1	
Splenic cyst	1	0	
Malignant lymphoma	3	4	

BMI, Body mass index.

*Data are median and range, or number of patients.

†By Chi-square test or Fisher exact test for categorical data and Mann-Whitney *U* test for nonparametric continuous data.

(SAS Institute Inc., Cary, NC). A *P* value of <.05 was considered statistically significant. The usefulness of individual prognostic values was estimated using the analysis of receiver operating characteristic (ROC) curves, which were constructed using the R statistical software from <http://www.r-project.org>.

RESULTS

Incidence of PSVT and comparison of perioperative factors. PSVT was diagnosed in 52.5% (21/40) patients. The number of patients and thrombus location were: 5 patients with both iPVT and dSVT, 6 with iPVT only, 3 with both iPVT and tSVT, 2 with tSVT only, 3 with dSVT only, and 2 with ePVT, iPVT, and tSVT. In our 40-case series, 4 patients were symptomatic: 4 had fever greater than 38°C, and 2 had abdominal pain of unknown cause.

There were no significant differences between patients with and without PSVT in terms of age, sex, BMI, operating time, blood loss, and platelet count (preoperative and POD 7 \pm 1), surgical procedure, indication for surgery (Table I). However, the weight of the resected spleen was significantly greater in patients with PSVT than those without PSVT (*P* = .028).

Preoperative SVD was then compared between patients with and without PSVT. As shown in Fig 2, preoperative SVD measured at all locations in patients with PSVT was significantly greater than in

patients without PSVT. Nine of 11 (82%) patients with preoperative SVD at location *b* of more than 10 mm had PSVT. Furthermore, in patients with preoperative SVD of 8 to 10 mm, 6 to 8 mm, and <6 mm measured at location *b*, the incidence of PSVT was 64% (7/11), 43% (6/14), and 0% (0/4), respectively. In patients with preoperative SVD of more than 10 mm at locations *a*, *b*, *c*, and *d*, the incidence of PSVT was 100% (6/6), 82% (9/11), 100% (8/8), and 89% (8/9), respectively.

Comparison of postoperative and post-treatment SVD. In order to compare the flow dynamics of portal venous system after splenectomy, we compared postoperative SVD in patients without PSVT and post-treatment SVD in patients with PSVT after recanalization. Of the 21 patients with PSVT, 4 received intravenous infusion or subcutaneous heparin (adjusted individually with the aim of 1.5- to 2-fold prolongation of the pretreatment activated partial thromboplastin time) followed by oral anticoagulation with warfarin, while 16 patients received only warfarin. The dose of warfarin was adjusted to achieve an international normalized ratio (INR) between 1.5 and 2.0. The last patient had dSVT only and did not receive any anticoagulation. Unresolved dSVT was found in 6 patients in spite of anticoagulation for 3 to 6 months; however, all other PSVTs resolved during the same period. One patient had short splenic vein stump after splenectomy; SVD in this patient could not be measured at locations *c* and *d*. The median preoperative SVD at locations *a*, *b*, *c*, and *d* in patients who

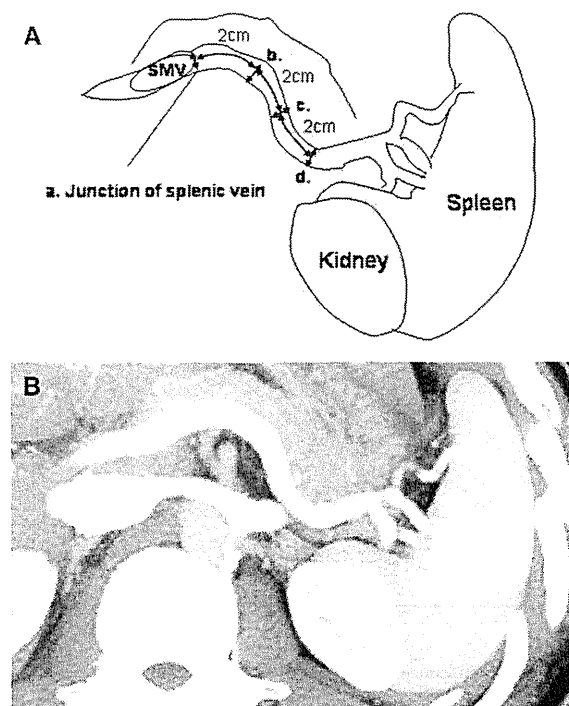


Fig 1. Measurement locations of splenic vein diameter and corresponding computed tomographic image. (A) Splenic vein diameter (SVD) was measured in the trans-axial images of computed tomography (CT) at the junction of portal vein (a), and 2 cm (b), 4 cm (c), and 6 cm (d) from the junction of portal vein. (B) Representative image of CT for measurement of SVD.

did not develop PSVT was 6.4, 7.2, 7.3, and 6.5 mm, respectively (Table II). The median postoperative SVD at the same locations after operation decreased to 6.1, 6.2, 5.9, and 5.7 mm, respectively, and these changes except for SVD at location *a* were significant (Table II). In patients with PSVT, the respective preoperative values were 8.4, 9.3, 9.6, and 9.5 mm, which decreased to 5.2, 5.5, 4.9, and 3.9 mm, respectively, after treatment (Table II). The reduction in SVD was significant for all 4 locations.

Fig 3 shows the rate of reduction of SVD following splenectomy. The median SVD reduction rate in patients without PSVT was less than 25% at each of the 4 locations, while that in patients with PSVT was significantly greater at each of the 4 locations. Differences in SVD following splenectomy were compatible with the results of rate of reduction (data not shown).

Multivariate analysis. Multiple logistic regression analysis was performed to summarize the development of PSVT and the multivariable associations with variables potentially considered as independent risk factors for PSVT following

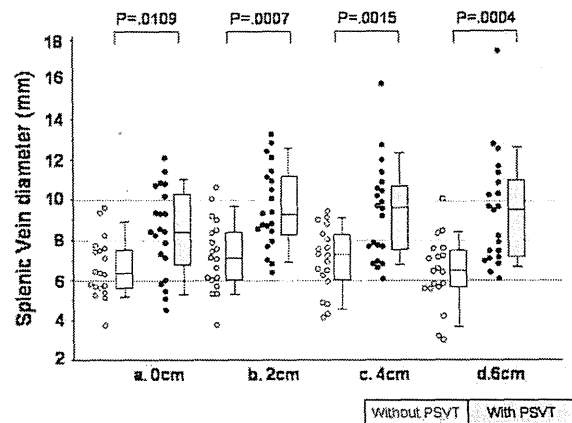


Fig 2. Box-and-whisker plots of preoperative SVD in patients with and without portal or splenic vein thrombosis (PSVT). Symbols: values of individual patients, boxes: group data. Open symbols and boxes: patients without PSVT, Gray symbols and boxes: patients with PSVT. In these plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. Statistical analysis was performed using the Mann-Whitney U test.

splenectomy. Ten factors identified by univariate analysis (listed in Table I) and preoperative SVD values measured at 4 different locations from the junction of PV were considered candidate covariates that could be included in the multivariate analysis. The type of surgical procedure (LS vs HALS) was excluded from the multivariate analysis, because information on surgical procedure is covered or carried by that of spleen weight in this study, ie, HALS was performed in patients with spleen weight greater than 650 g while patients less than 650 g underwent LS. Each value of preoperative SVD measured at 4 locations (SVD *a*, *b*, *c*, and *d*) was included separately in the analysis, since the 4 measurements had quite similar information to each other and, in particular, averaging them in the multivariate analysis is inappropriate due to problems of multi-collinearity as well as interpretability in multivariate fitting. Based on these considerations, the 10 factors: age, sex, BMI, malignant versus benign, operating time, blood loss, platelet counts before operation and at POD 7, spleen weight and a preoperative SVD value measured at 1 of the 4 locations, to sufficiently describe differences in characteristics of patients and/or confounders, were included in the multivariate analysis.

Table III shows the results of multiple logistic regression analysis when such analysis included one

Table II. Changes in splenic vein diameter after surgery

Location	No PSVT (n = 20)			PSVT (n = 21)		
	Before surgery	After surgery	P value*	Before surgery	After treatment	P value*
a	6.4, 5.1–9.6	6.1, 4.2–7.8	.0663	8.4, 4.5–12.1	5.2, 3.6–8.2 (n = 20)	<.0001
b	7.2, 3.8–10.6	6.2, 4.0–8.5	.0150	9.3, 6.4–13.3	5.5, 2.0–7.9 (n = 20)	<.0001
c	7.3, 4.2–9.5	5.9, 4.1–7.9	.0004	9.6, 6.2–15.9	4.9, 2.2–7.5 (n = 19)	<.0001
d	6.5, 3.3–10.0	5.7, 2.0–7.8	.0095	9.5, 6.1–17.7	3.9, 1.4–5.4 (n = 15)	<.0001

Data are median, range (mm).

*By Mann-Whitney U test.

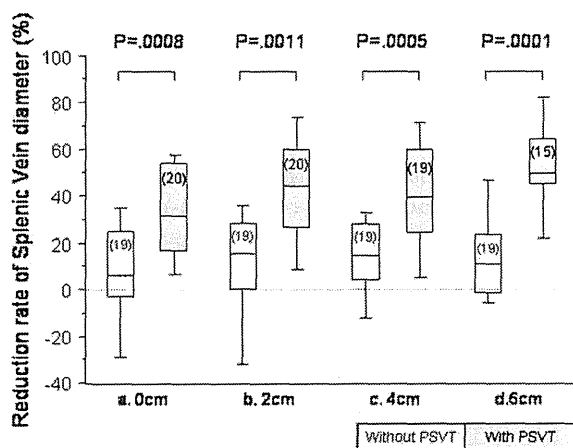


Fig 3. Box-and-whisker plots of the rate of reduction of SVD in patients with and without PSVT. Reduction rate (%) = (preoperative SVD – postoperative SVD)/preoperative SVD \times 100. Numbers in parentheses indicate the number of SVDs measured. Symbols: values of individual patients, boxes: group data. Open symbols and boxes: patients without PSVT. Gray symbols and boxes: patients with PSVT. In these plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. Statistical analysis was performed using the Mann-Whitney U test.

preoperative SVD value measured at any of the 4 locations. Preoperative SVD measured at location *b* was identified as the only significant determinant of PSVT ($P = .014$), while the other factors were not ($P > .05$) under the sample size of this study. In addition, the results of multivariate analysis using any of the preoperative SVD measured at the other 3 locations also identified preoperative SVD as the only significant risk factor ($P = .022$, $.004$, and near 0 for SVD *a*, *c*, and *d*, respectively), while all the other factors did not achieve statistical significance. These results indicate that preoperative SVD, irrespective of the site of measurement,

directly influenced the development of PSVT, while all other 9 factors, especially spleen weight, did not. To further explain the above findings, we included only two variables in the multiple logistic regression models: spleen weight and preoperative SVD values measured at the 4 locations (which were identified as significant factors in univariate analysis). The results of such analysis also identified each of the preoperative SVD value as significant determinant of PSVT ($P = .027$, $.003$, $.007$, and $.0006$ for SVD *a*, *b*, *c*, and *d*, respectively) but not spleen weight ($P = .456$, $.643$, $.958$, and $.773$, respectively).

Results of ROC analysis. Since multivariate analysis identified preoperative SVD value measured at each of the 4 locations as significant determinant of postoperative PSVT, we next analyzed the cutoff values of preoperative SVD that can best predict the development of PSVT by multiple logistic regression model that included preoperative SVD grouped at a cutoff value and the other 9 covariates used in the multivariate analysis. For such analysis, we used 8 mm as the cutoff value for preoperative SVD *b*, because it was found to be the best determination when searching the integer values (6, 7, 8, 9, 10, and 11 mm) with clinical convenience. As a risk estimate for the development of PSVT in preoperative SVD *b* grouped at the cutoff value of 8 mm, the adjusted odds ratio of patients with SVD ≥ 8 mm to those with < 8 mm was 5.221 (95% confidence interval [95% CI] 1.017–33.46). Fig 4 shows the results of ROC analysis for prediction of PSVT, based on this logistic regression model, including preoperative SVD *b* grouped at a cutoff value of 8 mm. The AUC was 0.8552 (95% CI 0.821–1.000).

DISCUSSION

Splenomegaly is considered as a risk factor for PSVT after splenectomy.^{6,9,10,12} A large stump of splenic vein tends to enhance thrombosis probably due to blood stasis and turbulence.^{1,2} Although splenomegaly indirectly indicates splenic vein

Table III. Results of multivariate logistic regression analysis of post-splenectomy PSVT

	Estimate (log-OR)	95% confidence interval	P value*
Age	0.0078	−0.047, 0.063	.781
Sex (M:0, F:1)	−0.0193	−2.858, 2.820	.989
BMI (kg/m ²)	−0.3075	−0.769, 0.154	.164
Malignant vs benign	−1.1937	−4.618, 2.231	.496
Operating time (min)	−0.0105	−0.034, 0.013	.374
Blood loss (ml)	−0.0006	−0.025, 0.024	.964
Platelet count: preoperative (× 10 ⁴ /μL)	0.0075	−0.007, 0.022	.295
Platelet count: POD 7 (× 10 ⁴ /μL)	0.0003	−0.005, 0.006	.916
SVD at location <i>b</i> (mm)	0.6280	0.052, 1.204	.013
Splenic weight	0.0029	−0.004, 0.010	.238

SVD, Splenic vein diameter.

*The relationships between the parameters and incidence of PSVT was examined by logistic regression models in the single-variate and multivariate analyses.

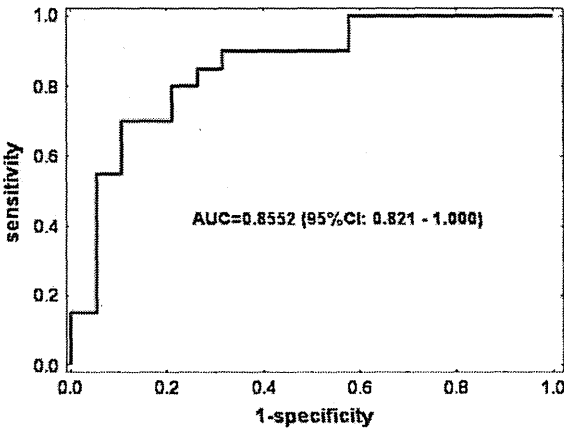


Fig 4. Receiver operator characteristic curve prediction of post-splenectomy PSVT using preoperative SVD value. Values for the area under the curve (AUC) and 95% confidence intervals are provided.

dilation, the direct relationship between SVD and post-splenectomy PSVT has rarely been studied. Possible reasons are (1) the majority of all retrospective analyses have been evaluated by ultrasonography and retrospective data collection was impossible, (2) measurement of SVD was not included in the prospective trials. Since we have been studying postsplenectomy PSVT with contrast helical CT, we were able to review and collect data of SVD pre- and postoperatively. Moreover, as we described in our previous study,¹³ the usefulness of CT for the diagnosis of PSVT after splenectomy has been emphasized previously.^{2,4,10} We prefer to perform CT rather than ultrasonography to detect PSVT, because it provides precise and objective information on PSVT for the entire portal venous system as well as on other lesions such as bowel

edema and ascites. The drawbacks of CT are exposure to radiation and use of contrast material.

Our analysis demonstrated that preoperative SVD in patients who later developed PSVT was significantly larger than that of patients who did not develop PSVT irrespective of surgical treatment, and that the incidence of PSVT after laparoscopic splenectomy correlated with preoperative SVD. Especially, the incidence of PSVT in patients with preoperative SVD greater than 8 mm was very high. Multivariate analysis identified preoperative SVD, irrespective of the location of measurement, as a significant independent risk factor for PSVT. Because measurement of SVD is very simple and easy, preoperative SVD can be a very useful indicator in the prediction of post-splenectomy PSVT. We recommend measuring SVD preoperatively in patients elected to undergo splenectomy.

We found significant differences in preoperative SVD at all 4 locations. For practical purposes, SVD should be measured at about 2 cm distal from the PV junction, because it is visually easier to recognize this portion on transaxial CT images than other locations. Eguchi et al¹⁴ demonstrated that SVD was significantly larger in patients with idiopathic portal hypertension (IPH) than in those with liver cirrhosis (16.2 vs 14.2 mm), and the incidence of PSVT was higher in patients with IPH than those with liver cirrhosis. Although their investigation was conducted in patients with portal hypertension, our results in patients with normal portal pressure are consistent with their data in terms of SVD and tendency for PSVT. They concluded that a decrease in blood flow in the portal vein might be associated with the development of PSVT in patients with IPH. De Cleva et al¹⁵ also found low blood flow in portal veins of patients with PSVT after esophago-gastric devascularization with splenectomy for

hepatosplenic infestation with *Schistosoma mansonia*. However, the difference in the reduction of portal flow was not significant between patients with and without PSVT (42 ± 16 vs $33 \pm 30\%$). Since reduction in SVD correlates with reduction in portal or splenic vein blood flow, we speculate that the greater the reduction rate of SVD is, the greater the incidence of PSVT. Our data on the reduction rate of SVD clearly showed that the reduction rate of SVD in patients with PSVT was significantly greater than in patients without PSVT.

We also found that the reduction of the size of SVD in patients with PSVT were greater than those without PSVT. Since thrombosis exacerbates narrowing of the splenic vein, these results might not be the cause, but rather the effect of thrombosis. However, we have one possible explanation for that. After splenectomy, splenic vein flow is reduced, followed by a decrease in SVD. These changes enhance the development of PSVT. A marked decrease in splenic vein flow volume following splenectomy requires sufficient narrowing of the splenic vein in order to maintain splenic vein flow speed; otherwise, thrombosis develops in the splenic vein, leaving little space for drainage of decreased blood flow. Thus, a greater reduction rate of SVD and larger reduction in size was noted in patients with PSVT. On the other hand, moderate or mild decrease in splenic vein flow volume is not associated with narrowing of the splenic vein and hence protects against thrombosis. Our results that the reduction rate of SVD and reduction in size were smaller in patients without PSVT are consistent with this hypothesis.

Before the present study, many patients who underwent laparoscopic splenectomy had uneventful recovery without any treatment for PSVT. However, it was reported that progression of the thrombus to occlude the portal and mesenteric veins could cause acute hypertension in the splanchnic circulation and intestinal infarction, which negatively influences the outcome in these patients.² Accordingly, we believe that prevention of further thrombus propagation is vital, even in patients asymptomatic at the time of a diagnosis. Since we do not know the natural history of PSVT and there is no supporting evidence on no-treatment, we are in the situation that all PSVT would better be treated unless clinically contraindicated. We have recently reported that patients with total splenic vein thrombosis are more likely to develop clinical symptoms and are thus candidate for aggressive treatment.¹⁶ Importantly, since CT can detect small thrombi, not all patients with PSVT detected by CT are symptomatic. Further research

on patients with asymptomatic PSVT must be carried out.

In conclusion, preoperative SVD was identified as a significant independent determinant of post-splenectomy PSVT, with a calculated cutoff value of 8 mm. We currently perform pre- and post-operative CT routinely to detect PSVT. However, there are many issues to be answered. For example, should all patients be screened? Should asymptomatic PSVT be treated? Should prophylaxis be carried out for patients with greater SVD? We plan prospective studies to resolve such issues. Future prospective studies related to post-splenectomy PSVT need to include measurement of pre- and post-operative SVD to clarify these issues. Because hemodynamic changes in the portal system play pivotal role and can be influenced by pneumoperitoneum, measurement of splenic vein flow is also mandatory. Prospective investigation of post-splenectomy PSVT to evaluate these factors would answer questions on postoperative surveillance, efficacy of prophylaxis, and treatment of PSVT.

This study did not demonstrate the risk of laparoscopic surgery on post-splenectomy PSVT. Since pneumoperitoneum-associated hemodynamic changes and coagulation impairment have been postulated recently as etiologic factors for PSVT,¹⁷⁻²⁰ prospective randomized studies that compare the incidence of PSVT between laparoscopic and open splenectomy with similar patient background stratified by SVD are necessary.

In conclusion, preoperative SVD is a risk factor for post-splenectomy PSVT. We recommend measurement of SVD preoperatively in patients elected to undergo splenectomy. Patients with preoperative SVD greater than 8 mm should be closely monitored after splenectomy because they are at high risk for portal or splenic vein thrombosis.

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Adenovirus-Mediated Gene Expression of the Human *c-FLIP_L* Gene Protects Pig Islets Against Human CD8⁺ Cytotoxic T Lymphocyte-Mediated Cytotoxicity

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ABSTRACT

Cell-mediated immunity, especially of human CD8⁺ cytotoxic T lymphocytes (CTLs) is believed to have an important role in the long-term survival of pig islet xenografts. Protection against human CD8⁺ CTL cytotoxicity may reduce the direct damage to pig islets and enable long-term xenograft survival in pig-to-human islet xenotransplantation. We have previously reported that *c-FLIP_{SIL}* genes, which are potent inhibitors of death receptor-mediated proapoptotic signals through binding competition with caspase-8 for recruitment to the Fas-associated via death domain (FADD), markedly suppress human CD8⁺ CTL-mediated xenocytotoxicity. In addition, the cytoprotective effects of *c-FLIP_L* seem to be significantly stronger than those of *c-FLIP_S*. Accordingly, in the present study, expression of *c-FLIP_L* was induced in intact pig islets by adenoviral transduction. Consequently, the cytoprotective capacity of the transgene in pig islets was examined in in vitro and in vivo exposure to human CD8⁺ CTLs. Cells from untransduced islets or mock islets were sensitive to CD8⁺ CTL-mediated lysis (59.3% ± 15.9% and 64.0% ± 8.9% cytotoxicity, respectively). In contrast, cells from pig islets transduced with the *c-FLIP_L* gene were markedly protected from lysis (30.5% ± 3.5%). Furthermore, prolonged xenograft survival was elicited from pig islets transduced with this molecule as assessed using an islet transplant model using the rat kidney capsule. Thus, these data indicate that intact pig islets can be transduced to express *c-FLIP_L* with adenovirus. Pig islets expressing *c-FLIP_L* are significantly resistant to human CTL killing and further exhibit beneficial effects to prolong xenograft survival.

THE EDMONTON PROTOCOL for human allogeneic islet transplantation can successfully restore endogenous insulin production and glycemic stability in patients with type 1 diabetes mellitus. However, insulin independence is usually not sustained despite islet infusions from two or more donors.¹ The current supply of islets from deceased human donors will almost certainly never meet

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the demand. Xenotransplantation using pig islets could potentially resolve the shortage of donor islets. Pigs are the favored donor species because of (1) their similar physiology to human beings; (2) unlimited availability owing to a short generation interval because of the high number of pregnancies; and (3) somatic cloning is possible, and, thus, production of transgenic animals can be substantially enhanced.² In particular, pig islets have precise glycobiochemical advantages because they lack α -gal epitopes, which induce complement activation, hyperacute rejection, and acute vascular rejection of xenografts. Therefore, successful short-term xenograft survival of these islets is the result of prevention of hyperacute rejection. However, after initial survival of pig islets, infiltrating cells into pig islet xenografts, including natural killer cells, macrophages, and CD8⁺ CTLs, are directly cytotoxic to the islets.^{3–6} In previous studies, we reported that direct cytotoxicity of human CD8⁺ CTLs to pig islets is mediated in major part by the Fas/FasL apoptotic pathway.^{4–6} Cellular FLICE inhibitory protein (c-FLIP) was originally identified as an inhibitor of death-receptor signaling through binding competition with caspase-8 for recruitment to the Fas-associated via death domain.^{7–9} Two major c-FLIP variants result from alternative messenger RNA splicing: a short 26-KDa protein (c-FLIP_S) and a long 55-KDa form (c-FLIP_L).^{7–9} We have demonstrated that the overexpression of *c-FLIP_{S/L}* genes markedly suppresses human CD8⁺ CTL-mediated xenocytotoxicity. In addition, the cytoprotective effects of c-FLIP_L seem to be significantly stronger than those of c-FLIP_S.⁶ The cytoprotective effect of c-FLIP_L in pig islet cells remains controversial. Accordingly, in the present study, we evaluated the cytoprotective activities of the c-FLIP_L molecule by adenovirus-mediated gene expression in pig islets.

MATERIALS AND METHODS

Pig Islet Isolation

Adult pig pancreases were removed at a slaughterhouse that handles young market-weight pigs (Large White–Landrace X–Duroc; age, 2 years; weight, 200–300 kg). Pig islets isolated using the modified Ricordi method as previously described^{10,11} were maintained in complete medium 199 containing 10% heat-inactivated pig serum. Pig islet purity was assessed using the percentage of dithizone-positive cells.

Construction of Adenovirus Vector

The pAdex1CAwt adenovirus vector, regulated by the CAG (chicken β -actin) promoter, containing the open reading frame of the human *c-FLIP_L* gene, was purchased from RIKEN BioResource Center, Wako, Japan. The adenovirus was propagated by infection of human embryonic kidney 293 cells. Subsequently, adenovirus was purified using a Cesium/Tris gradient, separated into aliquots, and stored at -80°C until use. The titer of recombinant adenoviruses (multiplicity of infection [MOI]) was measured using the 50% tissue culture infectious dose method.¹² The “empty” control adenovirus, which lacks the human c-FLIP_L insert, was also amplified in the same manner.

Transduction of Pig Islets by Adenovirus Vector

Freshly obtained adult pig islets represented in 500 μL of serum-free medium 199 were exposed to adenovirus encoding human

c-FLIP_L at a MOI of either 10 or 30 for 1 hour at 37°C . The transfected pig islets were then rinsed with serum-free RPMI (Roswell Park Memorial Institute) medium and resuspended in complete medium 199. Pig islets transfected with empty control adenovirus, which lacks the complementary DNA fragments of human c-FLIP_L (ie, mock islets), were used as the vehicle control. Western blot analysis was performed to identify the expression of this molecule in pig islets.

Generation of Human CD8⁺ CTLs

To generate human CD8⁺ CTLs, peripheral blood mononuclear cells, freshly obtained from the blood of healthy volunteers were separated. Then 10 to 15×10^6 cells of separated peripheral blood mononuclear cells were cocultured for 14 days with irradiated pig endothelial cell monolayers in the presence of 50 U/mL of recombinant human IL-2 as previously described.^{5,6} Subsequently, human CD8⁺ CTLs were positively selected using magnetic beads (Dyna Beads AS, Oslo, Norway) and subjected to an in vitro cytotoxicity assay.

In Vitro Cytotoxicity Assay

The cytotoxic activity of human CD8⁺ CTLs against pig islets was assessed using a chromium 51 (^{51}Cr) release assay.¹³ Either parental pig islets, mock islets, or transfected pig islets were incubated with $\text{Na}_2^{51}\text{CrO}_4$ for 24 hours (1 μCi of ^{51}Cr per 100 islets). Subsequently, ^{51}Cr -labeled pig islets were plated in 96-well plates as target cells for admixture with human CTLs isolated using magnetic beads at various effector-to-target ratios. The ^{51}Cr released from dead islet cells was measured in the supernatants.

Transplant Studies and Immunohistochemical Analysis

To prove the in vivo effectiveness of this molecule to prolong xenograft survival, parental, mock, or transfected pig islets were transplanted under the kidney capsule in 8- to 10-week-old Lewis rats (Oriental Yeast Co, Ltd, Tokyo, Japan). The animals were randomly distributed between the three experimental groups. Rats preimmunized intraperitoneally with 250 mg of pig kidney membranes three times at 1-week intervals were the recipients.^{5,6} In each case, 3000 IEQ of either parental, mock, or transfected pig islets were transplanted under the kidney capsule in the absence of immunosuppression. Transplant recipient rats were monitored until the time of harvest at day 3 or day 5 posttransplantation. Each grafted kidney was analyzed at immunohistochemistry. Kidney specimens were cut into small blocks, fixed in formalin, and embedded in a single paraffin block. After quenching endogenous peroxidase activity by exposure to 3% hydrogen peroxide–methanol, paraffin sections were stained with anti-pig insulin antibody (DAKO, Glostrup, Denmark) to detect surviving pig islet xenografts. The sections were rinsed and incubated with link antibody, followed by incubation of with horseradish peroxidase–conjugated streptavidin. Immunostaining was visualized with 0.02% diaminobenzidine (Sigma-Aldrich Corp, St Louis, Missouri) as the chromogen.

Statistical Analysis

Data were evaluated using the *t* test, with *P* < .05 considered significant. Data are presented as means (SD).

RESULTS

Protein Expression of Human c-FLIP_L in Pig Islets

No protein expression of c-FLIP_L was observed in parental pig islets (Fig 1A). The MOCK pig islets transduced with

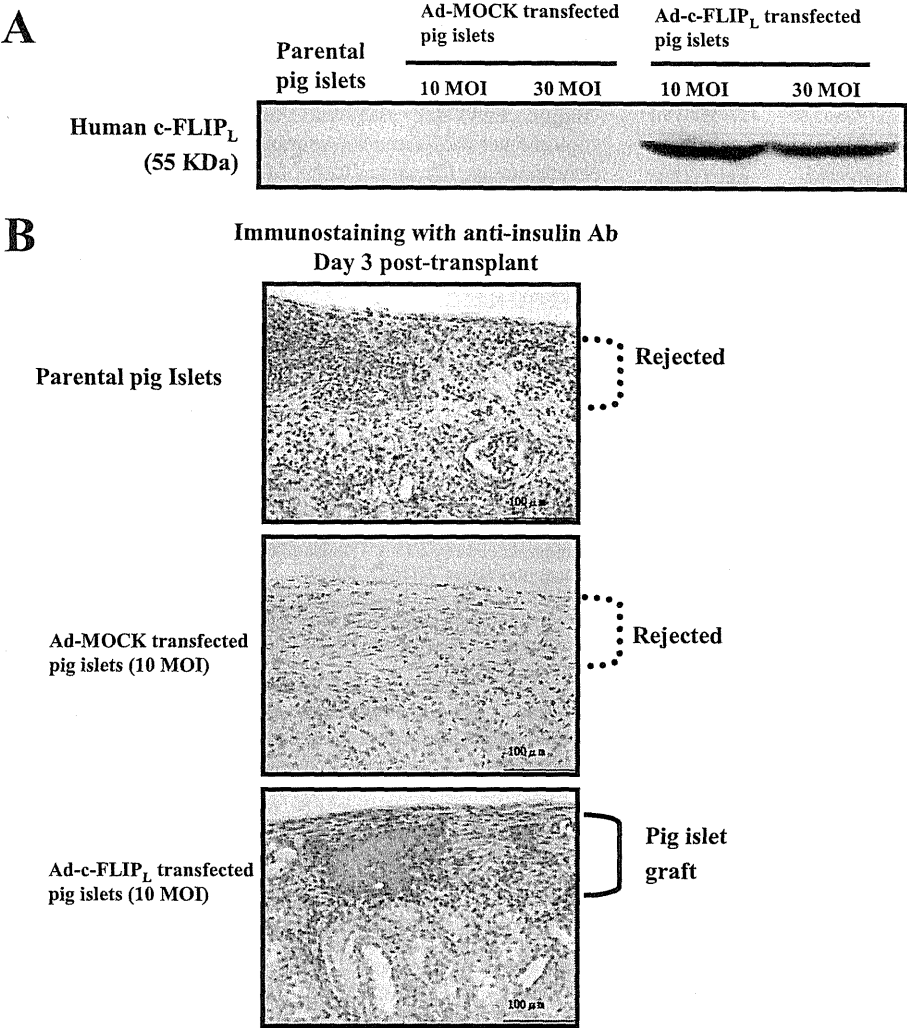


Fig 1. (A) The intracellular expression of c-FLIP_L protein. Adenovirus-mediated gene expression of human c-FLIP_L protein was assessed by Western blot analysis. **(B)** Immunohistological findings of rat kidney tissue of pig islets transplanted rats. Immunostaining with anti-pig insulin Ab for transplanted pig islet xenografts obtained at day 3 post-transplant. The black bars in each picture indicated 100 μm.

control adenovirus vector did not exhibit protein expression of c-FLIP_L at adenovirus concentrations of 10 and 30 MOI. In contrast, transduction with adenovirus vector containing complementary DNA of c-FLIP_L resulted in distinct expression of this molecule at 10 and 30 MOI. The expression level of c-FLIP_L was similar compared with adenovirus transduction of 10 and 30 MOI (Fig 1A).

Adenovirus Expression of c-FLIP_L Effectively Inhibits Cytotoxicity of Human CD8⁺ CTLs Against Pig Islet Cells

Human CD8⁺ CTLs generated by in vitro culture exhibited strong direct killing against parental and mock islets. Approximately, 60% lysis of both parental and mock islets was evident in these human CTLs at an effector-to-target ratio of 50:1 (Table 1). In contrast, the cytotoxicity was significantly reduced against pig islet cells transduced with the c-FLIP_L adenovirus vector, that is, 52% inhibition at an effector-to-target ratio of 50:1 (Table 1).

Prolonged Xenograft Survival Was Elicited From c-FLIP_L-Transfected Pig Islet Cells

To determine whether adenovirus expression of c-FLIP_L in pig islets can prolong xenograft survival, we transplanted pig islets under the kidney capsule in rats. The results of immunohistochemical analysis are shown in Fig 1B. At day 2 posttransplantation, parental, MOCK, and transfected pig islet xenografts survived under the kidney capsule (data not shown). At day 3 posttransplantation, parental and MOCK pig islet xenografts were completely rejected (Fig 1B). In contrast, pig islet xenografts expressing c-FLIP_L survived intact as judged by insulin staining (Fig 1B). At day 5 posttransplantation, pig islet xenografts expressing c-FLIP_L still exhibited insulin staining despite reduced graft size (data not shown). These findings demonstrate the beneficial effects of both in vitro and in vivo cytoprotection of pig islet xenografts expressing c-FLIP_L.

Table 1. ^{51}Cr Release in Pig Islets

Pig Islets	Adenovirus Concentration, MOI	Percent Cytotoxicity at ^{51}Cr Release Assay, Mean (SD)	
		E/T Ratio 50:1	E/T Ratio 25:1
Parental		59.3 (15.9)	47.6 (8.2)
Mock (control)	10	64.0 (8.9)	48.7 (14.8)
adenovirus	30	59.0 (1.4)	43.3 (5.5)
transfected pig islets)			
c-FLIP _L transfected	10	30.5 (3.5)*	24.3 (1.6)*
pig islets	30	23.6 (11.6)*	21.0 (11.0)*

Abbreviations: ^{51}Cr , chromium 51; E/T, effector-target; cFLIP_L, cellular FLICE-like inhibitory protein, long form; MOI, multiplicity of infection.

Amelioration of human CD8⁺ cytotoxic T lymphocyte-mediated cytotoxicity by transduced pig islets was assessed by ^{51}Cr release assay. Control parental and mock pig islets were estimated at the E/T Ratio of either 25:1 or 50:1. Values are given as the mean (SD) from five independent experiments.

*Difference statistically significant ($P < .05$, c-FLIP_L-transfected pig islets vs parental and mock pig islets).

DISCUSSION

In the present study, we determined that the expression of human c-FLIP_L can be induced in pig islet cells using adenovirus vectors. Pig islet xenografts expressing this molecule were markedly protected from direct human CD8⁺ CTL-mediated lysis. Furthermore, beneficial effects of in vivo prolongation of pig islet xenografts with adenoviral expression of c-FLIP_L were demonstrated.

It is generally thought that the adenoviral vector is not able to penetrate more than a few cell layers. In a previous study, we demonstrated that the virus vector used was able to infect more than 80% of islet cells, as assessed using fluorescence-activated cell sorting, and that protein expression in big islets was restricted to the outer cell layers.^{13,14} In addition, because the new DNA is not integrated into the genome of the infected cells, the gene expression is only transient. The strategy of adenovirus-mediated expression in pig islet cells may have only restricted application to clinical islet xenotransplantation. Another strategy would be to generate transgenic pigs expressing the c-FLIP_L molecule in the islet cells. However, in the study in which islets isolated from transgenic pigs expressed high levels of human decay-accelerating factor on endothelial cells, no or only minimal levels of this factor were detected on the islet cells.¹⁵ Therefore, these findings indicate that transgenic pigs, in which the gene constructs containing c-FLIP_L may be regulated by, for example, the insulin promoter, will have to be created to provide sufficient cytoprotection against CD8⁺ CTL cytotoxicity in pig islet xenotransplantation.

In this pig islet transplant model, large infiltrations of both CD8⁺ T cells and macrophages were detected. A large number of macrophages infiltrating pig islet xenografts secrete inflammatory cytokines including IL-1 β , tumor necrosis factor- α , and interferon- γ , which may induce β -cell damage through activation of several intracellular stress-signaling pathways.¹⁶ Our preliminary data suggest

that pig islet cells expressing c-FLIP_L induce resistance against cytokine exposure containing 100 U/mL of IL-1 β , 1000 U/mL of tumor necrosis factor- α , and 1000 U/mL of interferon- γ , as assessed using both the tetramethylrhodamine ethyl ester assay and the colorimetric methyl tetrazolium salt Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega Corp, Madison, Wisconsin). Future experiments will be required to further confirm the role of c-FLIP_L expression in pig islet cells.

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LETTER TO THE EDITOR

Important Matters to Identify Robust Markers for Metastasis and Recurrence in Solid Cancer

IN REPLY

We appreciate the interest of Prof. Liakakos and colleague in our article on the importance of the identification of robust genetic markers and targets to predict solid-cancer metastasis and recurrence.

Liakakos et al. describe two prospective randomized trials which concluded that locoregional control plus extended surgical lymphadenectomy and adjuvant chemotherapy significantly improved clinical outcome in the treatment of stages II and III gastric cancer.^{1,2} However, in spite of the significant efforts that have been made, peritoneal recurrence as well as distant metastasis remain major problems in the treatment of gastric cancer. This observation led us to conclude that there were undetectable isolated tumor cells in the circulatory system and disseminated in the bone marrow, and we could not expect further improvements in clinical outcome until these problems are overcome. Therefore, major efforts are required to identify specific biomarkers that predict recurrence and metastasis. With regard to potentially curative treatments for gastric cancer, Liakakos et al. suggested that it was important to be able to predict the risk of distant and peritoneal dissemination, and also to identify novel therapeutic targets for the prevention of dissemination and recurrence. They also stated that, considering the number of steps required for solid-tumor metastasis, multiple markers (rather than a single marker) would be preferable. Ideally, markers should also be tailored for each patient.

We completely agree with the points they made. However, we would like to point out that, from a practical viewpoint, new markers must reside in patients' serum because circulating markers offer convenience and reduce the cost of testing. We also emphasize the report of Grey et al., who found that cells disseminate very early and evolve to metastatic disease independent from the primary

tumor.³ Thus, we must identify markers at the earliest possible phase of solid-tumor development.

With regard to finding a marker which reliably and specifically predicts metastasis and recurrence, we should reconsider the classical pathway of metastasis. Several recent studies identified multiple factors which contribute to metastasis and recurrence, as shown in Table 1. As for tumor-specific factors in Table 1, we examined markers indicating the presence of isolated tumor cells, such as circulating tumor cells and disseminated tumor cells to clarify the clinical significance of their expression.^{4–8} However, we found that presence of isolated tumor cells did not accurately predict metastasis.⁴ Thus, we focused on identifying cancer stem cells in solid tumors and determining whether cancer stem cells have the capacity for metastasis.^{9,10} It is possible that the presence of cell surface markers in the circulatory system which are characteristic of cancer stem cells may accurately predict metastasis or recurrence. Moreover, the predictive markers for angiogenesis or invasion [vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs)] may well be indicators for metastasis. In fact, membrane type 1 (MT1)-MMP appears to be the best marker to predict distant metastasis compared with other MMP family members.¹¹ Furthermore, a recent study focused on the epithelial mesenchymal transition (EMT) as a critical event leading to metastasis and the avoidance of apoptosis in peripheral blood and bone marrow.¹²

Note that metastasis and tumor recurrence also require host side cells and factors (Table 1). After receiving signals from primary cancer cells, recruitment of hematopoietic progenitor cells from bone marrow or endothelial progenitor cells in peripheral blood was observed in premetastatic niches.^{13,14} Then, isolated tumor cells from primary cancers actively moved toward sites of premetastatic niche with chemokine production, thereby leading to metastasis.¹⁵ Therefore, cell surface markers indicative of immature host cells, chemokines, and their receptors may predict metastasis and recurrence. Houghton et al. demonstrated that chronic inflammation in the gastric mucosa recruited bone-marrow-derived progenitor cells for tissue repair.¹⁶ Therefore, we must identify both malignant cell-specific markers as well as niches and specific signals fostering metastasis.

Finally, we should focus on the presence of microRNA, which can mainly transactivate or inhibit proteins classified into both the cancer-cell-side as well as the host-side factors (Table 1).¹⁷ Therefore, further studies will be required

TABLE 1 Aspects of the metastatic cascade

Tumor-specific factors	Host-side factors		
	Cellular factors	Local or systemic signals	Circumstances
Disseminating cancer cells	Osteoblastic niche	Chemokines	Microenvironmental niches
Circulating cancer cells	Vascular niche	Cytokines	Hypoxia
Cancer stem cells			pH
Induction of angiogenesis	Hematopoietic progenitor cells		Inflammation
Induction of lymphangiogenesis	Endothelial progenitor cells		
Metastatic (invading) ability	Mesenchymal cells		
Epithelial mesenchymal transition			
MicroRNA			

to search for the combination between miR and its regulating genes to predict clinical magnitude in bone marrow (BM) and peripheral blood (PB) from solid-cancer cases.

In conclusion, we believe that it is imperative that future studies identify biomarkers pertinent to the prevention and treatment of recurrence or metastasis of solid cancers. If both cancer-side and host-side factors are considered simultaneously, it should be possible to improve clinical outcome.

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Published Online: 24 January 2009

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